EVALUATION OF THE ANTI-FUNGAL ACTIVITIES O

OIL EXTRACT OF AZADIRACHTA INDICA A. J

OINTMENT FORMULATIONS

BY

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CHAPTER ONE

1.0 INTRODUCTION

The history of the relationship between man and plants is as old as the history of the creation of the world. Man used plants for different purposes. The use of plants in the alleviation and cure of bodily ills goes as far back as the history of the human race itself (Achterberg, 2013). The universal role of plants in the treatment of disease is exemplified by their employment in all the major systems of medicine irrespective of the underlying philosophical premise (Evans, 2002).

Use of herbal medicines in the developing world has continued to rise because they are rich sources of novel drugs and their bioactive principles form the basis in medicine, nutraceuticals, pharmaceutical intermediates and lead compounds in synthetic drugs (De N and Ifeoma, 2002 ; Ncube *et al*., 2008). More importantly in Africa, particularly West Africa, new drugs are often beyond the reach of the poor such that up to 80 % of the population uses medicinal plants as remedy against infections and diseases (Ezekiel *et al.,* 2009).

Various parts of Neem tree are claimed to have significant medicinal uses: which include uses in leprosy, malaria, asthma, intestinal worms (Biswas *et al*., 2002). Neem oil used in cosmetic industries is a product of the neem tree and is employed in soap making (Girish *et al*., 2008).

Lack of standardization of dosage of many plant drugs is one of the major problems associated with their use in many traditional settings. This problem could lead to wide variations in the content of the active ingredient(s) of the traditional medicinal remedies, therapeutic failure, toxicity, emergence of drug resistance and many other deleterious effects on consumers

(Olowosulu and Ibrahim, 2005). Another problem with the use of traditional medicinal remedies is non-adherance to Good manufacturing Practice (GMP) during their formulations. Frequently, herbal formulations are associated with alterations in the physical characteristics, stability and potency of medicament (Olowosulu and Ibrahim, 2005).

1.1 Statement of Problem

Fungal infection (Mycosis) constitutes a major health problem, especially in tropical and sub-tropical developing countries including Nigeria. Transmission of these ringworm-causing organisms from infected animals to people has been reported (Wabacha *et al*., 1998). Diseases caused by fungi include superficial infections of the skin by dermatophytes in the Microsporum, Trichophyton or Epidermophyton genera. These dermophytic infections are named for the site of infection rather than the causative organism (Ayesh, 2013).

Trichophyton mentagrophytes among other dermatophytes is a major causative agent of superficial dermatomycoses and is known to account for as much as 69.5 % of all dermatophytic infections (Pranab *et al.,* 2003). *Trichophyton mentagrophytes* have developed resistance to many antifungals which has resulted in clinical problems (Vanden Bossche *et al*., 1994).

Traditional antifungals are becoming ineffective; this has resulted to terbinafine resistant dermatophyte strains existence and may on occasion be the reason for lack of clinical response to the drug (Pranab *et al.,* 2003). There is also report of resistance of *Trichophyton spp* to fluconazole and conflicting cure rates have been reported in patients who have used it to treat dermatophytosis (Korting and Abeck, 1995). Resistance of *Trichophyton mentagrophytes* has increased due to indiscriminate use of commercial antifungal drugs (Bueno *et al*., 2010). The zoonotic nature of the infection demonstrates the potential for animal-derived ringworm in

humans, and indicates the need for an integrated approach to control of ringworm infections (Wabacha *et al.*, 1998).

Azadirachta indica A. Juss seed oil has been shown to have antifungal activity against *Trichophyton mentagrophytes* in vitro (Natarajan *et al*., 2003). A concentration of 15µg/mL of the oil was observed to distort the growth pattern of the organism (Natarajan *et al*., 2003). The oil is messy, smelly and often present problem of cleaning. More-over, it causes allergic reaction on application to the skin. The tendency of the oil to wash off easily when used alone and when formulated into cream has called for its formulation into an ointment. However, there is limited information on the evaluation of the antifungal activities of neem seed oil ointment formulation, particularly on dermatophytes *in-vivo*.

1.2 Justification

Dermatophytosis is a zoonotic skin infection of keratinized tissues caused by a specialized group of fungi named dermatophytes (Mukherjee *et al*., 2011). Many such fungi live only in the topmost layer of the epidermis (stratum corneum) and do not penetrate deeper thus causing superficial mycosis but occasionally the organisms do invade subcutaneous tissues resulting in kerion development (Lademann, 2013). It has worldwide distribution and a public health problem all over the world. *Trichophyton mentagrophytes* is a keratinophilic fungus belonging to a homogeneous group of fungi called the dermatophytes (Oyeka, 2000). The dermatophytes cause a variety of cutaneous infections in humans and animals (Chermette *et al*., 2008). Several human outbreaks of *T. mentagrophytes* infection have been reported so far by direct contact with infected individual or indirect contact with infectious propagules in the environment (Hashimoto, 1991). The increasing prevalence of fungal infection worldwide is as a result of increasing rate of resistance of most pathogenic fungi to existing orthodox antifungals and the existing chemicals

that destroy the epidermal layers. *Azadirachta indica* A. Juss plant is found in different parts of Nigeria. The plant seed oil has been shown to have antifungal *in-vitro* activity against *Trichophyton mentagrophytes* (Natarajan *et al*., 2003) and as such can be evaluated *in-vivo. In-situ* activity needs to be evaluated to enable its deployment on the clinical treatment of dermatophytic infections

1.3 Main objective

The main objective of this study is:

- To evaluate the *in-situ* efficacy of neem oil formulation and its potential uses as an antifungal preparation.

1.4 Specific objectives

The specific objectives of this study are to

i. isolate and characterize dermatophytes from infected skins.

ii. extract neem oil from the seeds by the Soxhlet and cold maceration methods using petroleum ether and n-hexane solvents, as well as fractionate it using different solvent systems and chromatograhic technique.

iii. determine the physicochemical properties of neem seed oil according to AOAC method (1990).

iv. determine the antimicrobial activity of the extracted oil and its fractions against the isolates by the agar diffusion and dilution methods.

v. formulate ointment preparation containing the seed oil and its fractions and evaluate their *in-situ* antifungal properties.

1.5 Research Hypothesis

Hypothesis (H_0):

- Ointment formulation containing Oil extract of *Azadirachta indica* A. Juss seed does not possess antifungal activity against dermatophytes.

Alternate Hypothesis (H_1):

- Ointment formulated with Oil extract of *Azadirachta indica* A. Juss seed can be useful as an antifungal product.

1.6 Research limitations

- Only dermatophytes and non dermatophytes isolated from *Tinea corporis* (ringworm) were used in this study.
- Study was limited to ointment formulations of the seed oil and its fractions.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Azadirachta indica* A. Juss

Scientific classification –

Kingdom: Plantae,

Division: Magnoliophyta

Order: Sapindales

Family: Meliaceae

Genus: *Azadirachta*

Species: ***A. indica*** (Girish *et al*., 2008).

2.2 Description of Plant

Neem tree *Azadirachta indica* belongs to the family Meliaceae. It is a tree in the mahogany family with broad dark stem and wide spreading branches. It is a tall evergreen tree with clear foliage originally native of India. *Azadirachta indica* is one of the most successful of the introduced tree species in Nigeria. It is extensively naturalized in drier parts of Nigeria, and a successful shade and fuel plantation tree. Neem tree has a hardy and the stem reaching between 20 to 24 meters in height. The shoot are divided into numerous leaflets, each resembling a full grown leaf, small white flowers which are auxiliary bunches from 1.5 to 2 cm in length, green or yellow fruits each containing a seed. It is commonly used in arid and sub-arid zone in afforestation programmes (Kaura, 1998).

The fruit is a smooth (glabrous) olive-like drupe which varies in shape from elongate oval to nearly roundish, and when ripe are 1.4–2.8 centimetres (0.55–1.10 in) by 1.0–1.5 centimetres (0.39–0.59 in). The fruit skin (exocarp) is thin and the bitter-sweet pulp (mesocarp) is yellowish-white and very fibrous. The mesocarp is 0.3–0.5 centimetres (0.12–0.20 in) thick. The white, hard inner shell (endocarp) of the fruit encloses one, rarely two or three, elongated seeds (kernels) having a brown seed coat.

2.3 Origin and geographical locations

The exact native range of this species is obscure, but it is thought to be native to the Indian sub-continent (India and Bangladesh) and South-East Asia. It is naturalized distribution globally include Northern Australia, Tropical Asia, Africa, Fiji, Mauritius, Puerto Rico, the Caribbean and many countries in South and Central America. It is invasive in parts of Nigeria, Niger and Chad. It is distributed widely in the drier parts of Nigeria (Northern part) (Gahukar, 2014).

Plate 1: ***Azadirachta indica*** **A. Juss fresh fruits and leaves**.

Plate 2: ***Azadirachta indica*** **A. Juss dried seeds**

2.4 The chemistry of *Azadirachta indica* A. Juss

More than 135 compounds have been isolated from different parts of the neem tree, the bark, the heartwood, the leaves, the fruits, and seeds (Biswas *et al*., 2002). Nearly 100 protolimonoids, limonoids or tetranortriterpenoids, pentanortriterpenoids, hexanortriterpenoids and some nonterpenoid constituents have been isolated from various parts of the Neem tree (Koul *et al*., 1990). The most important bioactive principal constituent is azadirachtin (Isman *et al*., 1990). These compounds are classified into two major groups- iso-prenoids and others. The iso-prenoids include diterpenoids and triterpenoids containing protomeliacins, liminoids, azadirone and its derivatives, genudin and its derivatives, vilarin type of compounds and csecomeliacins such as nimbin, salannin and azadirachtin. The first compound to be studied was nimbin. The non-isoprenoids include proteins (amino acids) and carbohydrates (polysaccharides), sulphurous compounds, poly-phenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds, phenolic acids, etc (Biswas *et al*., 2002). Neem seeds contain alkaloids, saponins, triterpens, steroids, tannins and phenols (Kosma *et al*., 2011).

2.4.1 Triterpenoids

All the well-characterised compounds identified in neem tree belong to the class triterpenoids (Ochoa-Villarreal *et al*., 2015). By definition triterpenes are hydrocarbons and possess no heteroatoms, functionalized triterpenes should instead be called triterpenoids (Schwarzbauer *et al.*, 2015); however this distinction is not always adhered to in scientific literature, with the two terms often being used interchangeably. Triterpenoids obtain from plants by biosynthetic studies have shown to be derived from acetate, via mevalonate and squalene, which is the cyclized. From the stereochemical arrangement of the methyl groups at C- 10, C-13 and C-14 and the side

chain at C-17, the known triterpenoids of neem are all derived from tetracyclic triterpenoids, tirucallol with a tirucallanane structure.

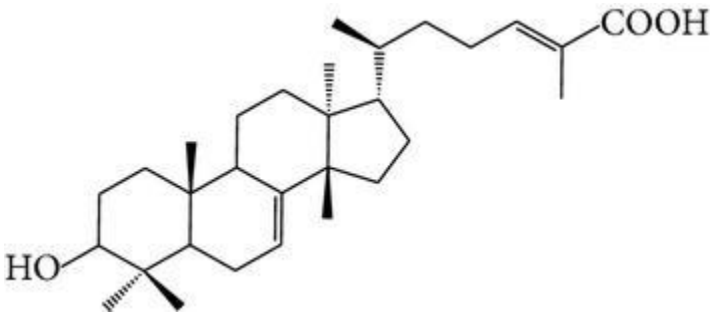

Figure 2.1: Molecular structure of Tirucallol (Paraschos *et al*., 2012)

Tirucallol has not been isolated from any neem product but all the substances that have been characterised can be considered as successive rearrangement and oxidation products of tirucallol. These can be divided into

I. Triterpenoids with the side chain intact but in which C-24 to C-27 have been lost (as in tetranortriterpenoids), and further altered products (penta- and hexa-nortriterpenoids)

II. Those in which the remaining carbon atoms are cyclized into a furan ring, the limonoids

III. Opening of the rings or rearrangement of the skeleton e.g. cleavage reaction involving fission of C -12 and C-13 in triterpenoids.

2.4.1.1 *Intact triterpenes*

A number of oxidized intact triterpenes have been isolated from other species of Meliaceae. These protolimonoids appear to be biosynthetic precursors of the limonoids because of their biological occurrence and oxidation pattern. The only protolimonoids isolated from neem tree are meliatriol isolated by Levie in 1967 (Subrahmanyam, 1990) and nimbocenon isolated by

Siddiqui *et al*., 1986. Both compounds show a preparatory step towards the formation of furan ring.

2.4.1.2 *Limonoids*

I. Intact ring –C system

Limonoids are usually defined as triterpenes derived from which four sides chain carbons have been lost and the remainder have been cyclized to a furan ring, hence the alternative name, tetranortriterpenoids. As well as the changes in the side chain, they also show a rearranged carbon skeleton, with migration of the double bond to a different ring and the C-30 methyl groups from C-14 to C-8. This arrangement has been demonstrated *in-vitro* by the conversion of turraeanthin from *Turraeanthus africanus* into a simple limonoid. A number of simple limonoids from neem which are closely related to tetracyclic triterpenoids such as Azadiradione (nimbinin) and azadirone were isolated by Kraus and Cramer 1987 (Siddiqui *et al*., 2009).

II. C- Ring opened limonoids

The most characteristic groups of triterpene-derived compounds so far identified in neem tree are those limonoids in which ring C has been open by oxidation, it is in this group of compounds that the interesting biological properties begin to appear. The first limonoid to be purified from neem is nimbin which is present in many parts of the tree. It is isolated as a crystalline solid from seed oil, root and bark. Hydrolysis of the three ester moieties gave deacetylnimbin, nimbinic acid and nimbin at C-2, C-3 double bond and sublimation of nimbic acid gave pyronimbic acid. Other compounds isolated include nimbolin A and B, salannin, nimbidinin, nimbolide (Gutierrez, 2011).

There is also A- and D- ring opened limonoids, but these are less common in the plant parts, examples are 4α, 6α-dihydroxy-A-homoazadirone for A-ring opened limonoids and Gedunin for D-ring opened limonoid (Gutierrez, 2011)

III. Products without a furan ring

There are a number of examples now known which would otherwise be classed as limonoids, but which do not contain the furan ring. The first of such compounds contained C-20, C-23 lactones, regarded as photo-oxidation products, possibly formed during isolation e.g the nimbin and salannin derivatives respectively (Gutierrez, 2011).

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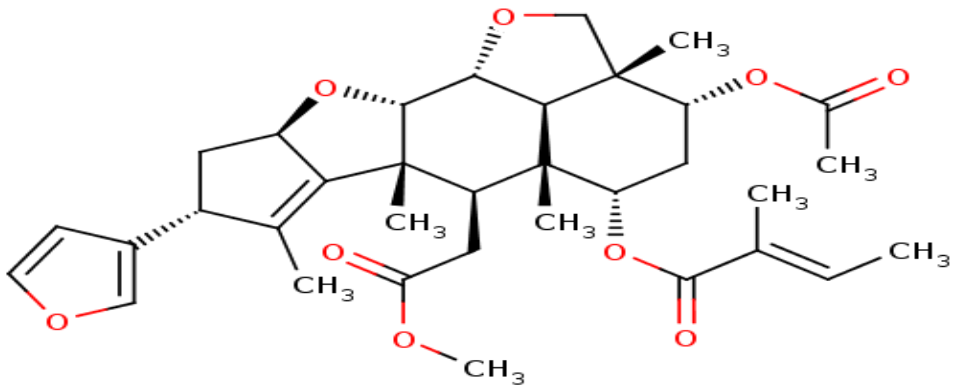


Figure 2.2: Molecular structure of salannin (Tan and Lou, 2011).

