THERAPEUTIC POTENTIALS OF NATURAL COMPOUNDS FOR CUTANEOUS CANDIDIASIS TREATMENT USING MURINE MODELS

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NNAMDI AZIKIWE UNIVERSITY, AWKA

MAY, 2021

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A DISSERTATION SUBMITTED TO THE DEPARTMENT OF APPLIED MICROBIOLOGY AND BREWING, FACULTY OF BIOSCIENCES, NNAMDI AZIKIWE UNIVERSITY, AWKA, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY (PhD) DEGREE IN MEDICAL MICROBIOLOGY.

SUPERVISOR: PROF. C. A. OYEKA

MAY, 2021

CERTIFICATION

This is to certify that this dissertation titled **"Therapeutic Potentials of Natural Compounds for Cutaneous Candidiasis Treatment using Murine Models"** was carried out by me, Udemezue, Onyekachukwu Izuchukwu (with registration number 2014487013P) in the Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University, Awka. No part of this work has been submitted in part or full for an award of a degree or diploma in this or any other institution.

……………………………………….. **………………………….**

Udemezue, Onyekachukwu Izuchukwu **Date**

APPROVAL PAGE

This dissertation titled **"Therapeutic Potentials of Natural Compounds for Cutaneous Candidiasis Treatment using Murine Models"** carried out by Udemezue, Onyekachukwu Izuchukwu with registration number 2014487013P has been approved for the award of Doctor of Philosophy (PhD) Degree in the Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka.

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DEDICATION

I sincerely dedicate this work to Almighty God for his grace, mercy, blessings, guidance and protection.

To him be all the glory.

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ABSTRACT

The increased risk of fungal diseases especially opportunistic fungal infections such as candidiasis, emerging fungal pathogens, limited range of antifungal drugs, high level of toxicity and development of resistance to the available antifungal drugs have increased the demand for more clinically effective and safer antifungal agents. The continuous search for more potent, cheaper and non-toxic raw materials to feed the pharmaceutical industries is inevitable. The aim of this work was to evaluate the therapeutic potentials of natural compounds (potash compounds and natural stones such as palm ash, potash alum, trona, blue stone, black stone and sulphur stone) for cutaneous candidiasis treatment using murine models. Four hundred and fifty high vaginal swab specimens were collected from patients suspected of having vulvo-vaginal candidiasis. These were inoculated on Sabouraud dextrose agar supplemented with 50µg/ml chloramphenicol and incubated aerobically at $25^{o}C$ for 48hours. The yeast isolates were identified based on their morphological, physiological, biochemical and molecular characteristics. Antimicrobial activity of the natural compounds on the *Candida* isolates were evaluated using *in vitro* (agar-well diffusion) and *in vivo* methods (albino rat model). The Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) of the agents were comparatively determined using broth dilution and micro-dilution methods. Full blood count of the test animals before and after immunosuppression was analysed using hemoanalyser. The organic and inorganic chemical constituents of the natural compounds were determined using GC-MS and X-ray fluorescence technology respectively. The *in vivo* toxicity screening of the natural agents was done using Draize's skin irritancy test, skin sensitization test and necropsy. The *in vivo* antimicrobial assay was done using eight-month old healthy albino rats weighing 220g – 240g. The animals were immunosuppressed intraperitoneally using 0.1ml cyclophosphamide (100mg/kg body weight). They were inoculated with a loopful of the isolates (equivalent to 1×10^{8}cfu/g) percutaneously and carefully observed for clinical signs and symptoms of candidiasis. Out of the 450 HVS specimens collected, 188 (42%) were positive for *Candida* growth. *Candida albicans*, 96 isolates (51%), *C. tropicalis,* 53 isolates (28%), *C. glabrata*, 28 isolates (15%) and *C. parapsilosis*, 11 isolates (6%). Among the potash compounds analysed, trona gave the highest inhibition zone diameter (IZD), 31.00±1.41mm against *C. glabrata*, potash alum, 30.00±1.41mm against *C. parasilopsis* and palm ash, 17.50±0.71mm against *C. glabrata* at 200mg/ml. Blue stone was the best among natural stones, 31.50±0.71mm against *C. tropicalis*, sulphur stone, 18.50±0.71mm against *C. parapsilosis* and Black stone, 0.00mm for all the isolates at 200mg/ml. The positive control ketoconazole (at 200mg/ml) gave the IZD of 21.00±1.41mm against *C. albicans*, 21.50±0.71mm against *C. tropicalis*, 20.50±0.71mm against *C. glabrata* and 19.00±1.41mm against *C. parapsilosis*. The MIC and MFC of the test agents varied among the *Candida* isolates. The MIC of blue stone for *C. albicans* was (50mg/ml), Potash alum (50mg/ml), Trona (100mg/ml), Sulphur stone (200mg/ml), Black stone (200mg/ml), Palm ash (100mg/ml) and Ketoconazole (50mg/ml). The combined activity of the agents (at 1:1 ratio) using their MIC and fractional inhibitory concentration (FIC) values against the isolates also varied. The agents exhibited synergism against *C. albicans* (FIC<1), indifference for *C. tropicalis* (FIC between 1 and 2) while antagonism for *C. glabrata* and *C. parapsilosis* (FIC>2). Palm ash + trona and palm ash + potash alum + trona combinations were antagonistic against all the isolates (FIC>2). During the *in vivo* studies, the signs and symptoms of cutaneous candidiasis disappeared gradually 5 – 7days during treatment with various formulations of the natural compounds. The immunosuppressed group developed infection 7 days after inoculation while the normal group developed infection 9 days after

inoculation. There was a drastic decrease in the white blood cells count five days after the immunosuppression; Neutrophil count (53.1% to 25.4%), Monocyte (6.7% to 2.3%), Eosinophil (1.7% to 1.3%) and basophil (0.6% to 0.5%). Histological analysis of stained infected rat skin sections revealed intense peri-adnexal infiltration of inflammatory cells, inflammed sebaceous units and malformed collagen bundles. Tissue regeneration was much quicker in treated groups compared to the negative control groups. Ketoral cream (ketoconazole) was used as the positive control. Total yeast count was done at 3 days intervals during treatment to evaluate the effectiveness of the agents in reducing the yeast population *in vivo*. The yeast population declined gradually with time (from 10^8cfu/g to 10^2cfu/g), thus showing the *in vivo* effectiveness of the antimicrobial agents in treatment of cutaneous candidiasis. The major inorganic components of the natural compounds were Blue stone: Cu(43.7%), S(29.4%), As(0.0015%), Pb(0.002%), Ni(0.005%); potash alum: S(44.5%), Al(14.49), Pb(0.0009%), As(0%), Ni(0.0004%); Trona: Cl(5.81%), Si(22.6%), Pb(0%), As(0.003%), Ni(0.0007%); Sulphur stone: S(94.72%), Al(3.5%), As(0%), Pb(0.0006%), Ni(0.00008%); Black stone: S(1.6%), Cl(0.98%), Cu(0.234%), Pb(0.001%), As(0%), Ni(0.0021%) and Palm ash: K(28.84%), Cl(15.21%), S(3.36%), Urea(8.6%), Pb(0.00074%), As(0.00052%), Ni(0.0007%). The organic constituents are basically naturally occurring fatty acids including vaccenic, decanoic, oleic and linoelaidic acids. *In vivo* toxicity analysis of the natural compounds showed no observable histopathological signs as seen in the architecture of the collagen bundles, blood vessels and sebaceous units described in the stained skin sections. The findings revealed that potash compounds and natural stones are clinically safe and effective in treatment of cutaneous candidiasis and could serve as good alternatives to conventional antifungal antibiotics.

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CHAPTER ONE

1.0 INTRODUCTION

Yeasts are unicellular eukaryotic microorganisms. They are classified as members of the fungus kingdom. Some species have the ability to develop multicellular characteristics by forming strings of connected budding cells known as pseudohyphae or false hyphae. They are estimated to constitute 1% of all described fungal species. Yeast sizes vary greatly, depending on species and environment, typically measuring 3 – 4 µm in diameter, although some yeast can grow to 40 µm in size. Most yeasts reproduce asexually by mitosis, and many do so by the asymmetric division process known as budding (Kurtzman and Fell, 2011; Hoffman *et al*., 2015).

Yeasts are very common in the environment and are often isolated from sugar-rich materials. Examples include naturally occurring yeasts on the epidermal layers of fruits and berries (such as grapes, apples, or peaches), and exudates from plants (such as plant saps or cacti). Some yeasts are found in association with soil and insects. The ecological functions and biodiversity of yeasts are relatively unknown compared to those of other microorganisms. Yeasts, including *Candida albicans*, *Rhodotorula rubra*, *Torulopsis* species, have been found living between people's toes as part of their skin flora. Yeasts are also present in the gut of mammals and some insects and even deep-sea environments host an array of yeasts (Herrera and Pozo, 2010). The body normally hosts a variety of microorganisms, including bacteria and fungi. Some of these are useful to the body, some cause no harm or benefit, and some can cause harmful infections. Some fungal infections are caused by fungi that often live on the hair, nails, and outer skin layers. They include yeast-like fungi such as *Candida*. Sometimes, these yeasts penetrate beneath the surface of the skin and cause infection (Martins *et al*., 2014).

***Candida* yeast**

Yeasts of the genus *Candida*, can be opportunistic pathogens, causing oral and vaginal infections in humans, known as candidiasis. *Candida* is commonly found as commensal in the mucous membranes of humans and other warm-blooded animals. However, sometimes these same strains can become pathogenic. The pathogenic yeasts of candidiasis in probable descending order of virulence for humans are *C. albicans*, *C. tropicalis*, *C. stellatoidea*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. guilliermondii*, *C. viswanathii*, *C. lusitaniae*, and *Rhodotorula mucilaginosa*. *C. auris* has been recently identified. Over 200 species of *Candida* exist in nature; thus far, only a few species have been associated with disease in humans (Brunke and Hube, 2013).

The medically significant *Candida* species include the following:

- *C albicans,* the most common species identified (50%-60%)
- *Candida glabrata* (previously known as *Torulopsis glabrata*) (15%-20%)
- *C parapsilosis* (10%-20%)
- *Candida tropicalis* (6%-12%)
- *Candida krusei* (1%-3%)
- *Candida kefyr* (< 5%)
- *Candida guilliermondi* (< 5%)
- *Candida lusitaniae* (< 5%)
- *Candida dubliniensis,* primarily recovered from patients infected with HIV
- *Candida auris* (Martins *et al*., 2014; Pappas *et al*., 2016).

Candida virulent factors

Candida species contain their own set of well-recognized but not well-characterized virulence factors that may contribute to their ability to cause infection. The main virulence factors include the following:

- Surface molecules that permit adherence of the organism to other structures (eg, human cells, extracellular matrix, prosthetic devices)
- Acid proteases and phospholipases that involve penetration and damage of cell envelopes
- Ability to convert to a hyphal form (phenotypic switching) (Chaves *et al*., 2013; Zahir *et al*., 2013).

Host defense mechanisms against *Candida* infection

As with most fungal infections, host defects also play a significant role in the development of candidal infections. Host defense mechanisms against *Candida* infection and their associated defects that allow infection are as follows:

- Intact mucocutaneous barriers - Wounds, intravenous catheters, burns, ulcerations
- Phagocytic cells - Granulocytopenia
- Polymorphonuclear leukocytes - Chronic granulomatous disease
- Monocytic cells - Myeloperoxidase deficiency
- Complement - Hypocomplementemia
- Immunoglobulins - Hypogammaglobulinemia
- Cell-mediated immunity - Chronic mucocutaneous candidiasis, diabetes mellitus, cyclosporin A, corticosteroids, HIV infection
- Mucocutaneous protective bacterial flora - Broad-spectrum antibiotics (Hanna and Etzoni, 2011)

Candidiasis

Candida normally lives inside the body (in places such as the mouth, throat, gut and vagina) and on skin without causing any problems. Sometimes *Candida* can multiply and cause an infection if the environment inside the vagina changes in a way that encourages its growth.

Candidiasis in the vagina is commonly called a “vaginal yeast infection.” Other names for this infection are “vaginal candidiasis,” “vulvovaginal candidiasis,” or “candidal vaginitis” (Goncalves *et al*., 2016).

Candidiasis is an infection caused by *Candida* species, mostly by *Candida albicans*. These fungi are found almost everywhere in the environment. Some may live harmlessly along with the abundant "native" species of bacteria that normally colonize the mouth, gastrointestinal tract and vagina. *Candida* infections can cause symptoms in healthy people. Usually the yeast infections are limited to the mouth, genital area or skin. However, people with weakened system from illness or medications such as corticosteroids or anticancer drugs are not only more susceptible to topical infections; they also are more likely to experience a more serious internal infection (Pappas *et al*., 2016).

Pathogenesis

The first step in the development of a candidal infection is colonization of the mucocutaneous surfaces. All of the factors outlined above are associated with increased colonization rates. The mechanisms of candidal invasion include (1) disruption of a colonized surface (skin or mucosa), allowing the organisms access to the bloodstream, and (2) persorption via the gastrointestinal wall, which may occur following massive colonization with large numbers of organisms that pass directly into the bloodstream (Williams and Lewis, 2011).

Candidiasis risk factors

Factors that increase the risk of candidiasis include HIV/AIDS, mononucleosis, cancer treatments, steroids, stress, antibiotic usage, diabetes, and nutrient deficiency. Hormone replacement therapy and infertility treatments may also be predisposing factors. Use of inhaled corticosteroids increases risk of candidiasis of the mouth. Inhaled corticosteroids with other risk

factors such as antibiotics, oral glucocorticoids, not rinsing mouth after use of inhaled corticosteroids or high dose of inhaled corticosteroids put people at even higher risk. Treatment with antibiotics can lead to eliminating the yeast's natural competitors for resources in the oral and intestinal flora, thereby increasing the severity of the condition. A weakened or undeveloped immune system or metabolic illnesses are significant predisposing factors of candidiasis. Almost 15% of people with weakened immune systems develop systemic illness caused by *Candida* species. Diets high in simple carbohydrates have been found to increase rates of oral candidiasis (Martins *et al*., 2014; Gerard *et al*., 2015).

Signs and symptoms of candidiasis

Signs and symptoms of candidiasis vary depending on the area affected. Most candidal infections result in minimal complications such as redness, itching, and discomfort, though complications may be severe or even fatal if left untreated in certain populations. In healthy (immunocompetent) persons, candidiasis is usually a localized infection of the skin, fingernails or toenails (onychomycosis), or mucosal membranes, including the oral cavity and pharynx (thrush), esophagus, and the genitalia (vagina, penis, etc.); less commonly in healthy individuals, the gastrointestinal tract, urinary tract and respiratory tract are sites of candida infection (Martins *et al*., 2014; Wang *et al*., 2014; Erdogan and Raos, 2015; Patil *et al*., 2015; Felix *et al*., 2019).

Types of candidiasis and their unique signs and symptoms

Oropharyngeal candidiasis:

Individuals with oropharyngeal candidiasis (OPC) usually have history of HIV infection, wear dentures, have diabetes mellitus, or have been exposed to broad-spectrum antibiotics or inhaled steroids. Although patients are frequently asymptomatic, when symptoms do occur, they can include the following:

- Sore and painful mouth
- Burning mouth or tongue
- Dysphagia
- Thick, whitish patches on the oral mucosa

Physical examination reveals a diffuse erythema and white patches that appear on the surfaces of the buccal mucosa, throat, tongue, and gums (Williams and Lewis, 2011).

Esophageal candidiasis

Patients with esophageal candidiasis may be asymptomatic or may have one or more of the following symptoms:

- Normal oral mucosa (>50% of patients)
- Dysphagia
- Odynophagia
- Retrosternal pain
- Epigastric pain
- Nausea and vomiting

Nonesophageal gastrointestinal candidiasis

The following symptoms may be present:

- Epigastric pain
- Nausea and vomiting
- Abdominal pain
- Fever and chills.
- Abdominal mass (in some cases) (Lalla *et al*., 2013; Erdogan and Raos, 2015).

Genitourinary tract candidiasis:

The types of genitourinary tract candidiasis are as follows:

- Vulvovaginal candidiasis (VVC) - Erythematous vagina and labia; a thick, curdlike discharge; and a normal cervix upon speculum examination.

- *Candida* balanitis - Penile pruritus and whitish patches on the penis
- *Candida* cystitis - Many patients are asymptomatic, but bladder invasion may result in frequency, urgency, dysuria, hematuria, and suprapubic pain
- Asymptomatic candiduria - Most catheterized patients with persistent candiduria are asymptomatic
- Ascending pyelonephritis - Flank pain, abdominal cramps, nausea, vomiting, fever, chills and hematuria
- Fungal balls - Intermittent urinary tract obstruction with subsequent anuria and ensuing renal insufficiency (Pappas *et al.,* 2016).

Diagnosis

Diagnosis of a yeast infection is done either via microscopic examination or culturing. For identification by light microscopy, a scraping or swab of the affected area is placed on a microscope slide. A single drop of 10% potassium hydroxide (KOH) solution is added to the specimen. The KOH dissolves the skin cells, but leaves the *Candida* cells intact, permitting visualization of the pseudohyphae and budding yeast cells typical of many *Candida* species. For the culturing method, a sterile swab is rubbed on the infected skin surface. The swab is then streaked on a culture medium. The culture is incubated at 37°C for several days, to allow development of yeast or bacterial colonies. The characteristic (such as morphology and colour) of the colonies may allow initial diagnosis of the organism causing disease symptoms. Respiratory, gastrointestinal, and esophageal candidiasis require an endoscopy to diagnose. For gastrointestinal candidiasis, it is necessary to obtain a 3–5 milliliter sample of fluid from the duodenum for fungal culture. The diagnosis of gastrointestinal candidiasis is based upon the

culture containing in excess of 1,000 colony-forming units per milliliter (Guarner and Brandt, 2011; Erdogan and Rao, 2015).

Treatment

Mouth and throat candidiasis are treated with antifungal medication. Oral candidiasis usually responds to topical treatments, otherwise, systemic antifungal medication may be needed for oral infections. Candidal skin infections in the skin folds (candidal intertrigo) typically respond well to topical antifungal treatments (e.g., nystatin or miconazole). Systemic treatment with antifungals by mouth is reserved for severe cases or if treatment with topical therapy is unsuccessful. *Candida* esophagitis may be treated orally or intravenously; for severe or azole-resistant esophageal candidiasis, treatment with amphotericin B may be necessary (Williams and Lewis, 2011; Lalla *et al*., 2013)

Vaginal yeast infections are typically treated with topical antifungal agents. A one-time dose of fluconazole is 90% effective in treating a vaginal yeast infection. For severe nonrecurring cases, several doses of fluconazole is recommended. Local treatment may include vaginal suppositories or medicated douches. Other types of yeast infections require different dosing. Gentian violet can be used for thrush in breastfeeding babies. *C. albicans* can develop resistance to fluconazole, this being more of an issue in those with HIV/AIDS who are often treated with multiple courses of fluconazole for recurrent oral infections. For vaginal yeast infection in pregnancy, topical imidazole or triazole antifungals are considered the therapy of choice owing to available safety data. Systemic absorption of these topical formulations is minimal, posing little risk of transplacental transfer. In vaginal yeast infection in pregnancy, treatment with topical azole antifungals is recommended for 7 days instead of a shorter duration (Lalla *et al*., 2013; Pappas *et al*., 2016).

Systemic candidiasis occurs when candida yeast enters the bloodstream and may spread (becoming disseminated candidiasis) to other organs, including the central nervous system, kidneys, liver, bones, muscles, joints, spleen, or eyes. Treatment typically consists of oral or intravenous antifungal medications. In candidal infections of the blood, intravenous fluconazole or an echinocandin such as caspofungin may be used. Amphotericin B is another option (Pappas *et al*., 2016).

Drug Discovery and Development

The pharmaceutical industry is continuing to attempt double-digit growth rates driven by high market capitalization. Standard responses to this challenge have only provided limited impact. Besides scaling-up businesses through mergers or selective acquisitions of platform technologies or drug candidates, an increase of Research and Development (R&D) productivity still represents a sure approach to address this challenge (Harvey *et al*., 2015).

Drug discovery is a process which is intended to identify a small synthetic molecule or a large biomolecule for comprehensive evaluation as a potential drug. New drugs are continually required by the healthcare systems to address unmet medical needs across diverse therapeutic areas, and pharmaceutical industries primarily strive to deliver new drugs to the market through the complex activities of drug discovery and development. Discovery involves a number of processes like target identification and validation, hit identification, lead generation and optimization and finally the identification of a candidate for further development (Newman and Cragg, 2016).

Development, on the other hand, includes optimization of chemical synthesis and its formulation, toxicological studies in animals, clinical trials, and eventually regulatory approval. Both of these processes are time-consuming and expensive and currently the industry is under

pressure owing to the extremely stringent regulatory requirements, environmental concerns, and reduced incomes due to patent expirations. These issues have had an adverse bearing on the R&D productivity in recent years; hence there is a need for innovative approaches as well as increased collaboration between industry, academia, and governmental research institutions, with a common objective of constantly delivering quality medicines. Today's fast moving pharmaceutical market requires more efficient drug development and production. Pharmaceutical development is intended to design a quality product and a manufacturing process that can consistently deliver the product with its intended performance. A pharmaceutical product should be designed to meet patients' needs ((Roemer and Krysan, 2014; Sandeep and Divya, 2018).

Traditionally, many drugs and other chemicals with biological activity have been discovered by studying chemicals that organisms create to affect the activity of other organisms for survival. Despite the rise of combinatorial chemistry as an integral part of lead discovery process, natural products still play a major role as starting material for drug discovery. A 2007 report found that of the 974 small molecule new chemical entities developed between 1981 and 2006, 63% were natural derived or semisynthetic derivatives of natural products. For certain therapy areas, such as antimicrobials, antineoplastics, antihypertensive and anti-inflammatory drugs, the numbers were higher. In many cases, these products have been used traditionally for many years. Natural products may be useful as a source of novel chemical structures for modern techniques of development of antimicrobial therapies. (Harvey *et al*., 2015; Newman and Cragg, 2016; Ahn, 2017; Torre and Albericio, 2017).

These natural compounds include potash compounds and natural stones such as black stones, brimstone and blue stones.

Potash compounds

Potash is the common name for various mined and manufactured salts that contain potassium in water – soluble form. The name derives from "pot ash", which refers to plant ashes soaked in water in a pot, the primary means of manufacturing the product before the industrial era. Potash refers to potassium compounds and potassium-bearing materials. Commonly available potash compounds include potassium carbonate, potash alum and palm ash. Most potash compounds form a strong alkaline solution when dissolved in water at room temperature (Enper *et al*., 2011).

Trona (Akanwu)

Potassium carbonate otherwise called trona, akanwu or kanwa is a yellowish – white or grayish – white alkaline salt, soluble in water and insoluble in alcohol or acetone. It forms a strongly alkaline solution when dissolved in water. It can be made as the product of potassium hydroxide's absorbent reaction with carbondioxide. Potassium carbonate is deliquescent and is used in the production of soap and glass (Civitello, 2017).

Potassium carbonate was first identified by Antonio Campanella in 1742. It has been used and mined for hundreds of years for several different purposes. Other terms for potassium carbonate include: carbonate of potash, dipotassium carbonate, dipotassium salt, pearl ash, "potash", salt of tartar, or salt of wormwood. Today, potassium carbonate is prepared commercially by the electrolysis of potassium chloride. The resulting potassium hydroxide is then carbonated using carbondioxde to form potassium carbonate which is often used to produce other potassium compounds (Bulatovic, 2015; Civitello, 2017).

$$2KOH + CO_2 \longrightarrow K_2CO_3 + H_2O$$

Potash alum

Alum or Phitkari is a transparent salt like substance that is used in cooking as well as for medicinal purposes. There are various types of Alum such as Potassium Alum or Potash, Ammonium, Chrome, Selenate. Alum is both a specific chemical compound and a class of chemical compounds. The specific compound is the hydrated potassium aluminum sulfate (potassium alum) with the formula KAL $(SO_4)_2.12H_2O$. More widely, alums are double sulphate salts, with the formula $AM(SO_4)_2.12H_2O$, where A is a monovalent cation such as potassium or ammonium, and M is a trivalent metal ion such as aluminum or chromium (III). Aluminum potassium sulfate, potash alum ($KAL(SO_4)_2.12H_2O$) is used as an astringent and antisepsis in various food preparation processes such as pickling and fermentation and as a flocculants for water purification, among other things (Alzomor *et al*., 2014; Ali and Zaigham, 2017).

Palm ash

Ash is simply the dust left over particles of burnt biomass, not the charcoal. The palm ash is a white, grey or black left over after burning of palm tree parts (such as *Elaeis guineensis*). It is rich in potassium and soluble in water. Palm ash is commonly sourced from palm shell, palm fiber and bunches. It is an ideal fertilizer for crop production owing to its high potassium content (Kamka-Evans *et al*., 2013).

Blue stones

Copper (II) sulfate commonly called blue stones are the inorganic compounds with the chemical formula $CuSO_4(H_2O)_x$, where x can range from 0 to 5. The pentahydrate (x = 5) is the most common form. Older names for this compound include blue vitriol, bluestone, vitriol of copper, and Roman vitriol. Copper sulfate is highly soluble in water. The largest health benefit of copper sulfate is that it is used to control bacteria and fungus growth on fruits, vegetables, and

other crops, as it's been registered for pesticide use in the United States since 1956. This includes mildew, which can cause leaf spots and plant spoilage, as copper sulfate binds to the proteins in fungus, damaging the cells and causing them to die. Copper sulfate is produced industrially by treating copper metal with hot concentrated sulfuric acid or its oxides with dilute sulfuric acid (Zumdahl and Decoste, 2013).

Black stones

Black stone otherwise known as Snake stone or Serpent stone is a rocklike substance that extracts poison from the body when placed on the wound inflicted by poisonous creatures like snakes and scorpions and even relieves one from boils or other bodily blisters caused by germs (Szweda *et al.*, 2015).

Sulphur stones (brimstone)

Sulfur also known as brimstone or burning stone is a bright yellow, crystalline solid at room temperature. It forms near volcanic vents and fumaroles, where it sublimates from a stream of hot gases. Small amounts of native sulfur also form during the weathering of sulfate and sulfide minerals. It is the tenth most common element by mass in the universe, and the fifth most common on Earth. Though sometimes found in pure, native form, sulfur on Earth usually occurs as sulfide and sulfate minerals. Sulphur is sparingly soluble in water though more soluble in warm water. Sulfur (specifically octasulfur, S_8) is used in pharmaceutical skin preparations for the treatment of acne and other conditions. It acts as a keratolytic agent and also kills bacteria, fungi, scabies mites, and other parasites. Precipitated sulfur and colloidal sulfur are used, in form of lotions, creams, powders, soaps, and bath additives, for the treatment of acne vulgaris, acne rosacea, and seborrhoeic dermatitis. Common adverse effects include irritation of the skin at the

application site, such as dryness, stinging, itching and peeling (de Castro *et al.,* 2013; (Seyedmousav *et al.,* 2017).

Over the years, infectious diseases caused by microbes such as bacteria, fungi, viruses and parasites have been treated with drugs known as antimicrobial agents. They are substances that kill or inhibit the growth of microorganisms such as bacteria, fungi, viruses or protozoa. Antimicrobial agents can be synthesized, manufactured by chemical procedures independent of microbial activity. They can also be semi-synthetic, that is, natural antibiotics that have been chemically modified to make them less susceptible to inactivation by pathogens (Szweda *et al.*, 2015).

1.1. Statement of problem

The increased risk of fungal diseases particularly in immunocompromised patients, emerging fungal pathogens, limited repertoire of antifungal drugs, toxicity and the development of resistance to the available antifungal drugs, have increased the demand for the development of new and effective antifungal agents. These have resulted in intensified efforts on antifungal drug search to develop clinically effective and safer antifungal agents. Antifungal research is particularly challenging, with little achievements recently. It is noteworthy that the newest class of antifungal drugs, the echinocandins, was discovered in the 1970s and took 30 years to gain acceptance into clinical practice. Similarly, the gold standard therapy for cryptococcosis, one of the most prevalent invasive, life- threatening fungal infections worldwide, consists of two drugs (AmB and flucytosine) that were discovered over 50 years ago.

Thus, there is urgent need for new cheap, clinically safe and effective compounds with novel mechanisms of action that may overcome the limitations of presently available antifungals. The continuous search for more potent and cheaper raw materials to feed the pharmaceutical

industries is inevitable. The raw materials include natural compounds such as potash compounds and natural stones.

1.2. Aim of this research

The aim of the research was to evaluate the therapeutic potentials of natural compounds (potash compounds and natural stones) for cutaneous candidiasis treatment using murine models. The materials for investigation include palm ash, potash alum, trona, blue stone, black stone and sulphur stone.

1.3 Objectives of this research:

The objectives of this research were:

1. To identify *Candida* species from cutaneous candidiasis in the study area.
2. To determine the phylogenetic links of the *Candida* species.
3. To determine the anticandidal potentials (*in vitro*) of the natural compounds (palm ash, potash alum, trona, blue stone, black stone and sulphur stone).
4. To determine the minimal inhibitory and minimal fungicidal concentrations of the compounds.
5. To evaluate the chemical compositions of the natural compounds.
6. To evaluate the toxicity of the natural compounds using albino rat model.
7. To determine the anticandidal potentials (*in vivo*) of natural compounds using albino rat model.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Fungi

A fungus is a eukaryote that digests food externally and absorbs nutrients directly through its cell walls. Most fungi reproduce by spores and have a body (thallus) composed of microscopic tubular cells called hyphae. Fungi are heterotrophs and, like animals, obtain their carbon and energy from other organisms. Some fungi obtain their nutrients from a living host (plant or animal) and are called biotrophs; others obtain their nutrients from dead plants or animals and are called saprotrophs (saprophytes, saprobes). Some fungi infect a living host, but kill host cells in order to obtain their nutrients; these are called necrotrophs (Hawksworth and Lucking, 2017; Tedersoo *et al*., 2018).

The three major groups of fungi are:

- Multicellular filamentous moulds.
- Macroscopic filamentous fungi that form large fruiting bodies. Sometimes the group is referred to as 'mushrooms', but the mushroom is just the part of the fungus we see above ground which is also known as the fruiting body.
- Single celled microscopic yeasts

Moulds are made up of very fine threads (hyphae). Hyphae grow at the tip and divide repeatedly along their length creating long and branching chains. The hyphae keep growing and intertwining until they form a network of threads called a mycelium. Digestive enzymes are secreted from the hyphal tip. These enzymes break down the organic matter found in the soil into smaller molecules which are used by the fungus as food. Some of the hyphal branches grow into the air and spores form on these aerial branches. Spores are specialised structures with a

protective coat that shields them from harsh environmental conditions such as drying out and high temperatures. They are so small that between 500 – 1000 could fit on a pin head (Chandler, 2010; Willey *et al*., 2020).

Macroscopic filamentous fungi also grow by producing a mycelium below ground. They differ from moulds because they produce visible fruiting bodies (commonly known as mushrooms or toadstools) that hold the spores. The fruiting body is made up of tightly packed hyphae which divide to produce the different parts of the fungal structure, for example the cap and the stem. Gills underneath the cap are covered with spores and a 10 cm diameter cap can produce up to 100 million spores per hour (Casadevall and Heitman, 2012; Willey *et al*., 2020).

2.2 Yeasts

Yeasts are eukaryotic, single-celled microorganisms classified as members of the fungus kingdom. The first yeast originated hundreds of millions of years ago, and at least 1,500 species are currently recognized. They are estimated to constitute 1% of all described fungal species. Yeasts are unicellular organisms that evolved from multicellular ancestors, with some species having the ability to develop multicellular characteristics by forming strings of connected budding cells known as pseudohyphae or false hyphae. Yeast sizes vary greatly, depending on species and environment, typically measuring 3–4 μm in diameter, although some yeast can grow to 40 μm in size. Most yeasts reproduce asexually by mitosis, and many do so by the asymmetric division process known as budding. With their single-celled growth habit, yeasts can be contrasted with molds, which grow hyphae. Fungal species that can take both forms (depending on temperature or other conditions) are called dimorphic fungi (Hoffman *et al*., 2015).

2.2.1 The Yeast – *Candida*

The name *Candida* was proposed by Berkhout. It is from the Latin word *toga candida*, referring to the white toga (robe) worn by candidates for the Senate of the ancient Roman republic. The specific epithet *albicans* also comes from Latin, *albicare* meaning "to whiten". These names refer to the generally white appearance of *Candida* species when cultured (Ainsworth, 1976; Obladen, 2012).

2.2.2 Taxonomic classification

Kingdom: Fungi

Phylum: Ascomycota

Subphylum: Ascomycotina

Class: Ascomycetes

Order: Saccharomycetales

Family: Saccharomycetaceae

Genus: *Candida*

2.2.3 Description and natural habitats

Candida is a genus of yeasts and is the most common cause of fungal infections worldwide. Many species are harmless commensals or endosymbionts of hosts including humans; however, when mucosal barriers are disrupted or the immune system is compromised they can invade and cause disease, known as an opportunistic infection. *Candida* is located on most of mucosal surfaces and mainly the gastrointestinal tract, along with the skin (Manolakaki *et al*., 2010).

Many species are found in gut flora, including *C. albicans* in mammalian hosts, whereas others live as endosymbionts in insect hosts. Systemic infections of the bloodstream and major

organs (candidemia or invasive candidiasis), particularly in patients with an impaired immune system (immunocompromised), affect over 90,000 people a year in the US. *Candida* is a yeast and the most common cause of opportunistic mycoses worldwide. It is also a frequent colonizer of human skin and mucous membranes. *Candida* is a member of normal flora of skin, mouth, vagina, and gut. As well as being a pathogen and a colonizer, it is found in the environment, particularly on leaves, flowers, water, and soil. While most of the *Candida* spp. are mitosporic, some have known teleomorphic state and produce sexual spores (Spanakis *et al*., 2010; Menden *et al*., 2019).

2.2.4 Species

The genus *Candida* includes around 154 species. Among these, six are most frequently isolated in human infections. While *Candida albicans* is the most abundant and significant species, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, and *Candida lusitaniae* are also isolated as causative agents of *Candida* infections. Importantly, there has been a recent increase in infections due to non-albicans *Candida* spp., such as *Candida glabrata* and *Candida krusei*. Patients receiving fluconazole prophylaxis are particularly at risk of developing infections due to fluconazole-resistant *Candida krusei* and *Candida glabrata* strains. Nevertheless, the diversity of *Candida* spp. that is encountered in infections is expanding and the emergence of other species that were rarely in play in the past is now likely. *Candida antarctica* and *Candida rugosa* are a source of industrially important lipases, while *Candida krusei* is prominently used to ferment cacao during chocolate production. *Candida rugosa* is also used as an enzyme supplement to support fat digestion with its broad specificity for lipid hydrolysis (Menden *et al*., 2019).

Among *Candida* species, *C. albicans*, which is a normal constituent of the human flora, a commensal of the skin and the gastrointestinal and genitourinary tracts, is responsible for the majority of *Candida* bloodstream infections (candidemia). Yet, there is an increasing incidence of infections caused by *C. glabrata* and *C. rugosa*, which could be because they are frequently less susceptible to the currently used azole-group of antifungal. Other medically important species include *C. parapsilosis*, *C. tropicalis*, *C. dubliniensis* and the more recent upcoming pathogen *C. auris* (Gow and Hanson, 2017; Ghazi *et al*., 2019)

Candida species, such as *C. oleophila* have been used as biological control agents in fruit. Other species of *Candida* include: *C. albicans*, *C. ascalaphidarum*, *C. amphixiae*, *C. antarctica*, *C. argentea*, *C. atlantica*, *C. atmosphaerica*, *C. auris*, *C. blankii*, *C. blattae*, *C. bracarensis*, *C. bromeliacearum*, *C. carpophila*, *C. carvajalis*, *C. cerambycidarum*, *C. chauliodes*, *C. corydali*, *C. dosseyi*, *C. dubliniensis*, *C. ergatensis*, *C. fructus*, *C. glabrata*, *C. fermentati*, *C. guilliermondii*, *C. haemulonii*, *C. humilis*, *C. insectamens*, *C. insectorum*, *C. intermedia*, *C. jeffresii*, *C. kefyr*, *C. keroseneae*, *C. krusei*, *C. lusitaniae*, *C. lyxosophila*, *C. maltosa*, *C. marina*, *C. membranifaciens*, *C. mogii*, *C. oleophila*, *C. oregonensis*, *C. parapsilosis*, *C. quercitrusa*, *C. rhizophoriensis*, *C. rugosa*, *C. sake*, *C. sharkiensis*, *C. shehatea*, *C. temnochilae*, *C. tenuis*, *C. theae*, *C. tolerans*, *C. tropicalis*, *C. tsuchiyae*, *C. sinolaborantium*, *C. sojae*, *C. subhashii*, *C. viswanathii, C. utilis*, *C. ubatubensis* and *C. zemplinina* (Khunnamwong *et al*., 2015).

2.2.5 Macroscopic and microscopic features

The colonies of *Candida* spp. are cream colored to yellowish, grow rapidly and mature in 3 days. The texture of the colony may be pasty, smooth, glistening or dry, wrinkled and dull, depending on the species. The colonies emit a yeasty odor on agar plates at room temperature.

The microscopic features of *Candida* spp. also show species-related variations. All species produce blastoconidia singly or in small clusters. Blastoconidia may be round or elongate. Most species produce pseudohyphae which may be long, branched or curved. True hyphae and chlamydospores are produced by strains of some *Candida* spp. Although they are the members of the same genus, the various species do have some degree of unique behavior with respect to their colony texture, microscopic morphology on cornmeal tween 80 agar at 25°C (Dalmau method) and fermentation or assimilation profiles in biochemical tests (Elfeky *et al*., 2016; Spivak and Hanson, 2017).

2.3 Candidiasis

Candidiasis is a fungal infection caused by a yeast (a type of fungus) called *Candida*. Some species of *Candida* can cause infection in people; the most common is *Candida albicans*. *Candida* normally lives on the skin and inside the body, in places such as the mouth, throat, gut, and vagina, without causing any problems. *Candida* can cause infections if it grows out of control or if it enters deep into the body (for example, the bloodstream or internal organs like the kidney, heart, or brain). Some types of *Candida* are resistant to the antifungals used to treat them. Candidiasis that develops in the mouth or throat is called thrush or oropharyngeal candidiasis. Candidiasis in the vagina is commonly referred to as a yeast infection. Invasive candidiasis occurs when *Candida* species enter the bloodstream or affect internal organs like the kidney, heart, or brain (Coronado-Castellote and Jimenez-Soriano, 2013; Gow and Hanson, 2017).

2.4 Classification of candidiasis

2.4.1 Thrush or oropharyngeal candidiasis

This is candidiasis of the mouth and throat. Candidiasis in the esophagus (the tube that connects the throat to the stomach) is called esophageal candidiasis or *Candida* esophagitis.

Esophageal candidiasis is one of the most common infections in people living with HIV/AIDS. *Candida albicans* is the most commonly implicated organism in this condition. *C. albicans* is carried in the mouths of about 50% of the world's population as a normal component of the oral microbiota. This candidal carriage state is not considered a disease, but when *Candida* species become pathogenic and invade host tissues, oral candidiasis can occur. This change usually constitutes an opportunistic infection by normally harmless micro-organisms because of local (i.e., mucosal) or systemic factors altering host immunity (Kerawala and Newlands, 2010; Buchacz *et al*., 2016).

Aetiological agents of oral candidiasis

The causative organism is usually *Candida albicans*, or less commonly other Candida species such as (in decreasing order of frequency) *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, or other species (*Candida stellatoidea*, *Candida pseudotropicalis*, *Candida famata*, *Candida rugosa*, *Candida geotrichium*, *Candida dubliniensis*, and *Candida guilliermondii*). *C. albicans* accounts for about 50% of oral candidiasis cases, and together *C. albicans*, *C. tropicalis* and *C. glabrata* account for over 80% of cases. Candidiasis caused by non-*C. albicans*. *Candida* (NCAC) species is associated more with immunodeficiency. For example, in HIV/AIDS, *C. dubliniensis* and *C. geotrichium* can become pathogenic (Anil and Shubhangi, 2010; Williams and Lewis, 2011).

Symptoms of candidiasis in the esophagus usually include pain when swallowing and difficulty swallowing. Candidiasis in the mouth, throat, or esophagus is uncommon in healthy adults. People who are at higher risk for getting candidiasis in the mouth and throat include babies, especially those younger than 1 month of age, and people with at least one of these factors: wear dentures, diabetics, cancer patients, HIV/AIDS patients, take antibiotics or

corticosteroids, including inhaled corticosteroids for conditions like asthma, take medications that cause dry mouth or have medical conditions that cause dry mouth and smokers. Most people who get candidiasis in the esophagus have weakened immune systems, meaning that their bodies don't fight infections well. This includes people living with HIV/AIDS and people who have blood cancers such as leukemia and lymphoma. People who get candidiasis in the esophagus often also have candidiasis in the mouth and throat (Lalla *et al*., 2010; Pappas *et al*., 2016).

