CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

The use of medicinal plants has always been part of human culture and is wide spread in Africa. In some countries, like Ghana, government encourages the use of indigenous forms of medicine rather than expensive imported drugs. Also in Nigeria, a large percentage of the populace depends on herbal medicines because the commercially available orthodox medicines are becoming increasingly expensive and out of reach (Fasola*et al*., 2005).

Stress, basically is a reaction of mind and body, which has a significant impact on the immune response in general and today's world is full of stress. The immune system is known to be involved in the etiology as well as pathologic mechanisms of many diseases. Immunology is thus probably one of the most rapidly developing areas of biomedical research and has great promises with regard to the prevention and treatment of a wide range of disorders (Ghaisas *et al,* 2009)

Primary immunodeficiency diseases represent a class of disorders in which there is an intrinsic defect in the human immune systems (rather than immune disorders that are secondary to infection, chemotherapy, or some other external agent). In some cases, the body fails to produce any or enough antibodies to fight infection, in other cases, the cellular defences against infection fail to work properly.

Traditional and folklore medicines play an important role in health services around the globe (Pradhan *et al,* 2009). In India around 20,000 medicinal plant species have been recorded recently but more than 500 traditional communities use about 800 plant species for curing different diseases. Currently 80% of the world population depends on plant-derived medicine for

the first line of primary health care for human alleviation because it has no side effects (Sheetal *et al*, 2008). Plants are important sources of medicines and presently about 25% of pharmaceutical prescriptions in the United States contain at least one plant-derived ingredient. In the last century, roughly 121 pharmaceutical products were formulated based on the traditional knowledge obtained from various sources (Kanjwani*et al*, 2000).

Among the medicinal plants commonly used in Nigeria for management and treatment of various types of ailments is *M. lucida* Benth. The plant *M. lucida* falls under the family Rubiaceae known to have wide usage in traditional medicine (Karou et al, 2011).*M. lucida* is a tropical West African tree ofmedium-size of about 18–25 m tall, the bark is grey to brown in colour, flowers are white in colour, the fruit is a drupe, seed is ellipsoid, yellowish and soft (Zimudzi and Cardon, 2005). *M. lucida* which is commonly known as the Brimstone tree, is known in Ghana as Kon kroma in Twi (Addo-Fodjour *et al*, 2008) while in Nigeria it is known as Nfia in Igbo (Nweze, 2011). The leaves are used as "oral teas", which are usually taken orally for the traditional treatment of malaria, and as a general febrifuge, analgesic, laxative and anti-infections (Makinde *et al*, 1985). The leaves have also been reported to possess strong trypanocidal (Asuzu *et al*, 1990; Abubakar *et al*, 2016)and aortic vasorelaxant activities (Ettarh and Emeka, 2004). Further studies have shown that leaf and stem bark of *M. lucida* possess anticancer (Sowemimo *et al*,2007), hepatoprotective (Oduola*et al*, 2010), cytotoxic and genotoxic (Akinboro *et al*, 2005), antispermatogenic (Raji *et al*, 2005), hypoglycemic and antidiabetic activity (Daziel, 1973; Olajide *et al*, 1999). *M. lucida* is used as an astringent and antiseptic for ulcerating abscess, exudate is rubbed on affected area (Adomi and Umukoro, 2010). The crude ethanolic extract of the leaves is said to contain alkaloids, cardenolides and saponins (Adomi and Umukoro, 2010). The major constituents of *M. lucida* extract are the various types of alkaloids,

anthraquinones and anthraquinols (Adesogan, 1973). Two compounds (oruwalol and oruwal) and 10 anthraquinones have been isolated and characterized from the stem of the plant (Adewunmi *et al.*, 1984).

1.2 Statement of Problem

Herbal medicine is neglected because it lacks scientific analysis. Most plants have physiological effect on human but the chemical constituentsresponsible are not determined.Various allopathic drugs or medicines are used to modulate the immune system. However, these drugs are very expensive for poor people, they are not easily accessible, and in most cases they are associated with adverse drug reactions. As a result, the majority of people especially in the rural areas of the developing world turn to the use of alternative herbal medicines from medicinal plants such as *M. lucida.* In Africa and Asia, about 80% of the population have been reported to depend on traditional medicine for their primary health care needs including immunomodulation (Chan, 2008). There is need to determine the chemical entities that are responsible for the immunomodulatory activity in the body.