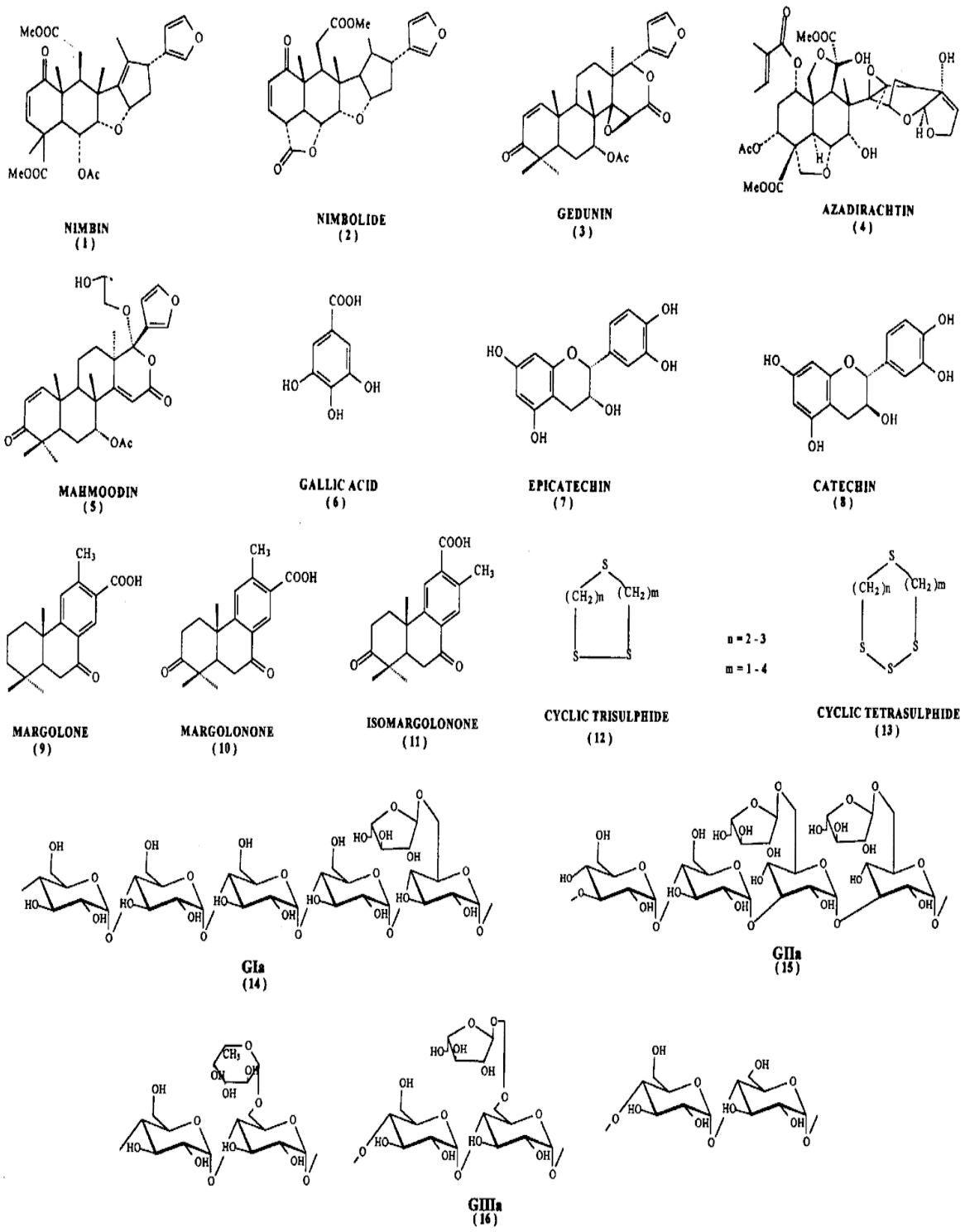


Fig 2.3: Some isolated chemical constituents from neem (Sudhir *et al*., 2010)

2.5 Chemical constituents of neem seed oil

The seed oil which is usually extracted by steam or solvents from crushed seed consists mainly of triglycerides and large amounts of triterpenoids (Kapazoglou *et al*., 2013). Nimbolide, gedunin and nimbin are chemical compounds with antifungal activity from neem seed oil (Hashmat *et al*., 2012). Nimbin which is a major crude bitter principle extracted from the oil of seed kernels of *A. indica* demonstrated several biological activities. From this crude principle some tetranortriterpenes, including, nimbinin, nimbidinin, nimbolide and nimbidic acid have been isolated (Agrawal, 2012).

2.5.1 Nimbin

Nimbin is a chemical compound classified as a triterpenoid isolated from *Azadirachta indica* (neem tree). Nimbin is thought to be responsible for much of the biological activities of neem, and is reported to have anti-inflammatory, antipyretic, antifungal, antihistamine and antiseptic properties. Nimbin demonstrated antifungal activity by inhibiting the growth of *Tinea rubrum* in *vitro*, it can completely inhibit the growth of *Mycobacterium tuberculosis* and was also found to be bactericidal (Rajarajan *et al*., 2012).

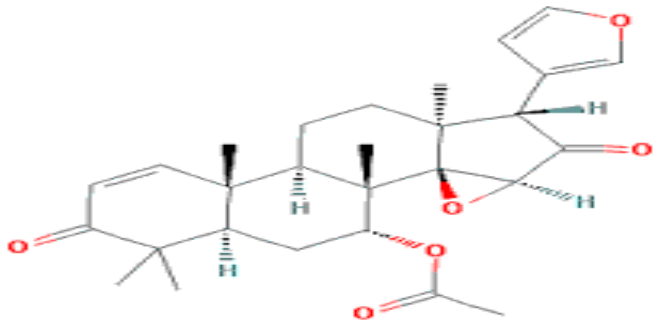


Figure 2.4: Molecular structure of Nimbinin (Bhowmik *et al*., 2010)

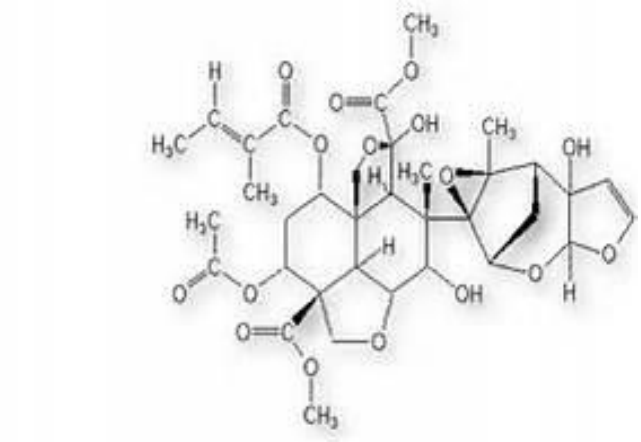


Fig 2.5: Molecular structure of nimbin (Sidhu *et al*., 2004)

Properties: The molecuar formula of nimbin is $C_{30}H_{36}O_9$ with a molar mass of 540.60mol^{-1}. Has a melting point of 205^{o}C (401^{o}F, 478K). The provisional naming was *nimbin* (sulphur-free crystalline product with melting point at 205 °C, empirical composition $C_7H_{10}O_2$), the structure of *nimbinin* (with similar principle, melting at 192 °C) to epoxyazadiradione which however differs in their optical rotation and *nimbidin* (cream-coloured containing amorphous sulphur, melting at 90–100 °C) was also isolated (Zahedi and Lohi, 2010).

2.5.2 Nimbolide

A terpenoid lactone derived from *Azadirachta indica* (Neem tree). Nimbolide has been shown to exert antimalarial activity by inhibiting the growth of *Plasmodium falciparum* (Murugan *et al*., 2013). Nimbolide also shows antibacterial activity against *S. aureus* and *S. coagulase* (Sukanya *et al*., 2009). Nimbolide from seed oil show antifungal, antimalarial and antibacterial activity including inhibition of *Mycobacterium tuberculosis* (El-Mahmood *et al*., 2008). It has a molecular formula of $C_{27}H_{30}O_7$ and a molecular weight of 466.5229g/mol.

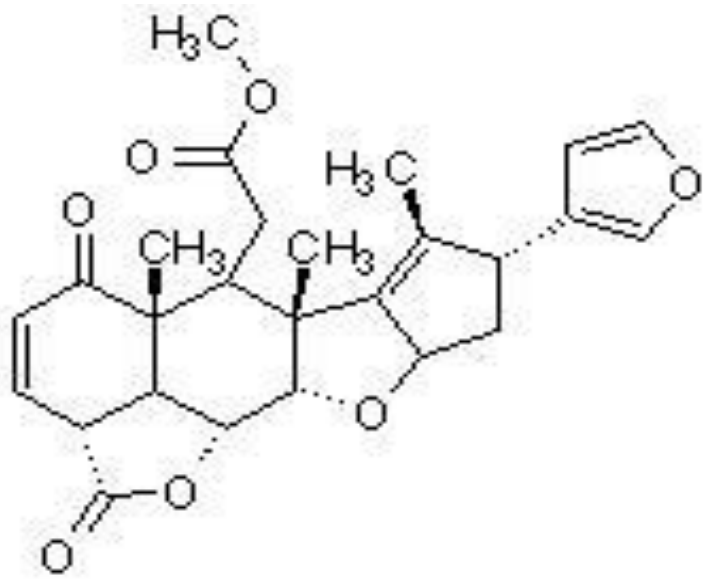


Fig 2.6: Molecular structure Nimbolide (Paul *et al*., 2011)

2.5.3 Gedunin

Gedunin is a tetranortriterpenoid ingredient obtained from the Indian neem tree (*Azadirachta indica*) with molecular weight of 482.57g/mol and molecular Formular $C_{28}H_{34}O_7$. Gedunin, isolated from neem seed oil has been reported to possess both antifungal and antimalaria activities (Biswas *et al*., 2002).

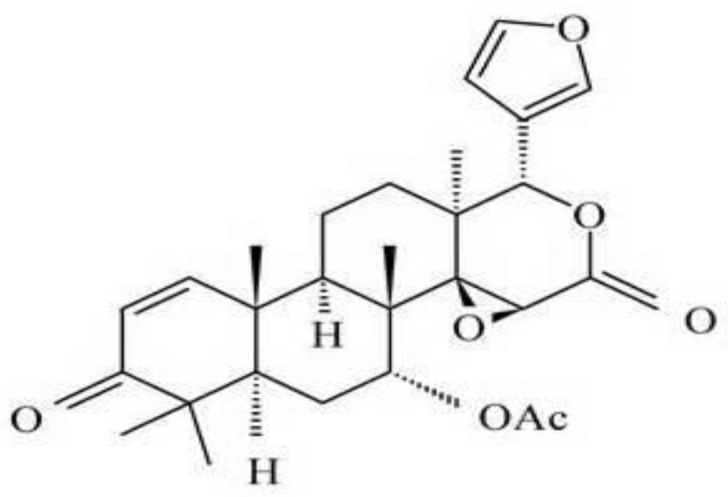


Fig 2.7: Molecular structure of Gedunin (Patwardhans et al., 2013)

2.5.4 Mahmoodin

Mahmoodin, a deoxygedunin isolated from seed oil, has been shown to possess moderate antibacterial action against some strains of human pathogenic bacteria (Biswas *et al.***,** 2002).

2.5.5 Margolonone and isomargolonone: They are active against *Klebsiella, Staphylococcus* and *Serratia* species (Biswas *et al*., 2002). These compounds have been isolated from neem stem bark. Sulphur-containing compounds such as cyclic trisulphide and tetrasulphide isolated from the steam distillate of fresh, matured neem leaves have antifungal activity against *Trichophyton mentagrophytes* (Biswas *et al*., 2002).

2.6 Medicinal Properties

Azadirachta indica is a multi-purpose timber tree from which high value products are extracted for use as multi-purpose medicines (Akula *et al*., 2003). It's effective to cure eczema and scabies; Lotion derived from neem seed, when locally applied, can cure these dermatological

diseases within 3-4 days in acute stage or a fortnight in chronic case. A paste prepared with neem and turmeric was found to be effective in the treatment of scabies (Akula *et al*., 2003).

The scientific literature is full of reports of studies on roots, stem bark, seeds, flowers and fruits of higher plants having bioactive substances such as peptides, alkaloids, tannins, phenols, sterols, flavonoids, glycosides amongst others which confer healing properties for their use in medicine (El-Mahmood *et al*., 2008). The therapeutic efficacies of the *A. indica* have been described by practitioners of traditional medicine. Some of the ethnomedicinal uses include treatment of skin disorders, rashes and boils, respiratory tract infections, sore gums and throat, eye and ear infections (Isman *et al*., 1990; Kaura *et al*., 1998; Akula *et al*., 2003). Extracts from Neem trees are very effective in treating various skin fungus conditions, including athlete's foot and lesions in the mouth and vagina (Tomar *et al*., 2009). Clinical studies with the dried neem leaf extract indicated its effectiveness to cure ringworm, eczema and scabies (Koul *et al*., 1990).

More serious diseases such as chicken pox and small pox have been treated with Neem tree pastes and even people suffering from herpes and hepatitis B viruses have obtained relief from Neem tree and its parts (Parida *et al*., 2000). Lotion derived from neem leaf, when locally applied, can cure these dermatological diseases within 3–4 days in acute stage or a fortnight in chronic case 98.99 %. A paste prepared with neem and turmeric was found to be effective in the treatment of scabies. In 97 % of cases, the paste was found to cure scabies within 3–15 days of treatment without any adverse effect (Koul *et al*., 1990).

2.7 Antifungal chemotherapeutic agents

Chemotherapy (often abbreviated to **chemo** and sometimes **CTX** or **CTx**) is a category of cancer treatment that uses chemical substances, especially one or more anti-cancer drugs (chemotherapeutic agents) that are given as part of a standardized chemotherapy regimen.

Chemotherapy may be given with a curative intent, or it may aim to prolong life or to reduce symptoms (palliative chemotherapy). In the case of infectious disease, it involves the use of drugs that will kill or limit the growth of microorganisms.

2.7.1. Mechanisms of action of antifungal drugs

Fungal cells are complex organisms that share many biochemical targets with other eukaryotic cells .Therefore, agents that interact with fungal targets not found in eukaryotic cells are needed (Debono,1994). The fungal cell wall is a unique organelle that fulfills the criteria for selective toxicity and the cell wall differs greatly from the bacterial cell wall and is not affected by antibacterial cell wall inhibitors such as the β-lactams or vancomycin (Debono, 1994).

Arrangement of the biomolecular components of the cell wall accounts for the individual identity of the organism. Although, each organism has a different biochemical composition, their gross cell wall structure is similar. There are three general mechanisms of action for the antifungal agents: cell membrane disruption, inhibition of cell division and inhibition of cell wall formation (Ghannoum *et al.*, 1999).