Treatment

Candidiasis in the mouth, throat, or esophagus is usually treated with antifungal medicine.The treatment for mild to moderate infections in the mouth or throat is usually an antifungal medicine applied to the inside of the mouth for 7 to 14 days. These medications include clotrimazole, miconazole, or nystatin. For severe infections, the most common treatment is fluconazole (an antifungal medication) taken by mouth or through a vein. If patient does not get better after taking fluconazole, healthcare providers may prescribe a different antifungal. The treatment for candidiasis in the esophagus is usually fluconazole. Other types of prescription antifungal medicines can also be used for people who can't take fluconazole or who don't get better after taking fluconazole (Pappas *et al*., 2016).

2.4.2 Vaginal candidiasis

Vaginal yeast infection, also known as candidal vulvovaginitis and vaginal thrush, is excessive growth of yeast in the vagina that results in irritation. The most common symptom is vaginal itching, which may be severe. Other symptoms include burning with urination, a thick, white vaginal discharge that typically does not smell bad, pain during sex, and redness around the vagina. Symptoms often worsen just before a woman's period (Ilkit and Guzel, 2011).

A vaginal yeast infection is a fungal infection that causes irritation, discharge and intense itchiness of the vagina and the vulva — the tissues at the vaginal opening. Also called vaginal candidiasis, vaginal yeast infection affects up to 3 out of 4 women at some point in their lifetimes. Many women experience at least two episodes. A vaginal yeast infection isn't considered a sexually transmitted infection. But, there's an increased risk of vaginal yeast infection at the time of first regular sexual activity. There's also some evidence that infections may be linked to mouth to genital contact (oral-genital sex) (Mendling and Brasch, 2012).

Causes

The fungus candida albicans is responsible for most vaginal yeast infections. Your vagina naturally contains a balanced mix of yeast, including candida, and bacteria. Certain bacteria (lactobacillus) act to prevent an overgrowth of yeast. But that balance can be disrupted. An overgrowth of candida or penetration of the fungus into deeper vaginal cell layers causes the signs and symptoms of a yeast infection. Overgrowth of yeast can result from: antibiotic use, pregnancy, uncontrolled diabetes, impaired immune system and oral contraceptive or hormone therapy. *Candida albicans* is the most common type of fungus to cause yeast infections. Yeast infections caused by other types of candida fungus can be more difficult to treat, and generally need more-aggressive therapies (Watson *et al*., 2012).

Diagnosis

Vulvovaginal candidosis is the presence of *Candida* in addition to vaginal inflammation. The presence of yeast is typically diagnosed in one of three ways: vaginal wet mount microscopy, microbial culture, and antigen tests. The results may be described as being either uncomplicated or complicated (llkit and Guzel, 2011).

Treatment

Treatment for yeast infections depends on the severity and frequency of your infections. For mild to moderate symptoms and infrequent episodes, your doctor might recommend:

- **Short-course vaginal therapy.** Taking an antifungal medication for three to seven days will usually clear a yeast infection. Antifungal medications — which are available as creams, ointments, tablets and suppositories — include miconazole (Monistat 3) and terconazole. Some of these medications are available over-the-counter and others by prescription only.
- **Single-dose oral medication.** Your doctor might prescribe a one-time, single oral dose of fluconazole (Diflucan). Oral medication isn't recommended if you're pregnant. To manage more-severe symptoms, you might take two single doses three days apart.
- **Long-course vaginal therapy.** Your doctor might prescribe an antifungal medication taken daily for up to two weeks, followed by once a week for six months.
- **Multidose oral medication.** Your doctor might prescribe two or three doses of an antifungal medication to be taken by mouth instead of vaginal therapy. However, this therapy isn't recommended for pregnant women.
- **Azole resistant therapy.** Your doctor might recommend boric acid, a capsule inserted into your vagina. This medication may be fatal if taken orally and is used only to treat *Candida* fungus that is resistant to the usual antifungal agents (*Sobel et al*., 2004; Pappas *et al.,* 2015).

2.4.3 Candidal balanitis

Balanitis is inflammation of the glans penis. When the foreskin is also affected, it is termed balanoposthitis. Signs and symptoms include: first signs – small red erosions on the

glans, redness of the foreskin, redness of the penis, rashes on the head of the penis, foul smelling discharge, painful foreskin and penis (Edwards *et al*., 2014).

Cause

Inflammation has many possible causes, including irritation by environmental substances, physical trauma, and infection such as bacterial, viral, or fungal. Some of these infections are sexually transmitted diseases. It is less common among people who are circumcised as in many cases the foreskin contributes to the disease. Both not enough cleaning and too much cleaning can cause problems. Diabetes can make balanitis more likely, especially if the blood sugar is poorly controlled. It is important to exclude other causes of similar symptoms such as penile cancer (Edwards *et al*., 2014).

Diagnosis

Diagnosis may include careful identification of the cause with the aid of a good patient history, swabs and cultures, and pathological examination of a biopsy (Pappas *et al*., 2015).

Treatment

Initial treatment in adults often involves simply pulling back the foreskin and cleaning the penis. Clotrimazole or miconazole are very helpful (Edwards *et al*., 2014).

Epidemiology

Balanitis is a common condition affecting 11% of adult men seen in urology clinics and 3% of children in the United States. Globally, balanitis may occur in up to 3% of uncircumcised males (Vaughan-Higgins *et al*., 2011; Edwards *et al*., 2014).

2.4.4 Candidal intertrigo

Candidal intertrigo refers to superficial skin-fold infection caused by the yeast, *Candida*. Candidal intertrigo is triggered by a combination of the following factors:

- The hot and damp environment of skin folds, which is conducive to the growth of *Candida* species, particularly *Candida albicans*
- Increased skin friction.
- Immunocompromise (Gray, 2010).

Clinical features of candidal intertrigo

Candidal intertrigo classically presents as erythematous and macerated plaques with peripheral scaling. There are often associated superficial satellite papules or pustules.

Affected areas may include:

- Skin folds below the breasts or under the abdomen
- Armpits and groin
- Web spaces between the fingers or toes (erosio interdigitalis blastomycetica) (Gray, 2010).

Diagnosis

Diagnosis of candidal intertrigo requires recognition of consistent clinical features. In cases of uncertainty, confirmation can be sought by way of fungal microscopy and culture of skin swabs and scrapings. Skin biopsy is usually not necessary.

Treatment

- Predisposing factors should be addressed primarily, such as weight loss, blood glucose control and avoidance of tight clothing.
- Patients should be advised to maintain cool and moisture-free skin. This may be aided by regular use of a drying agent such as talcum powder, especially if infection is recurrent.

- Topical antifungal agents such as clotrimazole cream are recommended as first-line pharmacological treatments.
- Severe, generalised and/or refractory cases may require oral antifungal treatments such as fluconazole or itraconazole (Karla *et al*., 2014; Metin *et al*., 2015).

2.4.5 Diaper Candidiasis

A diaper rash is a skin problem that develops in the area under an infant's diaper.

Causes

Diaper rashes are common in babies between 4 to 15 months old. They may be noticed more when babies begin to eat solid foods. Diaper rashes caused by infection with a yeast (fungus) called candida are very common in children. *Candida* grows best in warm, moist places, such as under a diaper. *Candida* diaper rash is more likely to occur in babies who:

- Are not kept clean and dry
- Are taking antibiotics or whose mothers are taking antibiotics while breastfeeding
- Have more frequent stools

Other causes of diaper rash include:

- Acids in the stool (seen more often when the child has diarrhea)
- Ammonia (a chemical produced when bacteria break down urine)
- Diapers that are too tight or rub the skin
- Reactions to soaps and other products used to clean cloth diapers (Bender and Chiu, 2020).

Symptoms

You may notice the following in your child's diaper area:

- Bright red rash that gets bigger
- Very red and scaly areas on the scrotum and penis in boys
- Red or scaly areas on the labia and vagina in girls
- Pimples, blisters, ulcers, large bumps, or sores filled with pus
- Smaller red patches (called satellite lesions) that grow and blend in with the other patches

Older infants may scratch when the diaper is removed.

Diaper rashes usually do not spread beyond the edge of the diaper (Gehris, 2018).

Diagnosis

The health care provider can often diagnose a yeast diaper rash by looking at your baby's skin. A KOH test can confirm if it is candida (Gehris, 2018).

Treatment of diaper rash

The best treatment for a diaper rash is to keep the skin clean and dry. This also helps prevent new diaper rashes. Lay your baby on a towel without a diaper whenever possible. The more time the baby can be kept out of a diaper, the better. Certain skin creams and ointments will clear up infections caused by yeast. Nystatin, miconazole, clotrimazole, and ketoconazole are commonly used medicines for yeast diaper rashes. For severe rashes, a steroid ointment, such as 1% hydrocortisone, may be applied. You can buy these without a prescription. But first ask your provider if these medicines will help (Gehris, 2018; Bender and Chiu, 2020).

2.4.6 Invasive candidiasis

What is invasive candidiasis?

Invasive candidiasis is an infection caused by a yeast (a type of fungus) called *Candida*. Unlike *Candida* infections in the mouth and throat (also called “thrush”) or vaginal “yeast infections,” which are localized to one part of the body, invasive candidiasis is a serious

infection that can affect the blood, heart, brain, eyes, bones, or other parts of the body (Magill *et al.*, 2018).

Candida normally lives inside the body (in places such as the mouth, throat, gut, and vagina) and on the skin without causing any problems. However, in certain patients who are at risk, *Candida* can enter the bloodstream or internal organs and cause an infection. A *Candida* bloodstream infection, also called candidemia, is the most common form of invasive candidiasis. In the United States, candidemia is one of the most common causes of bloodstream infections in hospitalized patients, and it often results in long hospital stays and death. It is also responsible for high medical costs (Kullberg and Arendrup, 2015). Antifungal medication can treat invasive candidiasis. Certain patients such as those with cancer or bone marrow or organ transplants might receive antifungal medication to prevent invasive candidiasis (Pappas *et al*., 2016).

Signs and symptoms

Signs and symptoms of invasive candidiasis are often non-specific and include fever and chills that do not respond to antibacterial treatment. Candidemia is the most common form of invasive candidiasis; other forms include endocarditis, peritonitis, meningitis, osteomyelitis, arthritis, and endophthalmitis. Invasive candidiasis is associated with an in-hospital all-cause mortality of approximately 30%. *Candida auris* has emerged globally since 2009, including in the United States from mid-2015, and is very concerning because it is highly drug-resistant, causes invasive infections associated with high mortality, and spreads easily between patients in healthcare settings (Pappas, 2016).

Etiologic agents

Candida albicans, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* are most common. Species distribution varies by patient population and geographic region. Most

infections arise from the endogenous flora of patients with risk factors following disruption of skin and mucosal barriers. Less commonly, *Candida* can be transmitted via healthcare workers' hands or contaminated medical devices. Another species called *C. auris* is emerging as a cause of invasive candidiasis around the world and in certain areas of the United States – read more about this concerning and often drug-resistant species (Kullberg and Arendrup, 2015).

Diagnosis

Invasive candidiasis is primarily diagnosed with blood culture. Newer culture independent diagnostic methods are promising but are not yet widely used. The Beta-D-glucan assay is approved as an adjunctive diagnostic tool but is not a very specific test for *Candida*. Determining the species of *Candida* causing the infection is important to guide appropriate antifungal treatment (Pappas *et al*., 2016).

Treatment of invasive candidiasis

The specific type and dose of antifungal medication used to treat invasive candidiasis usually depends on the patient's age, immune status, and location and severity of the infection. For most adults, the initial recommended antifungal treatment is an echinocandin (caspofungin, micafungin, or anidulafungin) given through the vein (intravenous or IV). Fluconazole, amphotericin B, and other antifungal medications may also be appropriate in certain situations. For candidemia, treatment should continue for 2 weeks after signs and symptoms have resolved and *Candida* yeasts are no longer in the bloodstream. Other forms of invasive candidiasis, such as infections in the bones, joints, heart, or central nervous system, usually need to be treated for a longer period of time (Kullberg and Arendrup, 2015).

Risk factors

Common risk factors for invasive candidiasis include:

- Critical illness with a prolonged intensive care unit stay
- Presence of central venous catheters
- Use of broad-spectrum antibiotics or total parenteral nutrition
- Having hematologic or solid organ malignancy, stem cell transplantation, neutropenia, or recent abdominal surgery (especially in the presence of an anastomotic leak)
- Being a pre-term infant with a very low birth weight
- Having renal failure or hemodialysis and injection drug use (Pappas *et al*., 2016).

Prevention

In healthcare settings, these measures are important to prevent invasive candidiasis:

- Adhering to hand hygiene recommendations
- Following recommendations for placement and maintenance of central venous catheters
- Practicing antibiotic stewardship (Kullberg and Arendrup, 2015).

2.4.7 Antibiotics or Iatrogenic Candidiasis

Antibiotics are used to kill off harmful bacteria in the body. But they can also destroy beneficial bacteria in the process, which may lead to a yeast infection. Vaginal yeast infections are fungal infections of the vagina. They happen when a type of fungus called *Candida*, which occurs naturally in the vagina, begins to grow out of control. Yeast infections can cause intense itching and irritation of the vagina and vulva — the outer part of the female genital area. Vaginas maintain their own balanced mix of yeast and bacteria. A type of bacteria called *Lactobacillus*

keeps the vagina slightly acidic, which is not welcoming to yeast. This slightly acidic environment keeps the yeast growing in the vagina under control (Sardi *et al.,* 2013).

2.5 Pathogenesis of candidiasis

An infection caused by *Candida* is termed candidiasis or candidosis. According to Sardi and his colleagues, the main transmission mechanism is through endogenous candidaemia, in which *Candida* species that constitute the microbiota of various anatomical sites under conditions of host weakness behave as opportunistic pathogens. Another mechanism for transmission is exogenous, and this occurs mainly through the hands of health professionals who care for patients. Also indicated in the spread of infection are health-care materials, such as contaminated catheters and intravenous solutions (Ingham *et al*., 2012; Sardi *et al.,* 2013).

However, *Candida albicans* is usually transmitted from mother to infant through childbirth, and remains as part of a normal human's microflora. The overgrowth of *C. albicans* leads to symptoms of disease, and it occurs when there are imbalances – for example, changes in the normal acidity of the vagina. *C. albicans* infections in rare cases are spread through sexual intercourse. The typical reservoir for *C. albicans* is in the normal human microflora, and is not found in animal vectors. People-to-people acquired infections mostly happen in hospital settings where immunocompromised patients acquire the yeast from healthcare workers (Cooper, 2011; Millsop & Fazel, 2016).

Candida species can infect different anatomical sites of the human host, even though evidence suggest that immune protection is site-specific for each species. The urinary tract is the anatomical site most conducive to the development of infections in hospitalized patients, although this remains a problem of questionable significance. For its pathogenicity, ovoid-

shaped budding yeast and parallel-walled true hyphae forms are the most important (Sardi *et al.,* 2013; Refai *et al.,* 2015).

Candida yeast cells adhere to host cell surfaces by the expression of adhesins (Hameed *et al.,* 2018). Upon contact to host cells the yeast-to-hypha transition and directed growth via thigmotropism is triggered (Mayer *et al.,* 2014). Addidtionally, expression of invasins mediates uptake of the yeast cell by the host cell through induced endocytosis. Adhesion, physical forces, and secretion of fungal hydrolases have been proposed to facilitate the second mechanism of invasion, i.e., fungal-driven active penetration into host cells by breaking down barriers (Mayer *et al.,* 2013). The attachment of yeast cells to abiotic (e.g., catheters) or biotic (host cells) surfaces can give rise to the formation of biofilms with yeast cells in the lower part and hyphal cells in the upper part of the biofilm (Xie *et al.,* 2012).

The human host uses a variety of protective mechanisms to prevent invasion by pathogenic *Candida spp*. These mechanisms consist of several barriers, which need to be overcome by the intruding pathogen. Epithelial cells, which in most cases are the first host cells to come into direct contact with the pathogen, play an important role as the first line of defense, by functioning as a passive physical barrier and restraining *Candida* from invasion of the underlying tissue (Yan *et al*., 2013). According to Yan and his colleagues, epithelial cell integrity ensured strong interepithelial cell connections (tight junctions), seals the gap between the cell surface and the mucosal lamina propria, thereby preventing interepithelial invasion of *C. albicans* (Yan *et al*., 2013).

In addition, several epithelial cell types, such as intestinal or vaginal epithelial cells, secrete mucins to form a mucus layer, which has protective properties in that it impedes direct contact of *C. albicans* with the epithelial cell surface (Hickey *et al*., 2011; Yan *et al.,* 2013;

Cassone, 2015). Inside the oral cavity, the flow of saliva also plays an important role in controlling an exceeding colonization, as the constant flushing and swallowing prevents adhesion to mucosal and dental surfaces (Conti and Gaffen, 2010; Bokor-Bratic *et al*., 2013). Furthermore, the presence antimicrobial agents, such lysozyme, lactoferrin, histatins, cathelicidins (including LL-37), calprotectins, and defensins in the saliva, serves as a chemical barrier that prevents Candida from attaching to the oral epithelium (Höfs *et al*., 2016). Gastrointestinal epithelial cells, too, can secrete antimicrobial agents such as lysozyme and ß-defensins, as well as various digestive enzymes.

During host interactions, *C. albicans* will have to cope with the entire immune response both innate and adaptive defenses of the host when the first line of defense has been breached during invasion into deeper tissues. Invasion of the human host is based on elaborated pathogenicity mechanisms, which are discussed in detail below. The interaction between *C. albicans* and bacterial members of the human microbiota is crucial during the normal commensal lifestyle of the fungus and plays a key role in keeping the fungus in check (Höfs *et al*., 2016). Also, Höfs *et al*. (2016) reported that *C. albicans* has been shown to be more virulent in the presence of specific bacterial pathogens.

2.6 General virulence factors of *Candida spp*

Virulence is a polygenic trait in *C. albicans* involving biochemical, physiological, genetic and morphogenetic characteristics (Dantas *et al*., 2016). *C. albicans* has a specialized set of proteins (adhesins, Invasins, and heat shock protiens) which mediate adherence and invasion of *C. albicans* cells to other microorganisms, to abiotic surfaces and to host cells (Naglik *et al.,* 2013). In addition, *C. albicans* forms highly structured biofilms composed of multiple cell types (round budding yeast-form cells, oval pseudohyphal cells, and elongated hyphal cells) encased in

an extracellular matrix, that has been strongly associated to cell adherence and resistance (Gulati & Nobile, 2016).

Adhesins are special sets of glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins that allow it to adhere to the surfaces of microorganisms. The best studied group of adhesins is the Als (agglutinin-like sequence) family (Mayer *et al*., 2013). The eight members that are encoded by the ALS gene family (ALS1-7 and ALS9) are cell surface proteins and are glycosylphosphatidylinositol (GPI)- linked to the ß-1,6-glucans of the fungal cell wall (Höfs *et al.,* 2016). ALS1-4 encode adhesins specific for germ tubes and hyphae, while Als5-7 and Als9 can be found on the surface of yeast cells (Modrzewska and Kurnatowski, 2015). For adhesion, the Als3 gene appears to the most important as it is up regulated during an infection of oral and vaginal epithelial cells. They are mostly required for epithelial binding. *C. albicans* displays adhesins to its cell wall which allow cells to attach to blood vessel walls and organs in systemic infections (Thompson *et al.,* 2011). Also, it helps with biofilm formation by helping with adhesion to each other (Murciano *et al.,* 2012).

Upon adhesion, Als3 proteins can function as invasins that help with the invasion of *C. albicans* into host epithelial and endothelial cells. Another important invasin gene is Ssa1, which normally codes for heat-shock proteins (Murciano *et al*., 2012). Basically, these specialized proteins such as E-cadherin and N-cadherin on the pathogen's surface mediate binding to host ligands (Yang *et al.,* 2014). Another hypha-associated GPI-linked protein, Hyphal Wall Protein 1 (Hwp1), also plays a significant role in mediating adhesion to host cells (Mayer *et al.,* 2013). The N-terminal region of Hwp1 serves as a substrate for epithelial cell-associated transglutaminases, resulting in covalent attachment of Candida to host epithelial cells via cross-linking of glutamine residues of the N-terminal region of Hwp1 to asyet unidentified host proteins (Höfs *et al.,* 2016).

After the adherence of yeast cells to the surface, there is development of hyphae cells in the upper part of the biofilm. Eventually, this leads to the dispersion of yeast cells, mature biofilms and a more resistant yeast cell altogether contributing to the pathogen's virulence. In the process of biofilm formation, Bcr1, Tec1 and Efg1 function as important transcriptional factors. Studies have shown that biofilms protect *C. albicans* colonization from neutrophil attack and deter the formation of reactive oxygen species (Xie *et al*., 2012). Morphology-independent proteins can also contribute to adhesion. These include GPI-linked proteins (Eap1, Iff4 and Ecm33), non-covalent wall-associated proteins (Mp65, a putative β-glucanase, and Phr1, a β-1,3 glucanosyl transferase), cell-sur- face associated proteases (Sap9 and Sap10) and the integrin-like surface protein (Dantas *et al.,* 2016).

In healthy individuals *C. albicans* is predominantly found as part of the gastrointestinal microbiome. Although the concentration of nutrients in this environment can be naturally high, growth of the fungus is believed to be controlled through competition with other members of the intestinal microbial flora. During disseminated candidiasis in susceptible individuals (like diabetic patients and immune compromised individuals), *C. albicans* gains access to the bloodstream (blood) which is relatively rich in glucose (6-8 mM), the preferred nutrient source of most fungi. Another method of invasion is the active penetration of *C. albicans* into host cells by an unknown mechanism involving hyphae (Hameed *et al.,* 2018).

Candida albicans can form biofilms on living and non-living surfaces, such as mucosal membranes and catheters, respectively (Srinivasan *et al.,* 2014). Phenotypic plasticity (switching) has been proposed to influence antigenicity and biofilm formation of *C. albican*. In addition to these virulence factors, several fitness traits influence fungal pathogenicity. They include a robust stress response mediated by heat shock proteins (Hsps); auto-induction of

hyphal formation through uptake of amino acids, excretion of ammonia (NH3) and concomitant extracellular alkalinization; metabolic flexibility and uptake of different compounds as carbon (C) and nitrogen (N) sources; and uptake of essential trace metals, e.g., iron (Fe), zinc (Zn), copper (Cu) and manganese (Mn) (Dantas *et al*., 2016).

2.7 Epidemiology of *Candida* infections

Fungal infections are currently among the most difficult diseases to manage in humans. Invasive and life-threatening fungal infections are an important cause of morbidity and mortality in patients who are immunocompromised, hospitalized with severe underlying diseases (e.g., acute myelogenous leukemia), require complex surgical procedures (e.g., for trauma), and need support in intensive care units (Slavin *et al.,* 2015). Overall, more than 300 million people are believed to suffer from a serious fungal infection, resulting in approximately 1.4 million deaths annually with most infections being caused by *Candida* and *Aspergillus* species (Brown *et al.,* 2012). Although superficial mycosis are the most common among fungal infections, affecting nearly 25% of the human population worldwide (Souza & Amaral, 2017), invasive fungal infections are of greater concern, since they are life-threatening, difficult to diagnose and account with a limited number of therapeutic options (Brown *et al.,* 2012).

The incidence of superficial and cutaneous fungal infections of the hair, skin, and nails, such as dermatophytosis, vaginal candidiasis, allergy, and mycotoxicosis, considerably exceeds that of invasive fungal infections IFIs (Seyedmousav *et al.,* 2017). However, the contribution of these diseases to morbidity worldwide is unrecognized (Calderone *et al.,* 2017). *Candida, Cryptococcus,* and *Pneumocystis* are responsible for more than 90% of all reported fungal-related deaths, although accurate incidence data is not officially available and may be underestimated (Brown *et al*., 2012). Particularly, *Candida albicans* is a major pathogen and is

responsible for 50–70% of cases of total fungal infections worldwide (Kullberg and Arendrup, 2015). Among the fungal infections, invasive candidiasis is one of the most common nosocomial fungal diseases with an estimate of 250 000 cases and more than 50 000 deaths worldwide per year (Ngo *et al.,* 2016).

It has been reported that vulvovaginal candidiasis (VVC) an infection caused by *Candida* species affects millions of women every year (Gonçalves *et al.*, 2016). *Candida* infections are mainly superficial, but in severely immunocompromised patients, serious systemic infections can occur (Kullberg and Arendrup, 2015). Most, if not all women carry *Candida* in vagina at some point of their lives, yet without symptoms of infection. *Candida* organisms gain access to the lower genital tract mainly from the adjacent perianal area (Gonçalves *et al.*, 2016). Many women develop VVC without any known predisposing factor, and therefore some groups of researchers have turned to genetic studies to reveal a possible association of VVC with genetic predispositions (Cassone, 2015). American studies have found that African-American women have increased prevalence of VVC and are more likely to be vaginal colonized by *Candida* compared to Hispanic and white women (Gonçalves *et al*., 2016). Gonçalves and his colleagues reported that this is because of decreased vaginal H_2O_2-producing Lactobacillus population, which is a protective barrier against *Candida* microorganisms among black women (Gonçalves *et al*., 2016).

A genetic-related predisposition has been proposed as a risk factor for VVC in black women. Furthermore, behavioral factors such as use of spermicides and/or condom (Amouri *et al*., 2011), use of intrauterine device (IUD) (Apalata *et al*., 2014), use of oral contraceptive pills (OCP) (Egbe *et al.,* 2011), Poor personal hygiene and sexual habits (Faraji *et al*., 2012), wearing of poorly ventilated clothing and/or synthetic underwear (Sobel, 2014) have been associated with

increased risk of VVC. *Candida parapsilosis* is well known for its threat to the pediatric population, as it is responsible for 17–50% of all fungemia in infants and neonates. *C. parapsilosis* is also second only to *C. albicans* in incidence as a cause of *Candida* endocarditis with mortality rates between 42 and 65% (Whaley *et al*., 2017). In the Asia- Pacific region, *C. tropicalis* has been reported to constitute 20–45% of *Candida* isolates (Pfaller *et al*., 2010). The high incidence of fungal infections is a problem that can be aggravated mainly by the increase of the elderly population and also by immunocompromised patients (Souza & Amaral, 2017).

2.8 Treatment of candidiasis

Different classes of antifungal drugs are available for the treatment of invasive cutaneous, and mucocutaneous candida infections. Despite an increase in the prevalence of fungal infections particularly in immunocompromised patients, only a few classes of antifungal drugs are available for therapy, and they exhibit limited efficacy in the treatment of life-threatening infections. These drugs include polyenes, azoles, echinocandins, and nucleoside analogs. Antifungal agents were grouped into four classes based on their site of action in pathogenic fungi (Seyedmousavi *et al.,* 2016).

Currently available drugs against invasive and cutaneous *Candida* infections include amphotericin B (AmB) and its derived lipid formulations (LFAmB), azoles (triazoles) including fluconazole (FLU), voriconazole (VOR), posaconazole (POS), and itraconazole, and echinocandins including caspofungin (CFG), micafungin (MIC), and anidulafungin. 5-fluorocytocine (5FC), a fluorinated pyrimidine analog has also antimycotic properties. Most *Candida* spp are susceptible to these agents, except for those with intrinsic or acquired resistance after exposure to other drugs (Flevari *et al.,* 2013).

2.8.1 Polyenes

AmB is a polyene macrolide antifungal regimen with fungicidal action, which was considered in the past to be the “gold standard” for the treatment of invasive fungal infections. Its antifungal activity was discovered in 1953, and it was approved for clinical use in the US in 1957 (Seyedmousav *et al.,* 2017). It has a high affinity for the sterols of fungal and bacterial membranes, forming small transmembrane channels which lead to monovalent ion leakage and cause fungal cell death (Gray *et al.,* 2012).

Derivatives of AmB were developed in order to limit toxicity, especially renal failure, which has been rated between 49%–65% (Flevari *et al.,* 2013). The use of polyenes is limited further as they are poorly absorbed through the gut and, therefore, topical application in the form of lozenges and oral suspensions are the principle means of administration in oral infection. Polyenes are frequently used in the treatment of chronic erythematous candidosis, and oral suspension of amphotericin B may be employed (Hamill *et al.,* 2013).

2.8.2 Nystatin

Nystatin is an amphoteric tetraene originally isolated from *Streptomyces noursei*. It is a polyene antifungal agent, which was first approved by the US Food and Drug Administration (FDA) in 1955 for the treatment of vaginal candidiasis (Seyedmousav *et al.,* 2017). Nystatin is not absorbed by intact mucosal surfaces. Following oral administration, it is passed unchanged in the feces; therefore, it is only active against yeasts present in the gastrointestinal tract (Hay, 2013). Oral or topical nystatin is well tolerated; however, patients with renal insufficiency receiving oral therapy with conventional dosage forms may experience toxicity occasionally. The spectrum of activity of nystatin includes *Candida spp., Cryptococcus neoformans, Trichosporon spp*., and *Rhodotorula spp* (Garcia-Cuesta *et al.*, 2014).

Topical and oral nystatin are recommended for the treatment of superficial Candida spp. infections of the skin, oral cavity, and esophagus including diaper dermatitis, angular cheilitis, and oral or vaginal candidiasis (Hay, 2013; Gonçalves *et al.,* 2016). Oral nystatin has also been used for the prevention of systemic candidiasis in patients who are specifically at risk such as those with hematologic malignancies and those undergoing induction of chemotherapy (Hope *et al.,* 2012).

2.8.3 Azoles

The azoles inhibit the fungal cytochrome P450 enzyme 14α-demethylase and prevent the conversion of lanosterol to ergosterol, which is essential for the fungal cell membrane integrity (Seyedmousav *et al.,* 2017). Subsequent depletion of ergosterol in the fungal cell results in inhibition of fungal growth and impairment of membrane permeability. The two most frequently administered azole antifungals in the treatment of oral candidosis are fluconazole (FLU) and itraconazole and these drugs have the advantage of being readily absorbed through the gut with the result that oral administration is an effective means of delivery (Seyedmousav *et al.,* 2017).

Fluconazole, voriconazole and pozoconazole have demonstrated activity against *Candida* spp. but reduce activity against *C. glabrata* and *C. krusei* (Williams and Lewis, 2011). FLU, with primarily fungistatic effect (800 mg or 12 mg/kg loading dose, followed by 400 mg or 6 mg/kg daily), has comparable efficacy to AmB for the treatment of candidemia, while it is indicated as empirical and curative treatment in non-neutropenic patients (Flevari *et al.,* 2013). Since azoles are fungistatic, complete resolution of the infection will be aided by simultaneously addressing predisposing host factors (Nimmi *et al*., 2010; Mourad & Perfect, 2018). Furthermore, fluconazole is secreted in high levels in saliva making the agent particularly suitable for treating oral infection (Williams and Lewis, 2011).

2.8.4 Echinocandins

Echinocandins (ECs) are a new class of antifungal agents that target the fungal cell wall by inhibiting 1, 3-β-D-glucan synthetase, leading to osmotic instability and cell death. They are semisynthetic amphiphilic lipopeptides formed during the fermentation of some fungi such as *Zalerion arboricola* or *A. nidulans* var. *echinulatus*. The ECs are considered to be safe drugs, with few reported side effects due to the fact, mammalian cells do not contain this polysaccharide target (1, 3-β- D - glucan). The three members of the group, CFG (loading dose of 70 mg, then 50 mg daily), MIC (100 mg daily), and anidulafungin (loading dose of 200 mg, then 100 mg daily), are all available only for parenteral use (Arendrup *et al.,* 2010; Seyedmousav *et al.,* 2017).

The echinocandins are highly active (fungicidal) against *Candida* spp. including isolates that are resistant to triazoles and *Candida* strains that form biofilms. These agents have modest activity (fungistatic) against *Aspergillus* spp. as well as dimorphic and melanized fungi. The echinocandins that are currently approved for clinical use are not orally bioavailable and, therefore, must be administered by slow intravenous infusion (1–2 h) (Seyedmousavi *et al.,* 2014; Seyedmousav *et al.,* 2017).

Micafungin was among the earliest marketed echinocandin synthesized the chemical modification of a fermentation product of *Coleophoma empetri.* Micafungin is approved for the treatment of esophageal candidiasis and prophylaxis of invasive *Candida* infections in patients undergoing hematopoietic stem cell transplantation. The indication for its use was later expanded to include candidemia, acute disseminated candidiasis, *Candida* abscesses, and peritonitis (de la Torre and Reboli, 2014). Another important issue, unique for the ECs, is the “eagle effect”, a

term used to describe the paradoxical in vitro and in vivo growth of *Candida* and *Aspergillus* isolates when the dose of the drug gets over the MIC level (Flevari *et al.,* 2013).

However, echinocandins are a good therapeutic option for the treatment of invasive candidiasis, despite their cost and the absence of an oral form. They possess fungicidal activity against most species of *Candida*, including those resistant to polyenes and to azoles; lack major adverse effects; and show no interaction with other drugs. Furthermore, echinocandins have been approved for the treatment of several fungal infections, including invasive candidiasis and candidemia, and are also indicated for empirical therapy in select high-risk patients (those with persistent fever and neutropenia) (Forastiero *et al*., 2015).

2.8.5 Nucleoside analogs

Flucytosine (5-fluorocytosine or 5-FC) is the only systemic antifungal agent belonging to the class of nucleoside analogs (Gulati & Nobile, 2016). It was the first agent used for the treatment of invasive mycoses in 1968 (Kneale *et al.,* 2016). Flucytosine is the fluorinated analog of cytosine and was dis- covered in 1957 as an analog of the cytostatic chemotherapeutic agent 5-fluorouracil (5-FU) used for antitumor therapy (Seyedmousav *et al.,* 2017). Flucytocine (or 5-fluorocytosine, 5FC) is an antimetabolite that acts as an antifungal against *Candida* spp*.,* *Cryptococcus* spp., and other fungi. 5FC enters the fungal cell via cytosine permease, and is metabolized to 5-fluorouracil, which is incorporated extremely closely into the fungal RNA, inhibiting both DNA and RNA synthesis (Gulati & Nobile, 2016). Flucytosine can be administered both orally and intravenously, and is well distributed in almost all body fluids including the lacrimal fluid, urine, and cerebrospinal fluid (CSF) (Nett and Andes, 2016).

Flucytosine is active against most clinically important yeast such as those of the *Candida* and *Cryptococcus* genera (Perfect *et al,* 2010) but has limited activity against melanized fungi

and *Aspergillus spp* (Nett and Andes, 2016). In addition, dimorphic fungi are resistant to flucytosine (Nett and Andes, 2016). However, Flucytosine in combination with an azole (fluconazole) may have a role in the treatment of disseminated *Candida* infections that are refractory to first-line antifungal agents (triazoles and lipid formulations of AmB) (Silva *et al.,* 2012). Rapid resistance occurs if flucytosine is used as monotherapy and therefore should never be used alone (Nett and Andes, 2016). Most of the drug is excreted unchanged in the urine, so that dose adjustment is necessary for patients with renal dysfunction. Considering the fact that 5FC is rarely administered as a single agent but in combination with other antifungal drugs (mainly LFAmB) for patients with IC, it is not suggested as a combination in elderly patients due to the accumulative nephrotoxicity risk (Flevari *et al.,* 2013; Kullberg *et al.,* 2015).

2.9 Antifungal drugs toxicity

Despite the availability of several effective agents in the antifungal drug arena, their therapeutic outcome is less than optimal due to limitations related to drug physicochemical properties and toxicity. Polyenes include the drugs, amphotericin B and nystatin and their mode of action is through direct binding to the sterol ergosterol found within fungal cell membranes. Polyene binding to ergosterol induces leakage of cytoplasmic contents leading to fungal cell death. The equivalent mammalian sterol is cholesterol, which has a lower binding affinity for polyenes and this makes host cells less susceptible to their toxic effects. Nevertheless, at higher therapeutic concentrations, polyenes do exhibit a degree of toxicity in humans (Bondaryk *et al*., 2013; Hamill *et al.,* 2013; Nett and Andes, 2016).

The primary drawbacks of AmB deoxycholate use are its dose- limited toxicity and significant side effects in various patients (Minnebruggen *et al*., 2010). The significant toxicities of AmB include infusion-related adverse effects (fever, chills, arrhythmia, hypotension, and

respiratory distress), nephrotoxicity, neurotoxicity, hematological side effects, and allergic reactions (Seyedmousav *et al.,* 2017). Also, administration of AmB-deoxycholate has historically been hampered by toxicity, bioavailability, and solubility issues; and hence the attempts to reformulate this agent (Ostrosky-Zeichner *et al.,* 2010).

2.10 Antifungal resistance

Antifungal resistance represents a major clinical challenge to clinicians responsible for treating invasive fungal infections due to the limited arsenal of systemically available anti-fungal agents. In addition current drugs may be limited by drug–drug interactions and serious adverse effects/toxicities that prevent their prolonged use or dosage escalation (Wiederhold, 2017). The mechanisms of antifungal drug resistance are either primary or secondary and are related to the intrinsic or acquired characteristics of the fungal pathogen that interfere with the antifungal mechanism of the respective drug/drug class or that lower target drug levels (Cowen *et al.,* 2014) Furthermore, resistance can occur when environmental factors lead to colonization or replacement of a susceptible species with a resistant one. Acquired resistance also appears to develop primarily when a large number of microorganisms are exposed to a fungistatic drug for a relatively long period (Pfaller, 2012).

Unfortunately, in recent years *Candida* resistance to azole antifungals has been detected and this can arise through several mechanisms including the over production of the lanosterol demethylase enzyme, an alteration in the demethylase enzyme structure that makes it less susceptible to azole inhibition, the use of multi-drug transporter pumps to remove azoles from the cell, and the incorporation of alternative sterols to ergosterol within the cell membrane (Williams and Lewis, 2011). Several NCAC species are inherently more resistant to azoles than other species. For example, 35% and 75% of *C. glabrata* and *C. krusei* isolates exhibit resistance

to fluconazole and this could be a reason why the prevalence of certain NCAC species in human disease has increased in recent years (Williams and Lewis, 2011).

Development of azole resistance in clinical isolates of *C. glabrata* has been almost exclusively linked to the presence of activating mutations in the zinc cluster transcription factor Pdr1 that lead to differential expression of downstream. Nearly all clinical isolates have been found to have PDR1 mutations, with such mutations found in the inhibitory domain, activating domain, middle homology region, and xenobiotic binding region (Whaley *et al*., 2017). The rapid acquisition of PDR1 mutations could be due to the high incidence of mutations in the mismatch repair gene MSH2, which results in a hypermutable phenotype (Healey *et al.,* 2016).

The worldwide incidence of fluconazole resistance in *C. parapsilosis* disseminated infections ranges between 2 and 5% (Pfaller *et al*., 2015). *C. krusei* exhibits intrinsic resistance to fluconazole, although there is some controversy whether its increased infection rate is related to fluconazole prophylaxis or previous treatment (Gong *et al.*, 2016). Rapid acquisition of resistance has also been observed in the case of a treatment regimen with the use of 5-fluorocytosine, which belongs to pyrimidine analogs (Szweda *et al*., 2015). Successful treatment of candidosis can be hampered where there is an established biofilm. *Candida* biofilms exhibit significantly higher tolerance to both antimicrobial mouthwashes and also traditional antifungal agents (Williams & Lewis, 2011). *Candida* cells within a biofilm are highly resistant to antifungal compounds. The factors responsible for the increased resistance of biofilm cells are manifold, including a reduced growth rate of the cells, upregulation of drug efflux pumps, the presence of persister cells and an impermeable extracellular matrix (Srinivasan *et al.,* 2014; Gulati and Nobile, 2016). Persister cells, which, due to their dormant stage, do not present an active target for antifungals contribute to antifungal resistance (Höfs *et al.,* 2016).

In *Candida albicans*, the ATP- binding cassette (ABC) transporters encoded by the CDR (*Candida* drug resistance) genes and the major facilitator (MF) superfamily encoded by the MDR (multidrug resistance) genes have been shown to contribute to drug resistance (Jeffery-Smith *et al.,* 2018). Expression of both CDR1 and CDR2, as well as expression of MDR1 and another transporter of the MF superfamily, FLU1, was shown to be up- regulated in azole resistant *C. albicans* strains (Gonçalves *et al.,* 2016; Khurana *et al.,* 2019).