1.3 Justification of the Study

Several studies in recent years have demonstrated the immunomodulatory effects of crude extracts of traditional herbal medicine (Sharma *et al*, 2011; Kumar *et al*, 2012). Such studies have identified potentially useful immunostimulatory and immune-suppressive plant extracts, in some cases pure lead compunds.

M. lucida have been reported to possess many pharmacological properties but the chemical fractions that are responsible for the immunomodulatory activity are not known. Hence the current study will be based on the isolation and structure elucidation of the immunomodulatory constituents of *M. lucida* leaf. Such detailed studies have not previously been reported but they are necessary in order to obtain data that might in future help in the development of immunomodulatory compounds from medicinal plants.

1.4 Research Hypothesis

The bioactive constituents isolated from leaf extract of *M. lucida* shows immunomodulatory activities.

1.5 Aim and Objectives of the Study

The aim of the study is to isolate and determine the chemical structures of immunomodulatory constituents of *M. lucida* leaf.

The specific objectives include;

- To extract and fractionate the crude extract of *M. lucida*
- To determine the immunomodulatory activity of the crude methanol extract, ethyl acetate fraction and butanol fraction of *M. lucida*
- To isolate the bioactive constituents present from the most active fraction of *M. lucida* leaf.
- To elucidate the structures of the active pure compounds using HPLC-DAD and nuclear magnetic resonance (NMR) techniques.

CHAPTER TWO

LITERATURE REVIEW

2.1 Scientific Classification of *Morinda Lucida* Benth

Family	Rubiaceae
Subfamily	Rubioideae
Tribe	Morindeae
Genus	Morinda
Species	*Morinda lucida* Benth

Vernacular Names

English	Brimstone trees
Igala	Ogele
Yoruba	Oruwo
Ibo	Nfia
Ebira	Ugigo

2.2 The Family, Rubiaceae

Rubiaceae is a family of flowering plants, variously called the madder family, bedstraw family or coffee family. The family takes its name from the madder genus Rubia. The plants included in the family are *Cofea arabica, Morinda lucida, Cinchona* (whose bark contains quinine). The Rubiaceae is one of the five largest plant families, including Orchidaceae, Compositae, Leguminosae, and Gramineae. The family includes trees, shrubs, climbers, herbaceous plants and

ant plants. Rubiaceae is subdivided into four subfamilies, namely Rubioideae, Cinchonoideae, Ixoroideae and Antirheoideae with about 50 tribes. According to the world Rubiaceae checklist, there are 611 genera and 13,143 species (Davis *et al.,* 2009). Psychotria, with 1834 species is the largest genus in the family. With many large and poorly defined genera, the family was not extensively researched as others. According to Karou *et al.* (2011), Rubiaceae are used as ornamental trees and in African folk medicine to treat several diseases including malaria, hepatitis, cough, hypertension, diabetes and sexual weakness. Following leads supplied by traditional healers, biological screening have shown many of the plants to possess antimicrobial, antidiabetic, antihypertension, antioxidant and anti-inflammatory activities. The family consists of many genera that serve as valuable medicinal plants. According to Karou *et al.* (2011), great attention has been paid to species such as *Nauclea latifolia, Morinda lucida, Mitragyna inerms* and *Crossopteryx febrifuga* but these plants have not been systematically studied and so, several compounds should be waiting to be discovered.

2.3 The Genus, *Morinda*

The genus, *Morinda*, is among the genera of Rubiaceae that serve as valuable medicinal plants. It comprises about 80 species and occurs throughout the tropics. In Africa 5 species are found and they are: *Morinda lucida Benth, Morinda citrifolia L., Morinda geminate DC, Morinda Longiflora and Morinda morindoides(Bak).* The distinctive characteristics of *Morinda lucida* is the comparatively small flowering and fruiting heads on long slender peduncles. *Morinda* species yield yellow and red dyes and are used to dye textiles in Nigeria, Ghana, Congo DR and Cote d'Ivoire.The *Morinda* species are bitter tasting and the bitter-tasting roots are used as flavouring for food and alcoholic beverages. The wood is excellent for making charcoal, and for

construction, mining props, furniture, canoes, poles and fuel wood. Many species, including those from Africa, are important medicinal plants, widely applied against various kinds of fevers and infections (Zimudzi and Cardon, 2005).

2.4 Origin and Geographic Distribution of *Morinda lucida*

In Africa, *M. lucida* occurs mainly in West Africa and Central Africa. In West Africa it occurs in the coastal countries including Nigeria (Zimudi and Cardon, 2005).