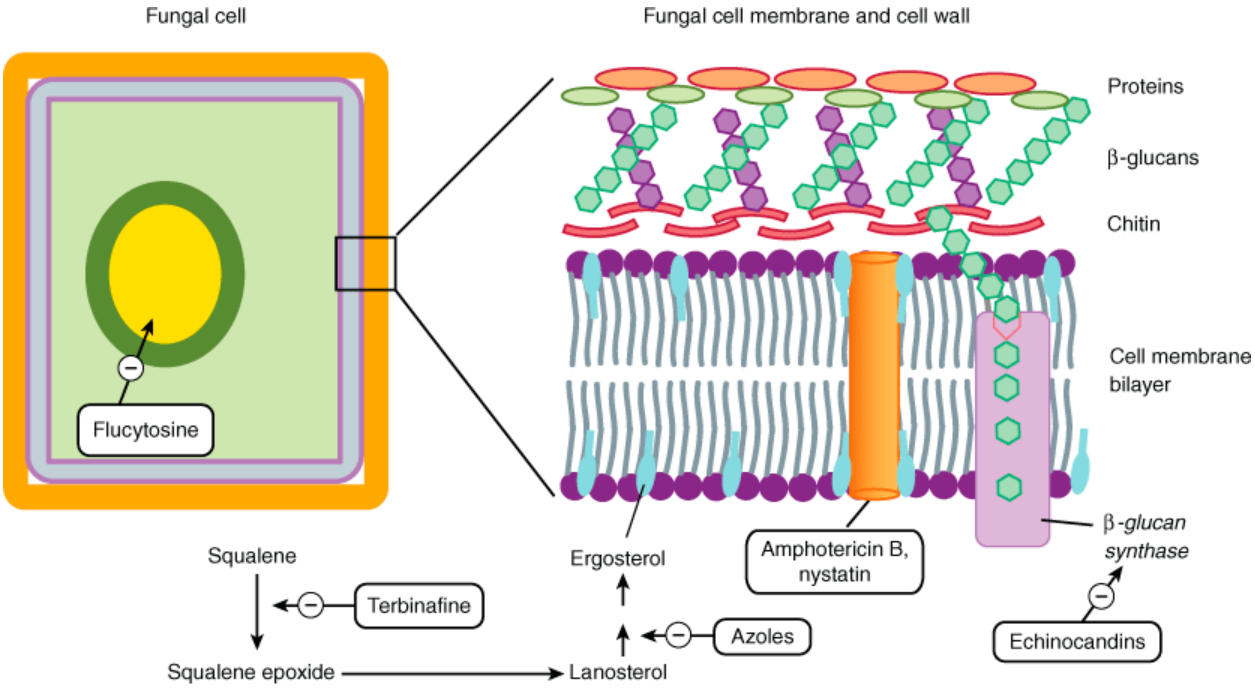


Figure 2.8: Mechanism of action of anti-fungal drugs (Aboh *et al*., 2014)

Table 2.1: Antifungal class, examples and their mechanism of action

Drug Class	Examples	Mechanism of action
Polyenes	Amphotericin B, Nystatin	Interacts with sterol in cell membrane forming channels that leak cellular contents
Antibiotics	Griseofluvin	Inhibit mitosis in the fungi
Azoles	Fluconazole, Itraconazole,	Inhibit ergosterol biosynthesis at the level of C14 demethylase
Allylamines	Terbinafine	Inhibit ergosterol biosynthesis at the level of squalene epoxidase
Thiocarbamate	Tonalftate	Inhibit ergosterol biosynthesis at the level of squalene epoxidase
Antimetabolites	Flucytosin	Inhibit DNA and RNA synthesis via conversion of 5- fluorocytosine to 5-fluoricil (5- fluouracil)
Profens	Ibuprofen	Damage to the cytoplasmic membrane

2.7.1.1 *Inhibition of cell wall formation*

Interference with fungal cell wall biosynthesis has not been as successful and effective as penicillin and cephalosporin against bacteria (Demain and Sanchez, 2009). Many chemicals have been discovered that interfere with various steps in fungal cell wall synthesis with excellent antifungal activity in vitro (Odds *et al*., 2003).Unfortunately, development of these agents into useful drugs has proven very difficult as many of these agents are developed to target β-glucan synthesis (Ghannoum *et al*.,1999).

2.7.1.2 *Cell membrane disruption*

Antifungal agents that disrupt the cell membrane do so by targeting ergosterol, either by binding to the sterol, forming pores and causing the membrane to become leaky (as with polyenes antifungals), or inhibiting ergosterol biosynthesis (as seen with azole antifungal agents (Avis, 2007). Ergosterol is similar to mammalian cholesterol, thus agents binding ergosterol may have acytotoxic effect in the host tissue. Ergosterol has two conjugated double bonds that are lacking in mammalian sterols (Sturleys, 2000).

2.7.1.3 *Inhibition of cell division*

Nucleoside antifungal agents affect cell division by targeting the microtubule effects in forming the mitotic spindle. They can also act by inhibiting DNA transcription (Stanton *et al.,* 2011).

2.7.2 Classification of antifungal agents

Based on their mechanism of actions (inhibition of cell division, cell membrane disruption and inhibition of cell wall formation) the major antifungal agents can be grouped into five classes: polyenes; azoles; allylamines; echinocandins; and other agents, including griseofulvin and flucytosine (Lewis, 2011).

2.7.2.1 *Polyenes antifungal agents*

The polyenes are a class of antifungal drugs that target the disruption of membranes. They bind to ergosterol in fungal membrane causing membrane to become leaky. Example of such class of drugs include amphotericin B and nystatin, are amphipathic. They are thought to have both hydrophobic and hydrophilic sides which intercalate into the membranes, forming a channel through which cellular components, especially potassium ions, leak and thereby destroying the proton gradient within the membrane. It has also been suggested that amphotericin B causes oxidative damage to the fungal plasma membrane (Lewis, 2011).

2.7.2.2 *Azole antifungal agents*

Azole antifungal agents are the largest class of synthetic antimycotics. Some used topically to treat superficial dermatophytic and yeast infections. Others used systemically to treat severe fungal infections. Antifungal activity stems from the presence of an aromatic five member heterocyclic, either an imidazole or a triazole (Shalini *et al*, 2011). The first members of the class were highly substituted imidazoles (clotrimazole, miconazole) were not absorbed orally. Ketoconazole introduced in 1984 was the first effective oral therapy for *Candida* (Como *et al*., 1994). Structure activity studies revealed that the imidazole ring can be replaced by a bioisosteric triazole ring without affecting the antifungal activity but achieving higher selectivity of the fungal targets vs. host. Itraconazole and Fluconazole are more potent, less toxic and provide effective oral therapy for many systemic fungal infections. These two are triazoles. Another triazole has been recently introduced (voriconazole). That said, amphotericin B is usually the preferred drug for life threatening systemic fungal infections. It is still the “gold standard”.

These imidazoles and triazoles inhibit CYP P450 14 α- demethylase in fungi. This enzyme is involved in the conversion of lanosterol to ergosterol. Other P450s in sterol biosynthesis may be affected. Five azole antifungals, miconazole, econazole, oxiconazole, sulconazole and tioconazole share a common general structure but differ by the additional structural elements attached to the central methylene carbon. All five antifungals are used in topical application only (Saag *et al*., 1988)

2.7.2.3 *Echinocandins*

There are three clinically important echinocandins and Caspofungin is the first to be licensed in Australia while Micafungin and Anidulafungin approved in United States and Australia respectively (Cappelletty *et al*., 2007)

2.7.2.4 *5-Flucytosine*

5-Flucytosine (5-FC) has an entirely distinct mode of action from the azoles. 5-FC is taken up into the cell by a cytosine permease and deaminated into 5-fluorouracil (FU) by cytosine deaminase. 5-FC is fungus specific since mammalian cells have little or no cytosine deaminase. FU is eventually converted by cellular pyrimidine-processing enzymes into 5-fluoro-dUMP (FdUMP), which is a specific inhibitor of thymidylate synthetase, an essential enzyme for DNA synthesis, and 5-fluoro-UTP (FUTP), which is incorporated into RNA, thus disrupting protein synthesis of the fungi (Barchiesi *et al*.,2000)

2.7.3 Antifungal Used in the Study.

2.7.3.1 *Terbinafine*

This is an allylamine with more limited spectrum of activity than the azoles and triazoles and is effective against dermatophytes (Denning and Hope, 2010). They are employed in the treatment of fungal infections of the skin and nails. This antifungal agent is reversible, non-competitive inhibitors of the first step in ergosterol biosynthesis, the conversion of squalene to squalene-2,3-epoxide by squalene epoxidase (Ruckenstuhl *et al*., 2005). The build-up of squalene in the cell membrane is toxic to the cell, causing pH imbalances and malfunction of membrane bound proteins.

Terbinafine comes as a tablet to be taken orally or as a topical cream. It is used to treat dermatophyte infections of the toenail or fingernail (onychomycosis, tinea unguium) caused by susceptible fungi. It is also effective in treatment of nail infections caused by most strains of *Trichophyton rubrum* and *T. mentagrophytes*. Although usually active in vitro against *Epidermophyton floccosum*, *Candida albicans*, and *Scopulariopsis brevicaulis*, efficacy in treatment of onychomycosis caused by these organisms has not been established in adequate and controlled studies (Balfour.and Faulds, 1992)

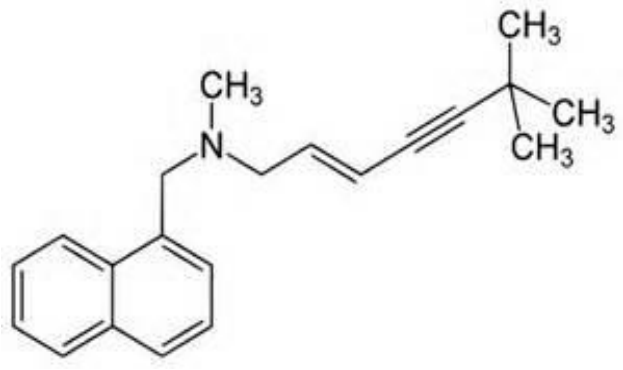


Figure 2.9: Chemical structure of Terbinafine (Sheng *et al*., 2006)

2.8 Physicochemical Parameters

Different physical and chemical parameters (Fattah *et al*., 2013) of non-edible oil are used to monitor the compositional quality of oils. These physicochemical parameters include organoleptic, iodine value (IV), saponification value (SV), acid value (AV), free fatty acid (FFA), refractive index (RI), viscosity, density and peroxide value (PV). Non-edible oils are one of the main constituents used in cosmetic purposes (Campanelli *et al*., 2010)

2.8.1 Acid value

It is defined as the weight of KOH in mg needed to neutralize the organic acids present in 1g of fat and it is a measure of the free fatty acids (FFA) present in the fat or oil. In chemistry, acid value (or "neutralization number" or "acid number" or "acidity") is the mass of potassium hydroxide (KOH) in milligrams that is required to neutralize one gram of chemical substance. It is an important tool that is used to indicate the quality, age, edibility and suitability of oil for use in industries (Pauli *et al*., 2014). It is used to indicate the previous lipase activity and other hydrolytic reaction or oxidation. It also measures the extent to which glyceride in the oil has

been decomposed by lipase and other physical factors such as light and heat. The acid number is a measure of the amount of carboxylic acid groups in a chemical compound, such as a fatty acid, or in a mixture of compounds.

2.8.2 Peroxide value

The peroxide value is an index of rancidity, thus indicates a poor resistance of the oils to peroxidation during storage. The maximum acceptable value of 10 meg peroxide/kg signifies its high oxidative stability (Zheng, 2003). Detection of peroxide gives the initial evidence of rancidity in unsaturated fats and oils. Other methods are available, but peroxide value is the most widely used. It gives a measure of the extent to which an oil sample has undergone primary oxidation, extent of secondary oxidation may be determined from p-anisidine test.

The double bonds found in fats and oils play a role in autoxidation. Oils with a high degree of unsaturation are most susceptible to autoxidation. The best test for autoxidation (oxidative rancidity) is determination of the peroxide value. Peroxides are intermediates in the autoxidation reaction. Autoxidation is a free radical reaction involving oxygen that leads to deterioration of fats and oils which form off-flavours and off-odours. Peroxide value, concentration of peroxide in an oil or fat, is useful for assessing the extent to which spoilage has advanced (Frank *et al*., 2011).

2.8.3 Iodine value

The iodine value is the measure of the degree of unsaturation of fatty acids in oil and could be used to quantify the amount of double bonds present in the oil which reflects the susceptibility of the oil to oxidation (Orhevba *et al.,* 2013). The higher the iodine value the higher the percentage

of the unsaturated fatty acids and the more C = C bonds present in the fat. The iodine value (or "iodine adsorption value" or "iodine number" or "iodine index") in chemistry is the mass of iodine in grams that is consumed by 100 grams of a chemical substance. Iodine numbers are often used to determine the amount of unsaturation in fatty acids. This unsaturation is in the form of double bonds, which react with iodine compounds. The higher the iodine number, the more C=C bonds are present in the fat. Very saturated oil is suitable for making soap while unsaturated oil on the other hand are well suited for making oil paints (Del Federico *et al* ., 2010).

2.8.4 Saponification value

Saponification value is a measure of the molecular weights of the triglycerides in oils and fats (Akbar *et al*., 2009). The triglycerides with high value of saponification value are considered to make better quality soaps than those with low saponification value (Atadashi *et al*., 2010). Saponification value (or "saponification number"/"Koettstorfer number", also referred to as "sap" in short) represents the number of milligrams of potassium hydroxide required to saponify 1 g of fat under the conditions specified. It is also a measure of the average molecular weight (or chain length) of all the fatty acids present. As most of the mass of a fat/tri-ester is in the 3 fatty acids, it allows for comparison of the average fatty acid chain length. The long chain fatty acids found in fats have a low saponification value because they have a relatively fewer number of carboxylic functional groups per unit mass of the fat as compared to short chain fatty acids. If more moles of base are required to saponify N grams of fat then there are more moles of the fat and the chain lengths are relatively small (Al-Amin *et al*., 2014).

2.8.5 Free fatty acid

Free fatty acid is the percentage by weight of a specified fatty acid (Arab, 2003). High concentrations of free fatty acids are undesirable in crude oils because they result in large losses of the neutral oil during refining. In chemistry, particularly in biochemistry, a fatty acid is a carboxylic acid with a long aliphatic tail (chain), which is either saturated or unsaturated. Most naturally occurring fatty acids have a chain of an even number of carbon atoms, from 12 to 28. Fatty acids are usually derived from triglycerides or phospholipids. When they are not attached to other molecules, they are known as "free" fatty acids. Fatty acids are important sources of fuel because, when metabolized, they yield large quantities of ATP. Many cell types can use either glucose or fatty acids for this purpose. In particular, heart and skeletal muscle prefer fatty acids. Despite long-standing assertions to the contrary, fatty acids can be used as a source of fuel for brain cells, at least in some rodents, in addition to glucose and ketone bodies (Crupi *et al*., 2013).

2.8.6 Viscosity

The viscosity of a fluid is a measure of its resistance to gradual deformation by shear stress or tensile stress. For liquids, it corresponds to the informal concept of "thickness". For example, honey has a much higher viscosity than water. Viscosity is a property arising from collisions between neighboring particles in a fluid that are moving at different velocities. When the fluid is forced through a tube, the particles which compose the fluid generally move more quickly near the tube's axis and more slowly near its walls: therefore some stress, (such as a pressure difference between the two ends of the tube), is needed to overcome the friction between particle layers to keep the fluid moving. For the same velocity pattern, the stress required is proportional to the fluid's viscosity.

A fluid that has no resistance to shear stress is known as an *ideal* or *inviscid* fluid. Zero viscosity is observed only at very low temperatures in superfluids. Otherwise, all fluids have positive viscosity, and are technically said to be viscous or viscid. In common parlance however, a liquid is said to be viscous if its viscosity is substantially greater than that of water; and may be described as mobile if the viscosity is noticeably less than water. A fluid with a relatively high viscosity, for example, pitch, may appear to be a solid (Dealy and Wissbrun, 2012).

2.9 Ointments

Ointment is a topical medication applied on the body surfaces. In medical terms, an ointment is defined as a homogeneous, viscous, semi-solid preparation with a high viscosity that is used for external application (Agrawal *et al*., 2012). An ointment has medicated ingredients which serve a protective, therapeutic, or prophylactic purpose when applied on the skin or mucous membranes (Asija, 2015).

2.9.1 Types of ointments

The medicated stuff or the ingredients present inside the ointment is actually the main base of ointments. There are 4 main ointment bases:

- Hydrocarbon bases, e.g. hard paraffin, soft paraffin, microcrystalline wax and ceresine
- Absorption bases like as wool fat, beeswax
- Water soluble bases like as macrogols 200, 300, 400
- Emulsifying bases like an emulsifying wax
- Vegetable oils such as coconut oil, sesame oil, olive oil, almond oil and peanut oil (Asija, 2015).

2.9.2 Ointment application

There are various parts of the body surfaces, skin and mucous membranes where ointment is applied for curing certain skin or disease conditions. Ointment is applied on hands, legs, face, eyes, ears, vagina, anus, throat etc. There are various problems when an ointment is suggested for treatment such as Ointment for burns, cuts, pain, itching, inflammation and pain, boils and scars, skin problems like eczema, dermatitis and psoriasis (Garg and Goyal, 2014).

2.9.3 List of ointments

An ointment may or may not be medicated. Some are easily over the counter while are some are prescribed by the doctor. Since they are very moisturizing, they are good for dry skin (Basler, 2000). Ointments also have a low risk of sensitization and low irritation risk. There is typically little difference between brands of generic and named brand drugs. Some popular ointments are listed below: Betamethasone, Norfloxacin, Soframycin, Ciprofloxacin, Ketoconazole, Ofloxacin, Tobramycin + Dexamethasone, Povidone- Iodine, Diclofenac, Polymyxin- B.