Reports of clinical isolates of *Candida spp* with decreased *in vitro* echinocandin susceptibility are still sporadic (1.7 to 2%), including among the vast majority of *C. krusei* isolates (Pfaller *et al.,* 2013). However, individual cases of *C. krusei* resistance to the echinocandins have been reported (Prigitano *et al.,* 2014). Acquired resistance to echinocandin therapy has been associated with amino acid substitutions caused by mutations in specific hot spot regions (HS1, HS2, and the recently described HS3 region) of the conserved FKS1 target gene in different *Candida species*, such as *C. albicans, C. tropicalis, C. krusei, C. glabrata, C. lusitaniae, and C. kefyr* (Johnson *et al.,* 2011; Beyda *et al.,* 2012), as well as in the FKS2 gene of *C. glabrata* (Fekkar *et al.,* 2013; Lewis *et al.,* 2013). These mutations have been related to prior echinocandin exposure and therapeutic failure in candidemic patients (Shields *et al.,* 2012).

2.11 Solutions to antifungal resistance

The increased risk of fungal diseases particularly in patients who are immunocompromised, emerging fungal pathogens, limited repertoire of antifungal drugs, toxicity and the development of resistance to the available antifungal drugs have increased the demand for the development of new and effective antifungal agents. These has resulted to intensified efforts on antifungal drug discovery to develop more clinically effective and safer

agents, especially against antifungal drug-resistant pathogens with low or no toxicity effect (Szweda *et al.*, 2015).

Development of effective and safe therapeutic treatment of fungal infections remains one of the major challenges for modern medicine. Resistance, toxicity and problems with administration are the most important limitations of therapies carried out with conventional antimycotics (de Castro *et al.,* 2013). The results for other active and safe chemotherapeutics which could be used in treatment of fungal infection clearly revealed that some of tested essential oils, e.g. isolated from *C. limon*, *O. basilicums* and especially from the bark of *C. cassia*, as well as propolis and silver nano- particles could be considered as alternative antifungal agents (Szweda *et al.*, 2015). Some of the investigated chemotherapeutics including silver, have shown high antifungal activity with low toxicity as the most important advantage (de Castro *et al.,* 2013).

Szweda and colleagues revealed that essential oils, propolis and silver nanoparticles strongly possess high potential for controlling and prevention candidiasis, including infections caused by the strains resistant to azoles, the group of chemotherapeutics commonly used for treatment and prevention of these infections. However, they suggested that a large variation in the composition (consequently antimicrobial activity) of natural products as well as the differences in the sensitivity of particular species of yeast of the genus *Candida* to silver nanoparticles should be considered for further research. So far, the most promising alternatives for conventional treatment of microbial (including fungal) infections are plant-derived compounds as well as gold and silver nano- particles (Rajeshkumar and Sundararman, 2012; Szweda *et al*., 2015).

2.12 Complimentary and alternative therapies

Some studies suggest that reducing sugar in the diet may help prevent yeast infections. Other foods that some practitioners believe may contribute to candidiasis include high amounts of milk, dairy products, and foods with high concentrations of yeast (cheese, peanuts, alcohol). The “candida diet” allows no alcohol, no simply sugars, no yeast, and very limited amounts of processed foods. Alternative therapies use natural antifungals or probiotics (“friendly” bacteria) as well as immune strengthening therapies to improve the body’s ability to keep *Candida* in check. There is conflicting evidence about whether eating yoghurt with live probiotic culture everyday can help prevent yeast infections, but it certainly does not hurt. Adding more garlic (fungicidal), nuts (essential fatty acids), whole grains (B vitamins), oregano, cinnamon, sage and cloves (antifungal spices) to your diet may help avoid yeast infections (Felix *et al*., 2019).

Probiotics: *Lactobacillus acidophilus* (1-10 billion live organisms per day) or *Bifidobacterium* (10-billion colony-forming units per day) help restore normal balance of bacteria in the bowel and mucous membranes. This helps prevent a build-up of *Candida* (Harvey *et al.,* 2015). Vitamin C (500-1,000mg per day), Vitamin E (200-400IV per day) and selenium (200mcg per day)-help reduce inflammation and keep your immune system strong. Essential fatty acids-help reduce inflammation. A mix of omega-6 (evening primrose) and omega-3 (fish oil) may be best. B-complex: B1 (50-100mg), B2 (50mg), B5 (100mg), B6 (50-100mg), B12 (100-1000mg), folate (400mg per day). Caprylic acid (1g with needs) is another type of fatty acid that may have antifungal properties (Cunha, 2010).

Propolis, a natural substance created by bees from pine resin, has antifungal properties in test tube studies. One study in humans showed that a special propolis preparation got rid of oral thrush in people who had denture stomatitis (mouth sores). People who are allergic to honey or

who have asthma should ask their doctor before taking propolis. Avoid overuse of antibiotics that kill off the friendly bacteria that normally keep *Candida* in check (Cunha, 2010).

2.12.1 Herbs

The use of herbs is a time-honored approach to strengthening the body and treating disease. Herbs, however, can trigger side effects and can interact with other herbs, supplements or medications. For these reasons, herbs should be taken with care, under the supervision of a health care provider. These herbs include:

1. Garlic (*Allium sativum*): garlic has good antifungal properties; though may increase the risk of bleeding, especially if you also take blood-thinners such as clopidogrel (plavix), warfarin (Coumadin), or aspirin. Garlic may interact with a number of medications including those needed to treat HIV.
2. The juice of the herb *Echinacea* (*Echinacea purpurea*) has been shown to help prevent recurrence of vaginal yeast infections (2-4ml per day). People with autoimmune diseases such as lupus or rheumatoid arthritis should not take *Echinacea*.
3. Tea tree oil (*Melaleuca alternifolia*) has antifungal properties in test tube. In one study, it was effective in treating oral thrush and should only be used as a mouthwash under your doctor's supervision.
4. Pomegranate (*Punica granatum*) gel was shown to be about as effective as miconazole gel in treating oral thrush associated with denture stomatitis (month sores) in one scientific study (Cunha, 2010; Harvey *et al*., 2015).

2.12.2 Homeopathy

Although few studies have examined the effectiveness of specific homeopathic therapies, professional homeopaths may consider remedies, based on their knowledge and experience, for

treating candidiasis. Before presenting a remedy, homeopaths take into account a person's constitutional types: your physical, emotional and psychological makeup. An experienced homeopath assesses all of these factors when determining the most appropriate treatment for each individual. Some of these common remedies used for candidiasis include:

1. Borax- for bleeding oral mucosa, especially with diarrhoea.
2. Belladonna - for bright red, inflamed skin that is not raw or oozing, but is painful, especially with irritability.
3. Chamomilla - for diaper rash, especially with irritability.
4. *Arsenicum album* - for burning, itching rashes, especially with anxiety
5. Graphites - for thick, cracked skin (corners of mouth or heels)
6. Kreosotum - for leukorrhea that cause itching and swelling (Deliopulus *et al*., 2010).

2.13 Drug discovery

Modern drug discovery involves the identification of screening hits, medicinal chemistry and optimization of those hits to increase the affinity, selectivity (to reduce the potential of side effects), efficacy/potency, metabolic stability (to increase the half-life) and oral bioavailability. Once a compound that fulfills all of these requirements has been identified, the process of drug development can continue, and, if successful, clinical trials are developed (Rask-Andersen *et al*., 2011; Lee *et al*., 2012).

Discovering drugs that may be a commercial success, or a public health success, involves a complex interaction between investors, industry, academia, patent laws, regulatory exclusivity, marketing and the need to balance secrecy with communication. Meanwhile, for disorders whose rarity means that no large commercial success or public health effect can be expected, the orphan

drug funding process ensures that people who experience those disorders can have some hope of pharmacotherapeutic advances (Warren, 2011).

Phenotypic screens have also provided new chemical starting points in drug discovery. A variety of models have been used including yeast, zebrafish, worms, immortalized cell lines, primary cell lines, patient-derived cell lines and whole animal models. These screens are designed to find compounds which reverse a disease phenotype such as death, protein aggregation, mutant protein expression, or cell proliferation as examples in a more holistic cell model or organism. Smaller screening sets are often used for these screens, especially when the models are expensive or time-consuming to run. In many cases, the exact mechanism of action of hits from these screens is unknown and may require extensive target deconvolution experiments to ascertain (Lee *et al*., 2012).

2.13.1 Discovery

Typically, researchers discover new drugs through:

- New insights into a disease process that allow researchers to design a product to stop or reverse the effects of the disease.
- Many tests of molecular compounds to find possible beneficial effects against any of a large number of diseases.
- Existing treatments that have unanticipated effects.
- New technologies, such as those that provide new ways to target medical products to specific sites within the body or to manipulate genetic material (Sandeep and Divya, 2018).

- At this stage in the process, thousands of compounds may be potential candidates for development as a medical treatment. After early testing, however, only a small number of compounds look promising and call for further study (Sandeep and Divya, 2018).

2.13.2 Development

Once researchers identify a promising compound for development, they conduct experiments to gather information on:

- How it is absorbed, distributed, metabolized, and excreted.
- Its potential benefits and mechanisms of action.
- The best dosage.
- The best way to give the drug (such as by mouth or injection).
- Side effects or adverse events that can often be referred to as toxicity.
- How it affects different groups of people (such as by gender, race, or ethnicity) differently.
- How it interacts with other drugs and treatments.
- Its effectiveness as compared with similar drugs.

- The vast majority of drugs available to date have at least one of the following drawbacks: (1) insufficient efficacy or increasing loss of effectiveness, (2) high level of toxicity, (3) inaccessibility, and/or (4) high costs. New drugs are desperately needed and will continue to be needed for the foreseeable future (Sandeep and Divya, 2018).

2.14 Natural Compounds with Antifungal Properties

Nature has provided scientists and researchers a virtual abundance of molecules to discover, and use as novel antifungal drugs.

2.14.1 Potash Compounds

Potash is the common name for various mined and manufactured salts that contain potassium in water-soluble form. The name derives from “pot ash”, which refers to plant ashes soaked in water in a pot, the primary means of manufacturing the product before the industrial era. Potash derives its name from potassium, and was first derived by electrolysis of caustic potash, in 1808, thus potash refers to potassium compounds and potassium-bearing materials. These potash compounds include:

1. Potash alum (KAL $(SO_2)_4$. $12H_20$)
2. Potassium carbonate (K_2C0_3) otherwise called trona, akanwu or kanwa
3. Palm potash (Amadi, 2020).

Alum

Alum or Phitkari, is a transparent salt like substance that is used in cooking as well as for medicinal purposes. Alum is a general name for a class of double sulphates containing aluminum and such metals as potassium, ammonium, iron, etc. They are commonly found in Nigeria, India, Egypt, Nepal, Italy, Philippines and many parts of Asian countries. There are a variety of commercially available alums; soda alum, Ammonium alum, Chrome alum, Selenate alum, Alumen exsiccatum (dried alum), Aluminium sulphate (also called papermaker’s alum, it is not technically alum but further processing and evaporation results in alum crystals (Ali *et al.,* 2017). Alum with the molecular formula, KAl(SO4)2.12H2O) is a colorless, odorless crystalline solid that turns white in air (Al-Talib *et al*., 2016). The resourcefulness of Alum individually or in synergism as; food preservative, medicine, water purifier, biotransformational and antimicrobial agents and sundry applications had been well documented (Amadi, 2020).

Notable advantages for the use of alum include cost effectiveness, availability, nontoxicity, reusability and ecofriendly (Brahmachari *et al*., 2019). The potency of alum as an

antimicrobial agent had been visibly demonstrated over the years through the myriads of its beneficial activities and relevance in a broad spectrum of human research and development (Ali *et al.,* 2017). Scientific data about the use of alum as a medicine for vaginal discharge is still limited, however its use as a natural remedy for the treatment of vaginal discharge because of its astringent properties is a common knowledge among the female folks. Recently, Shalli and colleagues reported that that alum has antimicrobial activity against pathogenic microbes, namely *C. albicans* and *N. gonorrhea* indicated by the presence of clear zones formed in the area of paper disks at concentrations of 20% and above (Shalli *et al.,* 2020).

Alum has demonstrated activity against oral microbiota. More recently, alum's activity against cariogenic streptococci, normal oral flora and periodontal pathogens including *Candida albicans*, *Streptococcus mutans* and *Pseudomonas aeruginosa*, aetiologic agents of oral thrush and mouth ulcers has significantly reduced the colonization ability these organisms have on enamel surfaces and decreasing the colloidal stability of oral bacteria (Rupesh *et al.,* 2010; Amadi, 2020). In another study, antifungal activity of crude Turmeric rhizome extracts and in combination with alum against five (5) fungal species were determined using disk diffusion (DD) and agar well diffusion methods (AWD). The results showed that the extracts and alum were active against all the test microbes (fungal isolates) and were dose-dependent. Strong antimycotic activity occurred with alum in the order *Aspergillus terreus* (17.5±1.0mm) > *A. flavus* (17±1.0mm) > *S. cerevisiae* (14mm) > *C. albicans* (12 ± 1.0mm) by DD respectively. With AWD, alum demonstrated highest antimycotic activity on *A. terreus* (35 ± 1.0mm) >*Penicillium crystallium* and *A. flavus* (33.0mm) >*S. cerevisiae* (24.0mm) (Amadi *et al.,* 2019). In Addition, Amadi reported that Alum demonstrated strong antimicrobial activity against both Gram positive and Gram negative bacteria as well as against yeasts and moulds. He argued that

this activity is dose and incubation period dependent and much more accentuated in synergism with plant extracts, inorganic substances or antibiotics (Amadi, 2020).

Alum mixed with wax has been used by women to remove unwanted hair. It is also beneficial for tightening and whitening of the skin due to its astringent property. Alum causes cells to shrink and removes excess oil from the skin making it effective for the reduction of acne scars and pigmentation marks. The topical application of Alum is found to be beneficial for mouth ulcers due to its strong healing activity (Roqaiya and Begum, 2015; Ali *et al.,* 2017). Health benefits of alum include: Treatment of dysentery, diarrhea, canker sores, athlete's foot, Eye abscess, lice, pimples, wrinkles, hair removal, water purification, tightening of vagina, treatment of body odour, prevention of premature ageing, stopping of bleeding cuts, relieving of muscle cramps, treatment of cracked heels and as mouth wash (Ali *et al*., 2017).

On the toxicological effects of Alum, histological studies have confirmed the safety of alum salt for mammalian consumption (Al-Talib *et al.,* 2016). It cannot be directly absorbed due to its negatively charged molecule, which are unable to pass through the cell membranes and therefore alum remain a harmless substance (Alzomor *et al.,* 2014). However higher concentration of alum might cause nephrotoxicity and intestinal bleeding (Bnyan *et al.,* 2014).

Trona (Akanwu)

Akanwu is the Local Nigerian name for Crystal of soda that is crystallized hydrous carbonate of sodium or Crystal potash that is crystallized hydrous carbonate of potassium. In Nigeria, Potash (Trona) is traditionally known as kaun among the Yoruba, kanwa among the Hausa and akanwu among the Igbos (Yaro *et al*., 2012) and Okanwa among Igalas and Ikoro among Egbira people Niger (Ajiboye *et al*., 2013). It is found in the Northern parts of Nigeria,

particularly Kano and Maiduguri States and neighboring countries such as Chad and Niger (Ajiboye *et al*., 2013).

Potash (akanwu) is a type of lake salt (sodium carbonate) that is dry and hydrated in nature. Studies reveal that the akanwu salt is the second most popularly used salt in Nigeria (Okpala, 2015). It is used for mixing water and oil while preparing local dishes like abacha, ugba and nkwobi (Adebayo and Imokhe, 2011). The medicinal use of potash for all sorts of ailments has been reported by few scholars. It is used in some concoction for curing cough and ameliorating toothache, stomach pains, and constipation. More so, it is administered to women postpartum to enhance maternal quality and quantity of breast milk (Okoye *et al.,* 2016). Akanwu identified as limestone exhibited antimicrobial activity against pathogenic bacteria and fungi organisms (*Bacillus substilis*, *Pseudomonas aerugenosa*, *Proteus spp, Staphylococcus aureus*, *Aspergillus fumigatus, Candida albicans*, *Candida pseudotropicalis and Penicillium expansium*) already implicated in the etiology and severity of human diseases (Ntukidem *et al.,* 2020).

Palm ashes (ngu)

Palm bunch ash (PBA), traditionally known as ngu in south eastern Nigeria, is used in place of trona (Akanwu) as food additive and tenderizer. It is also believed to be a non-purgative substance in preparing crude palm oil and African salad popularly known as Abacha. PBA produced by burning or ashing, which constitutes about 6.5% by weight of the empty fruit bunch (Okoye *et al.,* 2016), contains 30–40% K_2O and could thus be used as source of potassium fertilizer. PBA has high pH and contains varying amounts of other nutrients such as calcium (Ca), phosphorus (P), and magnesium (Mg) (Okoye *et al.,* 2016).

Unfortunately, as at the time of this write up, there are very limited data on the antimicrobial effect of Potash on pathogenic microorganisms. However, very recently, a comparative study in Nigeria on aqueous extract food grade ash from Furnace and Charred Plantain Peel and Palm bunches revealed that these ashes including Palm bunch ash have antimicrobial activity against the bacteria and fungi isolates. These microbial isolates include *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Proteus sp, Staphylococcus aureus*, *Aspergillus fumigates, Candida albicans*, *Candida pseudotropicalis and Penicillium expansuim* (Ntukidem *et al.,* 2020).

2.15.2 Natural stones

Blue stone

Copper Sulfate, also known as bluestone, has been used historically for home remedies, medical cures.Copper sulfate, also known as blue vitriol, Salzburg vitriol, Roman vitriol, blue copperas, or bluestone, is a chemical compound comprised of Copper, Sulphur and Oxygen whose formula is CuSO4 (Disabled-world, 2019). Copper Sulphate is an odorless crystalline substance, electric blue in color, highly toxic, and not safe to work with (Meena and Bansa, 2011). It is produced industrially and in laboratory by treating copper metal with hot concentrated sulfuric acid or its oxides with dilute sulfuric acid (Essa and Khallaf, 2016). The anhydrous form occurs as a rare mineral known as chalcocyanite. The hydrated copper sulfate occurs in nature as chalcanthite (pentahydrate), and two other rare ones: bonattite (trihydrate) and boothite (heptahydrate) (Pereira, 2010).

Copper sulfate was used in the past as an emetic (an agent that induces vomiting). It is now considered too toxic for this use, however, it is still listed as an antidote in the World Health Organization's Anatomical Therapeutic Chemical Classification System (Disabled-world, 2019).

Copper sulfate pentahydrate has reportedly been used as a fungicide and for the treatment of Athletes Foot or Tinea (Gava *et al.,* 2016; Jacobs *et al.,* 2017). Essa and Khallaf in 2016, reported the antifungal activity of different solutions of Cu-particles prepared by dissolving 200 mg of $CuSO_4$ in 200 mL deionized distilled H_2O measured against *Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum, Fusarium solani* and *Alternaria solani* (Essa and Khallaf, 2016). The results showed a remarkable growth inhibition of *A. flavus, A. niger, P. chrysogenum, F. solani* and *A. solani* by $CuSO_4$ (blue stones). The fungal growth was completely disappeared at the concentration 250 µg/mL while at 200 µg/mL the recorded percentage of the inhibition was 95.7 % for *Aspergillus niger*, 95.2 % for *Fusarium solani* and 97.4 % for *Alternaria solani* (Essa and Khallaf, 2016).

Sulfur stone (Brimstone)

Elemental sulfur has proved to be the most hydrophobic and fungitoxic compound among the natural compounds. Sulfur is one of the substances whose biocide properties (including antibacterial, antifungal and protocidal) have been indeed recognized for a long time (Nwachukwu *et al.,* 2012). Sulfur has been shown to play an established key role in plant defence, and therefore widely used nowadays in plant growing as a fungicide and acaricide (Ismail *et al.,* 2016). Presently, sulfur is a component of numerous cosmetics, for example, soap, crèmes, ointments, lotions. Sulfur ointment and other ointments, containing sulfur, sulfur-salicylic ointment, sulfur-tar ointment, sulfodekortem, Wilkinson ointment and others are used for treatment of various skin diseases of parasitic, mycotic, allergic and other nature-scabies, mycosis (keratomikosis, dermatophytes, etc.), psoriasis, seborrheic dermatitis and several others (Massalimov *et al.,* 2012). It is considered one of the safest medicines for treatment of the above mentioned diseases, seldom causing minor skin dryness. Its antiexudative effect is an important

component of anti-inflammatory therapeutic effect of sulfur-containing dermatological medicines (Massalimov *et al.,* 2012).

Medical drugs, based on elemental sulfur, are well demanded nowadays due to their high efficacy, absence of long-term effects and low cost. According to Ismail and colleagues, sulfur agents interact with organic substances and forms non-sulfides and pentatonic acid having antimicrobial and anti-parasitic activities. Massalimove *et al*. (2012) reported that Sulfur agents were able to suppress the growth of resistant *Candida albicans* at 70 mg/ ml micronized sulfur. In addition, when exposed to the mould fungi including dermatophytes, the concentration required to inhibit the colony is reduced by 3-4 times (Massalimove *et al*., 2012; Ismail *et al.,* 2016). Nwachukwu *et al.* (2012) suggested that the exact mechanism by which Sulfur defends plants against pathogens is that Sulfur is able to permeate external fungal hyphae and move into the cytoplasm where it impairs the mitochondrial electron transport chain, possibly causing the redirection of protons from O_2 to itself, and thus leading to the production of toxic H_2S. The Production of H_2S ultimately results in the alteration of oxidative phosphorylation and stops the germination and growth of conidial spores.

Black stone (Snake stone)

Black stone traditionally known as snake stone in some African communities has been used since antiquity to treat snake bites and local infections (Maregesi *et al.,* 2013). Its efficacy is debated since no clinical trial has been performed although a series of *in vivo* and *in vitro* experiments in a murine model snake bones has been carried out. The application of snake stone is popular in African and other continents. A stone is tied against incision made in the wounded area. These snake stone (black stone) originally from India, is applied to the site of the snake bite, are extremely absorbent and used to draw the venom out of snake bites, when place on the

wound sites, it adheres and extracts the venom and spontaneously and detaches after the venom is extracted limiting the venom circulation (Michael *et al.,* 2011).

Black stones are sold in market by herbalists or vendors. In rural areas most of families keep stones for emergency use. Despite snake stones remaining popular in various countries the method is not considered effective in modern medicine. Medical studies revealed that, black stone possess no efficacy to treat snake bites. However, after an extensive search of the available scientific literature as well as other online resources, very little evidence as to how Black stone is meant to work or its adverse effects was found. There was no evidence of its antimicrobial property either (Maregesi *et al.,* 2013)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

This study was carried out at the Laboratory Unit of Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Anambra State, South-East geopolitical zone of Nigeria. It is located between latitude 6°20"00" North and Longitude 7°00"00" East. The average temperature is 25.9^{o}C while the average rainfall in a year is 1386mm.

3.2 Specimens collection

3.2.1 High vaginal swab

Ethical clearance was obtained from the ethical committee of Chukwuemeka Odumegwu Ojukwu University Teaching Hospital, Awka. Four hundred and fifty swab specimens were aseptically and properly collected from patients suspected of suffering from candidiasis (vulvo-vaginal candidiasis). The swab specimens were collected by the assistance of the Medical Laboratory Scientist in the Hospital's Laboratory unit. Wet mounts of the specimens were prepared immediately using sterile grease-free microscope glass slide and normal saline (0.85% NaCl) to confirm the presence of yeast cells after which plates of Sabouraud dextrose agar media supplemented with chloramphenicol (50µg/ml) were inoculated and incubated aerobically at 25°C for 48 hours as described by Pagana *et al*. (2019).

3.2.2 Natural stones

The natural stones used for this study were black stones, blue stones and sulphur stones. These stones were hygienically selected and purchased at the Eke-Awka market in Awka South Local Government Area of Anambra State, Nigeria. The samples were transferred into sterile

containers, and transported to the laboratory for analysis. The method described by Kamka-Evans *et al*. (2013) was adopted.

The stones were taken to the Laboratory Unit of the Department of Geological Sciences, Nnamdi Azikiwe University, Awka, where they were identified by Mr. Preye Aseye.

3.2.3 Potash compounds

The potash compounds used for this study included potash alum (Tawas), Trona (akanwu or kanwa) and palm ash (ngu). These potash compounds were hygienically selected after purchase from the Eke-Awka market in Awka South Local Government Area of Anambra State, Nigeria. The samples were put in sterile containers and transported to the laboratory for analysis as described by Kamka-Evans *et al*. (2013).

3.3 Processing of the natural stones and potash compounds

Known weights of the natural compounds were soaked in water at room temperature and placed in the shaker at 60rpm at 40°C. The samples dissolved completely within two hours; except sulphur stone that dissolved within four hours.

3.4 Identification of yeast isolates

The yeast isolates were identified based on their morphological, physiological, biochemical and molecular characteristics as described by Campbell *et al*. (2013).

3.4.1 Morphological examination

Macroscopic appearance of the colonies: The colour, edges as well as the texture of the colonies were examined.

3.4.2 Microscopic examination

This was done as described by Pincus *et al*. (2007). Two drops of lactophenol cotton blue solution were placed on a clean grease-free glass slide. A small portion of the isolate was added

to the stain and emulsified using a sterile wire loop; then covered with a cover slip. This was examined under a microscope using the x10 and x40 objective lenses under low light intensity.

3.4.3 Growth on cornmeal tween-80 Agar

This test was carried out using the method described by Pagana *et al*. (2019). A light inoculum of the isolate was streaked by making a deep cut in the cornmeal agar (i.e. horizontal furrow), using inoculation needle. A flamed cover slip was placed along the line of inoculums and incubated for 24 hours at 25°C. The plates were examined microscopically (especially for the characteristic thick-walled round chlamydospores produced by *C. albicans*, in situ) through the cover slip, using low power objective x10 and x40 lenses.

3.4.4 Germ-tube test

The germ tube test also known as Reynold Brande's test provides a simple, reliable and economical procedure for the presumptive identification of *Candida albicans*. This test was carried out as described by Moya-Salazar and Rojasa (2018). A very little portion of the yeast isolate was taken from a 24hour-old culture colony using a sterile wire loop and inoculated into a sterile test tube containing 0.5ml of fresh sheep serum. The resulting mixture was incubated aerobically at 37°C for 2½ hours. After the incubation, a drop of the yeast-serum mixture was placed on a clean grease-free microscope slide, covered with a cover slip and examined using x10 and x40 objective lens. The appearance of small filaments projection, from the cell surface (without partition), confirmed the formation of germ tubes.

3.4.5 Growth on Chromogenic Agar

This test is used primarily as a screening test for obtaining a pure culture. This test was carried out as described by Cheesbrough (2018). The medium was heated at 45°C, allowed to cool and then poured into sterilized Petri-dishes. A loopful of the organism was taken aseptically

from a 24 hour old culture, inoculated on the agar by streaking and then incubated aerobically at 25^oC for 24 hours. Observation was made on the various colours produced by the organisms.

3.4.6 Sugar fermentation test

This test was carried out as described by Pagana *et al*. (2019). Fourteen sugars (1% each) were used for the test and included: D (+)-glucose, fructose, D (+)-galactose, D-xylose, trehalose, cellobiose, raffinose, inositol, dulcitol, sucrose, melibiose, maltose, lactose and dextrose. Each sugar (1g) was dissolved in 100ml of water in a conical flask (1%). Peptone (1.5g) and 2ml of bromothymol blue indicator were also added to each of the flasks after which 8ml of the final solutions were transferred into test tubes containing inverted Durham tubes. The test tubes were plugged with cotton wool and labelled accordingly. The test tubes and their contents were autoclaved at 115^oC for 10 minutes at $10ib/inch^2$ and then allowed to cool. A loopful of the test organism was inoculated into different sugars and incubated for 48hours at room temperature. Acid production was indicated by a yellow colour change of the indicator while gas production indicated by gas accumulation in the submerged Durham tubes.

3.4.7 The molecular identification tests

The underlisted procedures were conducted on the yeast isolates at Molecular Research Foundation for Students and Scientist, NAU, Awka; Bioinformatics Services, Ibadan and International Institute of Tropical Agriculture (IITA), Bioscience Center, Ibadan, Nigeria:

Genomic DNA Isolation of the fungal isolates.

Polymerase Chain Reaction Test

Nucleic acid Sequencing Analysis of the Amplicons as described by Todd (2012) and Morey *et al*. (2013).

Isolation/extraction of fungal genomic DNA

Genomic DNA was extracted using Quick-DNATM Fungal/Bacterial Miniprep Kit; Zymo Research), according to recommended protocol with slight modification. Pure fungi cells (100mg wet weight) that had been resuspended in up to 200*µl* of water was added to a ZR Bashing Bead Lysis Tube (0.1mm & 0.5mm). Bashing BeadTM Buffer (750 µl) was added to the tube and vortexed at maximum speed for ≥ 15minutes. ZR Bashing BeadTM Lysis Tube (0.1 & 0.5mm) was centrifuged in a microcentrifuge at 10,000 x *g* for 1 minute. Up to 400*µl* of the supernatant was added to a Zymo-SpinTM III-F Filter in a Collection Tube and centrifuged at 8000 x *g* for 1 minute. Genomic Lysis Buffer (1,200µl) was added to the filtrate in the Collection Tube from step 4 after which 800*µl* of the mixture was transferred to a Zymo-SpinTM IIC Column in a Collection Tube and Centrifuged at 10,000 x *g* for 1 minute. The flow through was discarded from the Collection Tube and the step repeated. DNA Pre-Wash Buffer (200µl) was added to the Zymo-SpinTM IIC Column in a new Collection Tube and Centrifuged at 10,000 x *g* for 1 minute. DNA Wash Buffer (500µl) was added to the Zymo-SpinTM IIC Column and centrifuged at 10,000 x *g* for 1 minute. The Zymo-SpinTM IIC Column was then transferred to a clean 1.5ml microcentrifuge tube and 60 *µl* DNA Elution Buffer was added directly to the column matrix. Centrifugation was at 10,000 x *g* for 30 seconds to elute the DNA.

PCR protocol

One Taq Quick-Load (12.5µl) 2X Master Mix with Standard Buffer (New England Biolabs Inc.), 0.5µl each of forward (ITS1: 5'- TCCGTAGGTGAACCTGCGG -3') and reverse primers (ITS4: 5' - TCCTCCGCTTATTGATATGC -3') were used. Nuclease free water (8.5µl) and 3µl of DNA template were used to prepare 25µl reaction volume of the PCR cocktail. The reaction was gently mixed and transferred to an Eppendorf nexus gradient Mastercycler

(Germany). Amplification conditions for the PCR were as follows: Initial denaturation for 30secs at 94°C, followed by 35 cycles of denaturation at 94°C for 20secs, primer annealing at 54°C for 45secs and strand extension at 72°C for 1 min. Final extension was at 72°C for 5 minutes. PCR products, the amplicons, were separated on a 2% agarose gel and DNA bands were visualized with Ethidium bromide on a gel Documentation System (Vilber, Germany).

Sequencing Protocol

PCR products (the amplicons) were cleaned using ExoSAP Protocol as follows:

1. The Exo/SAP master mix was prepared by adding the following to a 0.6ml micro-centrifuge tube:

a. Exonuclease I (Catalogue No. NEB M0293L) 20 U/ul 50 µl

b. Shrimp alkaline phosphatase (Catalogue No. NEB M0371) 1 U/ul 200 µl

2. The following reaction mixture was prepared:

a. Amplified PCR Product 10 µl

b. ExoSAP Mix (step 1) 2.5 µl

3. The above set up was well mixed and incubated at 37°C for 15 min

4. The reaction was put to a stop by heating the mixture at 80°C for 15 min

5. Fragments were sequenced using the Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 according to manufacturer's instructions:

https://www.nimagen.com/products/Sequencing/Capillary-Electrophoresis/BrilliantDye-Terminator-Cycle-Sequencing-Kit/

6. The labelled products were then cleaned with the ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053):

http://www.zymoresearch.com/downloads/dl/file/id/52/d4052i.pdf

7. The cleaned products were injected on the Applied Biosystems ABI 3500XL Genetic Analyser with a 50cm array, using POP7:

https://www.thermofisher.com/order/catalog/product/4406016

8. Sequence chromatogram analysis was performed using FinchTV analysis software:

https://www.softpedia.com/get/Science-CAD/FinchTV.shtml

3.5 Phylogenetic analysis of the *Candida* isolates

Bioedit sequencing software was used for the phylogenetic analysis.

3.6 *In vitro* evaluation of antifungal activity of the solutions

This assay was done using the agar-well methods as described by Bauer *et al*. (1966).

3.6.1 Preparation of stock solution

Stock solutions of the test agents (ie the natural stones and potash compounds) were prepared by dissolving 2g of the test agents in 10ml of sterile water to give 200mg/ml of the natural compounds. A double fold serial dilution of the stock solution was performed to obtain 100mg/ml, 50mg/ml and 25mg/ml concentrations.

3.6.2 The agar-well diffusion method

Plates of Mueller Hinton Agar supplemented with 2% glucose were aseptically prepared. Using 6mm cork-borer, 6mm diameter wells were made on the medium. The agar plates were seeded with 2 x 10^5cfu/ml (equivalent to 0.5 McFarland Standard) of the *Candida* isolates. This was done by inoculating 0.1ml of the above McFarland value on the prepared MHA using the spread plate method. Then, 0.5ml of the test solutions were added into the wells using sterile 1ml glass pipette. Positive and negative controls were prepared by adding 0.5ml of ketoconazole and distilled water respectively into the control wells. The experimental set up was incubated at 25^oC

for 24 hours. All of the experiments were performed in triplicates and the results reported as average of 3 experiments. Antifungal activity was determined by measuring the inhibition zone diameter (in mm) produced after 24hrs of incubation. The inhibition zone diameter was reported in Mean±Standard deviation as described by Jorgensen and John (2011).

3.6.3 Determination of MIC and MFC using broth dilution method

The method described by Gahlaut and Chhillar (2013) was adopted. Various concentrations of the test agents, from the stock solution of 200mg/ml, were made in Sabouraud dextrose broth by double fold serial dilution to obtain, 100mg/ml, 50 mg/ml, 25 mg/ml, 12.25mg/ml, 6.325 mg/ml, 3.125mg/ml and 1.5625 mg/ml. Each dilution in a test-tube was inoculated with 0.2 ml of the broth culture of test isolates diluted to 0.5 McFarland standard. All the tubes were incubated at 25°C for 24 hrs. The lowest concentration showing no visible growth (as compared with a negative control) was recorded as the minimum inhibitory concentration (MIC) for each organism.

The MFC was determined by transferring 1ml from each negative tube in MIC assay, onto the surface of freshly prepared Sabouraud dextrose agar plates (without the test agents) using the spread plate method and incubated at 25°C for 48 hrs. The lowest concentration showing no visible growth on SDA was recorded as minimum fungicidal concentration (MFC) for each organism.

3.6.4 MIC and MFC determination using the micro-dilution method

1. Double strength Mueller Hinton broth was prepared.
2. The microtitre plates were labeled according to Samples.

3. Different concentrations of the test solutions were prepared from 200mg/ml to 3.125mg/ml through double fold serial dilution using Mueller Hinton Broth as the diluents.
4. The inocula were standardized to 0.5 Mcfarland's standard.
5. The test solutions (75µl) (from each of the serially diluted tubes) were aseptically introduced into the microtitre tubes.
6. Also, the test isolates (75µl) were likewise introduced into same microtitre wells as above.
7. The set ups were incubated at 25°C for 24hours.
8. The absorbance of the various mixtures after the 24hours incubation was measured using the ELIZA plate analyser at 430nm and recorded as MIC (as percentage inhibition).
9. The MFC (as percentage inhibition) was also determined by reading the absorbance at 430nm after further 48hours incubation at 25°C as described by Elshikh *et al*. (2016).

3.7 Analysis of chemical (organic and inorganic) constituents of the test solutions

3.7.1 Gas Chromatography-Mass Spectroscopy (GC-MS) for organic constituents analysis

The GC–MS analysis of the solutions was done using Agilent Technologies GC systems with GC-7890A/MS-5975C model (Agilent Technologies, Santa Clara, CA, USA) equipped with HP-5MS column (30 m in length × 250 µm in diameter × 0.25 µm in thickness of film). Spectroscopic detection by GC–MS involved an electron ionization system which utilized high energy electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas with flow rate of 1 mL/min. The initial temperature was set at 50 –150°C with increasing rate of 3°C/min and holding time of about 10 min. Finally, the temperature was increased to 300°C at 10°C/min. One microliter of the prepared 1% of the extracts diluted with respective solvents was injected in

ansplitless mode. Relative quantity of the chemical compounds present in each of the extracts of was expressed as percentage based on peak area produced in the chromatogram. Bioactive compounds in the various solutions were identified based on GC retention time on HP-5MS column and matching of the spectra with computer software data of standards (Replib and Mainlab data of GC–MS systems) as described by Buss and Butler (2010).

3.7.2 X-Ray fluorescence spectroscopy for inorganic constituents' analysis

The samples size was first reduced to meet a ~10-µm particle size fraction. Once in powdered form, the sample was packed into a sample tray and analyzed by XRF. The XRF analysis provides a quantitative data for a suite of minerals. Identification of minerals is based on the location and intensity of peaks on the 2θ scale. Samples prepared were scanned with a Scintag® XFS2000 X-ray fluorescence using CuKα radiation at 40 kV and 30 mA. The majority of the scans were performed using a continuous scan mode from 2 to 34° 2θ with a 0.05 step size at 2 degrees per minute. The collected data were then analyzed using Jade 9+® software. To conclude the process, the results were assembled into easy-to-read spreadsheets, and the XRF trace for each sample was put into the form of a jpg image as described Maddix *et al*. (2002).

3.8 Toxicological screening of the test samples

Toxicological screening is very important for the development of new drugs and for the extension of the therapeutic potential of existing molecules.

3.8.1 The Draize's skin irritancy test

The Draize's skin irritancy test was used to measure the harmfulness of the natural compounds including natural stones and potash compounds in murines. In this test, various dilutions from 400mg/ml, 200mg/ml, 100mg/ml and 50mg/ml of the natural compounds each was applied to the skin surfaces (dorsally and ventrally) of healthy adult albino rat. The body

weights of the animals were measured prior to the test. During the observation period (14 days), signs such as erythema and edema were assessed and recorded as shown below. At the end of the study, the animals were sacrificed and pathological changes evaluated through detailed histological studies as described by Draize *et al*. (1944).

Grading of skin reactions during Draize's skin test

Erythema

0—None

1—Slight

2—Moderate

3—Severe

Edema

0—None

1—Slight

2—Moderate

3—Severe

3.8.2 Murine local lymph node assay (LLNA)

The LLNA method is used as an alternative to the Draize test and it is widely acceptable. The assay was carried out as described by Ramani *et al*. (2013). Various concentrations of the natural compounds each, ranging from 400mg/ml, 200mg/ml, 100mg/ml and 50mg/ml were applied on the surface of the ears of the rats for three consecutive days, and the proliferation of lymphocytes in the draining lymph node was measured at the end; and compared with a negative control. The lymphocyte counts before and after treatment was recorded.

3.8.3 Necropsy studies

This is postmortem examination of animals to determine the extent of a disease or the cause of death. Necropsy studies allows for a more detailed examination of the animal's skin surface. The necropsy was carried out as described by Auletta (2014). The rat skin was examined carefully to identify macroscopic findings and correlate them with in-life observations. Orientation marks (left, right, dorsal, and/or ventral) was made on the skin with permanent marker to help orient the specimen(s). Various concentrations, 400mg/ml, 200mg/ml, 100mg/ml and 50mg/ml of the natural compounds each was applied to the skin surfaces (dorsally and ventrally) of healthy adult albino rat. Untreated skin was sampled from the same general body area as treated skin to serve as negative control and minimize result interpretation errors.

Grading of skin reactions during necropsy studies

Erythema:

0—None

1—Slight

2—Moderate

3—Severe

Edema:

0—None

1—Slight

2—Moderate

3—Severe

Atonia:

A decrease in normal elasticity (resilience) of the skin

0 - None

1—Slight (slight impairment of elasticity)

2—Moderate (slow return to normal)

3—Marked (no elasticity)

Desquamation:

Scaling/flaking of the epidermis

0 - None

1—Slight (slight scaling)

2—Moderate (scabs and flakes)

3—Marked (pronounced flaking with denuded areas)

Fissuring:

Cracks in the skin

0 - None

1—Slight (definite cracks in epidermis)

2—Moderate (cracks in dermis)

3—Marked (cracks and bleeding)

Scab formation

0 - None

F—Focus/foci present

P—Patches present

Exfoliation:

0 - None

Sloughing of scabs/eschar tissue

P - Present

A - Absent

Tissue damage/Necrosis:

0 - None

Blanched or blackened dead tissue.

Absence of necrosis (Auletta, 2014).