2.5 Description of *Morinda lucida*

M. lucida (Benth) is an evergreen shrub of up to 18m height. The bark is grey in colour and the leaves are about 7-15 cm long by 3.5-7.5 cm broad. Flowers are bi-sexual, regular, heterostylous, and fragrant as shown figure 2.1. Calyx is cup-shaped, about 2 mm long, persistent. Corolla is salver-shaped, about 1.5 cm long, white or greenish yellow. Lobes are ovate-lanceolate; up to 5 mm x 2.5 mm. Ovary is inferior, 2-celled. Style is 8- 11mm long with stigma lobes 4-7 mm long. Stamens are 5, inserted in the Corolla throat, with short filaments. Fruits are a drupe; several together arranged into an almost globose succulent syncarp 1-2.5 cm in diameter, soft and black when mature (Zimudzi and Cardon, 2005)

Figure 2.1: *Morinda lucida* leaves with seeds.

Figure 2.2: *Morinda lucida* leaves sprouting flower

2.6 Ethnomedicine Information of *M. lucida*

Ethnomedicine from native point of view is the study of the indigenous way of treating and managing certain diseases affecting people living in a particular environment. This approach is particularly useful for the study of indigenous therapeutic agents since it allows the researcher to understand treatment patterns according to native explanatory models instead of only through the lens of biomedicine. *M. lucida* is a medicinal plant growing in African countries and widely used as medicine in West Africa. It is generally used as ingredients of fever teas, which are usually taken, for the traditional treatment of malaria. In West Africa,*M. lucida* is an important plant in traditional medicine. In Nigeria *M. lucida* is one of the four most used plants in the preparation of traditional medicines against fever. Decoctions and infusions or plasters of root, bark and leaves are recognized remedies against different types of fever, including yellow fever, malaria, trypanosomiasis and feverish condition during childbirth. In some cases, the plant is employed in the treatment of diabetes, hypertension, cerebral congestion, dysentery, stomach-ache, ulcers, leprosy and gonorrhea (Adesida *et al.,* 1972).In Côte d'Ivoire the bark or leaf decoction is applied against jaundice and in DR Congo, the decoction of the stem bark or leaf is combined with a dressing of powdered root bark against itch and ringworm (Abbiw, 1990). Adewunmi *et al.* (1984) reported in their work that the bark, root and leaf are bitter. They stated that the infusion or decoction of these parts is used for the treatment of yellow fever and other forms of fever. They also reported that the decoction of the leaf is applied to the breast of women at weaning of their infants to prevent infections. However, *M. lucida* is used generally for febrifuge, analgesic and laxative (Bever, 1996), while the decoction of the stem bark is used for the treatment of severe jaundice. As reported by Adjanohoun *et al.,* (1991) *M. lucida* is used locally in the treatment of irregular menstruation, insomnia and jaundice though the parts that are

useful for this purpose was not mentioned. Burkill, (1991) also stated that locally, *M. lucida* is used in the treatment of wound infections, abscesses and chancre (the primary syphilitic ulcer associated with swelling of local lymph glands and is painless, indurated, solitary and highly infectious). Also amongst the Igede People in Benue State, Nigeria, it was reported by Igoli *et al.,* (2005) and Ogaji *et al.,* (2006) that the decoction of the *M. lucida* is used twice or thrice daily as anti-diarrhea, while the leaves are used for treatment of infertility in women.

2.6.1 Phytochemistry

The major constituents of *M. lucida* extracts are various types of alkaloids, anthraquinones and anthraquinols (Adewunmi *et al.,* 1984). From the wood and bark of *M. lucida*, 18 anthraquinones have been isolated, including the red colorants 1- methylether-alizarin, rubiadin and derivatives, lucidin, soranjidiol, damnacanthal, nordamnacanthal, morindin, munjistin and purpuroxanthin. Two compounds (Oruwalol and Oruwal as well as ten anthraquinones) were isolated and characterized from the stem (Adesogan *et al.,* 1984).In addition to anthraquinones, tannins, flavonoids and saponosides have been isolated. Adesogan *et al.,* (1983), Rath *et al.,* (1995) and Koumagho *et al.,* (1992) isolated anthraquinones and oruwacin from the roots of *M.lucida*. Trease and Evans, (2002) also confirmed the presence of the above constituents in their publication. Two known triterpenic acids (Ursolic and oleanolic acids) were isolated from the leaves (Richard *et al.,* 2006). Three compounds (digitolutein, rubiadin 1-methyl ether and damnacanthal) were extracted from the stem bark (Koumaglo *et al.,* 1992).