2.9.4 Ointment used in this study

2.9.4.1 *Whitfield Ointment*

Whitfield's ointment is salicylic acid and benzoic acid in a suitable base, such as lanolin or Vaseline. The original ointment contains 3 % salicylic acid and 6 % benzoic acid, but other ratios are also used. It is used for the treatment of fungal infections, such as athlete's foot. Its effectiveness is unclear. It can have a slight burning effect that goes away after a few minutes. It is named after Arthur Whitfield (1868–1947), a British dermatologist. Whitfield ointment is highly effective in the treatment of tinea, of the chronic dry-scaling variety. The ingredient Benzoic Acid has antibacterial and antifungal properties. It is applied topically for

fungal skin infections such as ringworm and tinea. Salicylic acid has keratolytic and fungicidal properties. The Ointment should not be applied to broken or inflamed skin. Its use should be discontinued if excessive dryness or irritation of the skin occurs (Lamarre *et al*., 2009).

2.10 Characteristics of some fungal species

2.10.1 *Trichophyton mentagrophytes*

It is a zoophilic dermatophyte of wild and domestic rodents which is occasionally transmitted to man and other animals by direct contact with an infected animal or asymptomatic carrier or with contaminated material (hair and scales) from the environment (Aboh *et al*., 2014). On SDA plates they appear as flat, white to cream in colour, with powdery to granular surface. Conidia are numerous and single celled in dense clusters. Colonies are positive to hair perforation test (Brasch and Graser, 2005). *Trichophyton mentagrophytes* are the major causes of dermatomycosis (commonly known as ringworm) in many parts of the world. The infection is mainly spread by contact between infected and susceptible animals or via a contaminated environment such as bedding and walls (Aboh *et al*., 2014). *Trichophyton mentagrophytes* is also the second most commonly isolated fungus causing so-called tinea infections in humans, and the most common fungus that causes zoonotic skin disease (i.e. transmission of mycotic skin disease from humans to animals, and vice versa). The fungus has a major natural reservoir in rodents, but can also infect pet rabbits, dogs and horses (Oyeka, 2000)

2.10.2. *Candida albicans*

Candida albicans is a diploid fungus that grows both as yeast and filamentous cells and a causal agent of opportunistic oral and genital infections in humans (Aboh *et al*., 2014). On Sabouraud dextrose agar (SDA) it grows as cream coloured, smooth surfaced and glabrous colonies. It is

Gram positive and germ tube positive. Systemic fungal infections (fungemias) including those by *C. albicans* have emerged as important causes of morbidity and mortality in immunocompromised patients (e.g. AIDS, cancer chemotherapy, organ or bone marrow transplantation). *Candida albicans* biofilms may form on the surface of implantable medical devices. In addition, hospital-acquired infections by *C. albicans* have become a cause of major health concerns (Aboh *et al*., 2014).

Candida albicans is commensal and a constituent of the normal gut flora comprising microorganisms that live in the human mouth and gastrointestinal tract. *Candida albicans* can be found in 80% of the human population without causing harmful effects, although overgrowth of the fungus results in candidiasis (candidosis). Candidiasis is often observed in immunocompromised individuals such as HIV-infected patients. A common form of candidiasis restricted to the mucosal membranes in mouth or vagina is thrush, which is usually easily cured in people who are not immunocompromised (Aboh *et al*., 2014).

2.10.3. *Microsporon canis*

It is considered a zoophilic dermatophyte, given that it typically colonizes the outer surface of animal's body. Hence, animals, cats and dogs are believed to be the population hosts of this fungus, while humans are occasional hosts, in which the fungus can induce secondary infections. *Microsporum canis* has been identified as a causal agent of a ringworm infection (*Tinea corporis*) in humans, and children.

Microsporum canis is among the most common dermatophytes associated with tinea capitis and tinea corporis. Unlike some dermatophyte species, *M. canis* typically does not cause large

epidemics. Humans become infected as a result of direct or indirect contact with infected pets. *Microsporum canis* generally invades hair and skin; however, some nail infections have been reported. When hair shafts are infected, *M. canis* causes an ectothrix-type of infection where the fungus envelopes the exterior of the hair shaft without the formation of internal spores. This colonization of the hair shaft causes it to become unsheathed, resulting in characteristic round or oval non-inflammatory lesions they develop on the scalp. Infection triggers an acute leukocytic reaction in subcutaneous tissues, which gradually becomes highly inflammatory and leads to hair loss, in the case of *Tinea capitis* (Atzori *et al*., 2012).

2.10.4. *Aspergillus niger*

Aspergillus niger (A. niger) produce colonies that are of white or yellow in colour and is covered by dark asexually produced fungal spores. Mycelia, or threadlike, hyphae are divided by a septum and transparent. *A. niger* is a haploid filamentous fungus and is a very essential microorganism in the field of biology. In addition to producing extracellular enzymes and citric acid, *A. niger* is used for waste management and biotransformation. The fungi are most commonly found in mesophilic environments such as decaying vegetation or soil and plants (Gupta *et al*., 2012)

Genome sequencing of *A. niger* is important because of its involvement in producing citric acid as well as industrial enzymes, such as amylases, proteases, and lipases. The uses of these enzymes are essential because of its importance for transformation to food enzymes. Other properties of this species include pathogens that cause the spoilage of food and production of secondary metabolites, such as aflatoxin, that are toxic.

Aspergillus niger was isolated from the plant *Welwitschia mirabilis* in Namibia and Angola, a plant estimated to be about 3000 years old (Henschel and Seely, 2000). *A. niger* is easily isolated from common thing such as dust, paint, and soil. Commonly in labs, *A. niger* is isolated via chemostat cultures which can test positively or negatively for the fungi.

Aspergillus niger is relatively harmless compared to other filamentous fungi. Despite this fact, there have been some medical cases that have been accounted for, such as lung infections or ear infections in patients with weakened immune system, or an immune system that has been impaired by a disease or medical treatment. In the case of ear infections, *A. niger* invades the outer ear canal which can cause damage to the skin it came in contact with (Hidaka *et al*., 1998).

2.10.5. *Trichophyton rubrum*

Trichophyton rubrum is an anthropophilic dermatophyte. Many strains and varieties of *T. rubrum* have been described and opinion differs between mycologists as to the exact validity of many of these. For practical purposes two types may be distinguished: *T. rubrum* downy type and *T. rubrum* granular type. Microscopically, the downy type is characterized by the production of scanty to moderate numbers of slender clavate micro conidia and no macro conidia. Microscopically, the granular type is characterized by the production of moderate to abundant numbers of clavate to pyriform microconidia and moderate to abundant numbers of thin-walled, cigar-shaped macro conidia. The macro conidia may or may not have terminal appendages. Superimposed on these two microscopic morphologies are culture characteristics such as pigment production and surface typography. The downy strain has become the most widely distributed dermatophyte of man. It frequently causes chronic infections of skin, nails and rarely scalp. The granular strain is a frequent cause of tinea corporis in South East Asia and in Aborigines living in the Northern Territory of Australia. However, since the Vietman War, it has

been spread throughout the world, especially to those countries with returning troops or to those receiving refugees, where it has often been described as a new species. The granular strain represents the parent strain of the downy type; the later evolved by establishing a niche in feet (tinea pedis) when the former was imported into Europe around the turn of the century. It should be stressed that intermediate strains between the two types do occur and that many culture and morphological characteristics overlap (Sotirio *et al*., 2010).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Equipment

- Soxhlet extractor, {Quick- Fit, England}
- Chromatographic column, {Supelco, Italy}
- Biosafety cabinet class 2, {Esco, USA}
- Microscope, {Model XSZ-107BN, Olympus Optical Company, Ltd., Japan}
- Refrigerator, {Thermocool Engineering Company Ltd., Nigeria}
- Incubator, {Memmert Karl Kolb Scientific Technical Supplies, West Germany}
- Autoclave ,bench top, {Adelphi MFG Co Ltd, Portland}
- Electronic weighing balance, {Ohaus, AR2140, U.S.A,}.
- Water bath .{Gesellschaft fur Labortechnik, Germany}
- Rotary evaporator, {Buchi Rotavapor, U.S.A.}
- Oven, {Gallenkamp, England}.
- Spectrophotometer, Single-beam, {SPECTRONIC 20D; Milton Roy Company, Madrid, Spain}

3.1.2 Reagents.

- Petroleum Ether, {Sigma Aldrich- Missouri, U.S.A}.
- N-Hexane, {Sigma Aldrich- Missouri, U.S.A.}
- Chloroform, {Sigma Aldrich- Missouri, U.S.A.}
- Tween 80, {Sigma Aldrich- Missouri, U.S.A.}
- Silica gel G, {Merck, Germany}

- Dimethylsulfoxide, {Sigma Aldrich- Missouri, U.S.A.}
- Lactophenol cotton blue, {Sigma Aldrich- Missouri, U.S.A.}
- Soft Emulsifying wax, {BBH, England}
- Soft White Paraffin, {BBH, England}
- Liquid Paraffin, {BBH, England}

3.1.3 Microbiological Media

- Sabouraud Dextrose Agar, {Oxoid, Basingstoke, U.K.}
- Sabouraud Dextrose Broth, {Oxoid, Basingstoke, U.K.}
- Sabouraud Dextrose Agar + Chloramphenicol + Cycloheximide, {Cat No.21089.00 Deben Diagnostics Limited, U.K}

3.1.4 Reference Antifungals

- Terbinafine powder, {Cat No. F8929, Sigma Aldrich, U.S.A.}

3.2 Methods

3.2.1 Collection, identification and processing of plant material

The plant seeds were obtained from National Research Institute for Chemical Technology (NARICT), Zaria, Kaduna State, Nigeria. Identification and authentication of *Azadirachta indica* A Juss seed was done in the herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Kaduna State, Nigeria by Mal. Namadi Sunusi and given voucher specimen number 900151.

Neem seed was washed, sun-dried and foreign materials removed by winnowing. The cleaned neem seeds was oven dried at 50 ºC and then grinded into powder using milling machine at National Research Institute for Chemical Technology (NARICT), Zaria, Kaduna State.

3.2.2 Oil Extraction

3.2.2.1 *Soxhlet extraction*

Soxhlet extraction method was used to extract the oil from the processed seed. The produced material was placed in a 250 ml flask on a heating mantle. The solvents used were Petroleum-ether and n- hexane. 1500 g of the neem seed powder was packed inside a muslin cloth and placed in a thimble of Soxhlet extractor. A round bottom flask containing either of the solvents was fixed to the end of the extractor and a condenser was tightly fixed at the bottom end of the extractor. Extraction was done successively and exhaustively each time with each of the various solvents (n- Hexane followed by Petroleum ether) (Awolu *et al*., 2013).

The flask was then heated at 60 ºC with the use of a heating mantle. The solvent was vaporized and condensed into the evaporator. The process continued for 4 hours. Oil was recovered from the mixture (oil and solvent) by the use of rotary evaporating process. The oil was obtained and stored in a bottle for further processes.

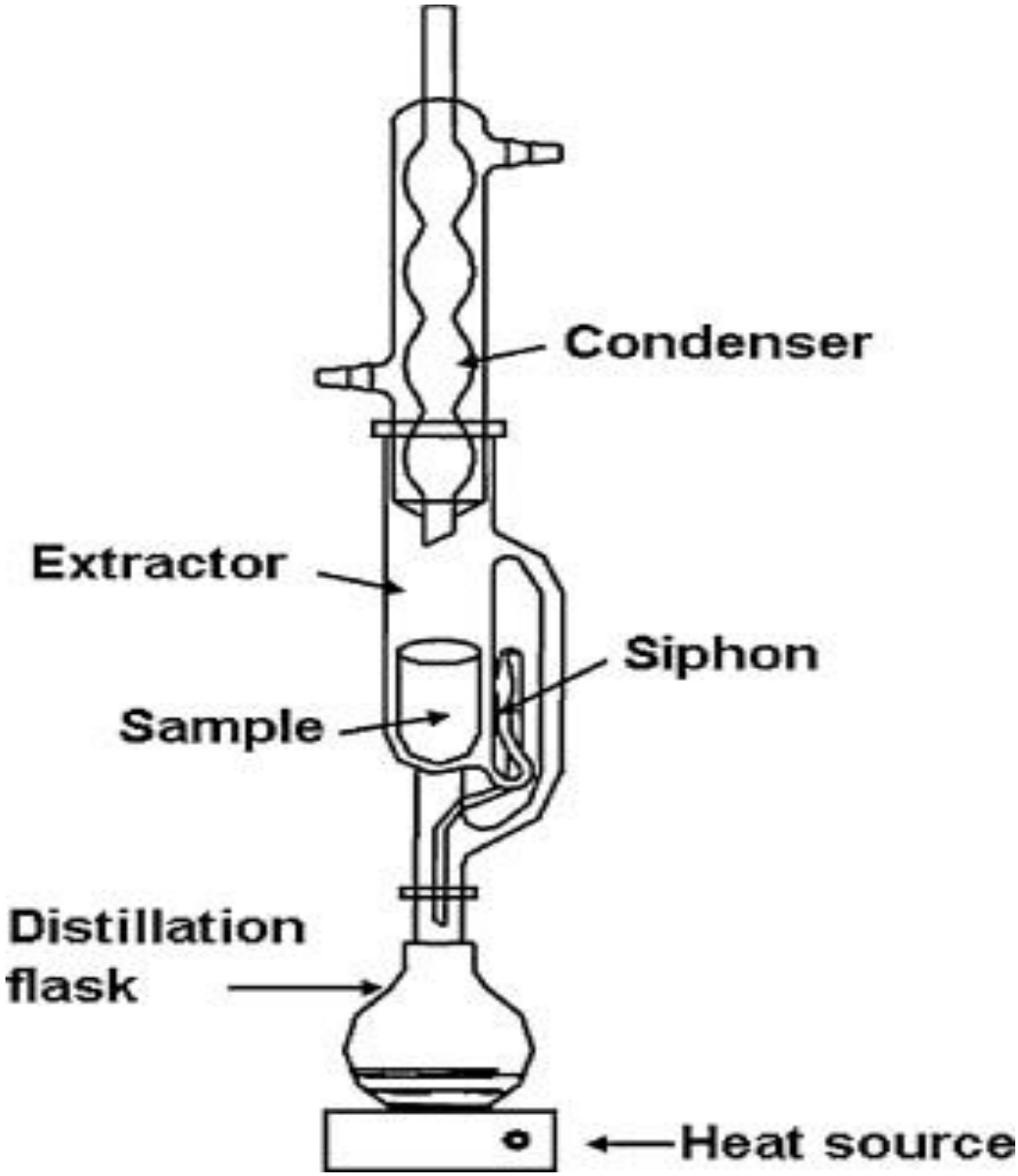


Figure 3.1 Soxhlet Extractor (Wang *et al*., 2010)

3.2.2.2 *Cold maceration*

In this process, 1500 g of the coarsely powdered crude plant seed was placed in a stoppered container (conical flask) with 1000 mL of the solvents (petroleum ether or n- hexane) and was allowed to stand at room temperature for a period of 3 days with intermittent agitation (stirring) until the soluble matter dissolved. The mixture was then strained, the marc (the damp solid material) pressed, and the oil extract was clarified by filtration after standing. The oil was recovered from the mixture (oil and solvent) by the use of rotary evaporator (Atabani *et al*., 2013).