3.9 *In vivo* evaluation of antifungal activity of the solutions

The *in vivo* antifungal potential of the natural stones and potash solutions on the fungal isolates was carried out using albino rats. Seventy-two eight-month old healthy albino rats (for each analysis) (males – 48; females – 24) weighing between 220g - 240g obtained from a private animal house at Awka, Anambra State, were used. The rats were caged and placed in a room with controlled cycles of 12 hours of light and 12 hours of darkness. Water and food were provided to the animals *ad libitum*. The animals were housed singly. This assay was done as described by Sasidharan *et al*. (2010).

The rats were divided into six groups of twelve rats each as follow:

Group 1: Infected and treated rats

Group 2: Immunosuppressed, Infected and treated rats

Group 3: Positive control A (Infected and Untreated rats)

Group 4: Positive control B (Immunosuppressed, Infected and Untreated rats)

Group 5: Negative control A (Infected Rats Treated with topical Antifungal Cream (ketoconazole).

Group 6: Negative control B (Uninfected rats).

The experiment was conducted in accordance with the internationally accepted principles of laboratory animals' use and care as described by Perry (2007).

3.9.1 Immunosuppression

The experimental animals' immune response was suppressed by injecting them (using insulin syringe) with 0.1ml of cyclophosphamide (100mg/kg body weight) intraperitoneally without anesthesia as described by Sasidharan *et al*. (2010). They were inoculated percutaneously (infected) with loopful of the test isolates (equivalent of 1 x 10^8 CFU/g) five days after immunosuppression.

3.9.2 Differential white blood cells count on immunosuppressed rats

Differential white blood cell count (DWBC) was conducted on the test animals before and after immunosuppression (i.e. day 0 before immunosuppression; and after day 5). Blood sample was collected carefully via cardiac and tail puncture. The DWBC count was determined using a hemoanalyser to check the immune status of the animals prior to inoculation or infection as described by Pagana *et al*. (2019).

3.9.3 Animal inoculation (Infection)

The experimental animals were infected percutaneously as described by Sasidharan *et al*. (2010). This was done carefully and aseptically to avoid contaminating the site. The dorsal area, just above the tail region, the perianal region, the penile and vaginal regions were gently disinfected with 70% (v/v) ethanol and shaved carefully using sterile surgical blades. The size of the shaved area was 2 x $2cm^2$. This area was gently, aseptically and carefully inoculated with a loopful of the isolates (equivalent to $1x10^8$ cfu/g), by gently smearing the yeast cell emulsified with normal saline, on the shaved surface. The animals were well fed and carefully observed till they developed signs of infections, characterized by reddening or continuous peeling-off of the skin with tiny reddish rashes (1-2.5mm diameter). The animals' behavior was carefully monitored immediately after the inoculation (infection).

3.9.4 Post inoculation analysis

Koch's postulate analysis

This was done to establish Koch's postulate in the disease process as described by Byrd and Segre (2016). Skin scrapings were carefully and gently collected (using sterile surgical blades) from the infected areas. This was immediately inoculated on SDA (without chloramphenicol) and Nutrient agar (without nystatin) and incubated aerobically at 25^oC for 24

hours. The colonies were further identified as described earlier based on their morphological and physiological characteristics.

3.9.5 Post-inoculation treatment

Antimicrobial formulations

The natural compounds were formulated for treatment using Vaseline jelly as the API base and carrier. This was done in the ratio of 4:1 (potash compound: Vaseline base; Natural stone: Vaseline base). The method was described by Alzomor *et al*. (2014).

Treatment

The experimental animals were treated daily. The negative control A, were treated with topical ketoconazole and Vaseline jelly, while the negative controls B were left untreated. The animals were carefully monitored for two weeks till full recovery as described by Sasihadran *et al*. (2010).

3.10 Total yeast count determination

The total yeast count from the infected skin tissue at three days intervals during treatment was done as described by Cheesbrough (2018) by collecting tissues from infected sites, and inoculating SDA plates supplemented with chloramphenicol (50µg/ml). The medium inoculation was done after serially diluting the tissue specimen in sterile water. The growth was recorded as colony forming unit per gram (CFU/g) of the tissue specimen.

3.11 Histological analysis

This was carried out as described by Slaoui and Fiette (2011); Ross and Pawling (2016) and Wick (2019). Skin tissues from the test sites were collected and transferred to 10% neutral buffered formalin (NBF) for 24hours at 40°C. The formalin-fixed tissues were then dehydrated through grades of alcohol and cleared in xylene, and then embedded in paraffin wax (60°C). The tissue sections (5mm) were deparafinized, stained with hematoxylin and counterstained with eosin. Histological analysis of the test animals' skin tissues was done before, during and after the treatment regimen.

3.12 Statistical analysis

The statistical analysis was done using SPSS version 25 to test the statistical significance of the data obtained.

CHAPTER FOUR

4.0 RESULTS

Frequency of the isolates from the HVS Specimens

A total of 450 High vaginal swabs were collected from patients attending Chukwuemeka Odumegwu Ojukwu University Teaching Hospital Awka for isolation of *Candida species*. Out of these, 188 (42%) yielded *Candida* spp which included *Candida albicans,* 96 isolates (51%), *C. tropicalis,* 53 isolates (28%), *C. glabrata,* 28 isolates (15%) and *C. parapsilosis,* 11 isolates (6%) as shown in figure 1.

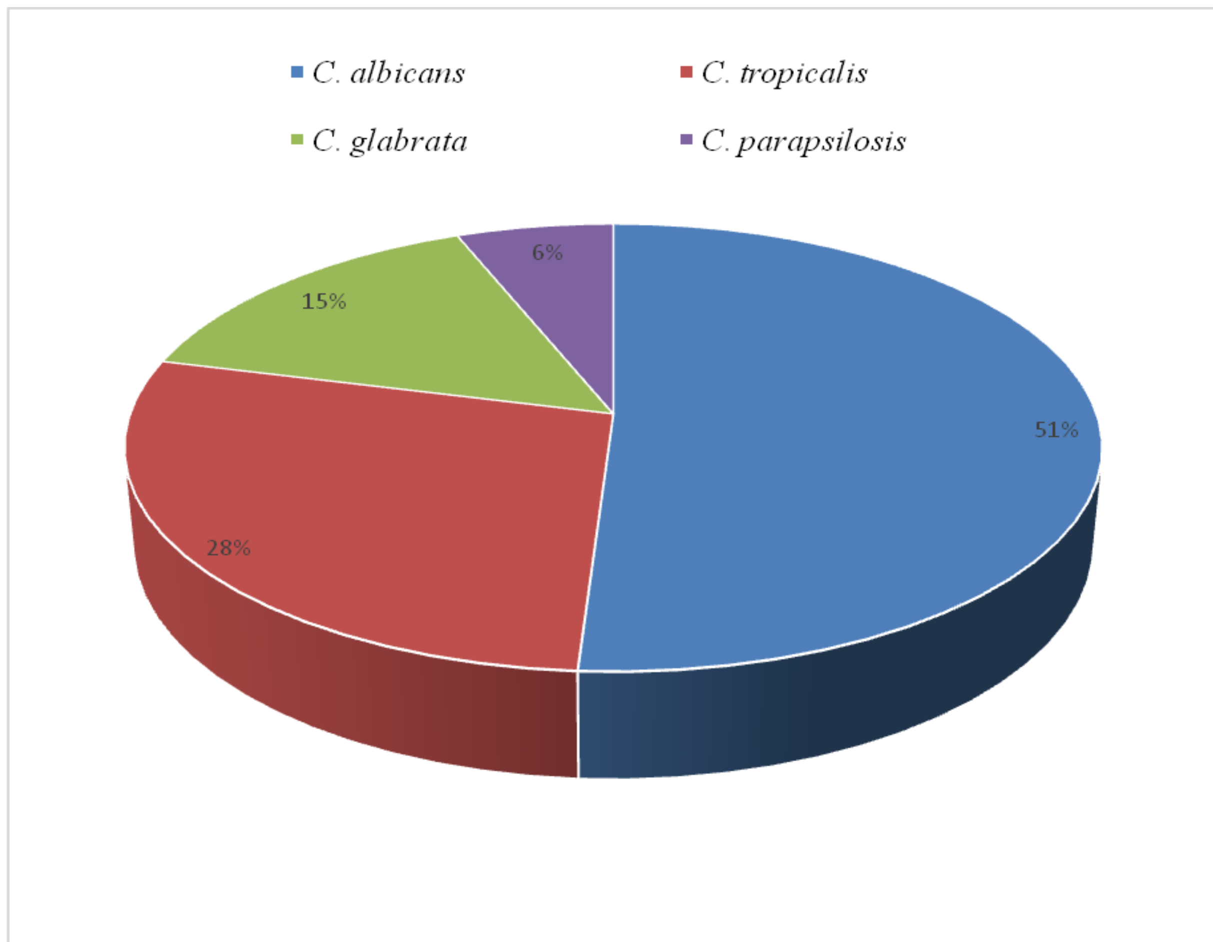


Figure 1: Frequency of the isolates from the HVS Specimens

Microscopic morphologies and germ tube test

Table 1 showed the morphological characteristics of the isolates after 24 to 48 hours growth on cornmeal tween-80 Agar at 25°C. *Candida albicans* and *C. tropicalis* produced chlamydospores, blastospores, pseudohyphae but did not produce arthroconidia. *C. parapsilosis* produced blastospores and pseudohyphae while *C. glabrata* produced only blastospores. None of the isolates produced arthroconidia. *C. albicans* was positive to germ tube test as shown in plate 1 while the other isolates were negative. Plates 2 – 5 showed the pure cultures obtained using the streak plate method while plates 6 – 9 were the micrographs of the isolates obtained after Gram staining. The micrographs showed the characteristic shape of the *Candida* isolates ranging from oval (*C. albicans, C. glabrata*), spindle (*C. parapsilosis*) to oblong shaped cells (*C. tropicalis*). *Candida species* are Gram positive as shown in the micrographs.

Table 1: Microscopic morphologies and germ tube test

Characteristic features	Isolates			
	C. albicans	***C. tropicalis***	***C. glabrata***	***C. parapsilosis***
Germ tube	+	-	-	-
Chlamydospores	+	+	-	-
Pseudohyphae	+	+	-	+
Blastospores	+	+	+	+
Arthrospores	-	-	-	-

+ = positive

- = Negative

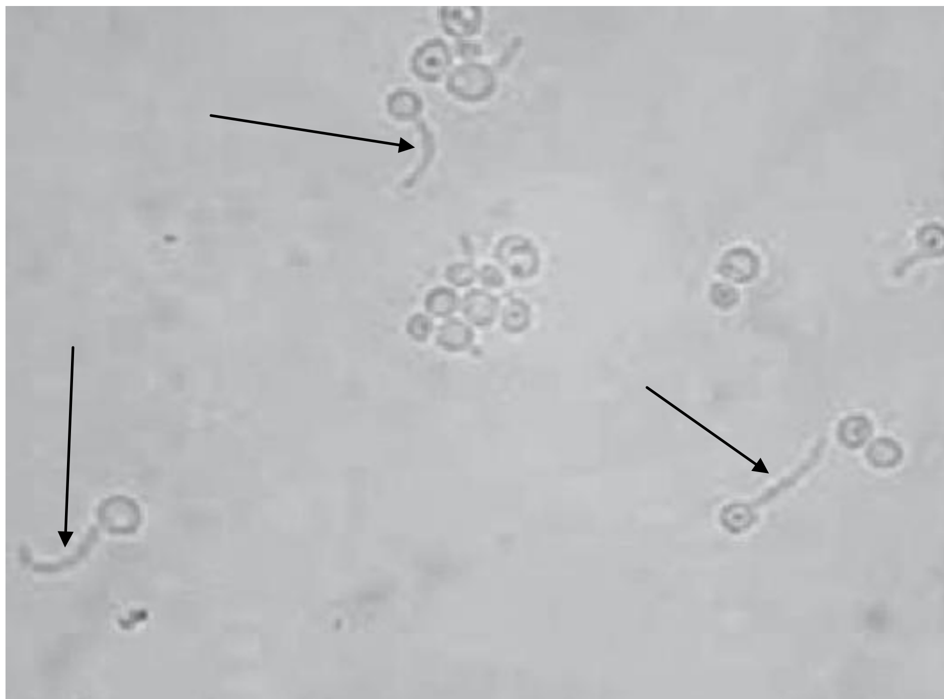


Plate 1: Germ tube production by *Candida albicans*

Plate 2: Pure Culture plate of *C. albicans*

Plate 3: Pure culture plate of *C. tropicalis*

Plate 4: Pure culture plate of *C. glabrata*

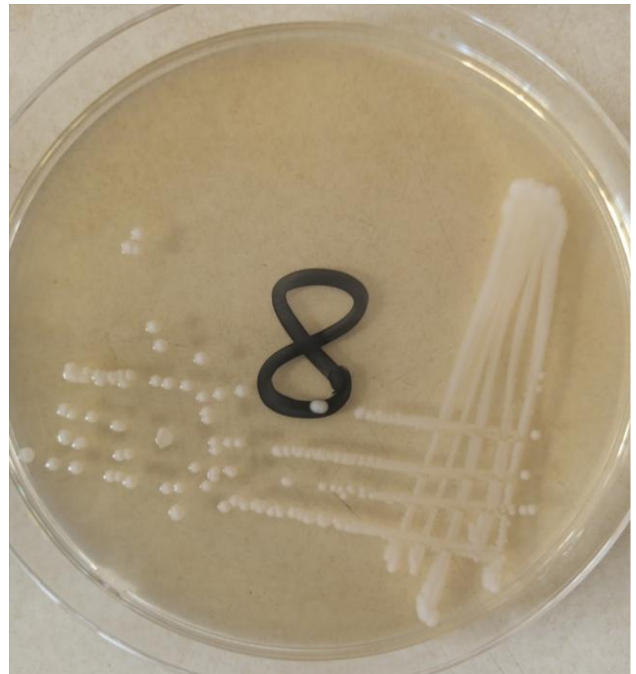
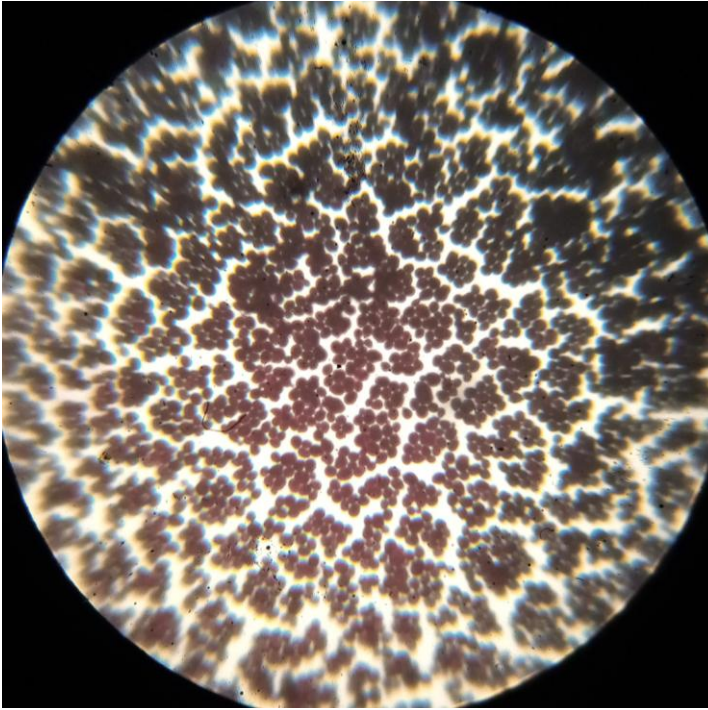


Plate 5: Pure culture plate of *C. parapsilosis*



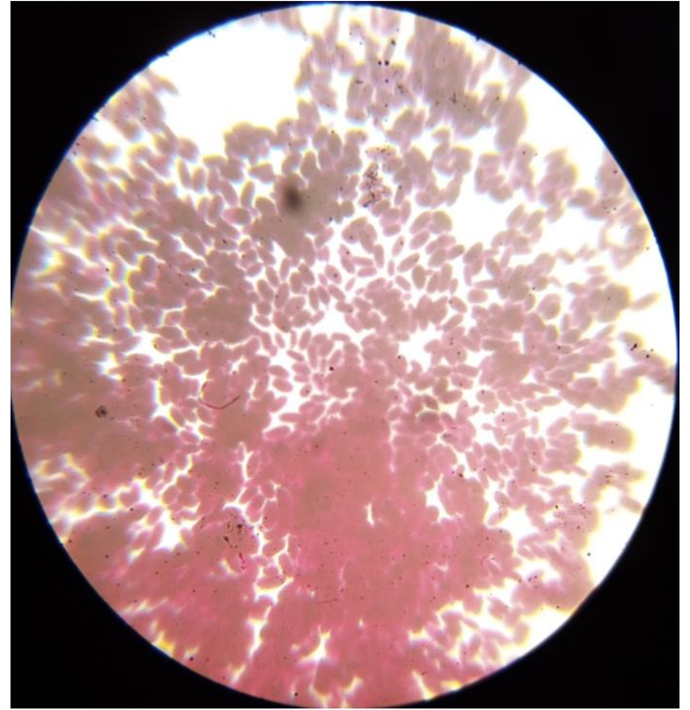


Plate 6: Micrograph of *C. albicans*

Plate 7: Micrograph of *C. tropicalis*

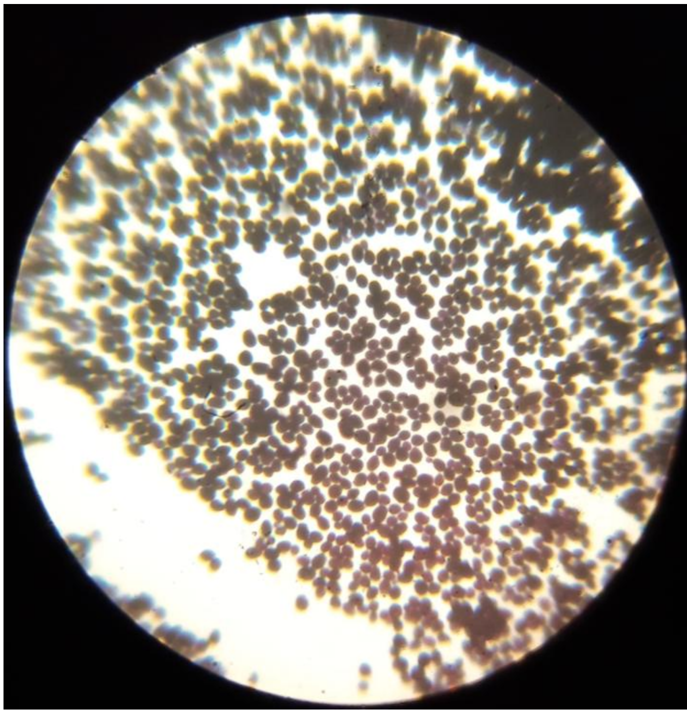


Plate 8: Micrograph of *C. glabrata*

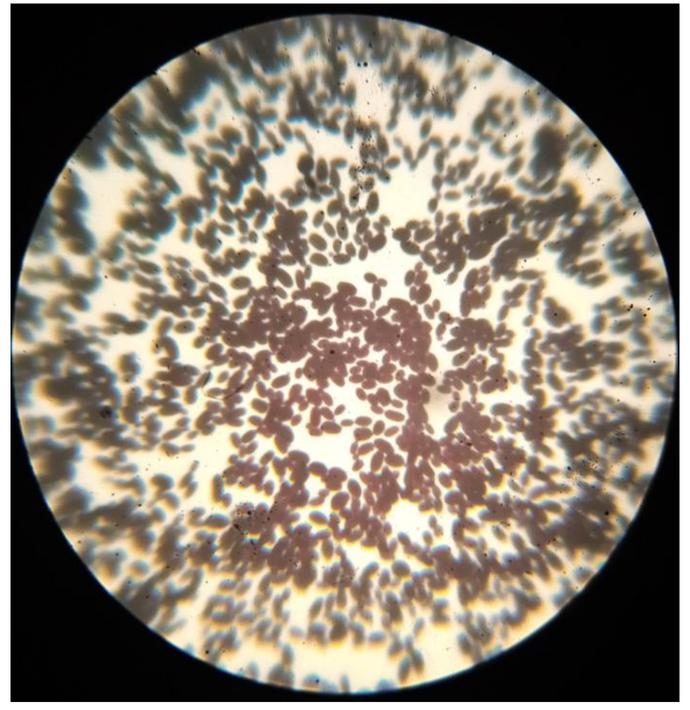


Plate 9: Micrograph of *C. parapsilosis*

Sugar fermentation test

Table 2 showed the ability of microorganisms to ferment various carbohydrates added in a basal medium, with the production of organic acid and gas as end products. Acid production was indicated by a yellowish colour change of the indicator (bromothymol blue) while gas production was indicated by gas accumulation in the submerged Durham tubes. The isolates exhibited varied response to the various carbohydrates. *Candida albicans* fermented 8 out of the 14 sugars, *C. tropicalis* 9, *C. glabrata* 4 and *C. parapsilosis* 8. All the isolates fermented fructose, glucose, dextrose and trehalose; while none fermented lactose, inositol, raffinose, dulcitol and melibiose.

Growth on chromogenic agar medium

CHROMagar is a differential ready-to-use chromogenic medium designed to identify *C. albicans*, *C. tropicalis,* and *C. krusei* by colony colour and morphology. It contains a chromogenic substratum for rapid detection and specific identification of *Candida* spp. Typically, on CHROMagar, colonies of *C. albicans* were green; *C. parapsilosis* are pale pink; *C. glabrata* were purple; while *C. tropicalis* were dark blue as shown in plate 10.

Phylogenetic analysis

The phylogenetic analysis revealed the evolutionary relatedness among the *Candida* isolates. The phylogenetic tree shown in figure 2 revealed that *C. albicans* and *C. parapsilosis* were very closely related more than the rest of the isolates. *C. glabrata* was closer, genetically to *C. albicans/C. parapsilosis* unlike *C. tropicalis* which was farther away from *C. albicans/C. parapsilosis*.

Table 2: Sugar fermentation test

Sugars	Characteristics of the isolates			
	C. albicans	***C. tropicalis***	***C. glabrata***	***C. parapsilosis***
Fructose	+	+	+	+
Sucrose	+	+	-	+
Lactose	-	-	-	-
Glucose	+	+	+	+
Inositol	-	-	-	-
Maltose	+	+	-	+
D-xylose	+	+	-	+
Galactose	+	+	-	+
Cellobiose	-	+	-	-
Dextrose	+	+	+	+
Trehalose	+	+	+	+
Raffinose	-	-	-	-
Dulcitol	-	-	-	-
Melibiose	-	-	-	-

Key: + = positive - = Negative

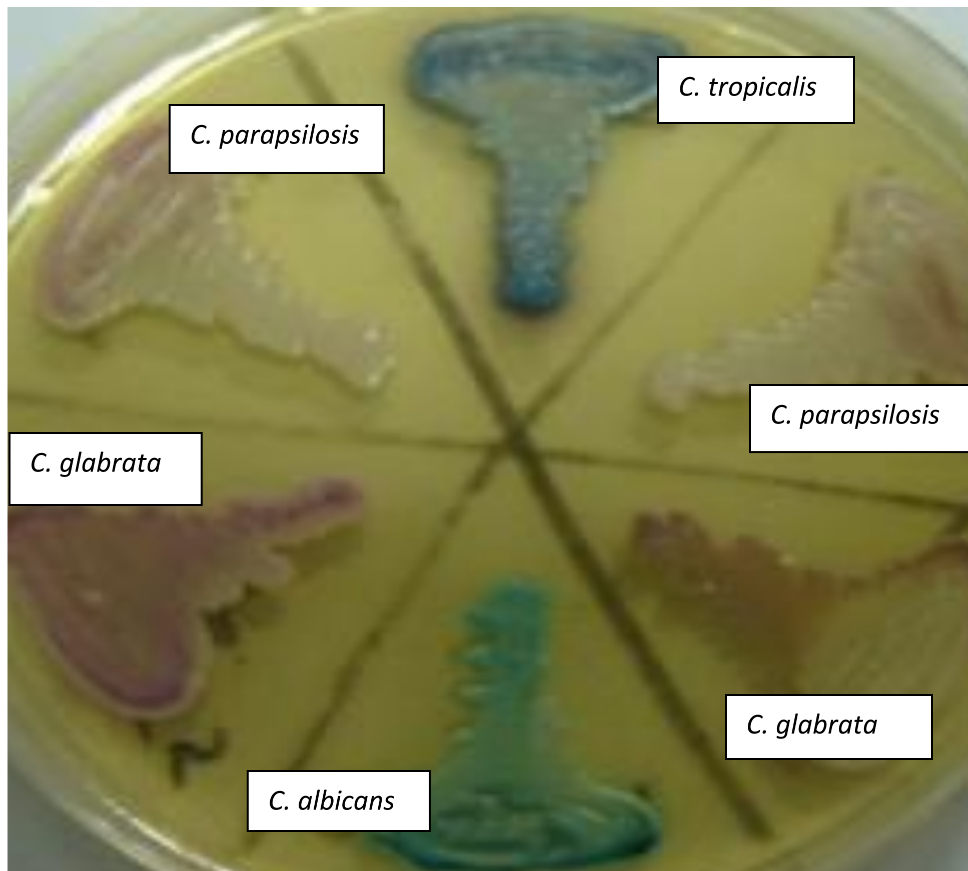


Plate 10: Growth of *Candida species* on Chromogenic agar medium

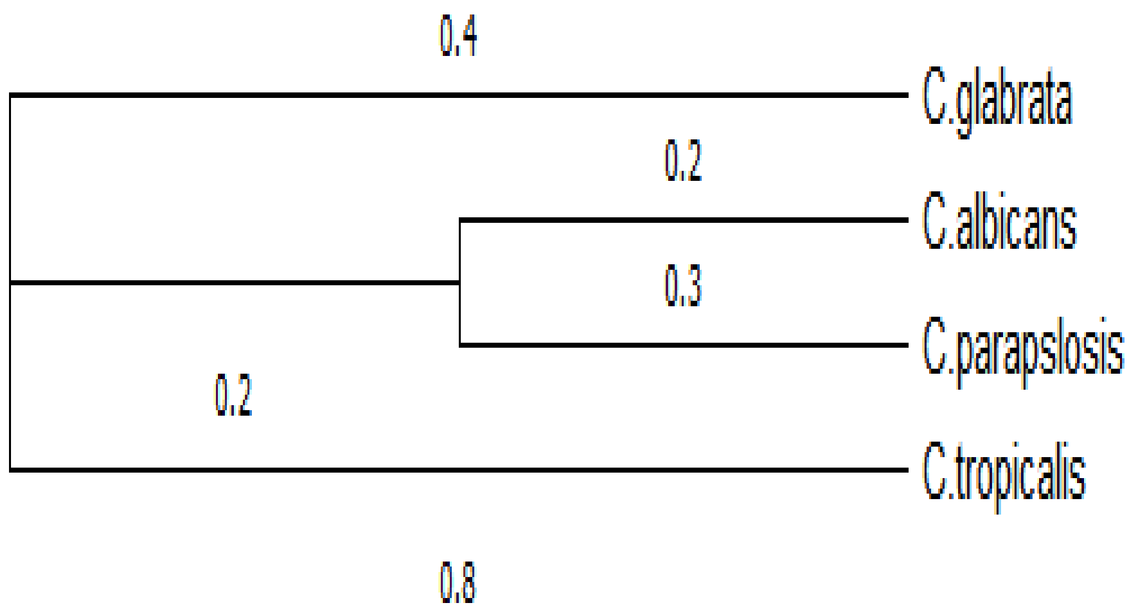


Figure 2: Phylogenetic tree developed using mega software

Antimicrobial actions of natural compounds against the isolates

Table 3 showed the inhibition zone diameter (IZD in mm) of the natural compounds (potash alum, trona, palm ash, blue stone, sulphur stone and black stone) on *Candida albicans* using the agar-well diffusion method. Potash alum and trona gave the best IZD (29.00±1.41mm) while blue stone gave the highest IZD among the natural stone (29.50±0.71mm). The isolate *C. albicans* was resistant to Black stone (IZD 0.00mm) as shown in Table 3 and Plates 11. Among the potash compounds, trona gave the best IZD (29.00±1.41mm) while blue stone gave the highest IZD among the natural stone (31.50±0.71mm). The isolate *C. tropicalis* was resistant to Black stone (IZD 0.00mm) as shown in Table 4 and Plates 11.

Trona gave the best IZD (31.00±1.41mm) while blue stone gave the highest IZD among the natural stone (26.00±1.41mm) against *C. glabrata.* The isolate was resistant to Black stone (IZD 0.00mm) as shown in Table 5 and Plates 11. Table 6 showed the inhibition zone diameter (IZD in mm) of the natural compounds on *Candida parapsilosis* using the agar-well diffusion method. Potash alum and trona gave the best IZD (30.00±1.41mm) while blue stone gave the highest IZD among the natural stone (30.50±0.71mm). *C. parapsilosis* was resistant to Black stone (IZD 0.00mm) as shown in Table 6 and Plates 11.

Table 3: Inhibition zone diameter of natural compounds against *Candida albicans* using the agar-well diffusion method

Concentration (mg/ml)	Potash alum (mm)	Trona (mm)	Palm ash (mm)	Blue Stone (mm)	Sulphur stone (mm)	Black Stone (mm)	Ketoconazole (mm)
12.5	-	-	-	13.00±1.41	-	-	-
25	13.50±0.71	12.00±1.41	-	17.00±1.41	-	-	-
50	19.50±0.71	18.00±1.41	10.00±0.00	21.00±1.41	11.00±1.41	-	14.50±0.71
100	27.50±0.71	24.50±0.71	12.50±0.71	24.50±0.71	14.50±0.71	-	19.00±1.41
200	29.00±1.41	29.00±1.41	16.50±0.71	29.50±0.71	17.00±0.00	-	21.00±1.41

Positive control for disc & well respectively = Ketoconazole.

Negative control (distilled water) = 00mm.

Table 4: Inhibition zone diameter of natural compounds against *Candida tropicalis* using the agar-well diffusion method

Concentration (mg/ml)	Potash alum (mm)	Trona (mm)	Palm ash (mm)	Blue Stone (mm)	Sulphur stone (mm)	Black Stone (mm)	Ketoconazole (mm)
12.5	-	-	-	-	-	-	-
25	12.00±1.41	-	-	13.00±1.41	-	-	-
50	16.00±1.41	12.00±1.41	-	19.50±0.71	11.00±1.41	-	14.00±1.41
100	20.00±0.00	17.50±0.71	11.50±0.71	24.50±0.71	14.50±0.71	-	16.50±2.12
200	26.00±0.00	29.00±1.41	16.00±1.41	31.50±0.71	16.00±0.00	-	21.50±0.71

Positive control for disc & well respectively = Ketoconazole.

Negative control (distilled water) = 00mm.

Table 5: Inhibition zone diameter of natural compounds against *Candida glabrata* using the agar-well diffusion method

Concentration (mg/ml)	Potash alum (mm)	Trona (mm)	Palm ash (mm)	Blue Stone (mm)	Sulphur stone (mm)	Black Stone (mm)	Ketoconazole (mm)
12.5	-	-	-	-	-	-	-
25	12.50±0.71	12.00±1.41	-	12.00±1.41	-	-	-
50	17.50±0.71	17.50±0.71	10.50±0.71	16.50±0.71	10.00±1.41	-	11.50±0.71
100	19.50±0.71	22.00±1.41	14.00±1.41	19.00±1.41	13.50±0.71	-	14.00±0.00
200	26.50±0.71	31.00±1.41	17.50±0.71	26.00±1.41	17.50±0.71	-	20.50±0.71

Positive control for disc & well respectively = Ketoconazole.

Negative control (distilled water) = 00mm.

Table 6: Inhibition zone diameter of natural compounds against *Candida parapsilosis* using the agar-well diffusion method

Concentration (mg/ml)	Potash alum (mm)	Trona (mm)	Palm ash (mm)	Blue Stone (mm)	Sulphur stone (mm)	Black Stone (mm)	Ketoconazole (mm)
12.5	-	-	-	11.00±1.41	-	-	-
25	11.00±0.00	11.50±0.71	-	15.50±0.71	-	-	-
50	16.00±2.83	16.50±0.71	10.50±0.71	21.00±1.41	10.50±0.71	-	12.00±1.41
100	22.00±1.41	23.00±1.41	14.00±0.00	26.00±1.41	14.00±1.41	-	14.50±0.71
200	30.00±1.41	30.00±1.41	16.50±0.71	30.50±0.71	18.50±0.71	-	19.00±1.41

Positive control for disc & well respectively = Ketoconazole.

Negative control (distilled water) = 00mm.

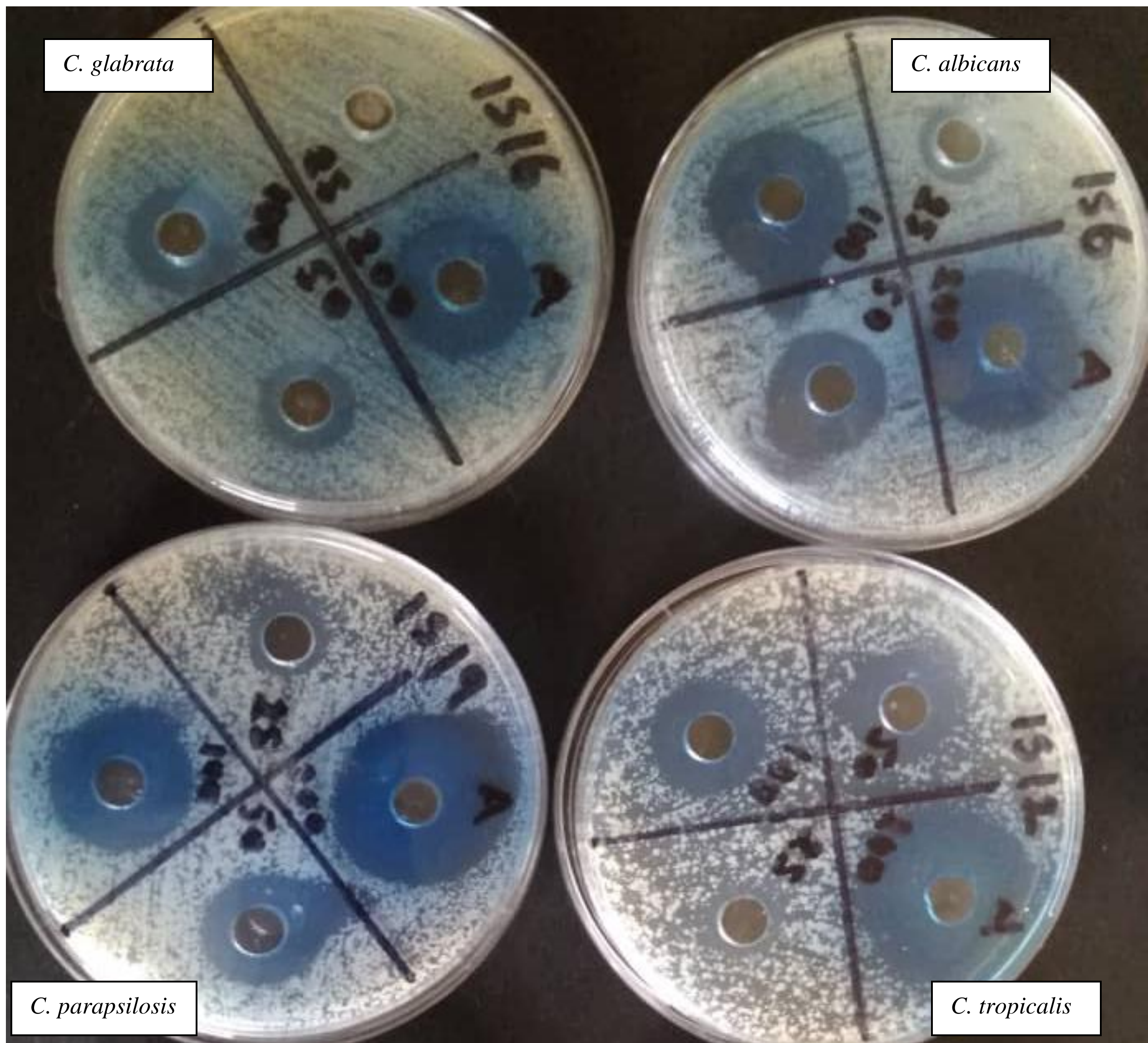


Plate 11a: Inhibition zone diameter of blue stone against the isolates

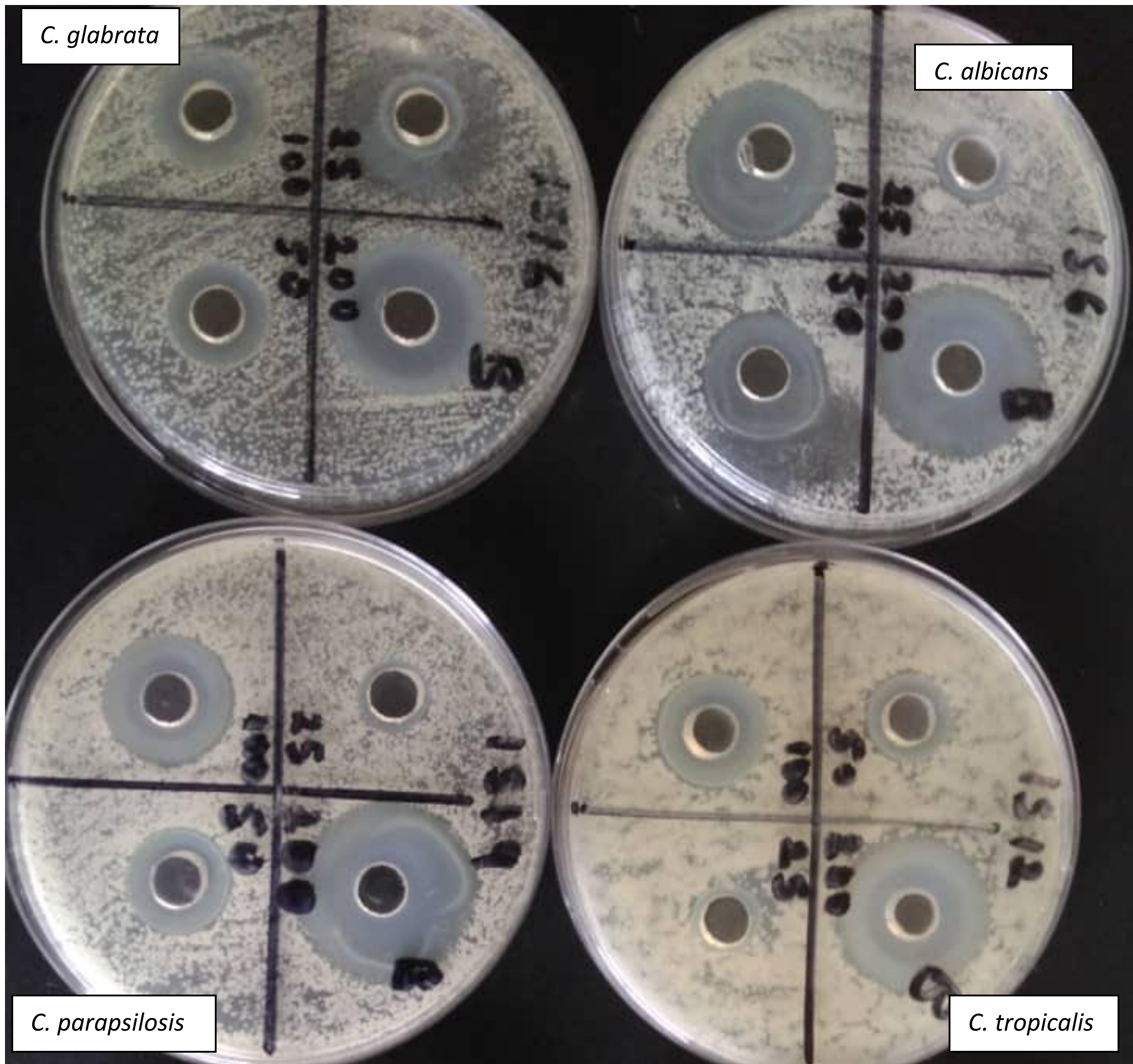


Plate 11b: Inhibition zone diameter of potash alum against the isolates

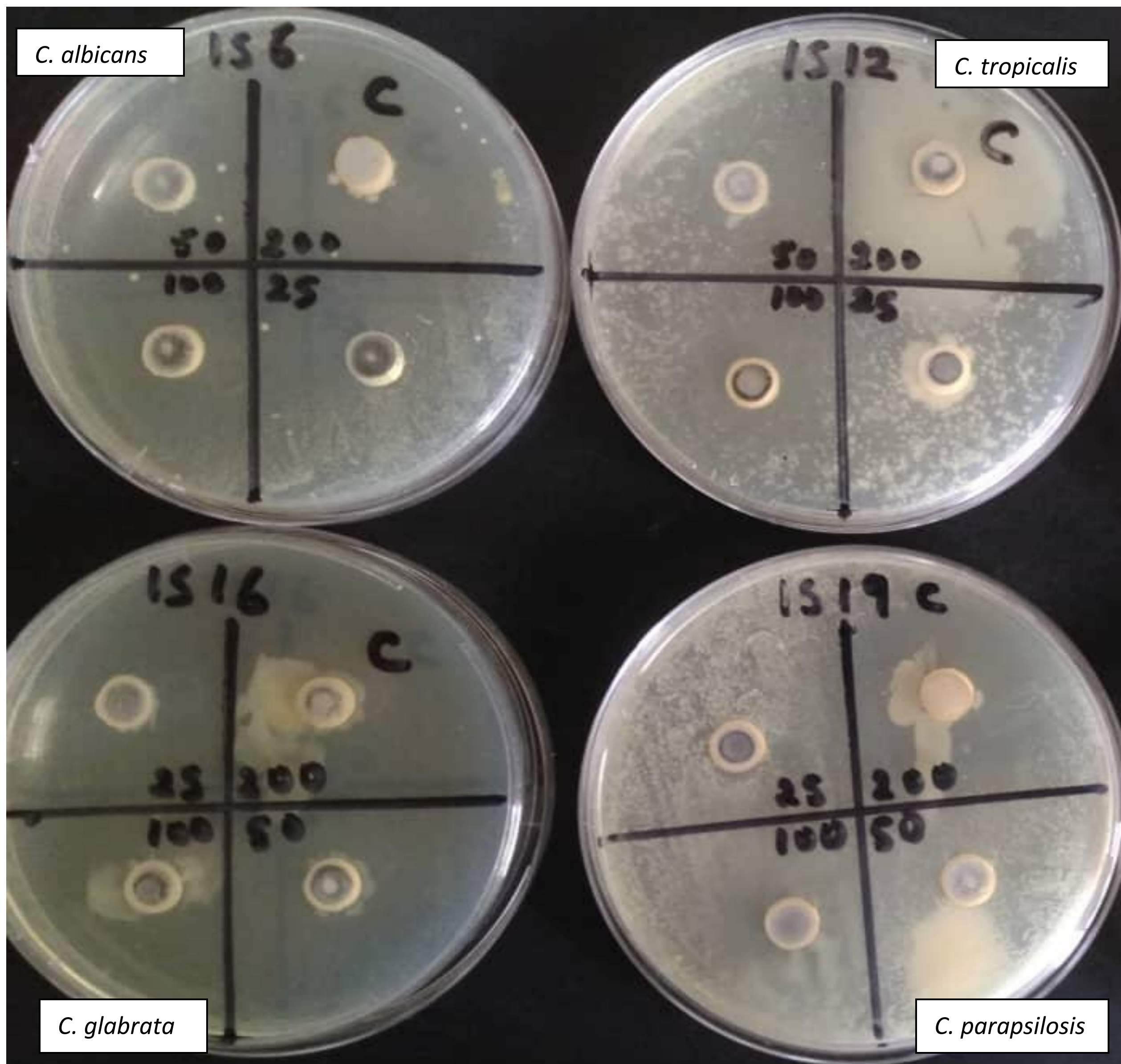


Plate 11c: Inhibition zone diameter of trona (akanwu) against the isolates

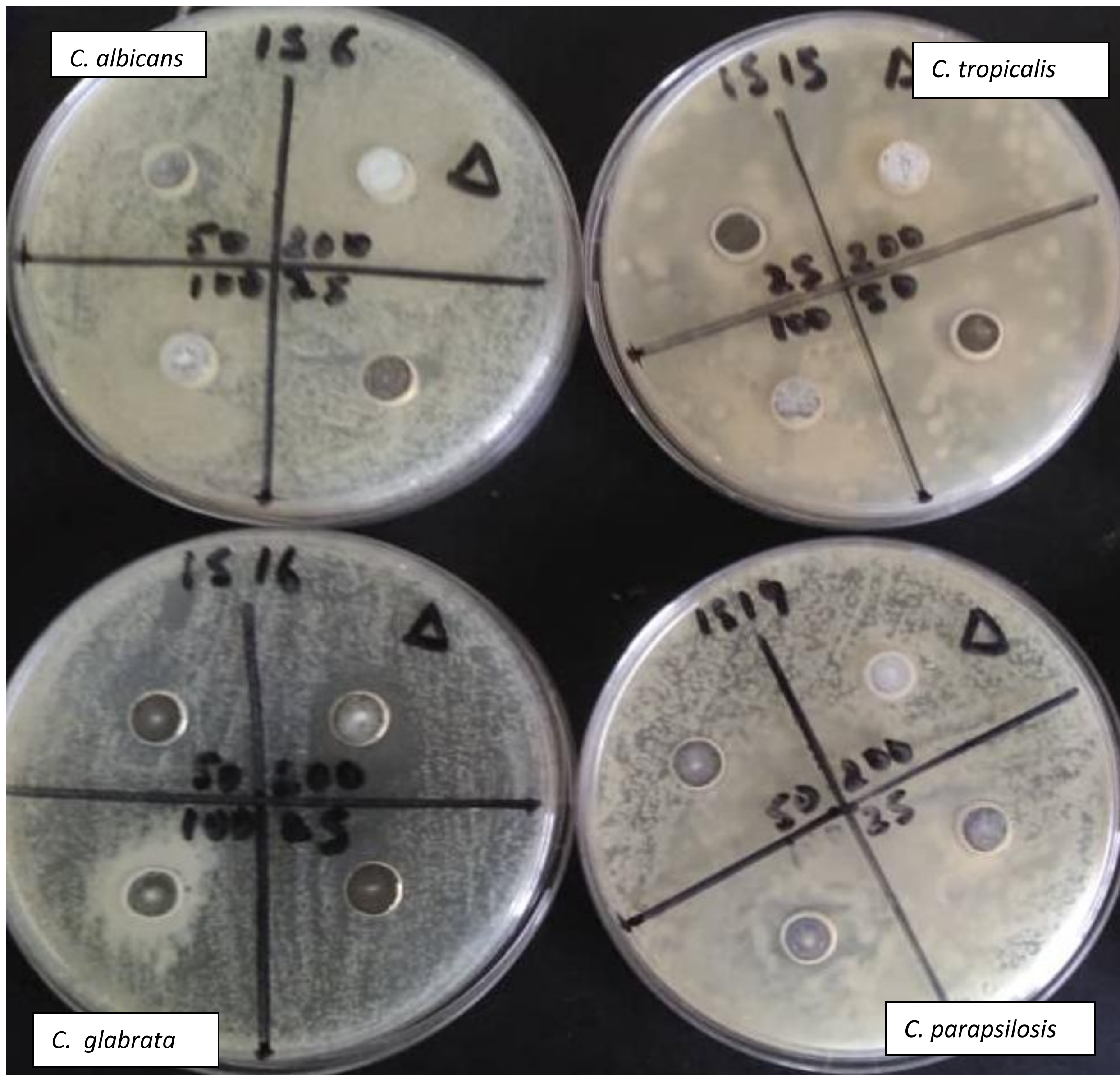


Plate 11d: Inhibition zone diameter of sulphur stone against the isolates

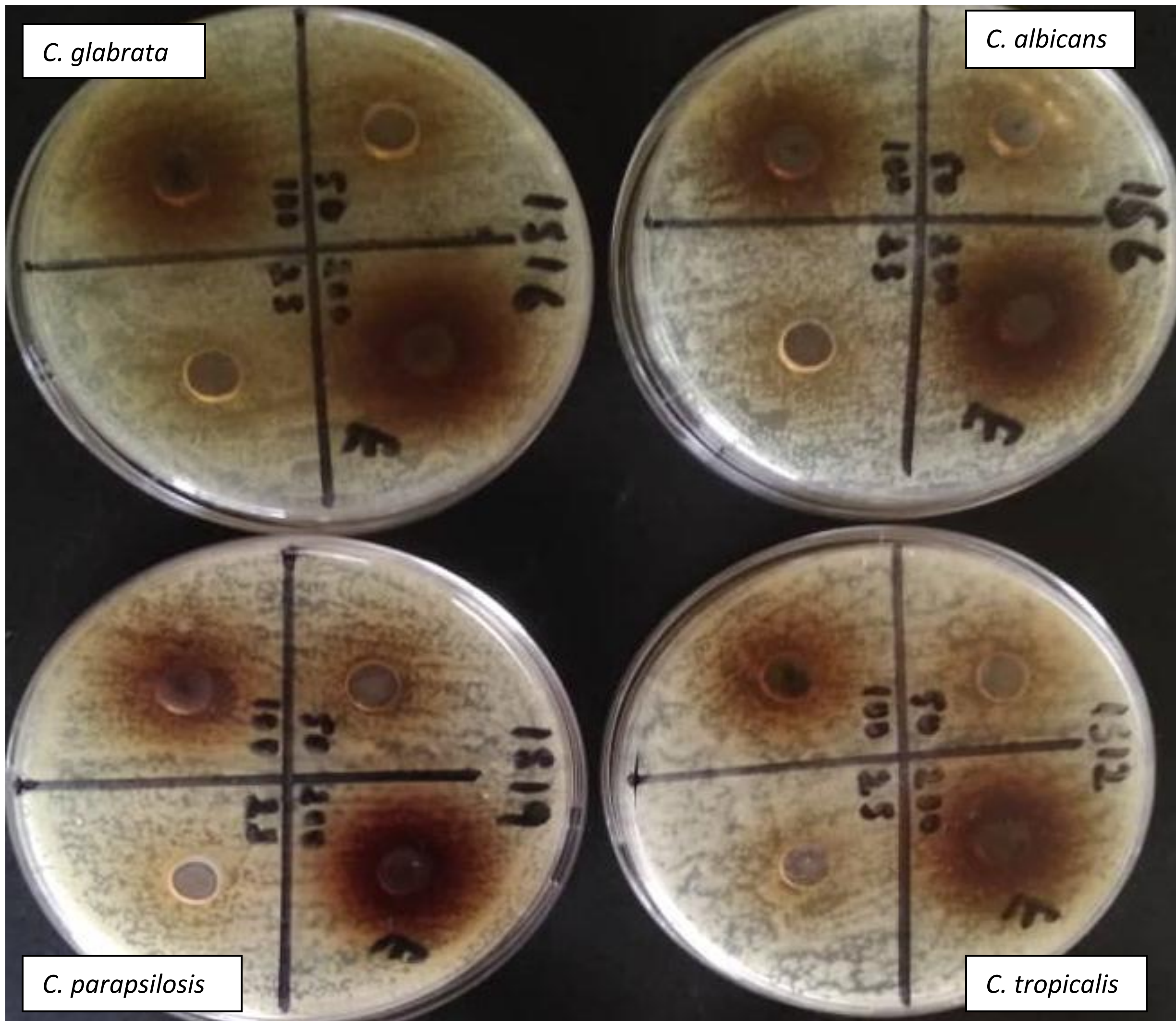


Plate 11e: Inhibition zone diameter of black stone against the isolates

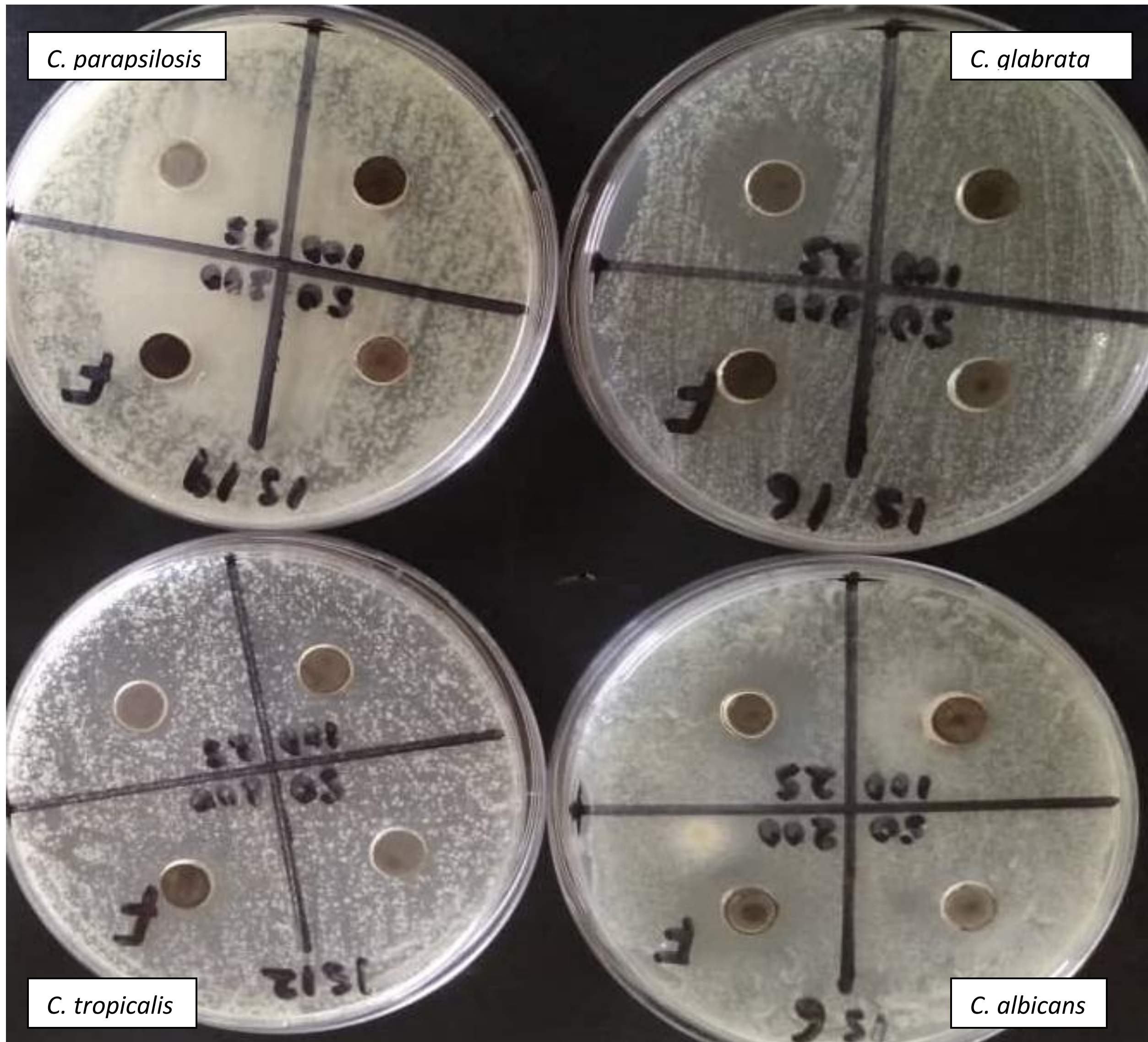


Plate 11f: Inhibition zone diameter of palm ash against the isolates

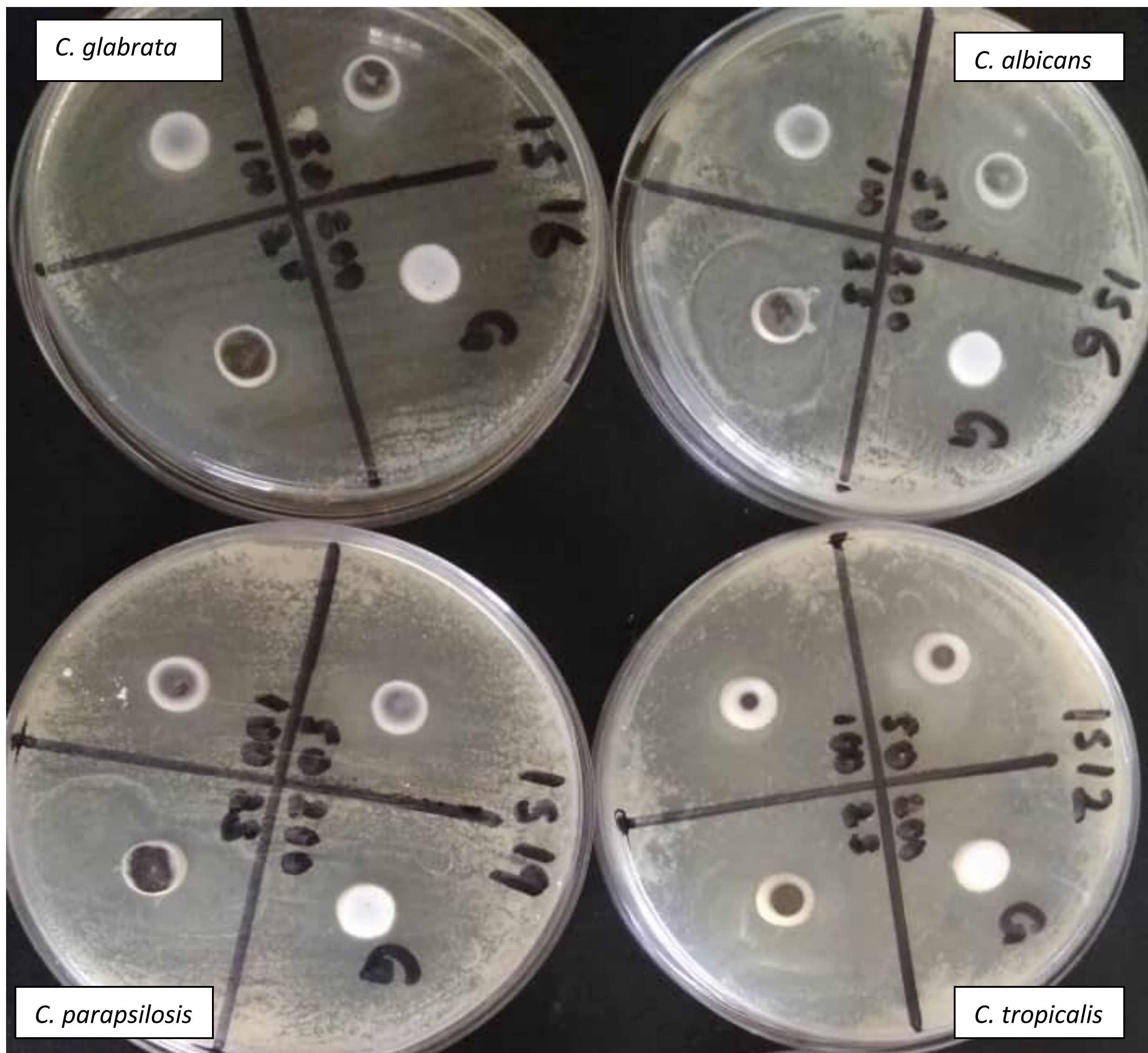


Plate 11g: Inhibition zone diameter of ketoconazole (+ control) against the isolates

MIC determination of the natural compounds on isolates using broth dilution method

Blue stone (50mg/ml) and potash alum (50mg/ml) had the highest MIC values while sulphur stone (200mg/ml) and Black stone (200mg/ml) had the lowest MIC values against *C. albicans*. The positive control, ketoconazole gave an MIC of 50mg/ml as shown in Table 7. Blue stone (25mg/ml) and potash alum (25mg/ml) had the highest MIC values while Black stone (200mg/ml) had the lowest MIC against *C. tropicalis*. The positive control, ketoconazole gave an MIC of 50mg/ml as shown in Table 8.

Table 9 showed the MIC of all the test agents against *C. glabrata*. Trona (6.25mg/ml) gave the best MIC value while Black stone (200mg/ml) was the least. The positive control, ketoconazole gave an MIC of 50mg/ml. Blue stone (12.50mg/ml), potash alum (12.50mg/ml), trona (12.50mg/ml) had the best MIC values while Black stone (200mg/ml) was the least against *C. parapsilosis*. The positive control, ketoconazole gave an MIC of 25mg/ml as shown in Table 10.

Table 7: MIC determination of all the test agents against *Candida albicans* using broth dilution method

Concentration (mg/ml)	Blue stone	Potash alum	Trona	Sulphur stone	Black stone	Palm ash	Ketoconazole (Control)
1.56	+	+	+	+	+	+	+
3.13	+	+	+	+	+	+	+
6.25	+	+	+	+	+	+	+
12.50	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+
50	-	-	+	+	+	+	-
100	-	-	-	+	+	-	-
200	-	-	-	-	-	-	-

+ Presence of growth

- No visible growth

Table 8: MIC determination of all the test agents against *Candida tropicalis* using broth dilution method

Concentration (mg/ml)	Blue stone	Potash alum	Trona	Sulphur stone	Black stone	Palm ash	Ketoconazole (Control)
1.56	+	+	+	+	+	+	+
3.13	+	+	+	+	+	+	+
6.25	+	+	+	+	+	+	+
12.50	+	+	+	+	+	+	+
25	-	-	+	+	+	+	-
50	-	-	-	-	+	+	-
100	-	-	-	-	+	-	-
200	-	-	-	-	-	-	-

+ Presence of growth

- No visible growth

Table 9: MIC determination of all the test agents against *Candida glabrata* using broth dilution method

Concentration (mg/ml)	Blue stone	Potash alum	Trona	Sulphur stone	Black stone	Palm ash	Ketoconazole (Control)
1.56	+	+	+	+	+	+	+
3.13	+	+	+	+	+	+	+
6.25	+	+	-	+	+	+	+
12.50	-	-	-	+	+	+	+
25	-	-	-	+	+	+	+
50	-	-	-	-	+	+	-
100	-	-	-	-	+	-	-
200	-	-	-	-	-	-	-

+ Presence of growth

- No visible growth

Table 10: MIC determination of all the test agents against *Candida parapsilosis* using broth dilution method

Concentration (mg/ml)	Blue stone	Potash alum	Trona	Sulphur stone	Black stone	Palm Ash	Ketoconazole (Control)
1.56	+	+	+	+	+	+	+
3.13	+	+	+	+	+	+	+
6.25	+	+	-	+	+	+	+
12.50	-	-	-	+	+	+	-
25	-	-	-	+	+	+	-
50	-	-	-	+	+	+	-
100	-	-	-	-	+	-	-
200	-	-	-	-	-	-	-

\+ Presence of growth

\- No visible growth

MFC determination of the natural compounds on isolates using broth dilution method

Table 11 showed the MFC of all the test agents against *C. albicans*. Potash alum (50mg/ml), trona (100mg/ml) had the highest MFC while sulphur stone (200mg/ml) and Black stone (200mg/ml) had the least MFC values. The positive control, ketoconazole gave an MFC of 50mg/ml. Blue stone (50mg/ml) and potash alum (50mg/ml) had the best MFC values against *C. tropicalis* while Black stone (200mg/ml) had the least value. The positive control, ketoconazole gave an MFC of 50mg/ml against *C. tropicalis* as shown in Table 12.

The MFC of Trona (12.50mg/ml) against *C. glabrata* was the best among the natural stones while potash alum (25mg/ml) was the best among the potash compounds. Black stone (200mg/ml) had the least MFC. The positive control, ketoconazole gave an MFC of 50mg/ml as shown in Table 13. Blue stone (25mg/ml), potash alum (25mg/ml) and trona (25mg/ml) had the highest MFC while Black stone (200mg/ml) had the least MFC against *C. parapsilosis*. The positive control, ketoconazole gave an MFC of 25mg/ml as shown in Table 14.

Table 11: MFC determination of all the test agents against *Candida albicans* using broth dilution method

Concentration (mg/ml)	Blue stone	Potash alum	Trona	Sulphur stone	Black stone	Palm ash	Ketoconazole (Control)
1.56	+	+	+	+	+	+	+
3.13	+	+	+	+	+	+	+
6.25	+	+	+	+	+	+	+
12.50	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+
50	+	-	+	+	+	+	-
100	-	-	-	+	+	-	-
200	-	-	-	-	-	-	-

+ Presence of growth

- No visible growth

Table 12: MFC determination of all the test agents against *Candida tropicalis* using broth dilution method

Concentration (mg/ml)	Blue stone	Potash alum	Trona	Sulphur stone	Black stone	Palm ash	Ketoconazole (Control)
1.56	+	+	+	+	+	+	+
3.13	+	+	+	+	+	+	+
6.25	+	+	+	+	+	+	+
12.50	+	+	+	+	+	+	+
25	+	+	+	+	+	+	-
50	-	-	+	+	+	+	-
100	-	-	-	-	+	+	-
200	-	-	-	-	-	-	-

+ Presence of growth

- No visible growth

Table 13: MFC determination of all the test agents against *Candida glabrata* using broth dilution method

Concentration (mg/ml)	Blue stone	Potash alum	Trona	Sulphur stone	Black stone	Palm ash	Ketoconazole (Control)
1.56	+	+	+	+	+	+	+
3.13	+	+	+	+	+	+	+
6.25	+	+	+	+	+	+	+
12.50	+	-	-	+	+	+	+
25	-	-	-	+	+	+	+
50	-	-	-	-	+	+	-
100	-	-	-	-	+	-	-
200	-	-	-	-	-	-	-

+ Presence of growth

- No visible growth

Table 14: MFC determination of all the test agents against *Candida parapsilosis* using broth dilution method

Concentration (mg/ml)	Blue stone	Potash alum	Trona	Sulphur stone	Black stone	Palm ash	Ketoconazole (Control)
1.56	+	+	+	+	+	+	+
3.13	+	+	+	+	+	+	+
6.25	+	+	+	+	+	+	+
12.50	+	+	+	+	+	+	+
25	-	-	-	+	+	+	-
50	-	-	-	+	+	+	-
100	-	-	-	-	+	+	-
200	-	-	-	-	-	-	-

+ Presence of growth

- No visible growth

Percentage inhibition of the natural compounds at different concentrations using the microdilution method

Blue stone and potash alum showed the best efficacy (95.9% inhibition) at 1.52mg/ml (MIC) while black stone was the least (0%) as shown in Table 15. Concentration inhibiting at least 20% fungal growth is considered MIC. Table 16 shows the percentage fungal inhibition of *C. tropicalis* at different dilutions of the test compounds with blue stone and potash alum showing the best efficacy (99.1% inhibition) at 1.52mg/ml while black stone was the least (36.6%) at 50mg/ml.

Blue stone showed the best efficacy (99.5% inhibition) against *C. glabrata* at 1.52mg/ml while black stone was the least (15.4%) at 12.50mg/ml as shown in Table 17. The percentage fungal inhibition of *C. parapsilosis* at different dilutions of the test compounds was shown in Table 18, with blue stone showing the best efficacy (65.2% inhibition) at 1.52mg/ml while black stone was the least (13.5%) at 25mg/ml. Concentration inhibiting at least 20% fungal growth is considered MIC.

Table 15: Percentage of fungal inhibition of *Candida albicans* at different concenmtrations of the test compounds

Concentration (mg/ml)	Blue stone	Potash alum	Trona	Sulphur stone	Black stone	Palm ash	Ketoconazole (Control)
1.56	95.9	95.9	-	-	-	-	95.9
3.13	95.9	95.9	-	-	-	-	95.9
6.25	95.9	95.9	-	-	-	75.6	95.9
12.50	95.9	95.9	-	-	-	95.9	95.9
25	95.9	95.9	95.9	-	-	95.9	95.9
50	95.9	95.9	95.9	95.9	-	95.9	95.9
100	95.9	95.9	95.9	95.9	-	95.9	95.9
200	95.9	95.9	95.9	95.9	-	95.9	95.9

NB. Concentration inhibiting at least 20% fungal growth is considered MIC

Table 16: Percentage fungal inhibition of *Candida tropicalis* at different concentrations of the test compounds

Concentration (mg/ml)	Blue stone	Potash alum	Trona	Sulphur stone	Black stone	Palm ash	Ketoconazole (Control)
1.56	99.1	99.1	-	-	-	-	99.1
3.13	99.1	99.1	-	-	-	13.3	99.1
6.25	99.1	99.1	-	7.3	-	63.7	99.1
12.50	99.1	99.1	22.4	26.6	-	71	99.1
25	99.1	99.1	80.7	80.7	-	99.1	99.1
50	99.1	99.1	99.1	99.1	36.6	99.1	99.1
100	99.1	99.1	99.1	99.1	37.8	99.1	99.1
200	99.1	99.1	99.1	99.1	80.4	99.1	99.1

NB. Concentration inhibiting at least 20% fungal growth is considered MIC

Table 17: Percentage fungal inhibition of *Candida glabrata* at different concentrations of the test compounds

Concentration (mg/ml)	Blue stone	Potash alum	Trona	Sulphur stone	Black stone	Palm ash	Ketoconazole (Control)
1.56	99.5	92.5	-	-	-	-	99.5
3.13	99.5	92.5	-	-	-	49	99.5
6.25	99.5	99.5	39.1	10.1	-	51	99.5
12.50	99.5	99.5	41	21.1	15.4	56	99.5
25	99.5	99.5	58.4	51	23.7	76	99.5
50	99.5	99.5	80.8	87	29.6	81.8	99.5
100	99.5	99.5	89.7	91	33.1	87.8	99.5
200	99.5	99.5	91	99.5	42.3	98.6	99.5

NB. Concentration inhibiting at least 20% fungal growth is considered MIC

Table 18: Percentage of fungal inhibition of *Candida parapsilosis* at different concentrations of the test compounds

Concentration (mg/ml)	Blue stone	Potash alum	Trona	Sulphur stone	Black stone	Palm ash	Ketoconazole (Control)
1.56	65.2	46.3	-	-	-	-	97.1
3.13	97.1	65.2	19.7	18.7	-	-	97.1
6.25	97.1	97.1	29.1	37.8	-	20.8	97.1
12.50	97.1	97.1	42.1	55.6	-	33.9	97.1
25	97.1	97.1	49.2	84.5	13.5	41.1	97.1
50	97.1	97.1	66.1	97.1	23.7	53.2	97.1
100	97.1	97.1	73.6	97.1	69.1	67.7	97.1
200	97.1	97.1	94.1	97.1	83.6	74.9	97.1

NB. Concentration inhibiting at least 20% fungal growth is considered MIC

Antimicrobial activity of combined natural compounds

The fractional inhibitory concentration index (FIC index) showed the outcome of the combination. If the FIC index is less than one, the combined activity is synergism, between 1 and 2 (Indifference) while above 2 is antagonism. In Table 19, all test agents were synergistic against *C. albicans* (FIC index < 1). Table 20 showed the antimicrobial activity of the combined natural compounds against *C. tropicalis.* All the test agents were indifferent against *C. tropicalis* (FIC index between 1 and 2) while Blue stone + Sulphur stone + Black stone combination was antagonistic (FIC index > 2) against the isolate.

All the test agents were antagonistic against *C. glabrata* (FIC index > 2) while Sulphur stone + Black stone combination was indifferent (FIC index between 1and 2) against the isolate as shown in Table 21. The combined activity against *C. parapsilosis* varied among the test agents as shown in Table 22: Potash alum + trona, potash alum + palm ash, Blue stone + Black stone and Blue stone + Sulphur stone + Black stone were all antagonistic (FIC index > 3); Blue stone + Sulphur stone (indifferent with FIC index between 1 and 2); while Sulphur stone + Black stone (Synergistic with FIC index < 1).

Table 19: Combined activity of natural compounds against *Candida albicans*

Agent	MIC A (mg/ml)	MIC B (mg/ml)	MIC C (mg/ml)	Combined MIC	FIC A	FIC B	FIC C	FIC index	INFERENCE
Potash Alum + Trona (mm)	50	100	-	17.89	0.3578	0.1789	-	0.5367	Synergism
Potash alum + palm ash	50	100	-	20.77	0.4154	0.2077	-	0.6231	Synergism
Blue stone + Sulphur stone	50	200	-	20.63	0.4126	0.10315	-	0.51575	Synergism
Blue stone + Black stone	50	200	-	35.7	0.714	0.1785	-	0.8925	Synergism
Sulphur stone + Black stone	200	200	-	35.1	0.1755	0.1755	-	0.351	Synergism
Blue stone + Sulphur stone + Black stone	50	200	200	32.71	0.6542	0.16355	0.16355	0.9813	Synergism

Key:

MIC A: Minimum inhibitory concentration of first agent in combination ratio.

MIC B: Minimum inhibitory concentration of second agent in combination ratio

MIC C: MIC A: Minimum inhibitory concentration of third agent in combination ratio

FIC: Fractional inhibitory concentration

FIC index= FIC A + FIC B

$$\text{FIC A} = \frac{\text{Mic of Drug A in combination with Drug B}}{\text{Mic of Drug A alone}} \qquad \text{FIC B} = \frac{\text{Mic of Drug B in combination with Drug A}}{\text{Mic of Drug B alone}}$$

NB. Synergism: FIC index<1.0; Additive: FIC index = 1.0; Indifference: FIC index between 1.0 & 2.0; Antagonism: FIC index >2.0 and; No activity: FIC index = 0.

Table 20: Combined activity of natural compounds against *Candida tropicalis*

Agent	MIC A (mg/ml)	MIC B (mg/ml)	MIC C (mg/ml)	Combined MIC	FIC A	FIC B	FIC C	FIC index	INFERENCE
Potash Alum + Trona (mm)	25	50	-	21.55	0.862	0.431	-	1.293	Indifference
Potash alum + palm ash	25	100	-	37.02	1.4808	0.3702	-	1.851	Indifference
Blue stone + Sulphur stone	25	50	-	20.76	0.8304	0.4152	-	1.2456	Indifference
Blue stone + Black stone	25	200	-	33.77	1.3508	0.16885	-	1.51965	Indifference
Sulphur stone + Black stone	50	200	-	56.14	1.1228	0.2807	-	1.4035	Indifference
Blue stone + Sulphur stone + Black stone	25	50	200	61.87	2.4748	1.2374	0.30935	4.02155	Antagonism

MIC A: Minimum inhibitory concentration of first agent in combination ratio.

MIC B: Minimum inhibitory concentration of second agent in combination ratio

MIC C: MIC A: Minimum inhibitory concentration of third agent in combination ratio

FIC: Fractional inhibitory concentration

Table 21: Combined activity of natural compounds against *Candida glabrata*

Agent	MIC A (mg/ml)	MIC B (mg/ml)	MIC C (mg/ml)	Combined MIC	FIC A	FIC B	FIC C	FIC index	INFERENCE
Potash Alum + Trona (mm)	12.5	6.25	-	21.55	1.724	3.448	-	5.172	Antagonism
Potash alum + palm ash	12.5	100	-	37.02	2.9616	0.3702	-	3.3318	Antagonism
Blue stone + Sulphur stone	12.5	50	-	20.76	1.6608	0.4152	-	2.076	Antagonism
Blue stone + Black stone	12.5	200	-	33.77	2.7016	0.16885	-	2.87045	Antagonism
Sulphur stone + Black stone	50	200	-	56.14	1.1228	0.2807	-	1.4035	Indifference
Blue stone + Sulphur stone + Black stone	12.5	50	200	61.87	4.9496	1.2374	0.30935	6.49635	Antagonism

MIC A: Minimum inhibitory concentration of first agent in combination ratio.

MIC B: Minimum inhibitory concentration of second agent in combination ratio

MIC C: MIC A: Minimum inhibitory concentration of third agent in combination ratio

FIC: Fractional inhibitory concentration

Table 22: Combined activity of natural compounds against *Candida parapsilosis*

Agent	MIC A (mg/ml)	MIC B (mg/ml)	MIC C (mg/ml)	Combined MIC	FIC A	FIC B	FIC C	FIC index	INFERENCE
Potash Alum + Trona (mm)	12.5	12.5	-	21.55	1.724	1.724	-	3.448	Antagonism
Potash alum + palm ash	12.5	100	-	37.02	2.9616	0.3702	-	3.3318	Antagonism
Blue stone + Sulphur stone	12.5	100	-	20.76	1.6608	0.2076	-	1.8684	Indifference
Blue stone + Black stone	12.5	200	-	33.77	2.7016	0.16885	-	2.87045	Antagonism
Sulphur stone + Black stone	100	200	-	56.14	0.5614	0.2807	-	0.8421	Synergism
Blue stone + Sulphur stone + Black stone	25	100	200	61.87	2.4748	0.6187	0.30935	3.40285	Antagonism

MIC A: Minimum inhibitory concentration of first agent in combination ratio.

MIC B: Minimum inhibitory concentration of second agent in combination ratio

MIC C: MIC A: Minimum inhibitory concentration of third agent in combination ratio

FIC: Fractional inhibitory concentration

Analysis of inorganic chemical constituents of the natural compounds using x-ray fluorescence technology

The inorganic components found in blue stone include oxides of copper (43.7%), sulphur (29.4%), aluminium (1.2%) etc. The toxic heavy metals which were present in very negligible amount include arsenic, lead and nickel (all <0.009%) as shown in figure 3. Potash alum comprises basically oxides of sulphur (44.5%), aluminium (14.49%) etc. The toxic heavy metals included oxides of lead, arsenic, nickel, all accounting for <0.0010% as shown in figure 4. The inorganic components found in trona included oxides of sulphur (2.14%), chlorine (5.8%), potassium (1.7%) etc while the toxic heavy metals included lead (0%), arsenic and nickel (all <0.003%) as shown in figure 5.

The major components of sulphur stone (brimstone) were elemental sulphur (94.72%) and aluminum (3.46%). lead, arsenic and nickel were less than 0.0007% as shown in figure 6.
Black stones comprised oxides of iron (1.9%), sulphur (1.64%), potassium (2%), copper (0.2%), chlorine (0.98%) etc. Oxides of nickel, lead and arsenic made up less than 0.002% as shown in figure 7. The inorganic components of palm ash included oxides of potassium (28.8%), sulphur (3.4%), calcium (2.8%), chlorine (15%) etc while the average concentration of toxic heavy metals was less than 0.0007% as shown in figure 8.