3.2.2.3 *Fractionation of Oil Extract*

A portion (50 ml) of crude n-hexane oil extract was subjected to column chromatography using silica gel G as an absorbent. The column was successively eluted with hundred milliliters (100 mL) of n-hexane (100 %), hexane: chloroform mixture (75:25 and 50:50 %) and 100 % chloroform. The crude petroleum ether oil extract was eluted with 100% petroleum ether, then petroleum ether: chloroform mixture (75:25 and 50:50%) and 100 % chloroform. The extraction steps are shown in Figure 3.2

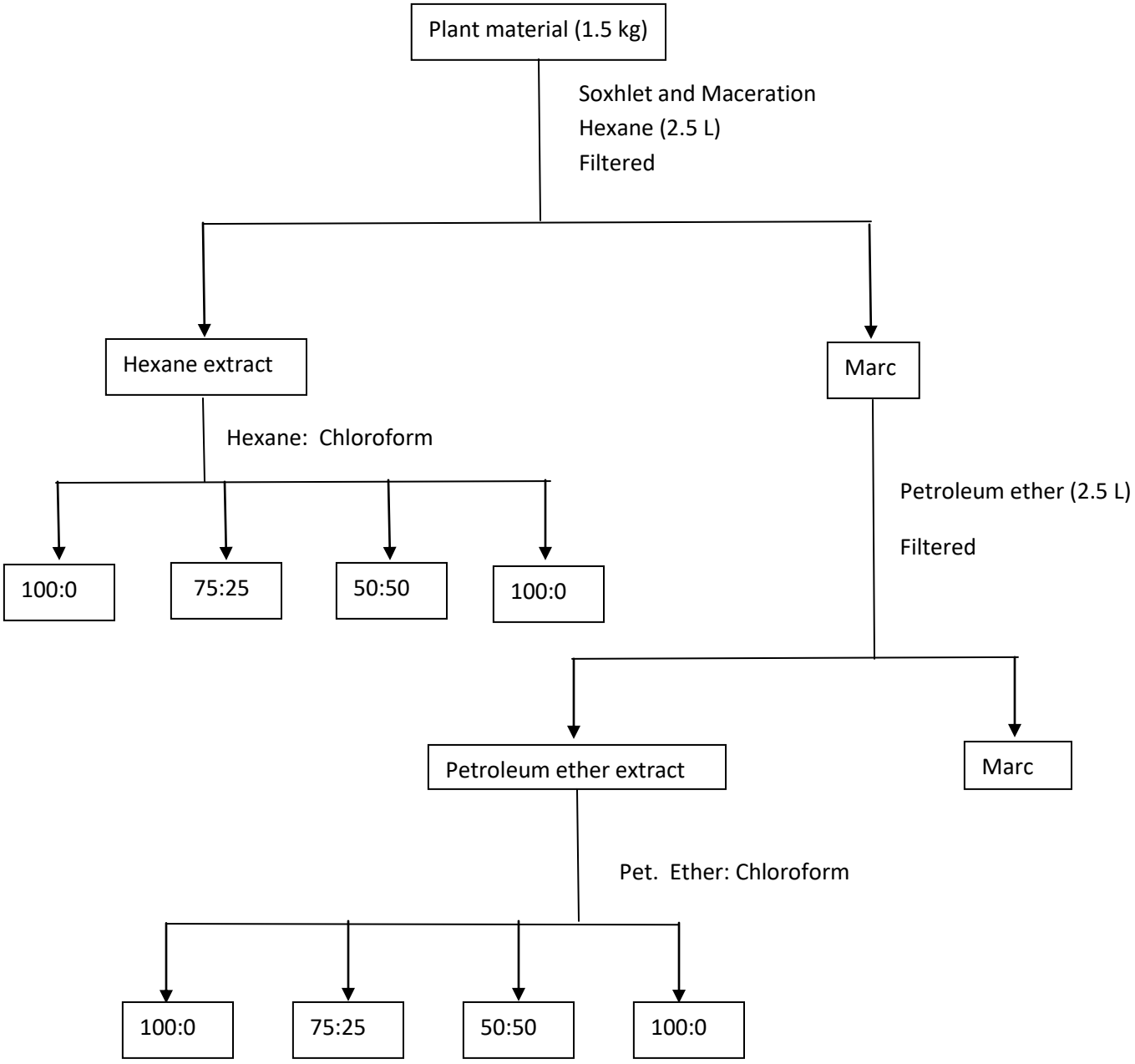


Figure 3.2 Extraction and Fractionation of Neem seeds oil

3.2.3 Physicochemical analysis

The following physicochemical analysis which involved organoleptic properties, density, viscosity, saponification value, iodine value, acid value, peroxide value, and free fatty acid of the neem oil were carried out as described by Association of Official Analytical Chemists methods (AOAC, 1990).

3.2.3.1 *Determination of iodine value* (IV)

The iodine value was determined by using Wijs method IUPAC (1979) (Paquot, 2013). 0.20 g of neem seed oil sample was weighed into a ground neck conical flask, 10ml carbon tetrachloride and 25 ml Wijs reagent was added, shaken and placed in the dark for an hour. Thereafter, 20 ml 10 % KI and 150 ml distilled water was added. This was titrated against 0.1 M sodium thiosulphate using 1 % starch solution as an indicator. A blank test was also carried out simultaneously under the same condition.

Iodine value = $\frac{12.69\ x\ T\ x\ (V1-V2)}{W}$ …….. 1

Where,

T = Molarity of sodium Thiosulphate solution

V_1 = Volume of Thiosulphate solution used for blank test

V_2 = Volume of thiosulphate solution used for test sample

W = Weight of the test sample

3.2.3.2 ***Determination of saponification value*** **(SV)**

This was carried out as described by IUPAC (1979) (Paquot, 2013). Two (2) gram of neem seed oil was weighed into a ground neck conical flask and 25ml ethanolic 0.5 M KOH was added. Antibumping chip was added and reflux condenser fitted. This was boiled for an hour. 0.5 ml of phenolphthalein was then added and titrated with 0.5M HCl solution after an hour. A blank test was carried out simultaneously.

$$\text{Saponification value} = \frac{56.1\ x\ T\ (V2 - V1)}{W} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad 2$$

Where,

T = Concentration (Molarity) of Hydrochloric acid used

V_2 = Volume of Hydrochloric acid used for the blank

V_1 = Volume of Hydrochloric acid used for the test sample

W = Weight of the test sample

3.2.3.3 ***Determination of acid value*** **(AV)**

This was carried out as described by IUPAC (1979). A solvent mixture of 25 ml 95 % ethanol and 25 ml diethyl ether was neutralised with 0.1 M ethanolic KOH using Phenolphthalein as indicator. Four (4) gram of the oil sample was dissolved in the neutralized solvent mixture and titrated with 0.1 M KOH.

$$\text{Acid value} = \frac{56.1\ X\ T\ X\ V}{W} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad 3$$

where,

T = Molarity of ethanolic KOH used

V = Volume of KOH use,   W = Mass in gram of the test sample

3.2.3.4 *Determination of peroxide value* (PV)

This was carried out as described by IUPAC (1979). Two gram each of the fermented and unfermented oil was weighed in ground neck conical flask and 10ml chloroform added to dissolve the oil. This was followed by addition of 15ml acetic acid and 1ml 5 % KI solution. The mixture was then shaken and kept in the dark for about 5 minutes. Seventy five millitre (75 ml) of water was added and titrated with 0.002 M Sodium thiosulphate.

Peroxide value = $\frac{V\ X\ T\ X\ 100}{W}$ ……..…… 4

Where,

V = Volume of sodium thiosulphate solution used

T = Molarity of thiosulphate

W = Mass in gram of test sample

3.2.3.5 *Determination of free fatty acid*

The FFA content of neem oil was determined volumetrically using aqueous sodium hydroxide (0.1 M) and phenolphthalein indicator (1 % ethanol) a neutral mixture of diethyl ether: ethanol (1:1) (50 ml) was used as a solvent. FFA values were reported as percentage oleic acid by weight And measured by the formula.

% FFA = $\frac{(2.82\ x\ volume\ of\ 0.1\ N\ NaOH\ in\ mL)}{W}\ X\ 100$…… 5

Where,

w = Weight of oil in gram

3.2.3.6 *Determination of refractive index*

Precisely 5 ml of the oil was filtered through a filter paper to remove impurities and traces of mixture. Steam of water was circulated through the instrument (butyro-refractometer) and the glass prism cleaned and dried. Few drops of the oil was placed in the prism, closed and allowed to stand for 2 minutes at a constant temperature. The butyro-refractometer and lightning were adjusted and the most distinct reading was obtained, refractive index determined.

$R = R^1 + K_1 (T^1 - T)$ …….. 6

Where,

R = Reading of refractometer reduced to a specified temperature

R^1= Reading at T^1

T^1= Temperature at which R^1 was taken

T = Specified temperature (40 ^oC)

K_1= constant (0.58)

3.2.3.7 *Determination of viscosity*

The capillary tube viscometer test method was used in determining the kinematic viscosity of neem oil. Five (5) ml of each oil was poured into a beaker and transferred into a clean and dried viscometer. The viscometer was cleansed with a non toxic solvent and dried.

The viscometer, containing the oil, was inserted, into the water bath at temperature of 30 ^oC .The pump was used to raise the level of the oil to the starting mark on the left hand limp of the viscometer; another finger was used to close the other limp to avoid the flow of the oil due to air. The finger was removed to allow the flow of oil down the capillary at that point, the time at

which the oil flow down was taken and recorded. The viscosity was then obtained by multiplying the constant of the viscometer by the time obtained. in centistokes:

$V = Ct + B/t$ [cSt] ,………………………………………………………….. 7

Where,

C = the instrument calibration constant ,

B = the instrument type constant depending on the capillary diameter,

t = efflux time in seconds.

3.2.3.8 *Determination of density*

Pycnometer was used to determine the density of the oil. The weight of empty Pycnometer and when filled with the oil was recorded. The density of the oil was calculated by dividing the weight of the oil by the volume of the pycnometer

3.2.3.9 *Determination of relative density*

The density of water was determined by the use of pycnometer and recorded. The relative density of the oil was calculated as the ratio of density of oil and that of water

3.2.3.10 *Spectroscopic analysis of A. indica oil and its fractions*

***Infra Red (I.R) Analysis*:** Infra Red (I.R) Analysis of the absorption spectra of *A. indica* oil and the fractions that have activity against dermatophytes was conducted at National Institute for Chemical Technology (NARICT), Basawa, Zaria, Kaduna state of Nigeria. A Fourier transform infrared (FTIR) spectrometer was used in which the sample was placed. The spectrometer directed beams of IR at the sample and measured how much of the beam and at which frequencies the sample absorbs the infrared light. The molecular identities were determined

through a reference database which houses thousands of spectra, so samples can be identified. The functional groups present in the oil and its fractions were determined by comparing the vibration frequencies in wave numbers of the samples spectrograph obtained from an FT-IR spectrophotometer.

3.2.4 Microbiological analysis

3.2.4.1 *Preparation of reference antifungal agents, culture media and solution*

a. Reference antifungal

Stock solutions of Terbinafine was prepared by dissolving appropriate quantity of the antifungal agent in dimethyl sulfoxide (DMSO) and later diluted to their required concentrations with broth.

b. Preparation of culture media

The required quantities of the dehydrated fungal media were weighed and prepared with distilled water according to the manufacturer's specifications. Where necessary, gentle heating was applied to aid dissolution and the media were dispensed into 10 or 20 ml sizes and sterilized by autoclaving at 121 ^{o}C for 15 minutes. These were kept aseptically until required.

c. Preparation of sterile solutions

Sterile distilled water: distilled water was dispensed in volumes of 100 ml and 200 ml in clean bottles and sterilized by autoclaving at 121 ^{o}C for 15 minutes. Sterile normal saline with 3 % Tween 80: 9 g of sodium chloride and 30 mL of Tween 80 were dissolved in 1 litre of distilled water. This solution was dispensed in volumes of 9 ml or 10 mL and sterilized by autoclaving at 121 ^{o}C for 15 minutes

3.2.4.2 *Isolation and characterization of dermatophytes*

a. **Ethical approval**.

The proposal for this study was submitted to the Ministry of Health Kaduna State. After obtaining ethical approval in line with WHO requirement, an informed consent form and questionnaire of each patient to willingly participate in the study was sought.

b. Specimen collection

Thirty (30) swab samples of T*inea corporis* (ringworm) were collected from the infected skins of 'Tsangaya Almajiri' pupils Kudan, Kaduna State using sterile scalpels. Affected areas were cleansed with 70%v/v ethanol, allowed to dry and light scrapings from the edge of the lesions were taken using a blunt sterile scalpel blade. The specimens were placed in clean white envelopes with each participants code labelled. Mycological analysis of the 30 specimens from the suspected infected sites of the participants was carried out in the Pharmaceutical Microbiology Laboratory of the Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, A.B.U Zaria.

c. Culture

The specimens were inoculated into 10 ml Sabouraud Dextrose broth and incubated for 48 hours. Growth from the broth was then streaked on Sabouraud Dextrose Agar containing chloramphenicol and cycloheximide and incubated at 37 ^{0}C for 14 days. Cultures were examined weekly for sporulation in case of the dermatophytes and *A. niger*. Pure colonies of dermatophytes were later subcultured on respective petri-dishes containing Sabouraud Dextrose Agar and incubated at 30 ^{o}C for 21 days (Wisselink *et al*., 2011)

d. Isolation and identification

Suspensions of the fungi spore were first made in Sabouraud Dextrose broth and later inoculated on Sabouraud dextrose Agar (SDA, Oxoid, England). The isolates were identified by routine mycological and biochemical procedures as modified by Mohammed *et al*., (2013).

- **Urease Test**: The Christensen's urea agar medium was prepared by mixing 29 grams of urea agar base with 15 grams of SDA and diluted with 1000 ml of distilled water. A phenol indicator was added to 10ml of the medium in two separate test tubes labelled A and B. *T. mentagrophytes* and *T. rubrum* were inoculated into test tube A and B respectively and incubated for 7 days (Mohammed *et al*., 2013).
- **Corn Meal:** The corn meal medium was prepared by mixing 8grams of corn meal with 4 grams of Agar and 2 grams of dextrose, the mixture was the diluted with 200mls of distilled water. *T. rubrum* isolates were inoculated into 10mls of the slant test tubes and incubated at room temperature until the dark red colour was observed (Mohammed *et al*., 2013).
- **Nutritional test:** Eight grams of rice grains were added to 25 ml of distilled water in conical flask and sterilized. 10 ml was dispensed in 20 ml sterile dispensing bottle and inoculated with the test sample (*microsporum canis*). The bottle was incubated at room temperature for 7 days until growth was observed (Mohammed *et al*., 2013).

Identification of dermatophyte species from positive culture was based on colony characteristics in pure cultures and microscopic morphology which include: the presence of conidia (macro and micro), and microscopic appearance (accessory structures) of the said conidia. Test organisms were aseptically purified, sub cultured and grown on 10 ml Sabouraud dextrose agar slants and thereafter kept in the refrigerator at 2 – 8 ^{0}C.

C*andida albicans* ATCC 10231 was obtained from the Department of Microbiology and Biotechnology, National Institute for Pharmaceutical Research and Development, Abuja, Nigeria. Subsequent dilutions were prepared from the above suspensions and were maintained in sterile distilled water at 4 °C until needed

3.2.4.3 *Determination of antifungal activity of Azadirachta indica* A. Juss

a. Cultivation and Standardization of Test Fungi

Eighteen-hour solid culture of the test *Candida spp*. was subcultured into sterile Sabouraud dextrose liquid medium. It was standardized according to Clinical Laboratory Standards Institute (CLSI, 2002) by gradually inoculating in normal saline to compare its turbidity to McFarland standard of 0.5 which is approximately 1.0×10^6 c.f.u/ml. However, for *Trichophyton mentagrophytes, Trichophyton rubrum, Microsporum canis, Candida albican and Aspergillus niger* fungal spores were harvested from 7 day old Sabouraud dextrose agar (SDA) slant cultures by washing with 10 ml sterile normal saline containing 3 % v/v Tween 80 with aid of sterile glass beads to help in dispersing the spores. Thereafter, the spore suspension were standardized to 1.0×10^5 spores / ml by using a single-beam spectrophotometer at 530 nm (OD530) adjusted to 70 -72 % transmittance for *Trichophyton mentagrophytes, Trichophyton rubrum* and *Microsporum canis* (Aboh *et al*., 2014). All adjusted suspensions were quantified by spreading 100 µl on Sabouraud dextrose agar plate and incubated at 30 °C for 72 hours for dermatophytes (Aberkane *et al*., 2002). Standardized isolates were maintained at 4 ºC (in the refrigerator) until required for use.

b. Antifungal Screening of the Seed oil Extract and Fractions

Eighteen hours cultures of *Candida albican* and suspension of spores prepared from fresh, mature (3- to 5-day-old) cultures (in some cases an extended incubation was required for proper sporulation of the isolate) in Sabouraud dextrose liquid medium were standardized to produce inoculum size of 10^6 c.f.u/ml for Candida albican and 10^5 c.f.u/ml for dermatophytes. 1 ml of the diluted culture of each test organism was used to flood Sabouraud dextrose agar media and excess aseptically drained.

The plates were allowed to dry at 30°C temperature in a sterilized incubator. Using the agar diffusion cup plate method, a sterile cork borer (6 mm) was used to bore holes in the agar plates. The bottoms of the wells (holes) were sealed with the appropriate molten Sabouraud dextrose agar. Using micropipette, 0.1 ml each of the different graded concentrations of the oil 'A', 'B', and 'C' were dispensed into the holes marked '1' (100 %v/v), '2' (90 %v/v), and '3' is 10 % DMSO used in diluting the oil extracts used as a negative control. These were allowed to diffuse into the agar at room temperature for one hour before incubation at 30 °C for 14 days. The diameters of zones of inhibition of the test organisms were measured to the nearest millimeter, using a well-calibrated meter ruler. The experiment was carried out in triplicates.

c. *Determination of Minimum Inhibitory Concentration (MIC)*

For fungal MIC determination, agar dilution method as modified by (Serban *et al*., 2011) was used.10 mL of the graded concentrations of the crude oil extracts was mixed with 10 ml of double strength Sabouraud dextrose agar supplemented with 0.3 % v/v Tween-80 and poured aseptically into sterile plates. The plates were allowed to set. 10 µL of the standardized organisms containing 10^6 cfu/mL was inoculated on the equidistantly placed sterile filter paper

disc. The plates were allowed to stand for one hour to allow interaction of the organism and test agent, and then incubated at 30 ^{o}C for 48 hours. The same procedure was repeated using Terbinafine. The lowest concentration of the agent that inhibited the visible growth of the test organisms was taken as the MIC.

d. *Determination of Minimum Fungicidal Concentration (MFC)*

The filter paper discs showing no visible growth from the determination of minimum inhibitory concentration was aseptically removed with the aid of a sterile forceps and transferred into 5ml sterile Sabouraud dextrose liquid medium and incubated at 30 ^{o}C for 48 hrs. Minimum fungicidal concentrations were determined as the lowest concentration resulting in no growth of subculture (Serban *et al*., 2011).

3.2.4.4 *Antifungal activity of A. indica oil ointment*

a. Preparation of emulsifying ointment base

Hundred (100) gram of emulsifying ointment was prepared by fusion method. Weighed amount of emulsifying wax was melted in evaporating dish on a water- bath. Calculated quantity of white paraffin (50 %w/w) was then added and stirred until all the ingredients were melted. The evaporating dish containing the content was removed from water-bath and appropriate amount of liquid paraffin added and stirred thoroughly until the mass cooled down and homogeneous product formed.

b. Preparation of medicated ointment

Ten (10) gram of ointment containing different concentrations of *Azadirachta indica* A Juss seed oil (AIO) was prepared using suitable Emulsifying Ointment base BP. (2005). 0 %, 30 %, 40 %,

50 %, 60 % ,70 % and 80 % w/w of *A.* indica seed oil was incorporated into 100 %, 70 %, 60 %, 50 %, 40 %, 30 %, and 20 % w/w of the emulsifying ointment bases respectively by trituration method (Collett and Aulton, 1990). The formulated products were subsequently distributed into sterile glass containers.

c. Preparation of terbinafine ointment

Ten (10) gram of ointment containing 0.05 % terbinafine was prepared using suitable ointment bases (Emulsifying ointment BP 2005). 0.05 % w/w of terbinafine was incorporated into 99.95 %w/w of the emulsifying ointment bases by trituration method (Collett and Aulton, 1990). The resulting product was subsequently distributed into sterile glass container.

Table 3.1: Formulation of ointment containing *A. indica* oil fractions and standard antifungals (10g)

Ingredients	A	B	C	D	E	F	G	H	I
A. indica oil	30	35	40	50	60	70	80	-	-
E/O	70	65	60	50	40	30	20	100	99.95
TFO	-	-	-	-	-	-	-	-	0.05

Key: E/O = Emulsifying ointment, TFO = Terbinafine ointment

Source: Laboratory work

d. Test for fungistatic activity

The agar well diffusion method was used in this determination. About 0.2 ml of the standardized microbial suspensions of test organisms was mixed with 20mls of the appropriate molten agar (40 ^{0}C) to give a population density of $10^4 – 10^5$ c.f.u/ml.

The seeded agar was aseptically poured into sterile Petri dishes and allowed to solidify. Holes (cups) measuring 6.0mm in diameter were bored into the over – dried agar using sterile cork borer. 0.1 g of the test ointment was filled into the cups using sterile spatulas. A pre-incubation diffusion time of 2 hours at room temperature was allowed. Thereafter, the plates were incubated at 30 ^{o}C for 21 days. The resulting diameters of zones of inhibition were measured in millimetre. The tests were carried out in triplicates.

e. Rate of kill studies / test for survival of test organisms in formulated Ointment

Ten gram (10 g) of the ointment containing 60 %w/w, 70 %w/w and 80 %w/w of neem seed oil fraction, terbinafine and whitfield ointments were inoculated with 0.1ml of spore suspension of dermatophytes containing approximately 10^6 spores/ml. The ointments were properly mixed with test organisms using sterile stirring rod to ensure even distribution of the test organisms.

Thereafter, 0.1 ml of the microbial suspensions was added to 10 ml of the sterile normal saline to serve as a control count of number of organisms added to the ointment. Approximately, 0.1g quantity of the ointment was removed at different time interval (0 – 12 days). Each withdrawn sample was carefully dispersed and then serially diluted with sterile normal saline containing 2% Tween 80. About 0.2 ml aliquots of the final dilution was then spread on triplicate plates. The colony forming units on each plate were counted and from this, the total number of viable

organisms per gram in the original ointment was computed. Plot of survival of the organisms against time in the formulated ointments and whitfield were made.

3.3 Statistical analysis

- All the data obtained from the studies were expressed as Mean ± SEM (Standard Error of Mean).
- Data were also presented in the form of figures tables and graphs**.**

CHAPTER FOUR

4.0 RESULTS

4.1 Extraction of oil from *Azadirachta indica* A. Juss seeds

The percentage yields of the oils from *A. indica* seeds using different solvents and extraction methods are shown in Table 4.1. Petroleum ether solvent produced higher yield of the oil compared with hexane, irrespective of the method of extraction, though, this was more pronounced by soxhlet method of extraction. Soxhlet method of extraction yielded more oil than the cold maceration method; about 45% higher in Hexane and 100% higher in petroleum ether.

4.2 Physicochemical characteristics of *Azadirachta indica* seeds oil

The physicochemical and organoleptic characteristics of the oils obtained via the two methods of extractions (soxhlet and maceration) using n-Hexane and petroleum ether solvents is shown in Table 4.2. Hexane oil from maceration recorded highest acid, iodine, saponification and free fatty acid values compared with values for the other three oils. Oils obtained by soxhlet method were generally lighter in colour; more viscous, denser with lower peroxide and acid values. Oils obtained by maceration using hexane as extraction solvent were associated with lower viscosity higher acid, iodine, and free fatty acid values.

Table 4.1: Percentage yield of oil extracts using various solvents and methods of extraction

Solvents	Methods	Yield (ml)	Percentage Yield (%)
Hexane	Soxhlet	220	13.33
	Maceration	154	09.33
Petroleum Ether	Soxhlet	331	20.00
	Maceration	180	10.67

Table 4.2: Physicochemical characteristics of the extracted seed oil

	Extraction method			
	Hexane		Petroleum Ether	
Oil/ Parameters	Soxhlet	Marc	Soxhlet	Marc
Colour	Light brown	Dark brown	Light brown	Dark brown
Smell	Pungent	Pungent	Pungent	Pungent
Taste	Bitter	Bitter	Bitter	Bitter
Viscosity (mm^2S^{-1}) /60	492.8	449.4	499.7	486.3
Relative Density (g/ml)	0.917	0.914	0.912	0.891
Density (g/ml)	0.911	0.908	0.906	0.885
Refractive Index	1.4652	1.4647	1.4645	1.4643
Acid value (mgKOH/g)	6.311	7.013	6.171	6.520
Peroxide value (mEq KOH/g)	0.10	0.26	0.07	0.28
Iodine value (mgI_2/g)	154.18	172.58	146.57	152.46
Saponification value (mgKOH/g)	207.54	223.03	211.78	214.52
Free fatty acid (mgKOH/g)	4.982	8.648	6.768	3.478

Key: Sox = soxhlet, marc = cold maceration.

4.3 Determination of the functional groups present in the oils and its fractions

The spectroscopic analysis (FT-IR) interpretation detecting functional groups of the oils and the fractions having antifungal activity is shown in Tables 4.3 and 4.4 respectively. Both the oils and fractions recorded class of compounds which include alkyl, alkanes, alkenes, aliphatic esters, ketone, carboxylic acid, amide and alkyl halide. However, there was a significant difference in FT-IR of the oil extract and its fractions, where the oil extract recorded the presence of aromatic compounds.

A strong and broad absorption band or frequency from 2500 – 3500cm^{-1} showed the presence of O – H stretch from carboxylic acid as seen in all the spectra, a strong absorption band from 1740 – 1755cm^{-1} revealed the presence of C = O bond for a five member cyclic ketone. A weak absorption band from 3400 – 3500 showed the presence of N – H stretch from an amides. The absorption frequency with a strong band from 1160 – 1210cm^{-1} revealed the presence of O = C – O – C from an aliphatic esters. A medium absorption band 1450 – 1600cm^{-1} showed the presence of C = C ring from an aromatic compounds and frequency bands of 200 – 500cm^{-1} revealed the presence of an alkyl halide.

Table 4.3 Functional groups present in the oil fractions

SAMPLE	ABSORPTION (cm^{-1})	CLASS OF COMPOUND
B2	420	Alkyl halide
	717.54	Alkenes
	1743.71	Ketone
	2863.42	Carboxylic acid
	2933.83	Alkyl
	3470.08	Amide
B3	364.81	Alkyl halide
	716.58	Alkanes/Alkyl
	1164.08	Aliphatic ester
	1744.67	Ketone
	2867.28	Carboxylic acid
	2930.93	Alkyl
	3478.81	Amide
C2	412. 78	Alkyl halide
	718.51	Alkenes
	1743.71	Ketone
	2862.46	Carboxylic acid
	2924.18	Alkyl
	3471.98	Amide

Extracts: B2 – 75 % Pet. ether and 25 % chloroform solvent system, B3 – 50 % Pet. ether and 50 % chloroform solvent system, C2 - 75 % Hexane and 25 % chloroform solvent system

Table 4.3 CONT Functional groups present in the oil fractions

SAMPLE	ABSORPTION (cm^{-1})	CLASS OF COMPOUND
C4	384.81	Alkyl halide
	715.61	Alkenes
	1743.71	Ketone
	2865.35	Carboxylic acid
	2929	Alkyl
	3472.95	Amide
D2	399.28	Alkyl halide
	715.61	Alkanes
	1165.04	Aliphatic ester
	1743.71	Ketone
	2869.21	Alkyl
	2930.93	Carboxylic acid
	3481.63	Amide

Extracts: C4 – 100% Hexane and 0 % chloroform solvent system, D2 – 75 % Pet. ether and 25 % chloroform solvent system

Table 4.4 Functional groups present in the oil extract

Sample	Absorption cm^{-1}	Class of compounds
PES	717.54	Alkyl/ Alkanes
	1165.04	Aliphatic esters
	1458.23	Aromatic compounds
	1743.71	Ketones
	2924.18	Carboxylic acid
	3471.98	Amide
PECM	717.54	Alkyl halide
	1165.04	Aliphatic esters
	1458.23	Aromatic compounds
	1743.71	Ketone
	2924.18	Carboxylic acid
	3471.98	Amide
HS	416.64	Alkyl halide
	1165.04	Aliphatic esters
	1458.25	Aromatic compounds
	1743.71	Ketone
	2924.18	Carboxylic acid
	3471.98	Amide
HCM	1165.04	Aliphatic ester
	1458.23	Aromatic compound
	1743.71	Ketone
	2924.18	Carboxylic acid
	3464.27	Amide

Key: HS = Oil obtained by n-Hexane using soxhlet, PES = Oil obtained using Petroleum ether using soxhlet, HCM = Oil obtained using n-Hexane using cold maceration, PECM = Oil obtained using petroleum ether using cold maceration

4.4 Isolation and characterization of dermatophytes with tinea corporis infections

Data presented in Table 4.5 shows that 70 % of the clinical samples yielded positive isolates with the dermatophytes constituting over 85 %. Four different organisms were isolated with *Trichophyton species* being the predominant dermatophyte.

4.5 Antifungal activities of *Azadirachta indica* A. Juss seeds oil extract

Table 4.7 shows the results of the susceptibility of the isolates to the undiluted oil extracts of *A.indica*. Hexane oil extract from soxhlet method of extraction had show the highest diameters of zones of inhibition against the isolated dermatophytes. Activity was exerted mostly on *Trichophyton mentagrophytes* and *Microsporum canis*. However, the levels of inhibition against the dermatophytes were far less compared with those exerted by terbinafine. No inhibitory activity was recorded against *Aspergillus niger* and *Candida albican.* Differences were also noted as with the level of activity among the oils. Seed oil obtained with n- hexane by the soxhlet method produced highest inhibitory zones while that by maceration using n-hexane was least inhibitory.

The level of inhibition also changed following dilution of the oil with DMSO. Table 4.8 shows the results of the susceptibility of the isolates to the oil extracts of *A.indica* when diluted with DMSO at 10%v/v,. Hexane oil extract from soxhlet and petroleum oil extract from maceration showed higher activities against isolated dermatophytes and still with no inhibitory activity of the oils recorded against *Aspergillus niger* and *Candida albican*.

Table 4.5: Isolation of dermatophytic species and their incidence in dermatophytes

Isolates	No.	(%)
Trichophyton mentagrophytes	10	47.6
Trichophyton rubrum	6	28.6
Microsporum canis	2	9.5
Total	18	85.7

Table 4.6: Biochemical and mycological tests of dermatophytes

Tests	TM	MC	TR
Urease test	+	-	-
Corn meal	-	-	+
Nutritional test	-	+	-

Key: TM = *Trichophyton mentagrophytes*, MC = *Microsporum canis*, TR = *Trichophyton rubrum*

Urease test, + = Pink colour

Corn meal, + = Dark red colour

Nutritional test, + = Growth

Table 4.7: Susceptibility of the test fungi to the oil extracts of *A. indica* at 100% v/v

Organisms	Zone of inhibition (mm)				
	A	B	C	D	TBF (32µg/ml)
TM	17.33 ± 0.58	14.67 ± 0.58	14.00 ± 0.00	16.33 ± 0.58	34.67 ± 0.58
MC	15.33 ± 0.58	13.00 ± 0.00	11.67 ± 0.58	12.00 ± 0.00	39.67 ± 0.58
TR	14.33 ± 0.58	12.00 ± 0.00	14.00 ± 0.00	14.67 ± 0.58	32.67 ± 0.58
AN	0	0	0	0	40.00 ± 0-00
CA	0	0	0	0	22.67 ± 0.58

*Values are mean inhibition zone (mm) ± S.D of three replicates cork borer- 6mm

Key: A = Oil obtained by n-Hexane using soxhlet, B = Oil obtained by Petroleum ether using soxhlet, C = Oil obtained using n-Hexane using cold maceration, D = Oil obtained using petroleum ether using cold maceration, TBF = Terbinafine, TM = *Trichophyton mentagrophytes*, MC = *Microsporum canis*, TR = *Trichophyton rubrum*, AN = *Aspergillus niger*, CA = *Candida albican*.