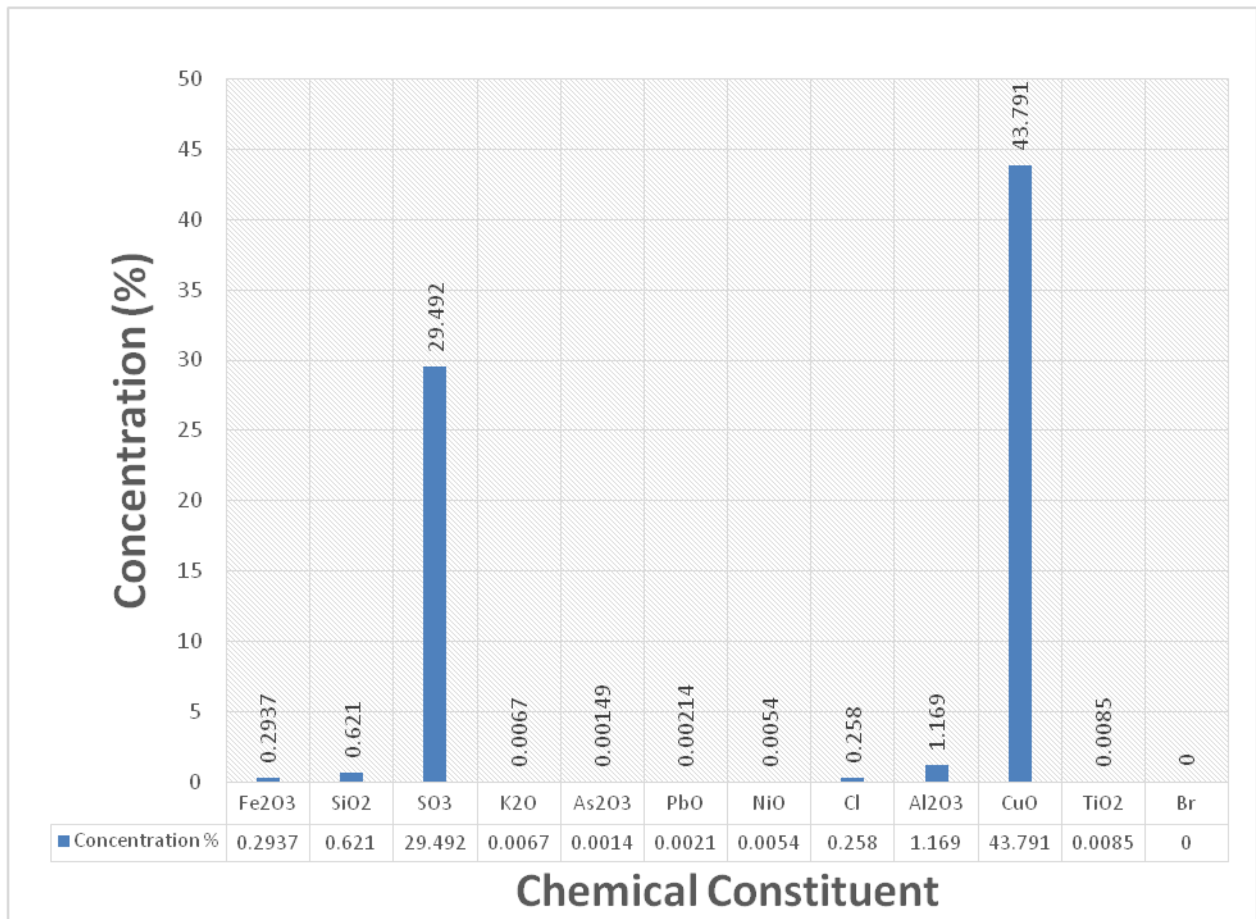


Figure 3: Inorganic chemical constituents of Blue stone (Blue vitriol)

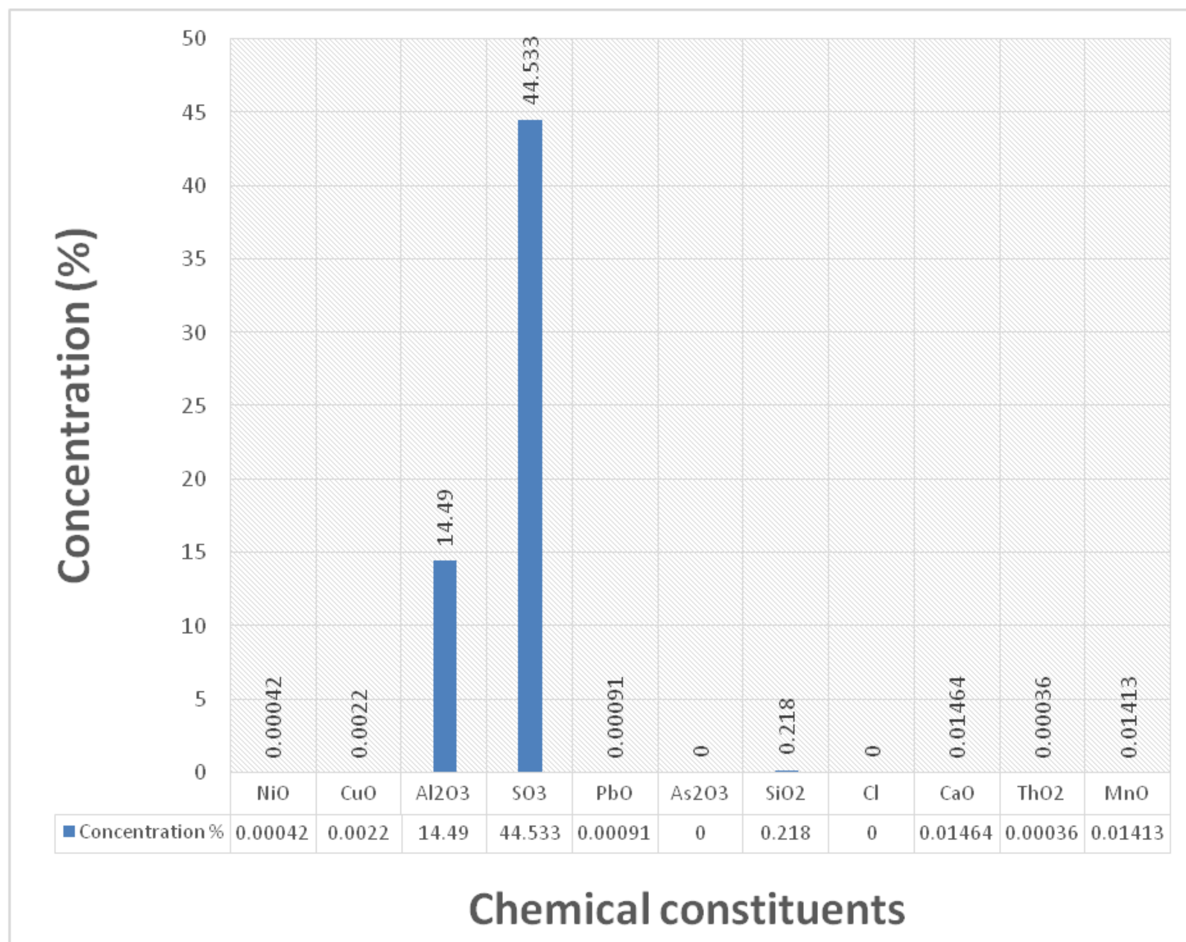


Figure 4: Inorganic chemical constituents of potash alum.

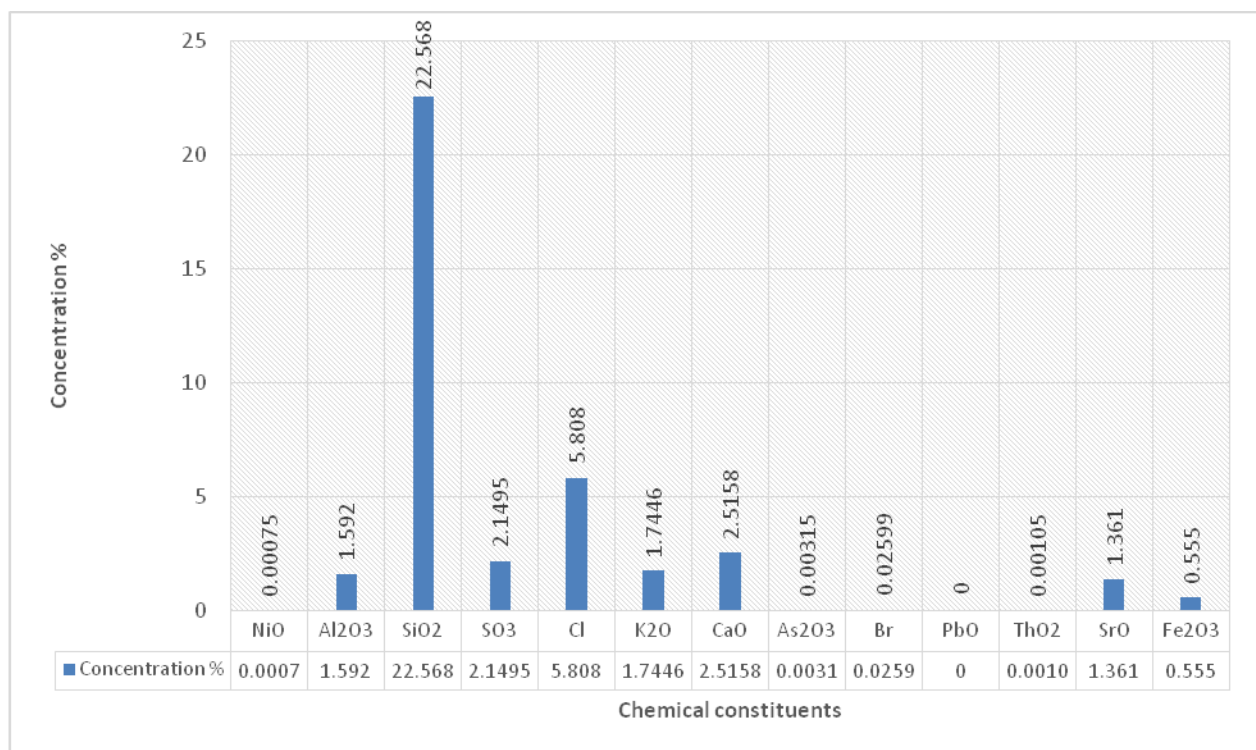


Figure 5: Inorganic chemical constituents of trona (Akanwu).

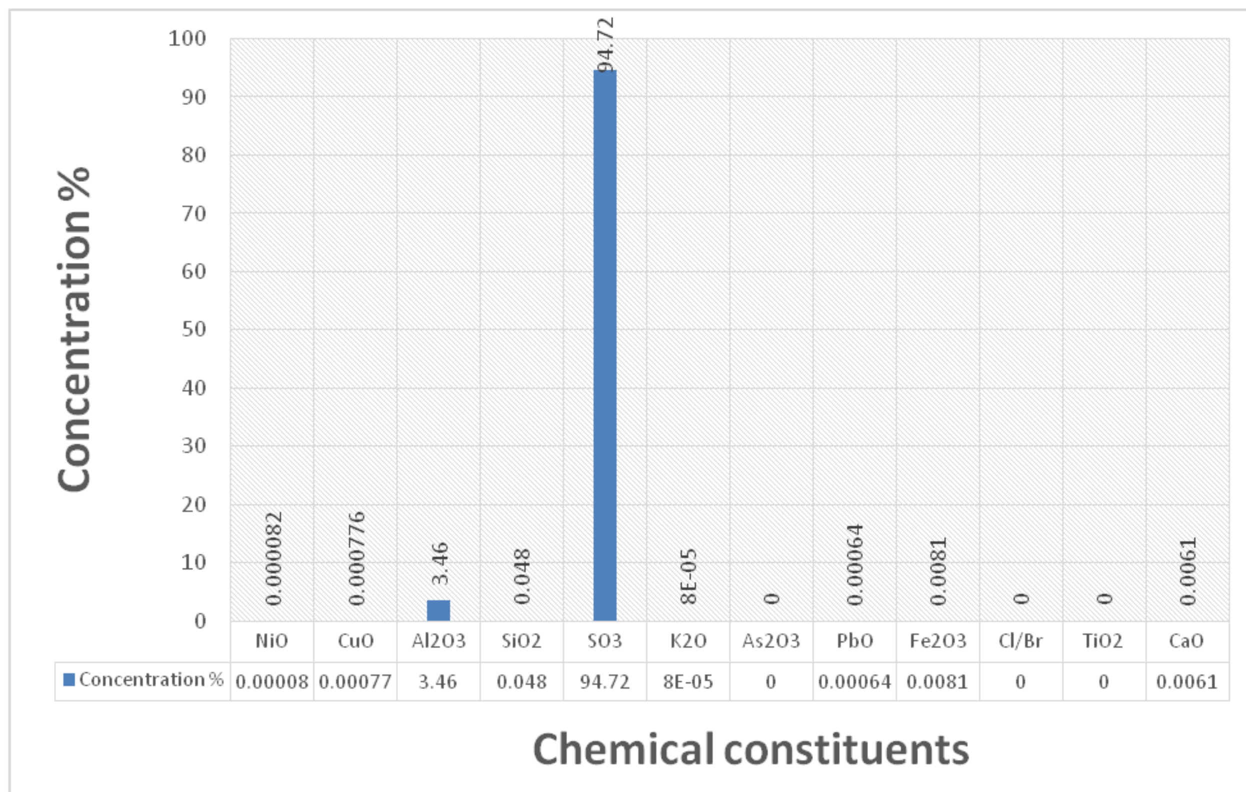


Figure 6: Inorganic chemical constituents of sulphur stone (Brimstone).

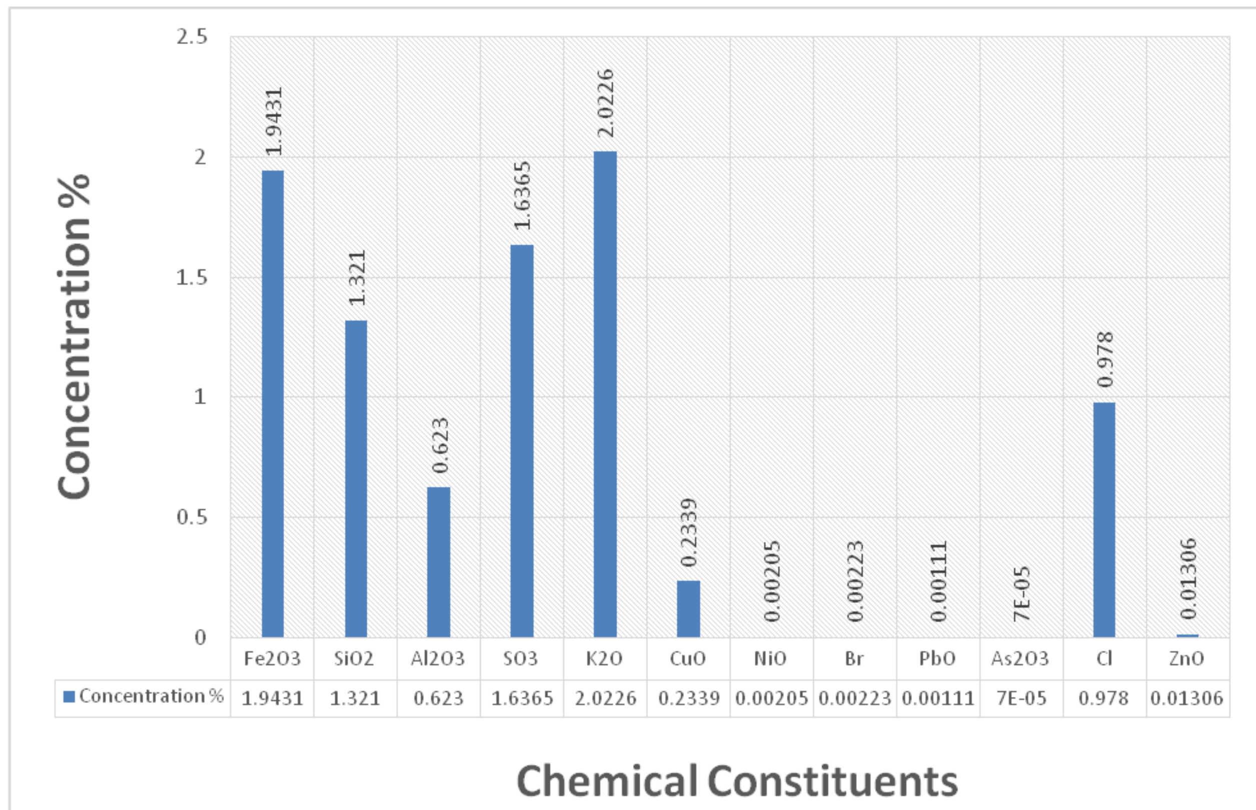
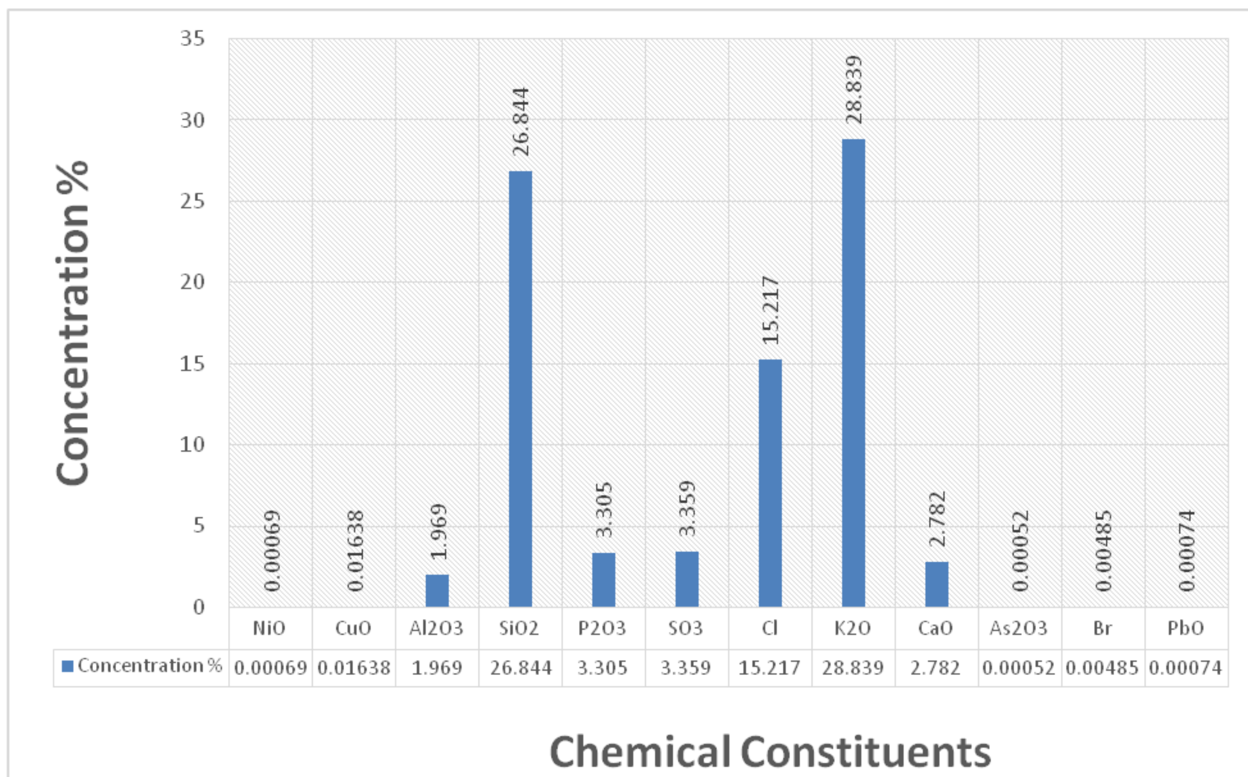


Figure 7: Inorganic chemical constituents of black stone (Snake stone).



	NiO	CuO	Al2O3	SiO2	P2O3	SO3	Cl	K2O	CaO	As2O3	Br	PbO
Concentration %	0.00069	0.01638	1.969	26.844	3.305	3.359	15.217	28.839	2.782	0.00052	0.00485	0.00074

Figure 8: Inorganic chemical constituents of palm ash (Ngu).

Analysis of organic chemical constituents of the natural compounds using GC-MS

The organic components found in blue stones were basically natural fatty acids such as linoelaidic acid (24.92%), oleic acid (12.97%), 6-octadecanoic acid (10.94%), trichloroacetic acid (0.81%), carbonic acid (0.66%) as shown in figure 9. The organic components of potash alum included cis-vaccenic acid (40.39%), hexadecanoic acid (13.21%), 9, 12-octadecadienoic acid (11.52%), Linoelaidic acid (9.84%), dimethylsulfoxide (0.69%) as shown in figure 10. Trona comprised linoelaidic acid (32.99%), 10, 12-hexadecadien-1-ol acetate, Nonanoic acid (14.81%), Hexadecanoic acid (6.8%) etc as shown in figure 11; while the organic components of sulphur stone were 9, 12-octadecadienoic acid (28.37%), oleic acid (10.26%), dimethylsulfoxide (1.01%) etc as shown in figure 12.

The organic components of black stone included 5-hexanoic acid (43.89%), D-glucopyranose (22.48%), thiirane (0.52%), acetic acid (0.4%) etc as shown in figure 13; while the organic components of palm ash included urea (8.65%), pentanoic acid (2.42%), 2-octyn-1-ol (1.17%) as shown in figure 14. Figure 15 showed the total yeast count at intervals of three days during treatment with blue stone. The yeast population gradually declined with time from 10^8 to 10^2.

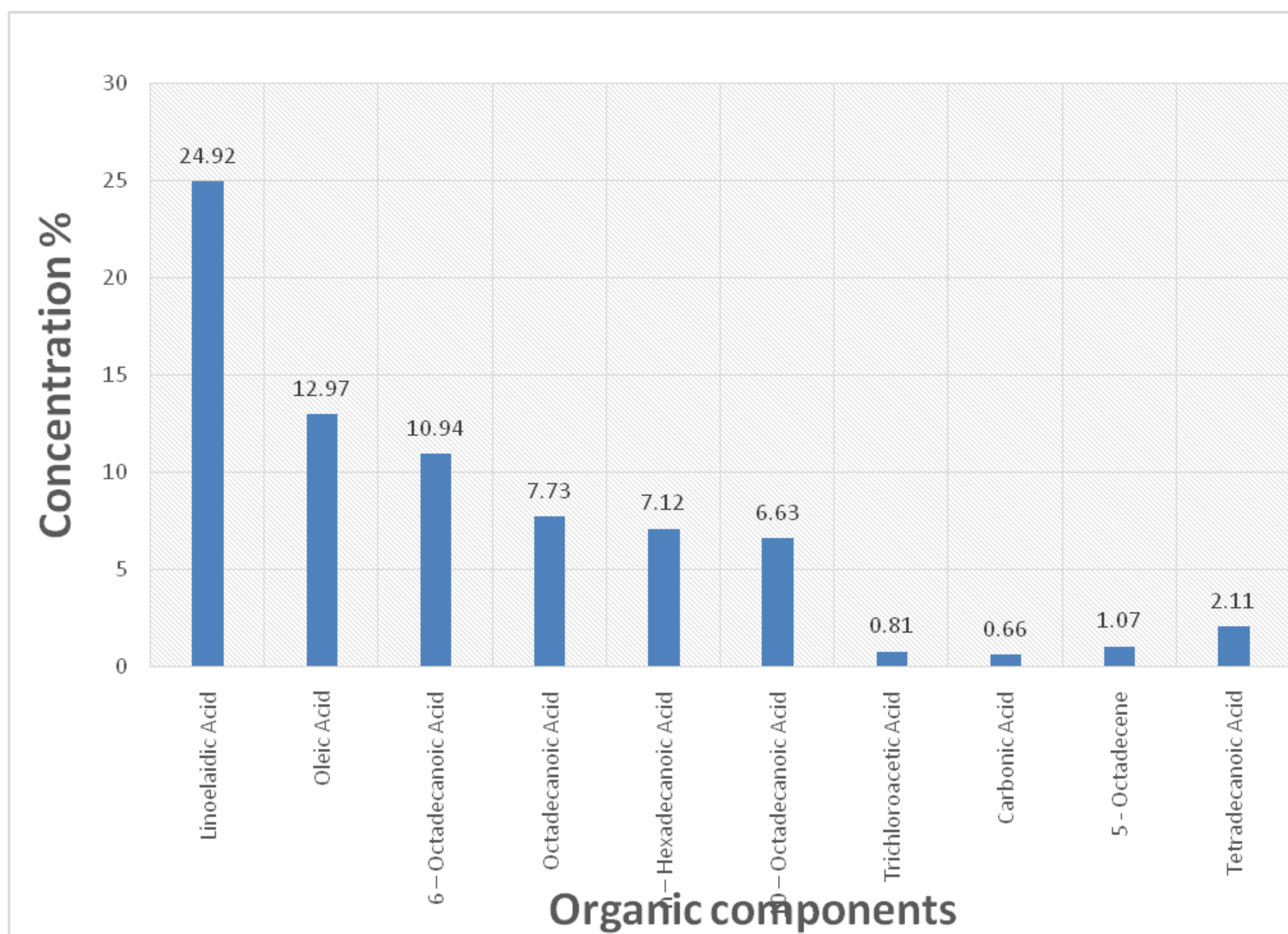


Figure 9: Organic chemical constituents of blue Stone (Blue vitriol).

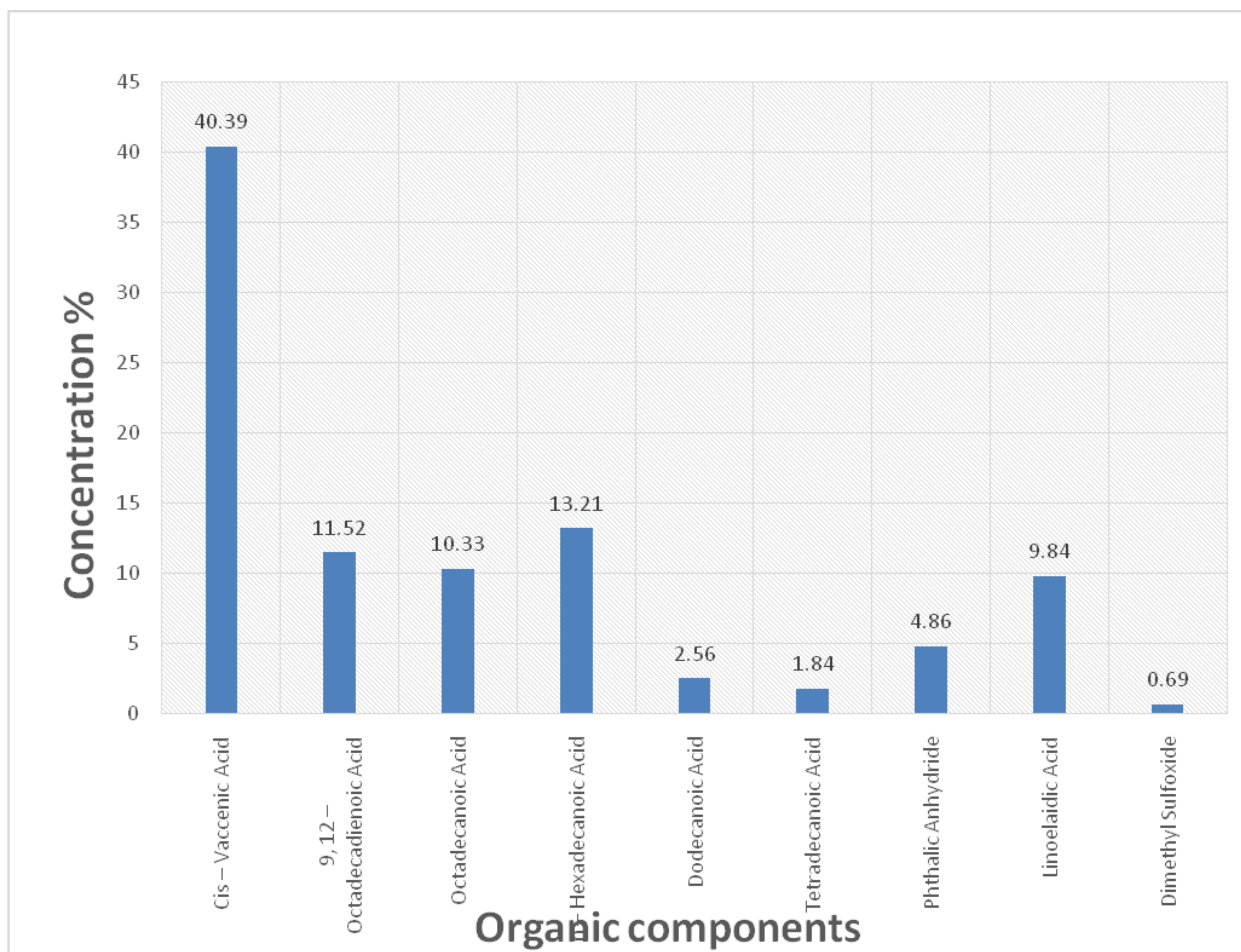


Figure 10: Organic chemical constituents of potash alum.

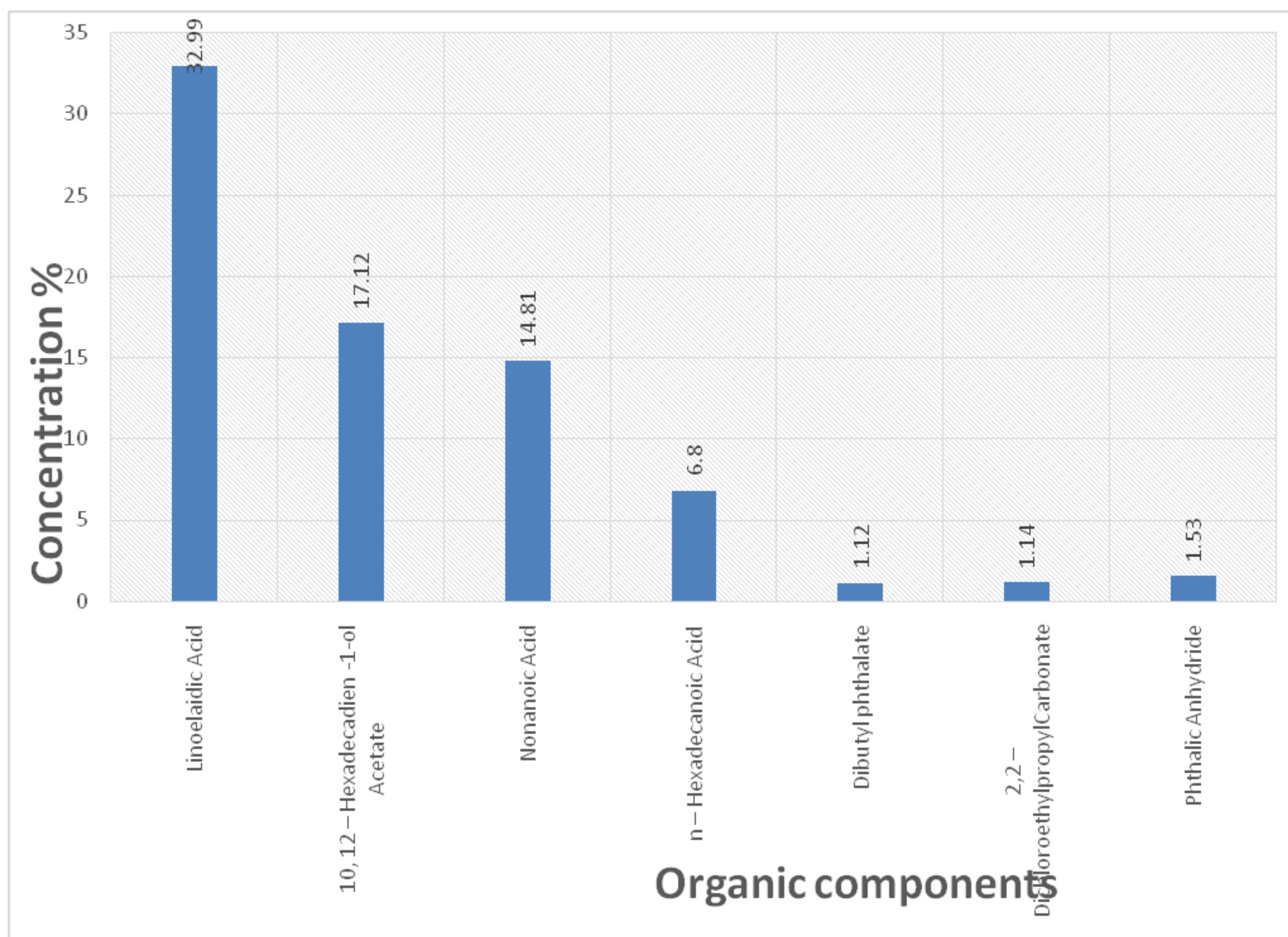


Figure 11: Organic chemical constituents of trona (Akanwu).

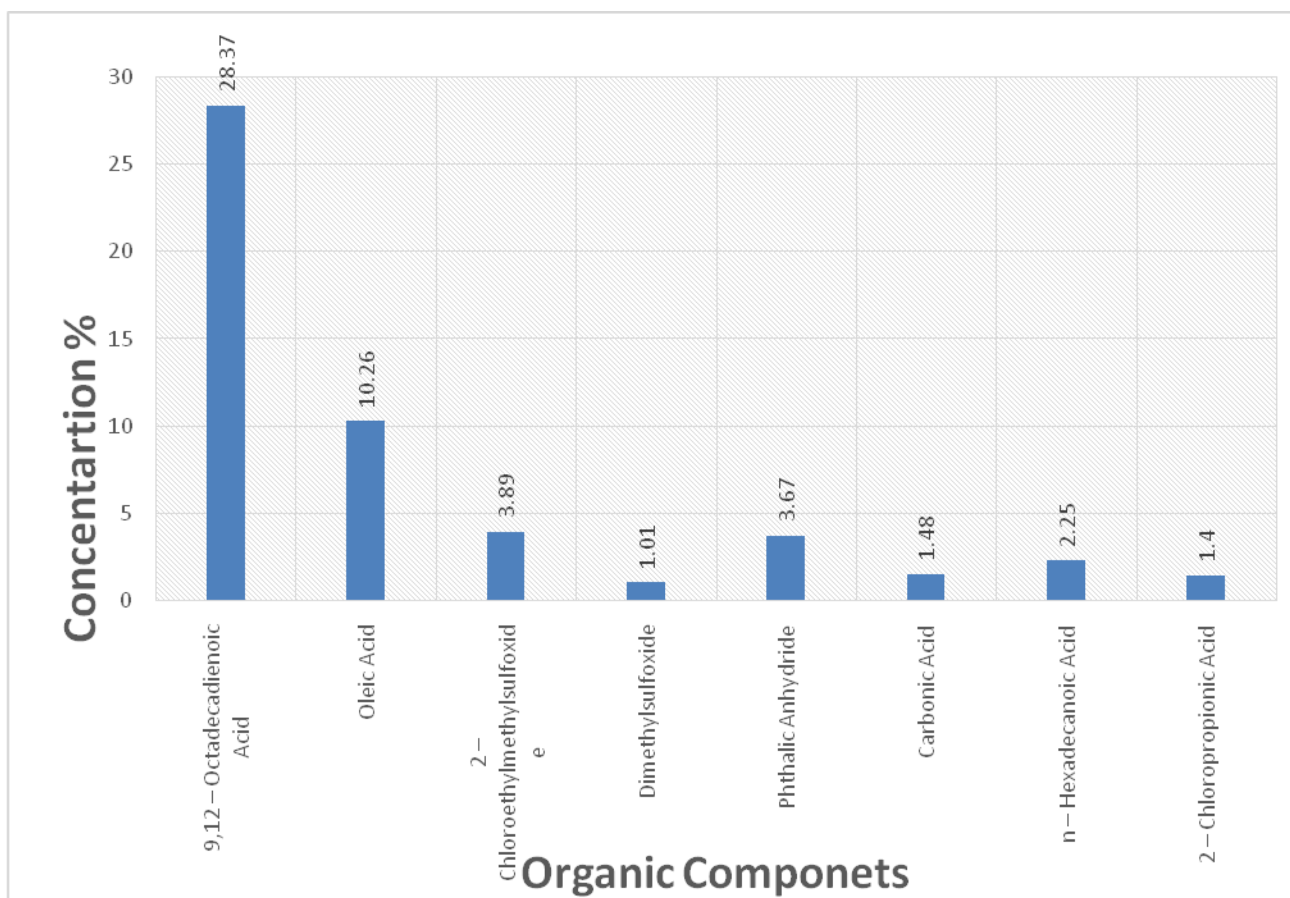


Figure 12: Organic chemical constituents of sulphur stone (brimstone).

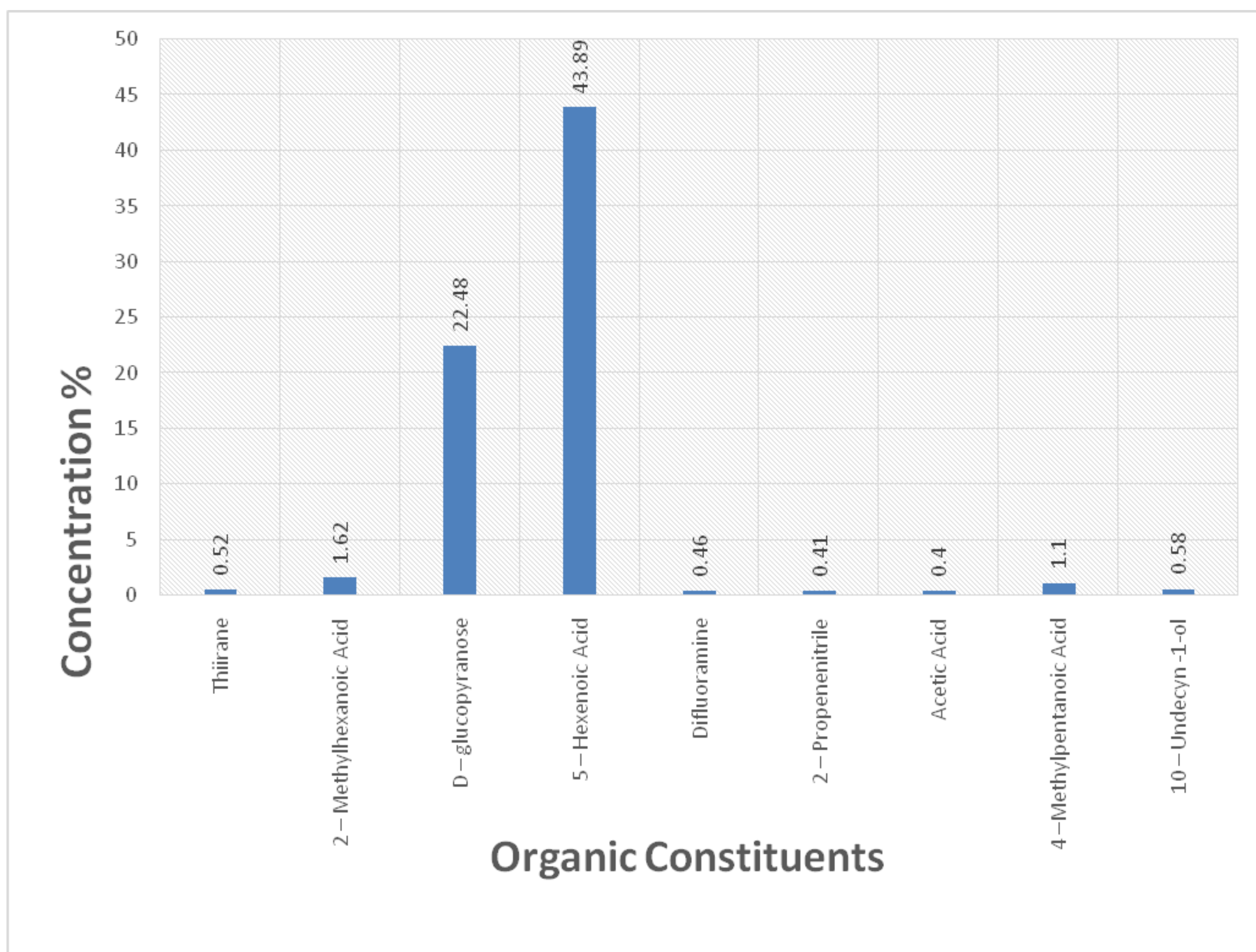


Figure 13: Organic chemical constituents of black stone (Snake stone).

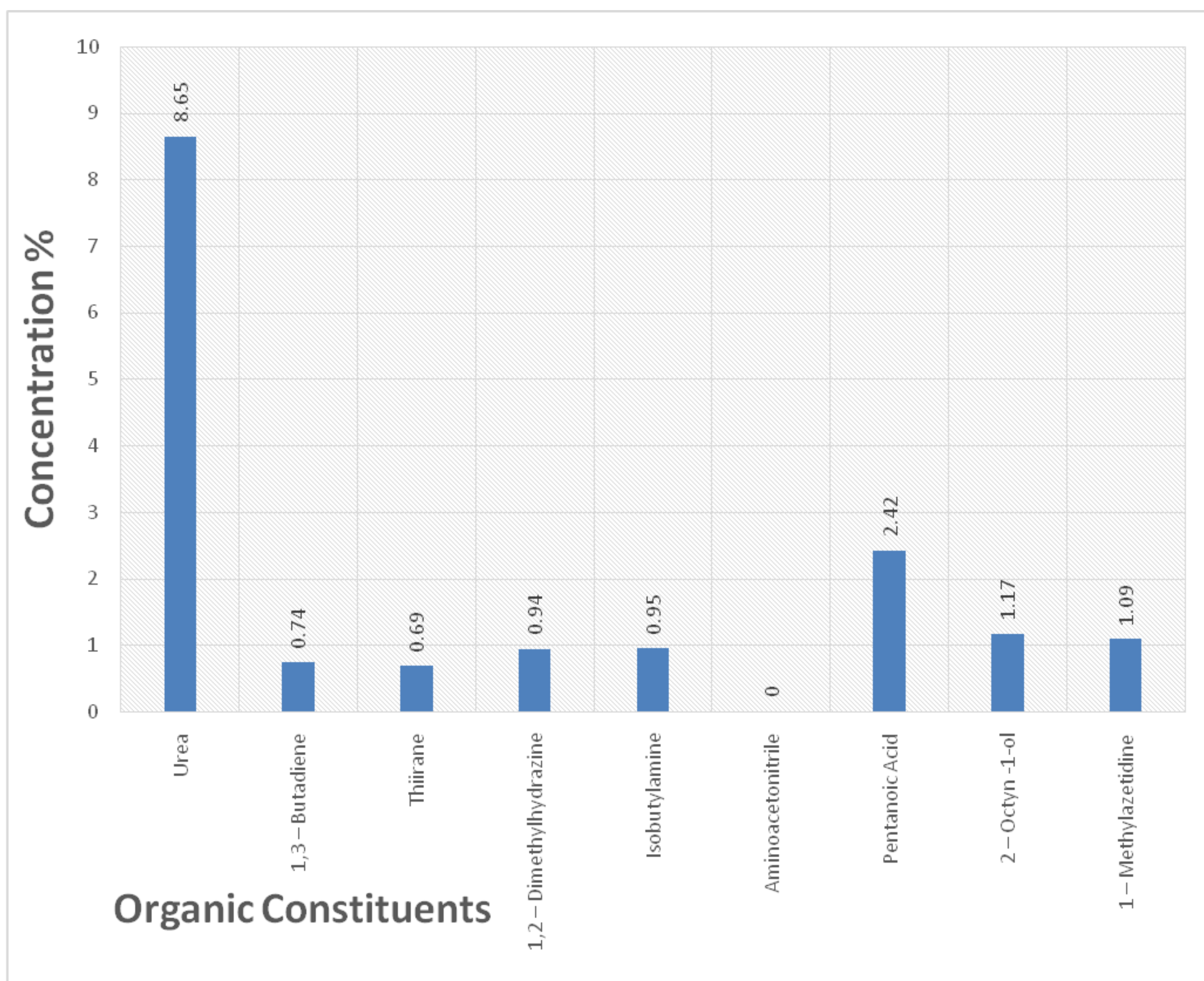


Figure 14: Organic chemical constituents of palm ash (Ngu).