Table 4.8: Susceptibility of the test fungi to the oil extracts of *A. indica* at 90% v/v

Organisms	Zone of inhibition (mm)				
	A	B	C	D	DMSO
TM	14.67 ± 0.58	12.33 ± 0.58	13.33 ± 1.16	14.00 ± 0.00	0
MC	16.00 ± 0.00	12.67 ± 0.58	12.00 ± 0.00	18.33 ± 0.58	0
TR	16.33 ± 0.58	12.33 ± 0.58	14.00 ± 0.00	14.00 ± 0.00	0
AN	0	0	0	0	0
CA	0	0	0	0	0

*Values are mean inhibition zone (mm) ± S.D of three replicates cork borer- 6mm

Key: A = Oil obtained by n-Hexane using soxhlet, B = Oil obtained using Petroleum ether, C = Oil obtained using n-Hexane using cold maceration, D = Oil obtained using petroleum ether using cold maceration, DMSO = 10% Dimethylsulfoxide, TM = *Trichophyton mentagrophytes*, MC = *Microsporum canis*, TR = *Trichophyton rubrum*, AN = *Aspergillus niger*, CA = *Candida albican.*

4.6 Antifungal Activities of the Oil Fractions

A total of 16 fractions were collected from the elution of the petroleum ether/n-hexane and chloroform of the four oil extracts. The results of their antifungal test are presented in Table 4.9. Antifungal activities of the fractions varied among the fractions. None of the fractions obtained from oil extracted by soxhlet method using Hexane as solvent had inhibitory activity against the test isolates.

On the other hand , fractions from the oil obtained by maceration using petroleum ether as solvent had activity only against the Trichophyton species (*T. mentagrophytes* and *T.rubrum*) , While the fractions obtained using Petroleum ether : Chloroform (50:50 %) by soxhlet with petroleum ether solvent had activity against all the three dermatophytes .

Table 4.9: Antifungal susceptibility profiles of test fungal isolates to fractions of *A. indica* oil

	Zone of inhibition (mm)		
Test organisms	TM	MC	TR
Solvent systems			
A 1	0	0	0
A2	0	0	0
A3	0	0	0
A4	0	0	0
B1	0	0	0
B2	15.33 ± 0.58	0	12.33 ± 0.58
B3	12.33 ± 0.58	11.00 ± 00	13.00 ± 0.0 0
B4	0	0	0
C1	0	0	0
C2	16.33 ± 0.58	0	0
C3	0	0	0
C4	12.33 ± 0.58	0	0
D1	0	0	20.67 ± 1.16
D2	16.67 ± 0.58	0	0
D3	0	0	0
D4	0	0	0

*Values are mean inhibition zone (mm) ± S.D of three replicates, cork borer- 6mm

Keys: TM = *Trichophyton mentagrophytes*, MC = *Microsporum canis*, TR = *Trichophyton rubrum*,

4.7 Minimum inhibitory / Fungicidal concentration values

The minimum inhibitory concentrations (M.I.C) of *A.indica Juss* seeds oil extracts results are shown in the Table 4.10. Most of the oils showed MIC at 50%v/v against *T. mentagrophytes* and *T. rubrum* while on *M. canis* it is as low as 3.13 %v/v.

The minimum fungicidal concentrations (M.F.C) of *A.indica Juss* seeds oil extracts results are presented in Table 4.11. The M.F.C of oil extracts on *T. mentagrophytes* and *T.rubrum* has shown to be greater than 50 %v/v while on *M. canis*, the M.F.C is as low as 12.5 %v/v.

Table 4.10: Minimum inhibitory concentrations of *A.indica* seeds oil extract (%v/v)

ORG	Oil Extracts				
	A	B	C	D	TBF (µg/ml)
TM	50.00 ± 0.00	50.00 ± 0.00	50.00 ± 0.00	>50.00 ± 0.00	< 2.00 ± 0.00
MC	3.13 ± 0.00	6.25 ± 0.00	12.50 ± 0.00	12.50 ± 0.00	4.00 ± 0.00
TR	50.00 ± 0.00	50.00 ± 0.00	25.00 ± 0.00	25.00 ± 0.00	32.00 ± 0.00
AN	NA	NA	NA	NA	16.00 ± 0.00
CA	NA	NA	NA	NA	< 2.00 ± 0.00

*Values are mean inhibition zone (mm) ± S.D of three replicates,

Key: A = Oil obtained by n-Hexane using soxhlet, B = Oil obtained using Petroleum ether using soxhlet, C = Oil obtained using n-Hexane using cold maceration, D = Oil obtained using petroleum ether using cold maceration, TBF = Terbinafine, TM = *Trichophyton mentagrophytes*, MC = *Microsporum canis*, TR = *Trichophyton rubrum*, AN = *Aspergillus Niger*, CA = *Candida albican*, NA = Not active

Table 4.11: Minimum fungicidal concentration of *A. indica* A. Juss seeds oil extracts (%v/v)

ORG	Oil extracts			
	A	B	C	D
TM	50.00 ± 0.00	>50.00 ± 0.00	> 50.00 ± 0.00	>50.00 ± 0.00
MC	12.50 ± 0.00	12.50 ± 0.00	25.00 ± 0.00	25.00 ± 0.00
TR	>50.00 ± 0.00	>50.00 ± 0.00	>50.00 ± 0.00	>50.00 ± 0.00
AN	NA	NA	NA	NA
CA	NA	NA	NA	NA

*Values are mean inhibition zone (mm) ± S.D of three replicates

Key: A = Oil obtained by n-Hexane using soxhlet, B = Oil obtained using Petroleum ether, C = Oil obtained using n-Hexane using cold maceration, D = Oil obtained using petroleum ether using cold maceration, TBF = Terbinafine, TM = *Trichophyton mentagrophytes*, MC = *Microsporum canis*, TR = *Trichophyton rubrum*, AN = *Aspergillus niger*, CA = *Candida albican*, NA = Not active

4.8: Antifungal Activities of the formulated Ointments

Antifungal activities of the oil fraction formulations varied among the formulations as presented in Table 4.12. The formulations from *Azadirachta indica* oil and the two standard ointments showed inhibitory activity against the isolated dermatophytes, Benzoic salicylic acid ointment however did not show inhibitory activity against *T. mentagrophytes*. An increase in the concentration of the oil fraction in the ointments shows an increase in the diameter zones of inhibition. Ointments containing 30 – 50 % of the seed oil did not produce inhibition zones against the test organisms. However, as the concentration of the oil increased from 60 % to 80 %, inhibitory activity became pronounced, for the three fungal species the level of inhibition, though less compared to those of terbinafine, it is more than that produced by the oils alone. (Table 4.7).

Antifungal activities of the unfractionated oil formulations varied among concentrations when incorporated, as presented in Table 4.12. The formulations from *Azadirachta indica* oil ointments showed a remarkable inhibitory activity against the isolated dermatophytes at higher concentrations. Ointment with crude oil concentration of 50 %w/w showed no inhibitory activity against *T. mentagrophytes* and *T.rubrum*.

Table 4.12: Antifungal susceptibility profiles of formulated ointments against isolated dermatophytes

Formulated ointments	Diameter zone of inhibition (mm)		
	TM	**MC**	**TR**
AIO ointment - I	15.67 ± 0.58	12.00 ± 0.00	11.00 ± 0.00
AIO ointment -II	16.67 ± 0.58	12.67 ± 0.58	14.00 ± 0.00
AIO ointment -III	18.67 ± 0.58	18.00 ± 0.00	21.00 ± 0.00
Benzoic/Salicylic acid ointment	0	20.67 ± 0.58	25.67 ± 0.58
0.05% Terbinafine ointment	37.65 ± 0.58	40.00 ± 0.00	35.00 ± 0.00
Emulsifying ointment BP	0	0	0

*Values are mean inhibition zone (mm) ± S.D of three replicates, cork borer- 6mm

Keys: TM = *Trichophyton mentagrophytes*, MC = *Microsporum canis*, TR = *Trichophyton rubrum*, AIO = *Azadirachta indica* oil fraction.

Formulations

I – 60% w/w AIO ointment IV - 50µg w/w Terbinafine ointment

II – 70% w/w AIO ointment V – 3/6%w/w Salicylic acid/ Benzoic acid ointment

III – 80% w/w AIO ointment

Table 4.13: Antifungal susceptibility profiles of the unfractionated oil formulated ointments against isolated dermatophytes

Formulated ointments	Diameter zone of inhibition (mm)		
	TM	**MC**	**TR**
AIO ointment - I	0	3.00 ± 0.00	0
AIO ointment -II	16.00 ± 0.00	12.67 ± 0.58	14.00 ± 0.00
AIO ointment -III	18.67 ± 0.58	15.00 ± 0.00	17.00 ± 0.00
AIO ointment – IV	20.67 ± 0.58	18.67 ± 0.58	20.67 ± 0.58
Emulsifying ointment BP	0	0	0

*Values are mean inhibition zone (mm) ± S.D of three replicates, cork borer- 6mm

Keys: TM = *Trichophyton mentagrophytes*, MC = *Microsporum canis*, TR = *Trichophyton rubrum*, AIO = *Azadirachta indica* oil.

Formulations

I – 50% w/w AIO ointment IV - 80% w/w AIO ointment

II – 60% w/w AIO ointment

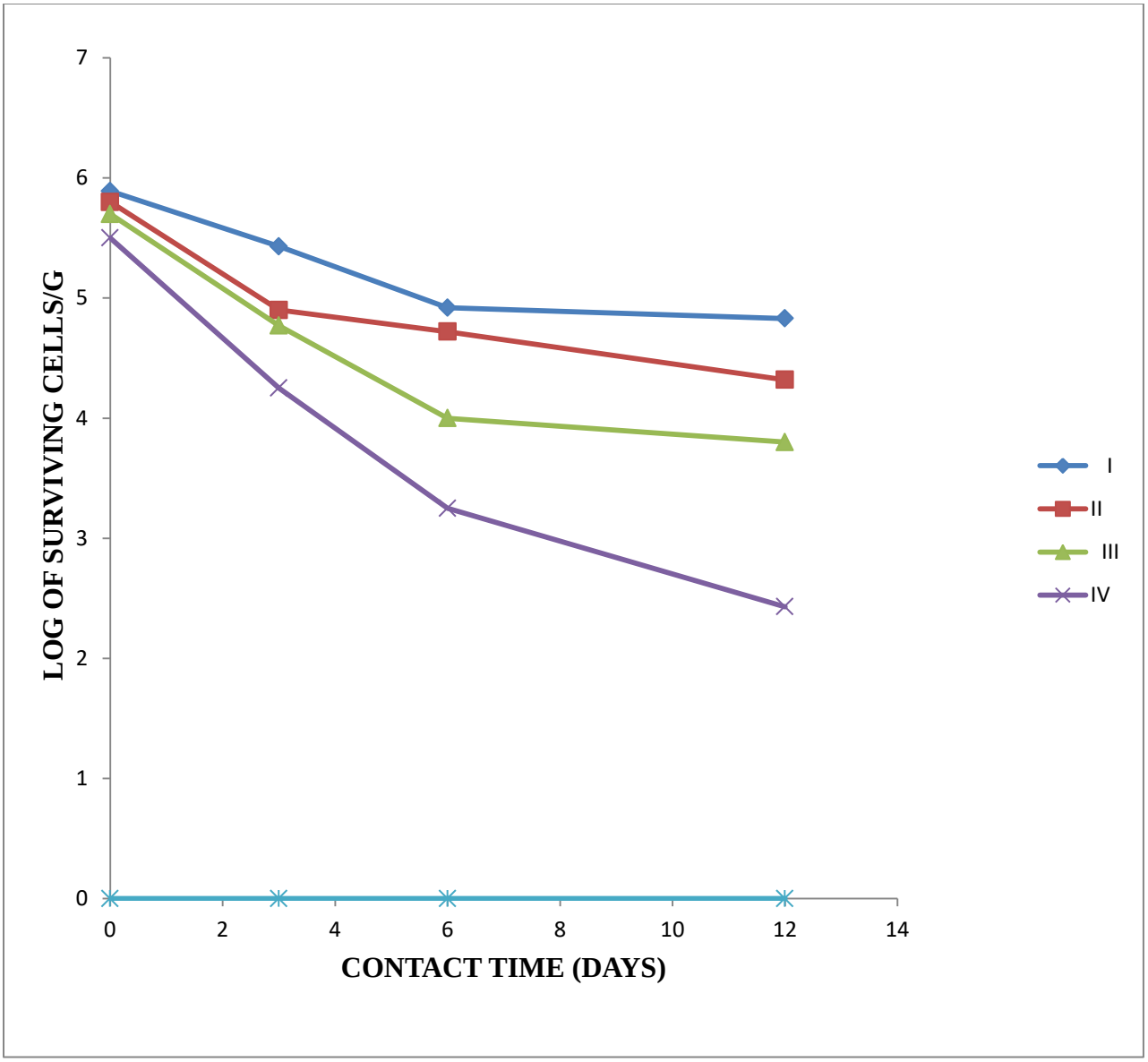
III –70% w/w AIO ointment

4.9 Survival of Test Organisms in Ointment formulations

The plotted graphs of different ointment formulations in Figure 4.1 showed a remarkable decrease in the log of surviving cells of *T. mentagrophytes* with increase in contact time. The higher the concentration of AIO in the formulation shows a decrease in the log of surviving cells as contact time increases.

The plotted graphs of different ointment formulations in Figure 4.2 showed a remarkable decrease in the log of surviving cells of *M. canis* with increase in contact time. The higher the concentration of AIO in the formulation shows a decrease in the log of surviving cells as contact time increase.

The plotted graphs of different ointment formulations in Figure 4.3 showed a remarkable decrease in the log of surviving cells of *T.rubrum* with increase in contact time. The higher the concentration of AIO in the formulation shows a decrease in the log of surviving cells as contact time increases.