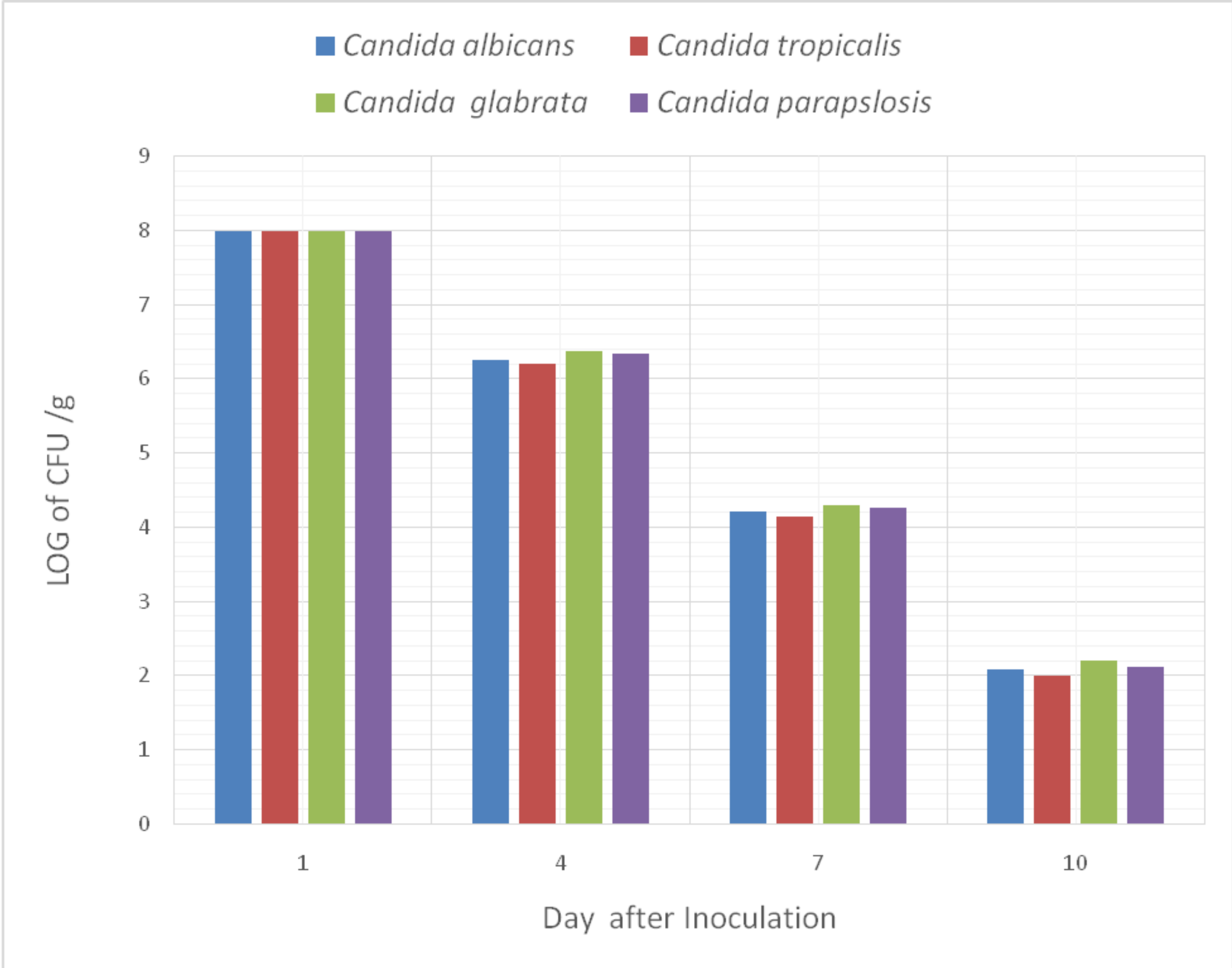


Figure 15: Total yeast count (during treatment with blue stone)

Full blood counts of the animals, pre and post-immunosuppression.

A detailed full blood counts of the test animals revealed a drastic decrease in the white blood cells (Neutrophils, Monocytes, eosinophils and basophils) and an increase in the lymphocyte count, five days after immunosuppression with 0.1ml of cyclophosphamide (100mg/kg body weight) (intraperitoneally) as shown in Tables 23 – 26. Neutrophil count (53.1% to 25.4%), Monocyte (6.7% to 2.3%), Eosinophil (1.7% to 1.3%) and basophil (0.6% to 0.5%) while the lymphocyte count increased from 35.4% to 70.5% probably due to exposure to exoantigens during the 2weeks acclimatization period.

Table 27 showed a comparative differential white blood cell count (after immunosuppression) using the manual and automated methods. The automated method was significantly more sensitive than the manual method ($p<0.05$).

Toxicity findings

Slight erythema and atonia were observed with potash alum and blue stone during skin irritancy tests. Others showed no observable sign of skin irritation according to Draize's test as shown in Table 28. There was no skin sensitization by the natural compounds as shown in Table. 29. There was no significant difference between day 1 and day 4. Slight erythema and atonia were observed with potash alum and blue stone during necropsy; Slight desquamation was seen with blue stone; Sulphur stone induced slight scab formation and exfoliation; while slight exfoliation was observed with palm ash as shown in Table 30.

Table 23: Full blood counts of the animals, pre and post-immunosuppression

	Rat 1		Rat 2		
Blood cells	**Before**	**After**	**Before**	**After**	**Ref. Range**
White blood cell					
Neutrophil	53.1%	25.4%	51.3%	27.0%	50.0 – 70.0
Lymphocyte	35.4%	70.5%	31.2%	67.8%	20.0 – 40.0
Monocyte	6.7%	2.3%	7.1%	2.8%	3.00 – 12.0
Eosinophil	1.7%	1.3%	2.3%	1.8%	0.50 – 5.00
Basophil	0.6%	0.5%	0.65%	0.6%	0.00 – 1.00
Red blood cell					
HGB	138g/L	134g/L	140g/L	138g/L	110 – 160
HCT	43.3%	42.2%	44.3%	43.6%	37.0 – 54.0
MCV	59.2fl	57.7fl	57.6fl	54.1fl	80.0 – 100.
MCHC	322g/L	318g/L	326g/L	317g/L	320 – 360
Platelet					
MPV	6.6fl	6.5fl	7.1fl	6.9fl	6.50 – 12.0
PDW	15.8	15.5	15.8	15.4	9.00 – 17.0
PCT	2.80ml/L	4.78ml/L	3.30ml/L	6.11ml/L	1.08 – 2.82

HGB: Hemoglobin; HCT: Hematocrit; MCV: Mean Corpuscular Volume; MCHC: Mean Corpuscular Hemoglobin Concentration; MPV: Mean Platelet Volume; PDW: Platelet Distribution Width; PCT: Procalcitonin.

Table 24: Full blood counts of the animals, pre and post-immunosuppression

	Rat 3		Rat 4		Ref. Range
Blood cells	**Before**	**After**	**Before**	**After**	
White blood cell					
Neutrophil	56.7%	28.6%	51.3%	27.9%	50.0 – 70.0
Lymphocyte	36.1%	67.2%	24.5%	57.5%	20.0 – 40.0
Monocyte	7.8%	2.8%	8.3%	3.7%	3.00 – 12.0
Eosinophil	1.0%	0.3%	7.1%	6.8%	0.50 – 5.00
Basophil	1.0%	1.1%	2.6%	4.1%	0.00 – 1.00
Red blood cell					
HGB	151g/L	146g/L	133g/L	129g/L	110 – 160
HCT	48.3%	46.5%	44.7%	41.4%	37.0 – 54.0
MCV	57.3fl	55.5fl	60.3fl	58.8fl	80.0 – 100.
MCHC	321g/L	313g/L	320g/l	312g/L	320 – 360
Platelet					
MPV	6.9fl	6.6fl	6.6fl	6.4fl	6.50 – 12.0
PDW	15.7	15.3	15.9	15.3	9.00 – 17.0
PCT	2.84ml/L	5.96ml/L	2.77ml/L	5.23ml/L	1.08 – 2.82

HGB: Hemoglobin; HCT: Hematocrit; MCV: Mean Corpuscular Volume; MCHC: Mean Corpuscular Hemoglobin Concentration; MPV: Mean Platelet Volume; PDW: Platelet Distribution Width; PCT: Procalcitonin.

Table 25: Full blood counts of the animals, pre and post-immunosuppression

Blood cells	Rat 5		Rat 6		
	Before	**After**	**Before**	**After**	**Ref. Range**
White blood cell					
Neutrophil	62.1%	39.6%	54.6%	29.7%	50.0 – 70.0
Lymphocyte	33.4%	56.1%	35.3%	63.1%	20.0 – 40.0
Monocyte	7.2%	2.7%	7.0%	2.4%	3.00 – 12.0
Eosinophil	2.6%	1.3%	1.9%	0.7%	0.50 – 5.00
Basophil	0.1%	0.3%	0.2%	0.4%	0.00 – 1.00
Red blood cell					
HGB	139.2g/L	125g/L	143g/L	136g/L	110 – 160
HCT	44.1%	41.9%	45.3%	43.2%	37.0 – 54.0
MCV	61.9fl	59.3fl	59.1fl	55.4fl	80.0 – 100.
MCHC	309g/L	298g/L	324g/L	317g/L	320 – 360
Platelet					
MPV	7.4fl	7.0fl	7.1fl	6.9fl	6.50 – 12.0
PDW	15.9	15.5	15.8	15.5	9.00 – 17.0
PCT	2.13ml/L	5.62ml/L	1.98ml/L	5.56ml/L	1.08 – 2.82

HGB: Hemoglobin; HCT: Hematocrit; MCV: Mean Corpuscular Volume; MCHC: Mean Corpuscular Hemoglobin Concentration; MPV: Mean Platelet Volume; PDW: Platelet Distribution Width; PCT: Procalcitonin.

Table 26: Full blood count of the animals, pre and post-immunosuppression

Blood cells	Rat 7 Before	Rat 7 After	Rat 8 Before	Rat 8 After	Ref. Range
White blood cell					
Neutrophil	49.8%	23.1%	50.7%	27.6%	50.0 – 70.0
Lymphocyte	33.1%	68.9%	32.3%	69.3%	20.0 – 40.0
Monocyte	6.0%	2.6%	7.9%	3.8%	3.00 – 12.0
Eosinophil	2.5%	1.9%	3.1%	2.1%	0.50 – 5.00
Basophil	0.85%	0.7%	0.87%	0.75%	0.00 – 1.00
Red blood cell					
HGB	138g/L	136g/L	144g/L	141g/L	110 – 160
HCT	44.1%	42.3%	45.8%	44.3%	37.0 – 54.0
MCV	58.3fl	53.5fl	60.1fl	54.3fl	80.0 – 100.
MCHC	324g/L	316g/L	331g/L	319g/L	320 – 360
Platelet					
MPV	7.2fl	6.7fl	7.8fl	7.0fl	6.50 – 12.0
PDW	15.6	15.3	16.2	15.6	9.00 – 17.0
PCT	2.75ml/L	5.18ml/L	3.10ml/L	6.01ml/L	1.08 – 2.82

HGB: Hemoglobin; HCT: Hematocrit; MCV: Mean Corpuscular Volume; MCHC: Mean Corpuscular Hemoglobin Concentration; MPV: Mean Platelet Volume; PDW: Platelet Distribution Width; PCT: Procalcitonin.

Table 27: Comparative (manual and automated) differential white blood cell count (after immunosuppression)

White Blood Cells (%)	Sample 1		Sample 2		Sample 3		
	Manual	Automated	Manual	Automated	Manual	Automated	Ref. Range
Neutrophil	16.50±0.71	24.20±1.70a	22.50±0.71	26.75±0.35a	19.5±0.71	28.05±078a	50.0 – 70.0
Lymphocyte	83.00±1.41	69.75±1.06a	77.50±0.71	67.90±0.14a	77.50±0.71	67.60±0.57a	20.0 – 40.0
Monocyte	0.00±0.00	2.15±0.21a	0.00±0.00	2.50±0.42a	0.00±0.00	2.50±0.42a	3.00 – 12.0
Eosinophil	0.00±0.00	1.15±0.21a	0.00±0.00	1.65±0.21a	2.50±0.71	0.90±0.85b	0.50 – 5.00
Basophil	0.00±0.00	0.35±0.21b	0.00±0.00	0.50±0.14a	0.00±0.00	1.00±0.14a	0.00 – 1.00

a - $P < 0.05$; b – $P > 0.05$

Table 28: Draize's skin irritancy test

Parameters (signs)	Blue stone	Potash alum	Trona	Sulphur stone	Black stone	Palm ash	Control (H_2O)
Erythema	1	1	0	0	0	0	0
Edema	0	0	0	0	0	0	0
Atonia	1	1	0	0	0	0	0
Fissuring	0	0	0	0	0	0	0

None = 0, Slight = 1, Moderate = 2, Severe = 3 @200mg/ml

Table 29: Skin sensitization test (Murine local lymph node assay)

Parameters	Blue stone	Potash alum	Trona	Sulphur stone	Black stone	Palm ash	Control (H_2O)
Day 1 (Lymphocyte count %)	32.50±0.71	31.50±0.71	30.50±0.71	32.50±0.71	29.50±0.71	30.50±0.71	32.50±0.71
Day 4 (Lymphocyte count %)	35.50±0.71	33.50±0.71	29.50±0.71	30.50±0.71	29.50±0.71	28.50±0.71	30.50±0.71

$P > 0.05$ @200mg/ml

Table 30: Necrospy findings

Parameters (@200mg)	Blue stone	Potash alum	Trona	Sulphur stone	Black stone	Palm ash	Control (H_2O)
Erythema	1	1	0	0	0	0	0
Edema	0	0	0	0	0	0	0
Atonia	1	1	0	0	0	0	0
Desquamation	1	0	0	0	1	0	0
Fissuring	0	0	0	0	0	0	0
Scab Formation	0	0	0	1	0	0	0
Exfoliation	0	0	0	1	0	1	0
Necrosis	0	0	0	0	0	0	0

None = 0, Slight = 1, Moderate = 2, Severe = 3

Treatment of vaginal and penile cutaneous candidiasis

Plates 12 – 14 showed the clinical signs of penile candidiasis caused by *C. albicans*. The signs include erythema, rash, exfoliation and desquamation. Blue stone was very effective in treatment of penile candidiasis as shown in the plates. There was a complete recovery after day 5 as shown. Plates 15 – 17 revealed the clinical signs of cutaneous candidiasis caused by *C. albicans*. The signs include erythema, rash and exfoliation. Blue stone was very effective in treatment of cutaneous candidiasis as shown in the plates. In Plates 18 – 20, the clinical signs of penile candidiasis (*C. tropicalis*) presented include slight erythema, mild rash and exfoliation. The test animals responded very well to treatment with Potash alum showing full recovery after day 6. Clinical signs of cutaneous candidiasis (*C. tropicalis*) such erythema, rash and exfoliation were observed 5 days after infection as shown in plates 21 – 23. Potash alum at 100mg/ml was very effective in treatment of the *Candida* infection.

Plates 24 – 26 showed the clinical signs of vaginal candidiasis caused by *C. glabrata*. The signs presented include whitish discharge, exfoliation and slight erythema. The test animals responded very well to treatment with 200mg/ml potash alum solution; showing full recovery after day 7. Plates 27 – 29 showed the clinical signs associated with vaginal and cutaneous candidiasis (*C. parapsilosis*). The observed signs include erythema, rash, exfoliation and whitish vaginal discharge. Complete recovery was observed after 6 days of treatment with sulphur stone. The signs observed were skin rash, slight erythema and intense exfoliation as shown in plates 30 – 32. Full recovery with complete hair coverage was observed after day 5 of treatment of penile candidiasis (*C. parapsilosis*) with trona at 200mg/ml. A black stone at 200mg/ml was used in treatment of cutaneous candidiasis caused by *C. albicans.* The clinical signs observed include mild exfoliation, rash and erythema as shown in plates 33 – 35. Complete recovery was observed after day 6. The clinical signs of penile candidiasis (*C. parapsilosis*) observed include mild erythema, mild rash and intense exfoliation as shown in plates 36 – 38. The signs resolved complete after day 6 of treatment with palm ash.

Plate 12: Day 1 before treatment with clinical signs of penile candidiasis (*Candida albicans*)

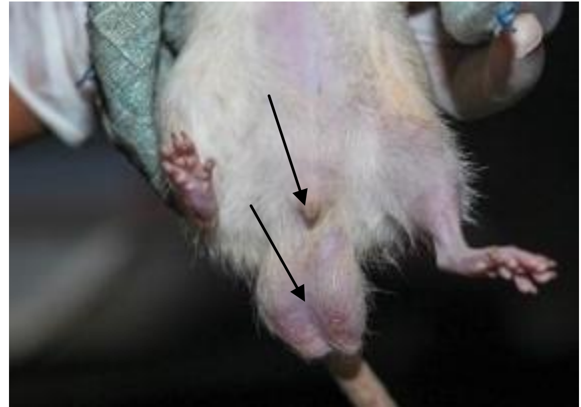


Plate 13: Day 3 after treatment with Blue stone (*Candida albicans*)

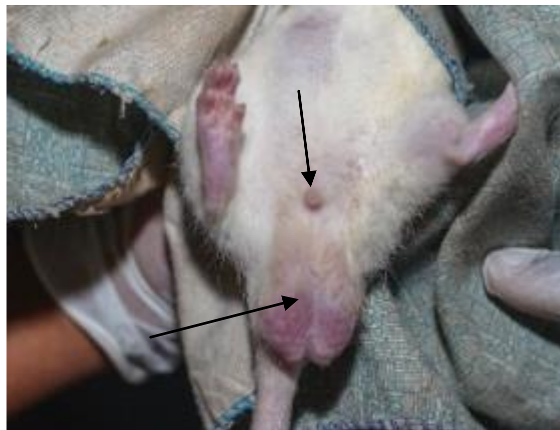


Plate 14: Day 5 after treatment with blue stone (*Candida albicans*)

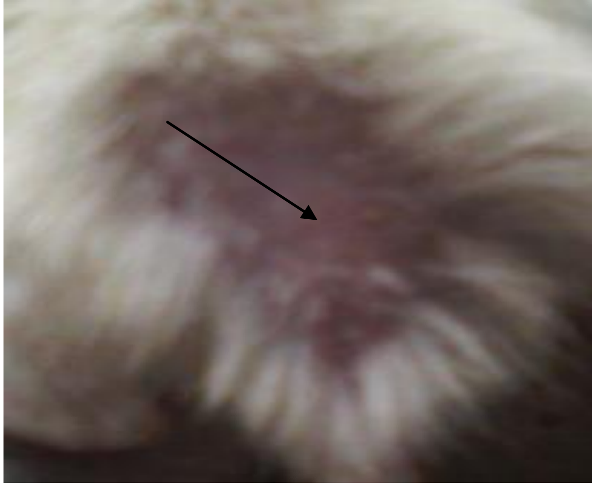


Plate 15: Day 1 before treatment with clinical signs of cutaneous candidiasis (*Candida albicans*)

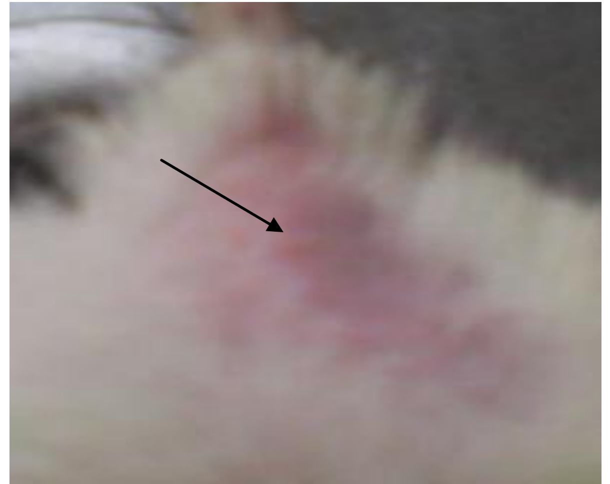


Plate 16: Day 3 after treatment with blue stone (*Candida albicans*)

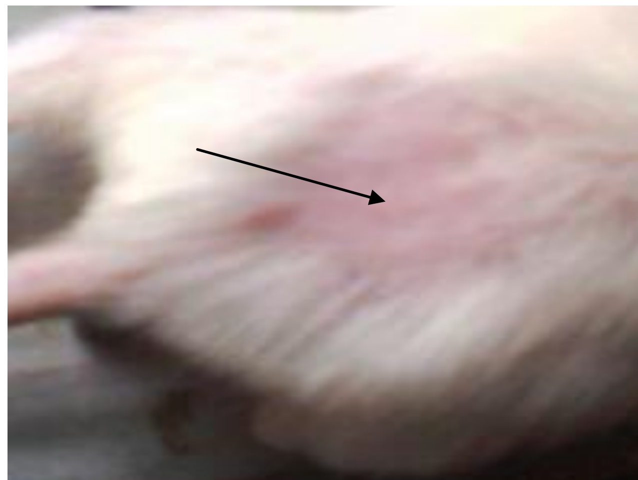


Plate 17: Day 5 after treatment with blue stone at (*Candida albicans*)

Plate 18: Day 1 before treatment; with clinical signs of penile candidiasis (*Candida tropicalis*)

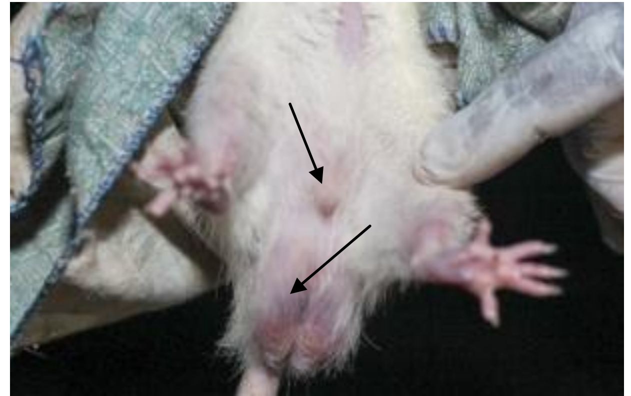


Plate 19: Day 3 after treatment with Potash alum (*Candida tropicalis*)

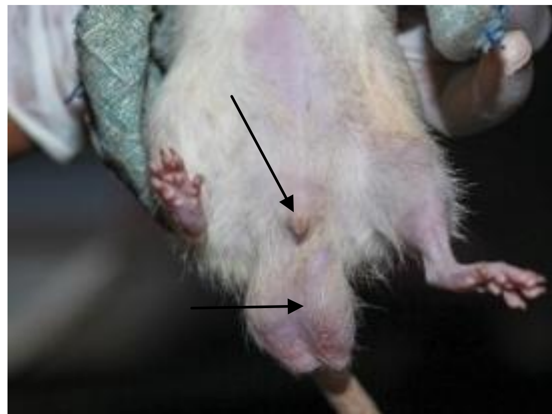


Plate 20: Day 5 after treatment with Potash alum (*Candida tropicalis*)

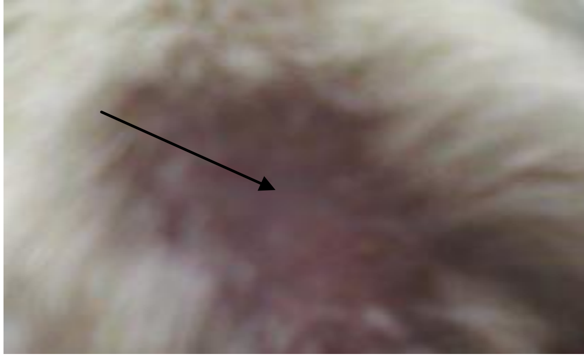


Plate 21: Day 1 before treatment; showing clinical signs of cutaneous Candidiasis (C. *tropicalis*)

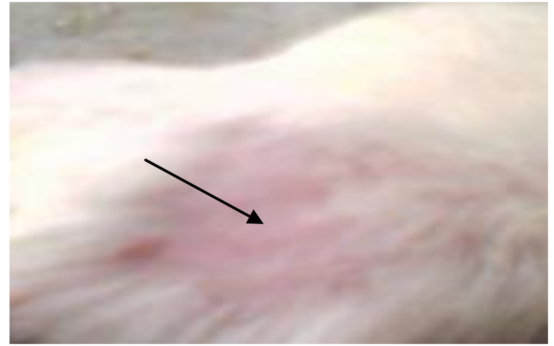


Plate 22: Day 3 after treatment with Potash alum (*C. tropicalis*)

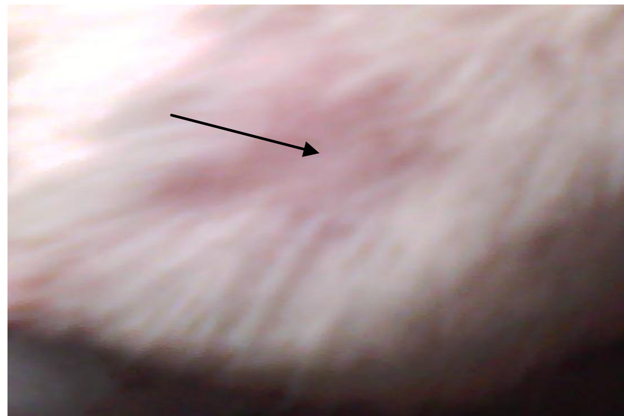


Plate 23: Day 5 after treatment with Potash alum (*C. tropicalis*)

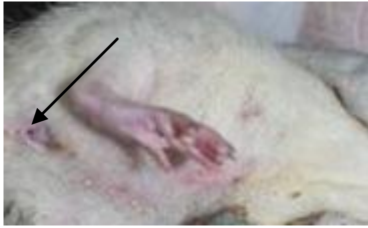


Plate 24: Day 1 before treatment, showing clinical signs of vaginal candidiasis (*C. glabrata*)

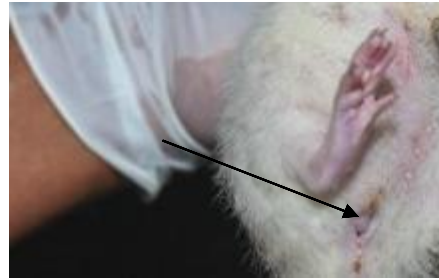


Plate 25: Day 3 after treatment with Potash alum (*C. glabrata*)

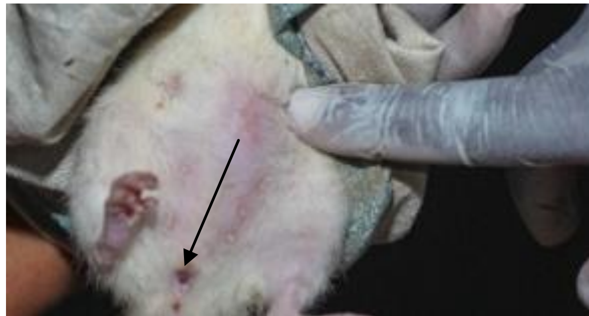


Plate 26: Day 5 after treatment with potash alum (*C. glabrata*)

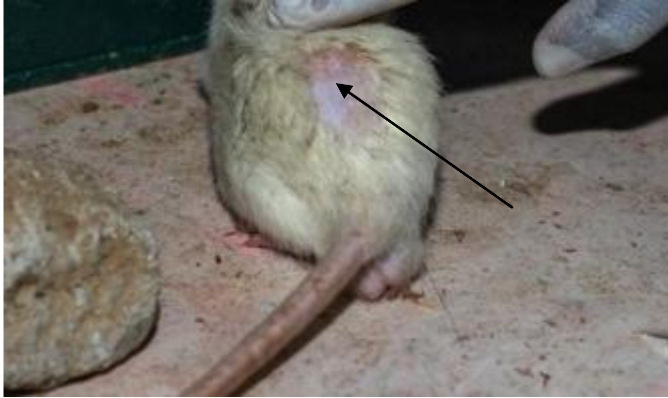


Plate 27: Day 1 before treatment showing clinical signs of cutaneous candidiasis (*Candida parapsilosis*)

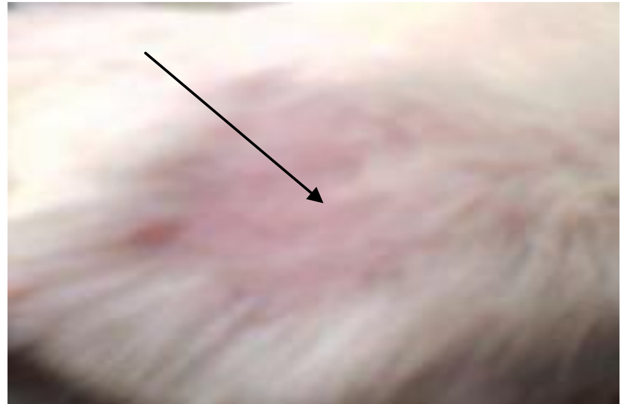


Plate 28: Day 3 after treatment with sulphur stone (*Candida parapsilosis*)

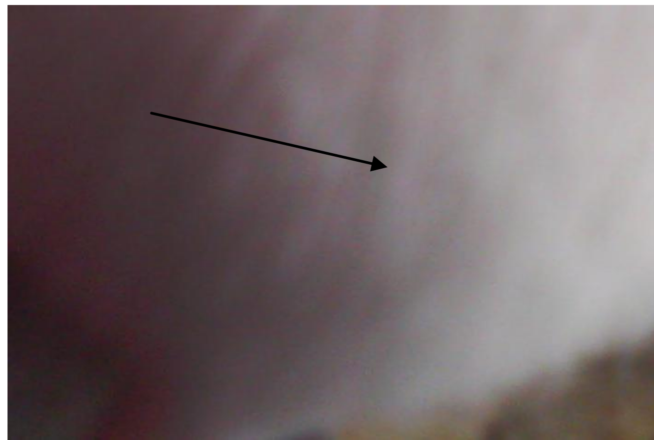


Plate 29: Day 5 after treatment with sulphur stone (*Candida parapsilosis*)

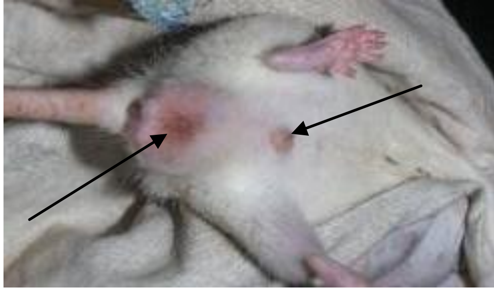


Plate 30: Day 1 before treatment showing clinical signs of penile candidiasis (*Candida parapsilosis*)

Plate 31: Day 3 after treatment with Trona (*Candida parapsilosis*)

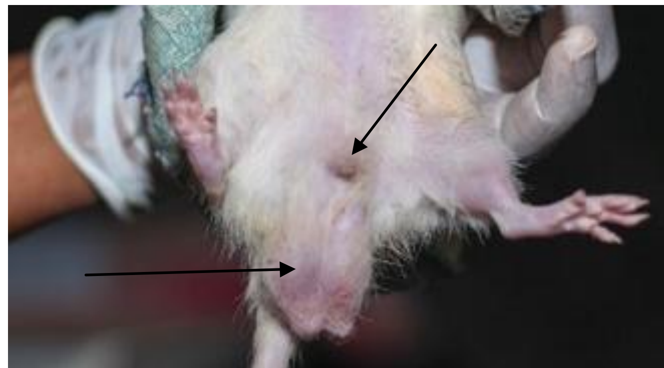


Plate 32: Day 5 after treatment with Trona (*Candida parapsilosis*)

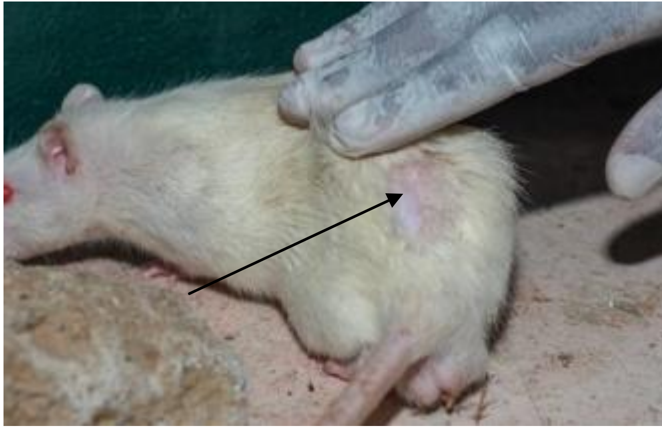


Plate 33: Day 1 before treatment showing clinical signs of cutaneous candidiasis (*C. albicans*)

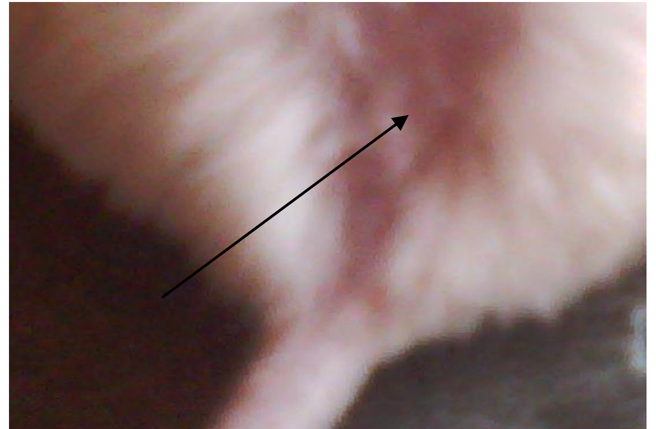


Plate 34: Day 3 after treatment with black stone (*C. albicans*)

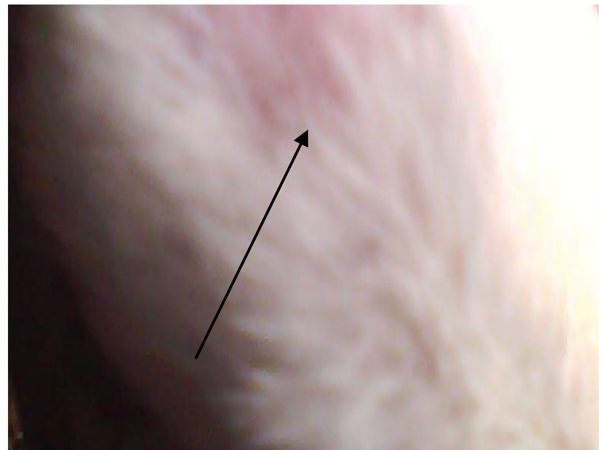


Plate 35: Day 5 after treatment with black stone (*C. albicans*)

Plate 36: Day 1 before treatment showing clinical signs of penile candidiasis (*Candida parapsilosis*)

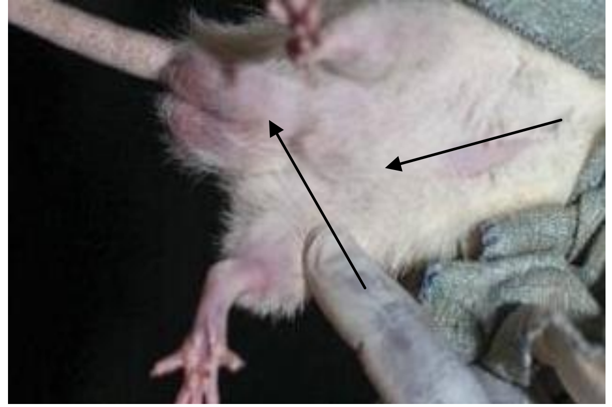


Plate 37: Day 3 after treatment with palm ash (*Candida parapsilosis*)

Plate 38: Day 5 after treatment with palm ash (*Candida parapsilosis*)

Post inoculation histopathology findings

Plate 39 revealed stained normal albino rat skin section before infection and treatment. The collagen bundles, blood vessel, sebaceous units and hair follicles are in their normal size and forms. Plate 40 showed infected stained skin section revealing intense peri-adnexal infiltration of inflammatory cells, inflammation of the sebaceous units with drastic loss of hair follicles. Plate 41 showed gradual restoration of the skin architecture and disappearance of the inflammatory cells, day 3 after treatment with Blue stone (potash alum and trona). Plate 42 showed almost complete restoration of the normal skin architecture, the blood vessels, collagen bundles, sebaceous units, with very little infiltrated inflammatory cells (lymphocytes); day 5 during treatment with blue stone (Potash alum and trona).

Histological findings during toxicology studies

Plates 43 and 44 showed stained skin sections of the test animals during toxicity screening of the natural compounds. No histopathological sign was observed from day 1 till day 5.

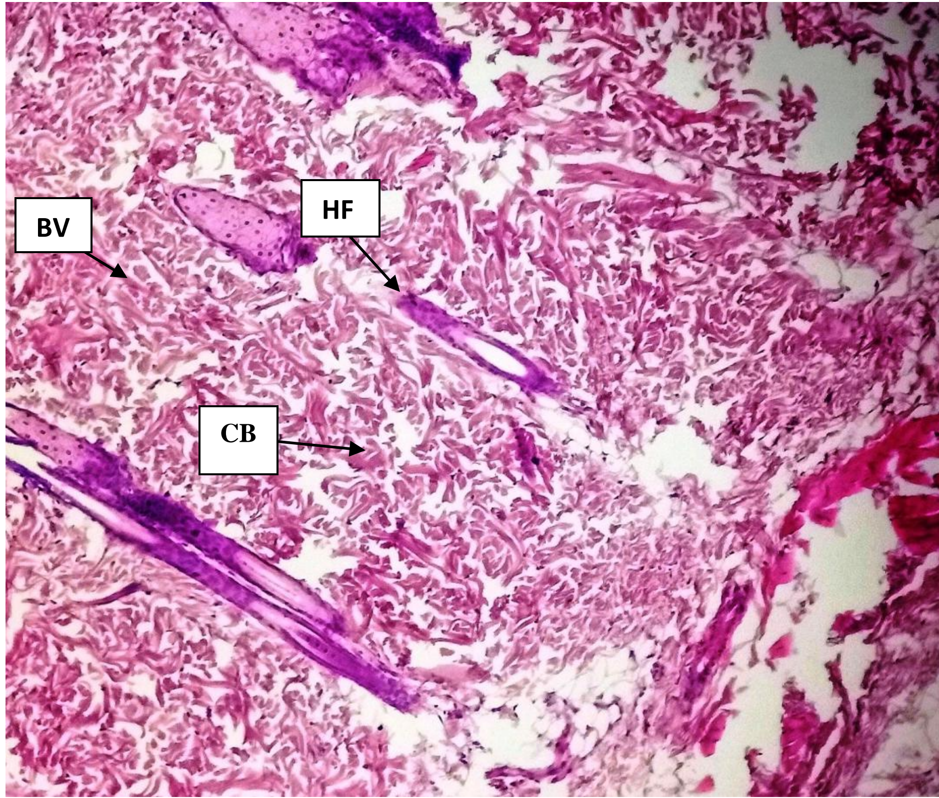


Plate 39: Normal albino rat skin section before infection and treatment

Key: HF: Hair follicles, BV: Blood Vessels, CB: Collagen bundles

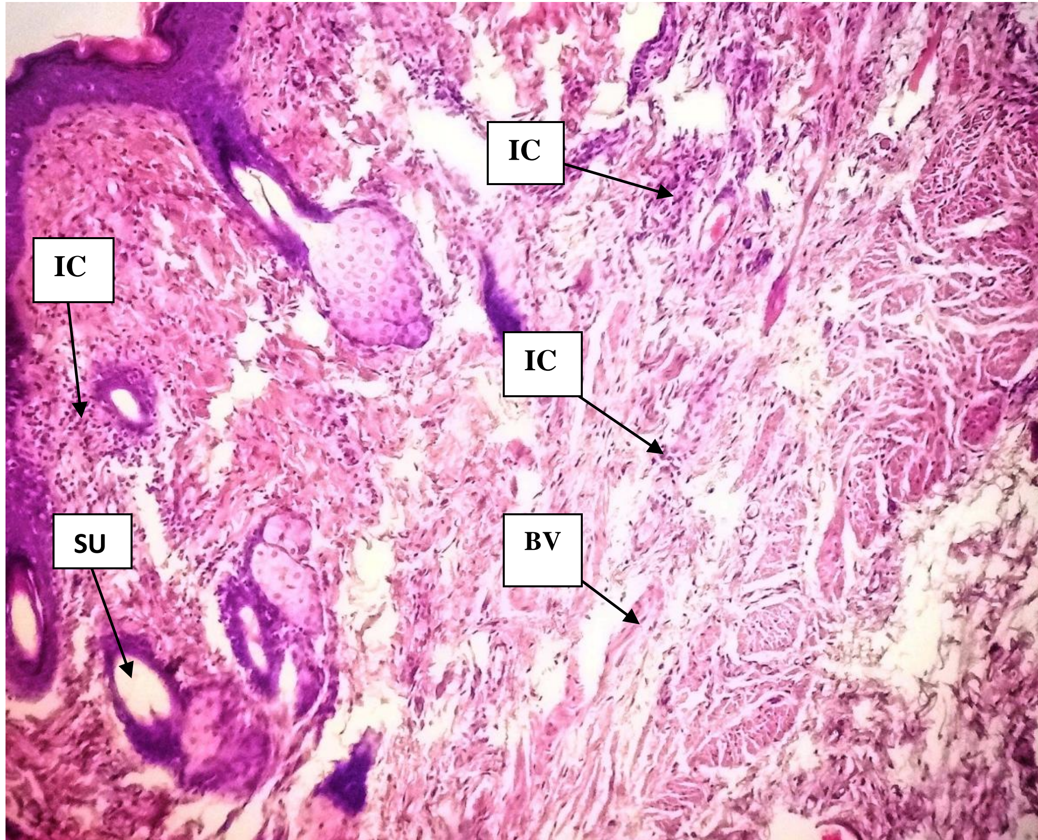


Plate 40: Day 1 before treatment showing intense peri-adnexal infiltration of inflammatory cells, inflammation of the sebaceous units with drastic loss of hair follicles.

Key: IC: Inflammatory cells, BV: Blood Vessels, SU: Sebaceous Units

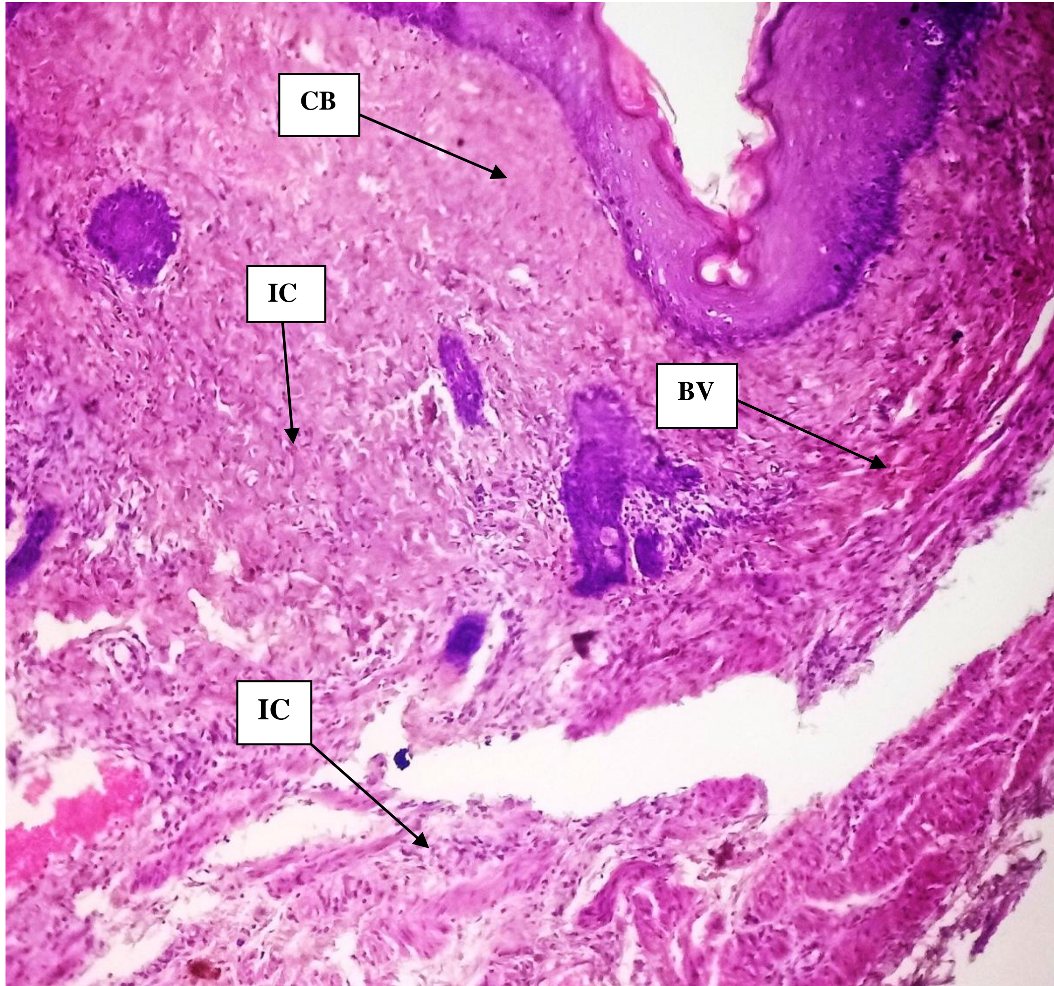


Plate 41: Day 3 after treatment with Blue stone (Potash alum and Trona) showing a gradual restoration of the skin architecture. The Collagen bundles are being restored with the gradual disappearance of the inflammatory cells.

Key: CB: Collagen Bundles, IC: Inflammatory Cells, BV: Blood Vessels

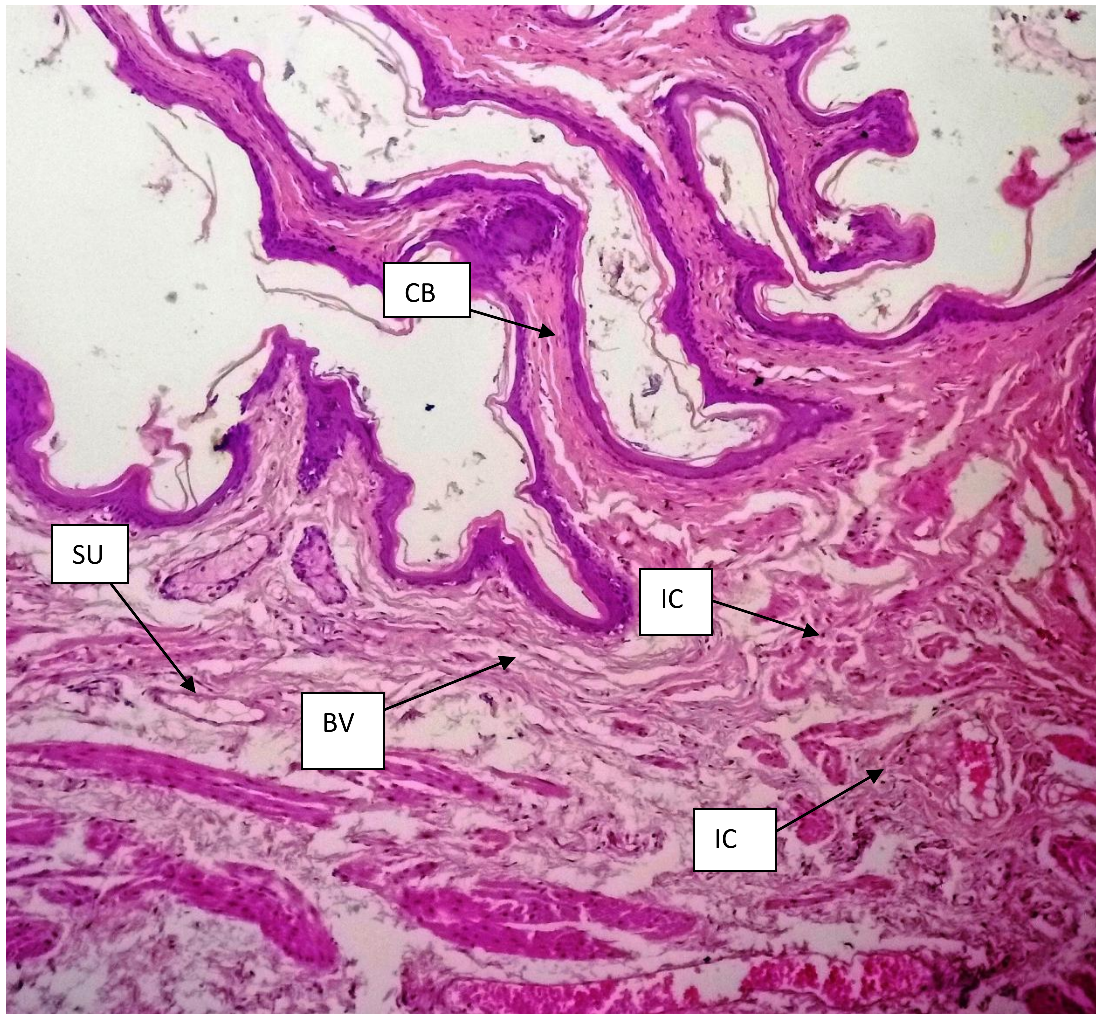


Plate 42: Day 5 during treatment with blue stone (Potash alum and Trona) showing almost complete restoration of the normal skin architecture, the blood vessels, collagen bundles, sebaceous units and with very little infiltrated inflammatory cells (lymphocytes).

Key: SU: Sebaceous Units, BV: Blood Vessels, IC: Inflammatory Cells

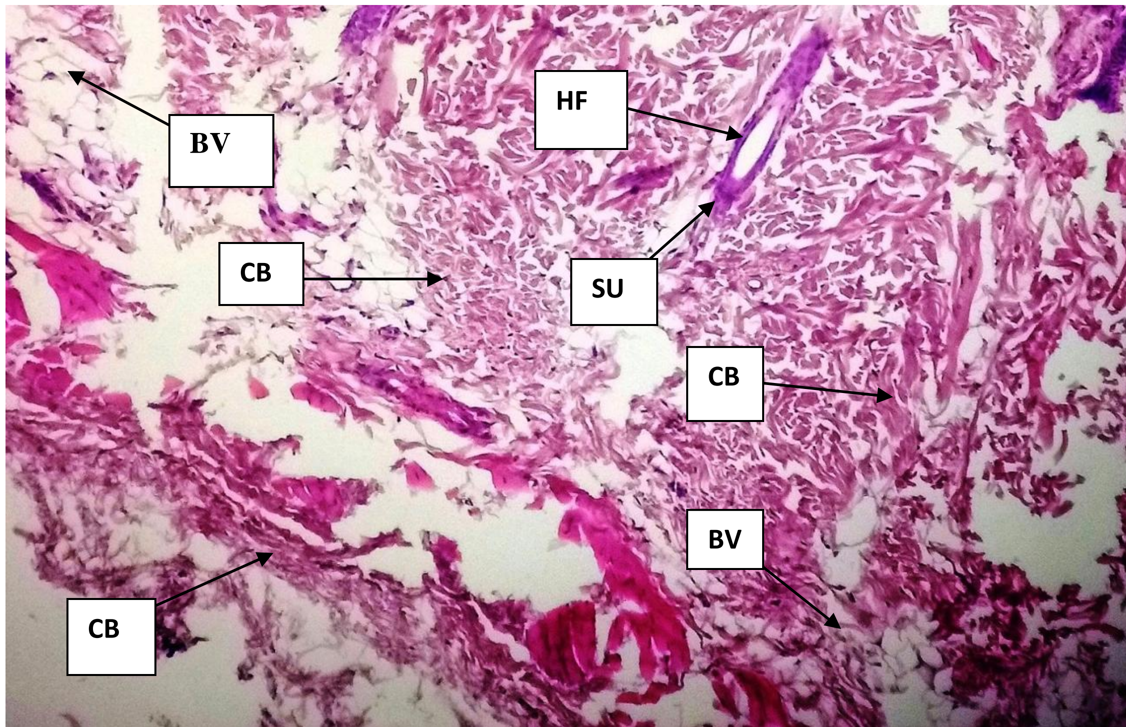


Plate 43: Skin section for day 1 (pre-exposure) till day 5 (post-exposure) during toxicology (for blue stone, potash alum and Trona). No observable histopathological sign shown.

Key: HF: Hair Follicles, BV: Blood Vessels, CB: Collagen Bundles, SU: Sebaceous Units

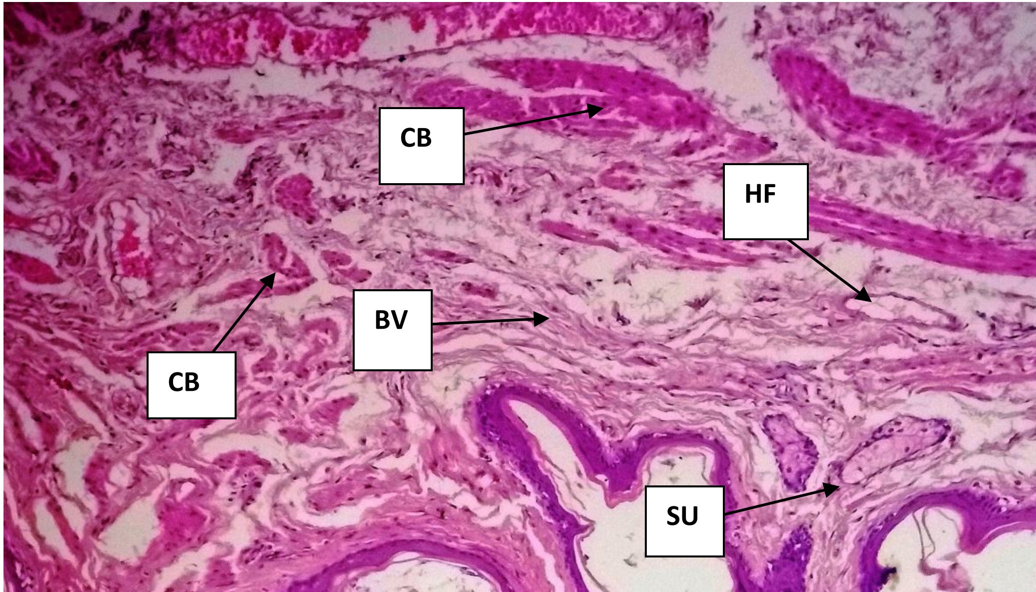


Plate 44: Skin section for day 1 (pre-exposure) till day 5 (post-exposure) during toxicology (for sulphur stone, black stone and palm ash). No observable histopathological sign shown.

Key: CB: Collagen Bundles, BV: Blood Vessels, SU: Sebaceous Units, HF: Hair Follicles

CHAPTER FIVE

5.0 DISCUSSION

This research investigated and analysed the toxicity and chemotherapeutic values of some selected natural compounds (potash compounds and natural stones: palm ash, potash alum, trona, blue stone, black stone and sulphur stone) in the treatment of cutaneous candidiasis using albino rat model. Four hundred and fifty high vaginal swab samples were collected and analysed. Out of these samples, one hundred and eighty – eight were positive to *Candida* growth. Fifty-one percent (51%) were *C. albicans, C. tropicalis* (28%), *C. glabrata* (15%) and *Candida parapsilosis* (6%) as shown in figure 1. Watson *et al*. (2012) also reported that *Candida albicans* is the most common type of fungus to cause yeast infections. The work on yeast infections (Watson *et al*., 2012) further revealed that the fungus, *C. albicans* is responsible for most vaginal yeast infections. Thus, *C. albicans* is the most aetiological agent of vaginal candidiasis (51%).

The findings above are in concordance with the fact that *Candida* is an opportunistic pathogenic fungus that normally inhabits various anatomical sites of the human body including the gastrointestinal and genitourinary tracts. Sharma *et al*. (2017) also reported that *Candida albicans* was the most prevalent isolate from clinical specimens making up 35% while non-albicans *Candida* make up 65%. They further revealed their species spectrum as follows: of the 100 isolates, 35 were *C. albicans*, 17 *C. tropicalis*, 6 *Candida glabrata*, 8 *Candida guilliermondi*, 1 *Candida kefyr*, 2 *Candida krusei*, 14 *Candida parapsilosis*, 2 *Candida lusitaniae,* and 1 *Trichosporon*. Kullberg and Arendrup (2015) also revealed that *Candida albicans* is responsible for 50–70% of cases of total fungal infections worldwide.

C. albicans was positive to germ tube test as shown in plate 1 while the other isolates were negative. Plates 2 – 5 showed the pure cultures obtained using the streak plate method

while plates 6 – 9 were the micrographs of the isolates obtained after Gram staining. The micrographs showed the characteristic shape of the *Candida* isolates ranging from oval (*C. albicans, C. glabrata*), spindle (*C. parapsilosis*) to oblong shaped cells (*C. tropicalis*). *Candida species* are Gram positive as shown in the micrographs.

The isolates exhibited varied response to fermentation of the various carbohydrates. *Candida albicans* fermented 8 out of the 14 sugars, *C. tropicalis* 9, *C. glabrata* 4 and *C parapsilosis* 8. All the isolates fermented fructose, glucose, dextrose and trehalose; while none fermented lactose, inositol, raffinose, dulcitol and melibiose as shown in Table 2. This notwithstanding, molecular identification tests involving genomic DNA extraction, polymerase chain reaction and nucleic acid sequencing of amplicons, provided a confirmatory result on the identity of the isolates. Figure 2 revealed the phylogenetic relationship between the isolates. *C. albicans* and *C. parapsilosis* are very closely related more, than the rest of the isolates. *C. glabrata* is closer, genetically to *C. albicans/C. parapsilosis* unlike *C. tropicalis* which is farther away from *C. albicans/C. parapsilosis*.

Potash compounds and natural stones are excellent antimicrobials, though with varying levels of potency and toxicity, *in vitro* and *in vivo*. Among the potash compounds analysed, trona (otherwise called kanwa or akanwu) gave the highest inhibition zone diameter, followed by potash alum and palm ash as shown in Tables 3, 4, 5 and 6. Ali *et al.* (2017) pointed out that the potency of alum as an antimicrobial agent had been visibly demonstrated over the years through myriads of its beneficial activities and relevance in a broad spectrum of human research and development. Shalli *et al.* (2020), stated alum has been used as a remedy for the treatment of vaginal discharge. Ntukidem *et al.* (2002) reported that trona exhibited antimicrobial activity

against pathogenic bacteria and fungi organisms including *Pseudomonas aeruginosa, Proteus spp, Candida albicans and C. pseudotropicalis.*

The natural stones also showed remarkable growth inhibition. Blue stone gave the highest inhibition zone diameter while black stone was totally inactive using the agar-well diffusion method. Sulphur stone (brimstone) showed a mild *in vitro* antimicrobial activity as shown in Tables 3, 4, 5 and 6. Blue stone has reportedly been used as a fungicide and for the treatment of athlete's foot or tinea (Gava *et al.,* 2016; Jacob *et al*. (2017). Essan and Khallaf (2016) also reported the antifungal activity of different solutions of copper sulphate in deionized distilled water against *Aspergillus flavus, A. niger, Penicillium chrysogenum, Fusarium solani* and *Alternaria solani.* Massalimov *et al.* (2012) reported that sulphur agents were able to suppress the growth of resistant *Candida albicans* at 70mg/ml. In addition, when exposed to the mould fungi including dermatophytes, the concentration required to inhibit the colony is reduced by 3 – 4 times.

Black stones traditionally known as snake stone in some African countries has been used since antiquity to treat snake bites and local infections, though there was no evidence of its antimicrobial property either (Maregesi *et al*., 2013). The comparative studies carried out showed that black stone exhibit some level of antimicrobial properties against the *Candida* isolates. The microdilution method, a highly sensitive assay procedure, showed the antimicrobial efficacy (MIC and MFC) of black stones as shown in Tables 7, 8, 9 and 10. The antimicrobial activities of the natural compounds were higher using the microdilution method than the broth dilution method. Nwachukwu *et al*. (2012) revealed that Sulphur has proven to be the most hydrophobic and fungitoxic compound among the naturals compounds. During the preparation of the stock solutions of the natural compounds, Sulphur was observed to be sparingly soluble in

water; though the solubility increases with time and temperature. The higher activity observed with the microdilution method may be due to the longer incubation time given which enabled for complete dissolution of the Sulphur stone. The same applies to black stones; thus the more soluble an agent is, the better its antimicrobial activity as seen above. All the *Candida* isolates shared similar sensitivity profile to the natural compounds *in vitro* and likewise *in vivo*. The natural compounds showed varying degrees of efficacy *in vitro* and *in vivo* against the *Candida* isolates. The results agreed with similar works done by Kamka-Evans *et al*. (2013) and Ali and Zaigham (2017).

The Combination studies of the various natural compounds gave totally different results. The combined activities of the agents, using their MIC and FIC values against the *Candida* isolates varied. The agents exhibited synergism against *C. albicans*, indifference against *C. tropicalis*, antagonism against *C. glabrata and C. parapsilosis*. Combination such as palm ash + trona and potash alum + palm ash + trona showed no zone of inhibition (resistance). This may be attributed to antagonistic reactions among the various combinations. The best activity was observed when the agents were analysed singly as shown in Tables 3 – 23. The results agreed with similar works done by Kamka-Evans *et al*. (2013).

Chemical constituents (organic and inorganic components) analysis was carried out on the natural compounds using Gas Chromatography-Mass Spectroscopy and X-ray fluorescence Technology. The inorganic components found in blue stone include oxides of copper (43.7%), sulphur (29.4%), aluminium (1.2%) as shown in figure 3. The heavy metals which are present in very negligible amount include arsenic, lead and nickel (all <0.009%). Even though Copper Sulphate is considered toxic for parenteral use, it is still listed as an antidote (an emetic) in the WHO Anatomical Therapeutic Chemical Classification system (Disabled World, 2019). The

organic components are basically natural fatty acids such as linoelaidic acid and oleic acid which are possibly contaminants from the source environment as shown in figure 9.

Potash alum comprises oxides of sulphur (44.5%), aluminium (14.49%) etc. The heavy metals include oxides of lead, arsenic, nickel, accounting for <0.0010% (very negligible) as shown in figure 4. The safety of alum as shown above was also reported by Brahmachan *et al.* (2019) who stated that notable advantages for the use of alum include cost effectiveness, availability, non-toxicity, reusability and ecofriendliness. The organic components include naturally occurring fatty acids (vaccenic acid, decanoic acid and linoelaidic acid) (figure 10) which exhibit some level of antimicrobial activity. These must have contributed to the antimicrobial efficacy of potash alum.

Studies revealed that trona (akanwu) salt is the second most popularly used salt in Nigeria (Okpala, 2015). The inorganic components found in trona as shown in figure 5 include sulphur (2.14%), chlorine (5.8%), and potassium (1.7%) while the heavy metals include lead (0%), arsenic and nickel (<0.003%). The organic components include linoelaidic acid, nonanoic acid and decanoic acid (figure 11). These organic fatty acids exhibit some level of antimicrobial properties. Parenteral use of trona is relatively safe while its topical application is considered very safe as shown in the findings.

Sulphur has been applied in the treatment of various skin diseases owing to its safety in topical applications (Massalimov *et al.*, 2012). Ismail *et al.* (2016) reported that medical drugs based in elemental sulphur, are well demanded nowadays due to their high efficacy and low cost. The major components of sulphur stone (brimstone) are elemental sulphur (94.72%), aluminum (3.46%) as shown in figure 6. Lead, arsenic and nickel are less than 0.0007%. Oleic acid, decanoic acid etc make up the organic components as shown in figure 12. They probably also

contribute to the antimicrobial activity of sulphur stone. Black stones comprises oxides of iron (1.9%), sulphur (1.64%), potassium (2%), copper (0.2%), chlorine (0.98%), nickel, lead and arsenic (all <0.002%) as shown in figure 7. Hexanoic acid, Pentanoic acid etc make up the organic components (figure 13). The above listed components must have contributed to the mild *in vitro* antimicrobial effect of black stone on the isolates.

Figure 8 showed the inorganic components of palm ash include potassium (28.8%), sulphur (3.4%), calcium (2.8%), chlorine (15%) etc while the average concentration of toxic heavy metals is <0.0007%. The organic components are urea and fatty acids as shown in figure 14. Urea-containing creams are used as topical dermatological products to promote rehydration of the skin. Urea preparation (40%) may also be used for non-surgical debridement of nails. It dissolves the intercellular matrix (Nicolaou and Montagnon, 2008). Urea and its derivatives have been reported by Patil *et al*. (2019) to exhibit some level of antimicrobial activity. Thus, the antimicrobial activity of palm ash is possibly due to sulphur, chlorine, urea and the fatty acids.

The *in vivo* toxicological analysis of the natural compounds showed that the agents are fit for topical applications. The skin sensitization test (Murine Local Lymph node Assay) result (Table 29) and necropsy findings (Table 30) also indicated that the agents are very safe for topical applications. These findings also agreed with the report by Ismail *et al*. (2016) who stated that sulphur-based drugs are well demanded nowadays due to their high efficacy, non-toxicity and low cost. On the same note, the histological studies by Al-Talib *et al*. (2016) confirmed the safety of alum salt for mammalian consumption. Alzomor *et al*. (2014) also reported that alum cannot be directly absorbed on the skin due to its negatively charged molecules which are unable to pass through the cell membrane and therefore, alum remains a harmless substance. However, higher concentrations of alum might cause nephrotoxicity and intestinal bleeding (Bryan *et al*.,

2014). The histopathological findings as described in the stained skin sections showed no observable histopathological signs as seen in the architecture of the collagen bundles, blood vessels and the sebaceous units.

A detailed hematology analysis of the test animals was carried out to determine their immune status. There was a drastic decrease in the white blood cells (Neutrophils, Monocytes, eosinophils and basophils) five days after immunosuppression with 0.1ml of cyclophosphamide (100mg/kg body weight) (intraperitoneally) as shown in Tables 23 to 26. There was an increase in the lymphocyte count which may result from exposure to environmental antigens during the one week period of acclimatization. The red blood cell count was normal (within the reference range) indicating that the animals are “relatively” healthy. A comparative (Manual and automated) differential white blood cell count (before and after immunosuppression) as shown in Tables 23 – 26 showed that automated method (using the computerized hemoanalyser) is very much more sensitive than the manual method.

The effectiveness of treatment using the various formulations of natural compounds was also evaluated. All the agents were very effective in the treatment of various forms of cutaneous candidiasis. The various signs of cutaneous candidiasis observed included erythema, rash, exfoliation, desquamation and whitish discharge (for vaginal candidiasis). The signs gradually disappeared 5 – 7 days during treatment.

Histological analysis was performed on normal albino rat skin (negative control) and infected rat skins. The normal rat stained skin sections (Plate 39) showed the collagen bundles, blood vessel, sebaceous units and hair follicles in their normal size and forms. The stained infected rat skins sections (before treatment) showed an intense peri-adnexal infiltration of inflammatory cells, inflammed sebaceous units and malformed collagen bundles as shown in

Table 40. The histological results revealed that tissue regeneration was very much quicker in the treated groups compared to the negative control groups. The increased cellular infiltration of inflammatory cells observed may be due to the presence of pathogens (*Candida spp*), but the antimicrobial activities of the natural compounds massively reduced the yeast population. This indirectly reduced the inflammatory cells in the infection sites. These findings are in line with the work done by Sasidharan *et al*. (2010) on the wound healing potentials of *Elaeis guineensis* jacq leaves on infected albino rat model. Vaseline gelly was used as a base in the drug formulation at ratio of 4:1. It was also used as the negative control (placebo) while ketoral (ketoconazole) was used as the positive control.

Total yeast count was done at interval of three days during treatment to evaluate the effectiveness of the agents in reducing the yeast population *in vivo*. It was observed that the yeast population declined gradually with time as shown in figure 15; thus showing the *in vivo* effectiveness of the antimicrobial agents in treatment of cutaneous candidiasis.

5.1 CONCLUSION AND RECOMMENDATION

Drug discovery is a chronological process which is intended to identify a chemical agent or biomolecule for comprehensive evaluation as a potential drug. New drugs are continually required by the healthcare systems to address unmet medical needs across diverse therapeutic areas and pharmaceutical industries primarily strive to deliver new drugs to the market through the complex activities of drug discovery and development.

Despite the availability of several effective antifungal agents, their therapeutic outcome is less than optimal due to limitations related to drug physicochemical properties and toxicity. Therefore, there is urgent need to intensify the current antifungal drug discovery efforts to develop more clinically effective, cheaper and safer agents, especially against antifungal drug-resistant pathogens. Such raw materials include potash compounds and natural stones.

This Research work has shown that natural compounds such as potash compounds and natural stones are clinically safe and effective in treatment of cutaneous candidiasis as shown in the in vitro and in vivo tests results. Thus, they could serve as good alternatives to conventional antifungal antibiotics. I therefore recommend that more research be done on other cheaper, readily available, clinically safe and effective natural compounds for treatment of fungal infections and other diseases.

CONTRIBUTIONS TO KNOWLEDGE

This study revealed that several species of *Candida* cause Candidiasis in the study area including non-albicans, namely *C. tropicalis, C. glabrata* and *C. parapsilosis*. These *Candida* species are susceptible to the anticandidal effects of natural compounds (potash compounds and natural stones) at concentrations ranging from 1.56mg/ml to 200mg/ml. This study therefore reveals the safety of such treatment in view of the high level of resistance to conventional drugs by these *Candida* species. Pharmaceutical industries can therefore exploit the use or incorporation of these natural compounds to save mankind. This is important as antifungal drugs seem difficult to develop.

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APPENDIX 1

Statistical Analysis

Oneway Anova: Skin Sensitisation test

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean
						Lower Bound
Blue_Stone	Day 1	2	32.5000	.70711	.50000	26.1469
	Day 2	2	35.5000	.70711	.50000	29.1469
	Total	4	34.0000	1.82574	.91287	31.0948
Potash_Alum	Day 1	2	31.5000	.70711	.50000	25.1469
	Day 2	2	33.5000	.70711	.50000	27.1469
	Total	4	32.5000	1.29099	.64550	30.4457
Trona	Day 1	2	30.5000	.70711	.50000	24.1469
	Day 2	2	29.5000	.70711	.50000	23.1469
	Total	4	30.0000	.81650	.40825	28.7008
Sulphur_Stone	Day 1	2	32.5000	.70711	.50000	26.1469
	Day 2	2	30.5000	.70711	.50000	24.1469
	Total	4	31.5000	1.29099	.64550	29.4457
Black_Stone	Day 1	2	29.5000	.70711	.50000	23.1469
	Day 2	2	29.5000	.70711	.50000	23.1469
	Total	4	29.5000	.57735	.28868	28.5813
Palm_Ash	Day 1	2	30.5000	.70711	.50000	24.1469
	Day 2	2	28.5000	.70711	.50000	22.1469
	Total	4	29.5000	1.29099	.64550	27.4457
Water	Day 1	2	32.5000	.70711	.50000	26.1469
	Day 2	2	30.5000	.70711	.50000	24.1469
	Total	4	31.5000	1.29099	.64550	29.4457

Descriptives

		95% Confidence Interval for Mean	Minimum	Maximum
		Upper Bound		
Blue_Stone	Day 1	38.8531	32.00	33.00
	Day 2	41.8531	35.00	36.00
	Total	36.9052	32.00	36.00
Potash_Alum	Day 1	37.8531	31.00	32.00
	Day 2	39.8531	33.00	34.00
	Total	34.5543	31.00	34.00
Trona	Day 1	36.8531	30.00	31.00
	Day 2	35.8531	29.00	30.00
	Total	31.2992	29.00	31.00
Sulphur_Stone	Day 1	38.8531	32.00	33.00
	Day 2	36.8531	30.00	31.00
	Total	33.5543	30.00	33.00
Black_Stone	Day 1	35.8531	29.00	30.00
	Day 2	35.8531	29.00	30.00
	Total	30.4187	29.00	30.00
Palm_Ash	Day 1	36.8531	30.00	31.00
	Day 2	34.8531	28.00	29.00
	Total	31.5543	28.00	31.00
Water	Day 1	38.8531	32.00	33.00
	Day 2	36.8531	30.00	31.00
	Total	33.5543	30.00	33.00

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Blue_Stone	Between Groups	9.000	1	9.000	18.000	.051
	Within Groups	1.000	2	.500		
	Total	10.000	3			
Potash_Alum	Between Groups	4.000	1	4.000	8.000	.106
	Within Groups	1.000	2	.500		
	Total	5.000	3			
Trona	Between Groups	1.000	1	1.000	2.000	.293
	Within Groups	1.000	2	.500		
	Total	2.000	3			
Sulphur_Stone	Between Groups	4.000	1	4.000	8.000	.106
	Within Groups	1.000	2	.500		
	Total	5.000	3			
Black_Stone	Between Groups	.000	1	.000	.000	1.000
	Within Groups	1.000	2	.500		
	Total	1.000	3			
Palm_Ash	Between Groups	4.000	1	4.000	8.000	.106
	Within Groups	1.000	2	.500		
	Total	5.000	3			
Water	Between Groups	4.000	1	4.000	8.000	.106
	Within Groups	1.000	2	.500		
	Total	5.000	3			

Differential White Blood Cell Count

One way Sample One

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean
						Lower Bound
Neutrophil	Manual	2	16.5000	.70711	.50000	10.1469
	Automated	2	24.2000	1.69706	1.20000	8.9526
	Total	4	20.3500	4.57056	2.28528	13.0772
Lymphocytes	Manual	2	83.0000	1.41421	1.00000	70.2938
	Automated	2	69.7500	1.06066	.75000	60.2203
	Total	4	76.3750	7.71767	3.85884	64.0945
Monocytes	Manual	2	.0000	.00000	.00000	.0000
	Automated	2	2.1500	.21213	.15000	.2441
	Total	4	1.0750	1.24733	.62367	-.9098
Eosinophil	Manual	2	.0000	.00000	.00000	.0000
	Automated	2	1.1500	.21213	.15000	-.7559
	Total	4	.5750	.67515	.33758	-.4993
Basophil	Manual	2	.0000	.00000	.00000	.0000
	Automated	2	.3500	.21213	.15000	-1.5559
	Total	4	.1750	.23629	.11815	-.2010

Descriptives

		95% Confidence Interval for Mean		
		Upper Bound	Minimum	Maximum
Neutrophil	Manual	22.8531	16.00	17.00
	Automated	39.4474	23.00	25.40
	Total	27.6228	16.00	25.40
Lymphocytes	Manual	95.7062	82.00	84.00
	Automated	79.2797	69.00	70.50
	Total	88.6555	69.00	84.00
Monocytes	Manual	.0000	.00	.00
	Automated	4.0559	2.00	2.30
	Total	3.0598	.00	2.30
Eosinophil	Manual	.0000	.00	.00
	Automated	3.0559	1.00	1.30
	Total	1.6493	.00	1.30
Basophil	Manual	.0000	.00	.00
	Automated	2.2559	.20	.50
	Total	.5510	.00	.50

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Neutrophil	Between Groups	59.290	1	59.290	35.083	.027
	Within Groups	3.380	2	1.690		
	Total	62.670	3			
Lymphocytes	Between Groups	175.563	1	175.563	112.360	.009
	Within Groups	3.125	2	1.563		
	Total	178.688	3			
Monocytes	Between Groups	4.623	1	4.623	205.444	.005
	Within Groups	.045	2	.022		
	Total	4.667	3			
Eosinophil	Between Groups	1.322	1	1.322	58.778	.017
	Within Groups	.045	2	.023		
	Total	1.367	3			
Basophil	Between Groups	.122	1	.122	5.444	.145
	Within Groups	.045	2	.023		
	Total	.167	3			

Oneway Sample 2

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean
						Lower Bound
Neutrophil	Manual	2	22.5000	.70711	.50000	16.1469
	Automated	2	26.7500	.35355	.25000	23.5734
	Total	4	24.6250	2.49583	1.24791	20.6536
Lymphocytes	Manual	2	77.5000	.70711	.50000	71.1469
	Automated	2	67.9000	.14142	.10000	66.6294
	Total	4	72.7000	5.55818	2.77909	63.8557
Monocytes	Manual	2	.0000	.00000	.00000	.0000
	Automated	2	2.5000	.42426	.30000	-1.3119
	Total	4	1.2500	1.46401	.73201	-1.0796
Eosinophil	Manual	2	.0000	.00000	.00000	.0000
	Automated	2	1.6500	.21213	.15000	-.2559
	Total	4	.8250	.96047	.48023	-.7033
Basophil	Manual	2	.0000	.00000	.00000	.0000
	Automated	2	.5000	.14142	.10000	-.7706
	Total	4	.2500	.30000	.15000	-.2274

Descriptives

		95% Confidence Interval for Mean		
		Upper Bound	Minimum	Maximum
Neutrophil	Manual	28.8531	22.00	23.00
	Automated	29.9266	26.50	27.00
	Total	28.5964	22.00	27.00
Lymphocytes	Manual	83.8531	77.00	78.00
	Automated	69.1706	67.80	68.00
	Total	81.5443	67.80	78.00
Monocytes	Manual	.0000	.00	.00
	Automated	6.3119	2.20	2.80
	Total	3.5796	.00	2.80
Eosinophil	Manual	.0000	.00	.00
	Automated	3.5559	1.50	1.80
	Total	2.3533	.00	1.80
Basophil	Manual	.0000	.00	.00
	Automated	1.7706	.40	.60
	Total	.7274	.00	.60

ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
Neutrophil	Between Groups	18.063	1	18.063	57.800	.017
	Within Groups	.625	2	.313		
	Total	18.688	3			
Lymphocytes	Between Groups	92.160	1	92.160	354.462	.003
	Within Groups	.520	2	.260		
	Total	92.680	3			
Monocytes	Between Groups	6.250	1	6.250	69.444	.014
	Within Groups	.180	2	.090		
	Total	6.430	3			
Eosinophil	Between Groups	2.722	1	2.722	121.000	.008
	Within Groups	.045	2	.023		
	Total	2.767	3			
Basophil	Between Groups	.250	1	.250	25.000	.038
	Within Groups	.020	2	.010		
	Total	.270	3			

One way Sample 3

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean
						Lower Bound
Neutrophil	Manual	2	19.5000	.70711	.50000	13.1469
	Automated	2	28.0500	.77782	.55000	21.0616
	Total	4	23.7750	4.97351	2.48676	15.8610
Lymphocytes	Manual	2	77.5000	.70711	.50000	71.1469
	Automated	2	67.6000	.56569	.40000	62.5175
	Total	4	72.5500	5.73963	2.86981	63.4170
Monocytes	Manual	2	.0000	.00000	.00000	.0000
	Automated	2	2.5000	.42426	.30000	-1.3119
	Total	4	1.2500	1.46401	.73201	-1.0796
Eosinophil	Manual	2	2.5000	.70711	.50000	-3.8531
	Automated	2	.9000	.84853	.60000	-6.7237
	Total	4	1.7000	1.12250	.56125	-.0861
Basophil	Manual	2	.0000	.00000	.00000	.0000
	Automated	2	1.0000	.14142	.10000	-.2706
	Total	4	.5000	.58310	.29155	-.4278

Descriptives

		95% Confidence Interval for Mean		
		Upper Bound	Minimum	Maximum
Neutrophil	Manual	25.8531	19.00	20.00
	Automated	35.0384	27.50	28.60
	Total	31.6890	19.00	28.60
Lymphocytes	Manual	83.8531	77.00	78.00
	Automated	72.6825	67.20	68.00
	Total	81.6830	67.20	78.00
Monocytes	Manual	.0000	.00	.00
	Automated	6.3119	2.20	2.80
	Total	3.5796	.00	2.80
Eosinophil	Manual	8.8531	2.00	3.00
	Automated	8.5237	.30	1.50
	Total	3.4861	.30	3.00
Basophil	Manual	.0000	.00	.00
	Automated	2.2706	.90	1.10
	Total	1.4278	.00	1.10

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Neutrophil	Between Groups	73.103	1	73.103	132.312	.007
	Within Groups	1.105	2	.553		
	Total	74.208	3			
Lymphocytes	Between Groups	98.010	1	98.010	239.049	.004
	Within Groups	.820	2	.410		
	Total	98.830	3			
Monocytes	Between Groups	6.250	1	6.250	69.444	.014
	Within Groups	.180	2	.090		
	Total	6.430	3			
Eosinophil	Between Groups	2.560	1	2.560	4.197	.177
	Within Groups	1.220	2	.610		
	Total	3.780	3			
Basophil	Between Groups	1.000	1	1.000	100.000	.010
	Within Groups	.020	2	.010		
	Total	1.020	3			

APPENDIX 2

Molecular Identification