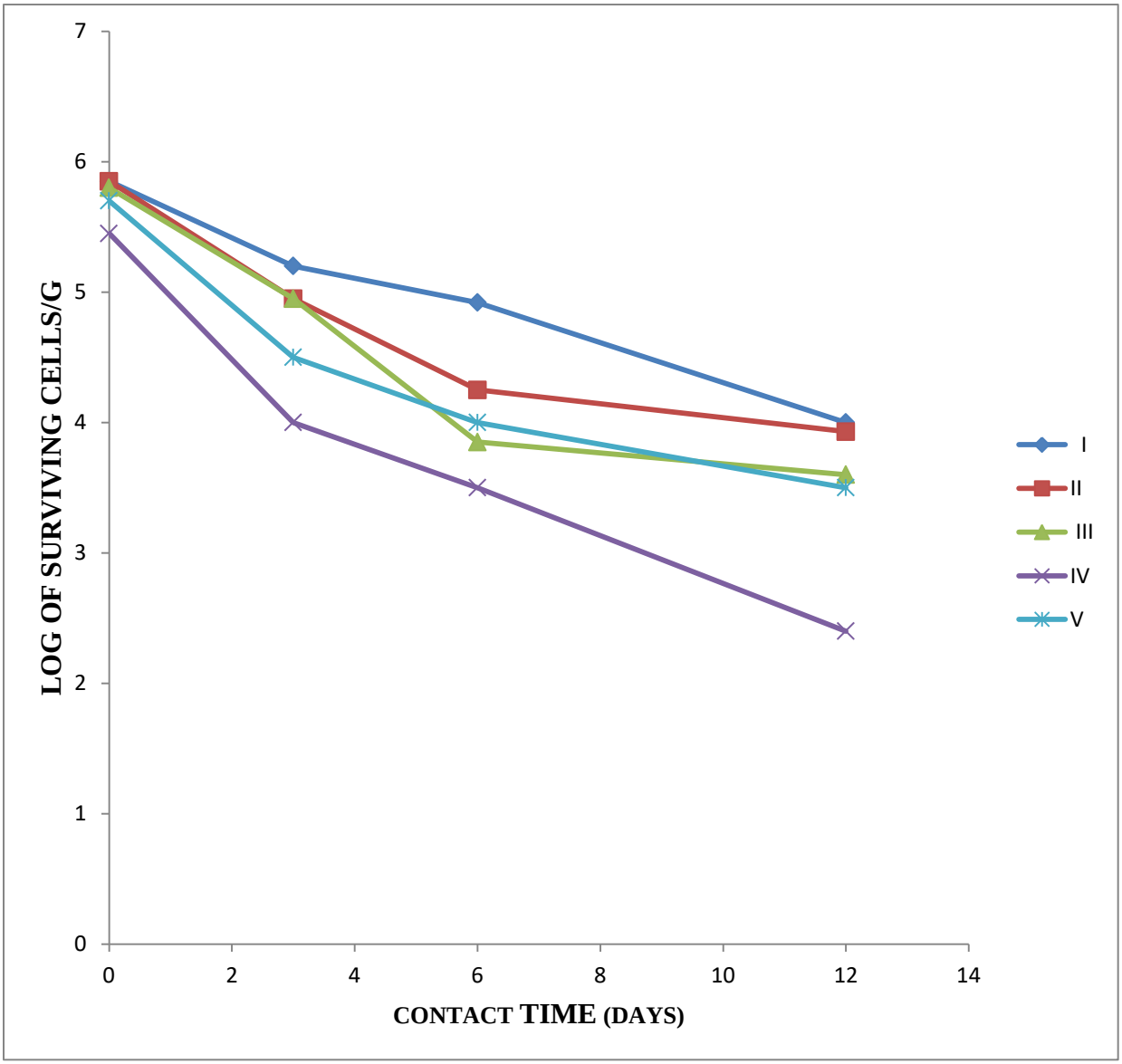


I – 60% w/w AIO ointment IV - 50µg w/w Terbinafine ointment (Control)

II – 70% w/w AIO ointment

III – 80% w/w AIO ointment

Figure 4.1: Survival of *Trichophyton mentagrophytes* in ointment formulated with different concentrations of *Azadirachta indica* oil



I – 60% w/w AIO ointment

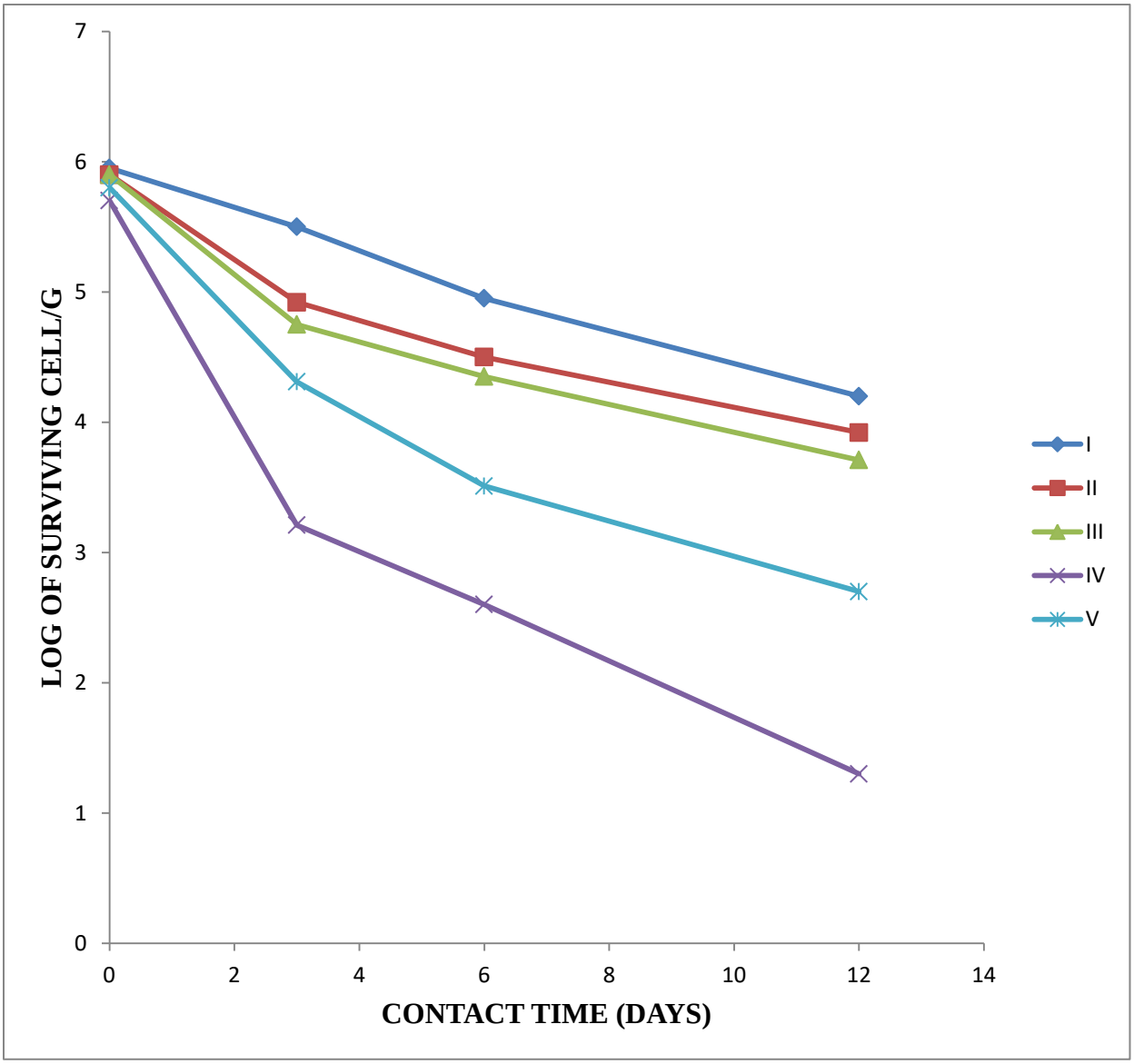
II – 70% w/w AIO ointment

III – 80% w/w AIO ointment

IV - 50µg w/w Terbinafine ointment (Control)

V – 3/6%w/w Salicylic acid/ Benzoic acid ointment (Control)

Figure 4.2: Survival of *M .canis* in ointment formulated with different concentrations of *Azadirachta indica* oil



I – 60% w/w AIO ointment IV - 50µg w/w Terbinafine ointment (Control)

II – 70% w/w AIO ointment V – 3/6%w/w Salicylic acid/ Benzoic acid ointment (Control)

III – 80% w/w AIO ointment

Figure 4.3: Survival of *T. rubrum* in ointment formulated with different concentrations of *Azadirachta indica* oil

CHAPTER FIVE

5.0 DISCUSSIONS

The two solvents and methods used in the extraction of *Azadirachta indica* A Juss *seeds* yielded different percentages of oil. The higher yields observed with both soxhlet and cold maceration of the petroleum ether oil extract over the n-Hexane oil extract might be due to the difference in their polarity. During extraction, solvents tend to diffuse into the solid plant material and thus solubilizing the compounds with similar polarity (Ormeno *et al.*, 2011). The determination of biologically active compounds from plant material is dependent on the type of solvent and method used in their extraction procedure (Das *et al*., 2010). In a study by Ndana *et al*., (2010), the percentage yield of the hexane oil extract of *A. indica* using soxhlet method of extraction was reported to be 47.0%. This is much higher than the percentage yield of 13.33% recorded in this study. In a study by Awolu *et al*., (2011) in Akure, Nigeria, a 49% yield using petroleum ether by soxhlet method of extraction was reported which is also higher than the percentage yield of 20%. This could be due to the difference in source and processing of the seed. Fresh seeds generally contain less oil because of the level of moisture contents. Geographical locations are also known to impact yield. Soxhlet extraction method which gave higher yield has an added advantage of ensuring solvent recovery. The yield and biological activity of extracts have been shown to be highly dependent on solvent polarity (Mohammedi and Atik, 2011).

The quality of oil is expressed in terms of physico-chemical properties (saponification value, iodine value, peroxide value, acid value, free fatty acid, specific gravity and kinematic viscosity) indicated that the four oil extracts are similar in many parameters.

The saponification value (a measure of the molecular weights of the triglycerides in oils and fats) neem seed oils, though varied among oils, is similar to the 213 reported by Warra *et al* (2011). Triglycerides with high values of saponification are considered to make better quality soaps than those with low saponification value (Atadashi *et al*., 2010). This implies that oil from cold maceration using Hexane solvent will be suitable for soap making. However, these values were slightly higher than the range (175 - 205) for non-edible oils (Pharmaceutical codex) .Generally, saponification value change depending on the origin of the lipid and what the weather conditions were for that particular plant material and the processing techniques used (Amaro *et al.,* 2011).

The iodine value obtained in this study indicates the extracted neem oils are relatively unsaturated and thus possesses the property of absorbing oxygen on exposure to the atmosphere with time. That is, they are susceptible to oxidative rancidity. Iodine value is useful in predicting the drying property of oils and the high values observed indicates that the oil obtained in this study are drying. Non drying liquid oil has iodine value range of 80-120 while semi drying oil have an iodine value range 120-150 and drying oils are normally greater than 150 (Mohamed, 2013).

The acid value which is a measure of the free fatty acids(FFA) present in the fat or oil, is an important tool used to indicate the quality, age, edibility and suitability of oil for use in industries (Pauli *et al*., 2014). It is also used to indicate the previous lipase activity and other hydrolytic reaction or oxidation. It measures the extent to which glyceride in the oil has been decomposed by lipase and other physical factors such as light and heat. The acid values found in the extracted

oils are within acceptable values for most non edible oils (Atabani *et al*., 2013). Acid values in two of the oil extracts are similar to the 4.80 – 6.37 as reported by Jessinta *et al,* (2014).

High concentrations of free fatty acids are undesirable in crude oils because they result in large losses of the neutral oil during refining, and also indirect susceptibility to rancidity. The value obtained for the oil extracted by maceration using hexane was close to the 8.70 reported by Ndana *et al* (2010) and also implies high rancidity.

The peroxide value (an index of rancidity and thus an indicator of poor resistance to peroxidation during storage) obtained in this study are below the maximum acceptable value of 10 meg peroxide/kg and signifies its high oxidative stability (Zheng, 2003). These values are relatively low compared with the peroxide values of other oils of wild plants with high peroxide values associated with high rancidity rate. Jessinta *et al*., (2014) in Malaysia recorded 8.49 and 1.67. These values are far more than the values obtained in this study and this might be due to geographical variation.

The oil extracts and some fractions revealed an antifungal activity against dermatophytes, the classes of compounds present include, alkyl, alkanes, alkenes, aliphatic esters, ketone, carboxylic acid, amide and alkyl halide and aromatic compounds. The presence of ketone C = O functional group from a five membered cyclic ring and O = C – O – C functional group from an aliphatic esters revealed that gedunin, nimbin, nimbinin and nimbolide constituents are likely present in the oil extracts and its fractions. Thus, these functional groups of ketone, hydroxyl, carboxyl and aliphatic ester may be responsible for the antidermatophytic activity of neem seed oil. The

constituents nimbinin, gedunin, nimbin and nimbolide are terpenoid compounds and a work done by Nuzhat and Vidyasagar, (2014) revealed that terpenoid compounds are responsible for antidermatophytic activity.

The predominance of *Trichophyton species* as the causative agent of *Tinea corporis* (Ringworm of the body) is not unexpected. Most studies found *T. rubrum* and *T. mentagrophytes* as the commonest etiological agents of dermatomycosis (Koksal *et al*., 2009). *Trichophyton spp* accounted for 76.2% of *Tinea corporis* in India (Kaur and Kakkar, 2010). *Trichophyton mentagrophytes* has been cited as the major causative agent for *Tinea corporis* and is known to account for as much as 47.6 – 69.5% of all dermatophytic infections (Pranab *et al.,* 2003). The isolation of *T. rubrum* in this study agrees with the report of Raghuramulu *et al*., (2011) in India, where 18.37% of *T. rubrum* was isolated. They also recorded 6.12% for *Microsporum canis* which is close to the 9.5% obtained in this study.

The level of antifungal activities of the test oil extracts varied from one test isolate to another. It was observed that there was a slight increase in antifungal activity when the oil extracts was diluted with 10% DMSO.

The increased antifungal activity of oil extract A and D over B is an indication that this method and solvent were able to extract oil having most of the active components of the plant seed responsible for the antifungal activity (terpenoid). This finding agrees with that of Ospina *et al*., (2014) in Colombia where the hexane - methanol fraction had inhibitory activity against *Trichophyton mentagrophytes*, *T. rubrum,* and *Microsporum canis* at higher concentrations. The findings of Ospina *et al.* (2014) are also in agreement with this research finding that a non-polar fraction of neem seed oil was more effective in inhibiting the growth of dermatophytes. Oil extracts obtained from petroleum ether using cold maceration tend to have the most activity

when compared to other oil extracts as this method of extraction and solvent probably extracted most of the components responsible for the antifungal activity (terpenoid).

It is worthy to note here that the oil extracts do not have antifungal activity on both *Candida albican* and *Aspergillus niger*, although the reports of Sunita and Mahendra in (2008) in India, on the contrary showed activity against *Aspergillus niger*. This could be due to the method and solvent used in extraction, the geographical location and time of collection of the plant seed.

It was also observed that the ointment formulations with oil concentrations of 50 – 80 % showed antifungal activities. This might be due to the inability of the oils at lower concentrations to diffuse to critical concentration required to exert antifungal activity. The higher activity observed with the formulation ointment showed that the ointment base used in this research is suitable for the formulation of both the oil extract and its fractions.

The level of reduction in the test cell counts as the contact time increases in the formulated ointment for the oil extract, its fraction and the reference antifungal agents is expected. – More cells are generally killed on longer contact with inhibitory agents. The survival curves which showed more cells killed with higher oil concentrations is in tandem with results obtained for the zones of inhibitions.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Neem seed oil extracted by soxhlet and maceration methods using n -hexane and petroleum ether as solvents have similar physico-chemical properties, with different percentage yields which are far lower than those reported by other workers. Using column chromatograhic technique, 16 fractions of the oil were obtained.

Neem oil is a non edible and drying oil, although it has a drying property with time and susceptible to rancidity due to high concentration of free fatty acid. The high level of triglyceride makes it suitable in soap making.

The oils and its fractions revealed the presence of alkyl, alkanes, aliphatic esters, ketone, carboxylic acid, amide and alkyl halide. The presence of these functional groups revealed the possible presence of nimbinin, nimbin gedunin and nimbolide constituents must likely present in the oil extracts and its fractions.

Dermatophytes isolated from children with *Tinea corporis*, and used as test organisms in this study are predominantly *Trichophyton species*, mostly *Trichophyton mentagrophytes* and few *Trichophyton rubrum*. Other species were *Microsporum canis* and a non dermatophytic organism *Aspergillus niger*. Characterisation was based on microscopic/macroscopic examinations and biochemical tests.

The crude oil extracts exhibited antifungal activities against the dermatophytes but not against non dermatophytes such as *Candida albican* and *Aspergillus niger*. The MIC and MFC ranged from 3.13 to >50%v/v and 12.50 to >50%v/v respectively.

Ointment formulations of the oils and its fractions containing 60 – 80%v/v of oils exhibited appreciable and significant anti-dermatophytic activities. The antifungal activities were relatively less compared with those of the standard terbinafine ointment.

There was substantial reduction in the number of viable fungal cells when inoculated into the ointment formulation.

6.2 Conclusion

Neem seed oil, especially extracted by soxhlet method using hexane had appreciable inhibitory activities against causative agents of *Tinea corporis* (ringworm). Only five of the sixteen fractions of these oils exhibited some level of antifungal activities.

Ointment formulations containing 60%v/v of the oil had appreciable *in – situ* dermatophytic activities. Ointment formulations containing the unfractionated oils had better antifungal activities compared with the fractions.

6.3 Recommendations

The findings of this study have shown the need for further investigation to establish the economic viability of exploiting *A.indica* plant for fungal bioactives.

- Toxicology test for safety especially skin sensitivity test should be carried out ,
- The release profile should be carried out on the ointment with the aim of ascertaining the exact concentration of the unbound drug release to exert therapy.
- The odour of the oil should be worked on by masking it with a suitable fragrance

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