2.6.2 Pharmacological activity

Test with animals confirms the attributed activity of several traditional medicinal applications of *M. lucida*. Extracts showed anti-inflammatory, antifever and pain-reducing activity in tests with rats and promoted gastric emptying and intestinal motility. Leaf extracts showed in vitro antimalarial activity against *Plasmodium falciparum* while in several other tests antidiabetic properties were confirmed. Inhibiting effects on cancer tumours in mice have also been reported. A leaf extract gave 100% mortality in the freshwater snail *Bulinus globulus* at a concentration of 100 ppm.

2.6.2.1 Antimalarial activity

Obih *et al.*, (1985) investigated the various extracts (stem bark, root bark and leaves extracts) of *M.lucida*for antimalarial activity in a 4 – day schizontocidal test against a chloroquine sensitive strain of *Plasmodium berghei* in mice. The result showed that the stem bark extracts had the most promising result with 96.4% suppression of parasitaemia. They also investigated the antimalarial activity of the leaves extract collected in the month of August as compared to chloroquine and pyrimethiamine on the early and established infections caused by *Plasmodium berghei* in mice for 4 days. The result showed that 1.0mg/kg of the leaf extract equivalent of chloroquine produced positive effect on early infection. Awe *et al.*, (1997) evaluated the antimalarial effect of the leaf extracts sample of *M.lucida* against *Plasmodium berghei* in mice during various seasons (March, June, September and December) of the year for schizontocidal activity during early and established infections in 4 – day test and in addition to repository test. The result showed that both the March and June samples were found to be active, while September sample was less active than June sample, the December sample was devoid of activity

in the entire test carried out on the early and established infections as well as repository test. Sittie *et al.,* (1999) studied the structure activity relationship *invitro* of anthraquinones isolated from the roots of *M. lucida.* The result showed that an aldehyde group at C-2 and a phenolic hydroxyl group at C-3 enhance the activity of anthraquinones against the growth of *Plasmodium falciparum*. Richard *et al.,* (2006) also discovered that the petroleum ether extracts from *M. lucida* leaves exhibited an*invitro* antiplasmodial activity against *Plasmodium falciparum* with IC_{50} at 3.9±0.3µg/ml. This result was attributed to the presence of Ursolic acid found in the leaves.Agomo *et al.,* (1992) studied the impact of extracts of some anti - malaria medicinal plants amongst which were the aqueous extracts of the leaves and barks of *M. lucida,* on cell populations in various organs of mice and compared the effect with chloroquine treated mice. The result showed that all infected mice treated with the plant extract exhibited chemosuppression activity of early parasitaemia which did not lead to their survival. The result further showed that the total number of nucleated cells in the liver, spleen and peripheral blood of malaria – infected mice increased enormously before the animals died. However, all infected mice treated with chloroquine survived and the number of nucleated cells in both the malaria infected and uninfected mice were decreased.

2.6.2.2 Trypanocidal activity

Asuzu *et al.,* (1990) investigated the effect of methanol extract ofthe dried leaves of*M. lucida* on *Trypanosoma brucei* infected mice. The result showed that the intraperitoneal injection of the extract significantly suppressed the level of parasiteamia after *Trypanosoma brucei* infection in mice which is dose dependent with 1000mg/kg producing the maximum effect. They however

concluded that the best trypanocidal activity was obtained when treatment with *M. lucida* extract commenced simultaneously with Trypanosome inoculation.

2.6.2.3 Antifungal activity

Rath *et al.,* (1995) investigated the anti-fungal activity of ten anthraquinones isolated from a dichloromethane extract of the roots of *M. lucida.* The result showed that four of these anthraquinones were active against *Cladosporium cucumerinum* and *Candida albicans*. The result concluded that the most potent anti – fungal anthraquinone was identified as alizarin -1-methyl ether, which exhibited activity against *Aspergillus fumigatus* and *Trichophyton mentagrophytes* at MIC dose of 100 and 50 μg/ml respectively.

2.6.2.4 Antidiabetic activity

Kamanyi *et al.,* (1994) investigated the hypoglycemic effect of the aqueous extract of the root of *M. lucida* in alloxan – induced diabetic mice. The result showed that the extract at a dose of 148mg/kg and 280mg/kg produced a significant hypoglycemic effect by causing a fall in blood sugar by 51% and 60%, respectively, after 4h of administration. The result concluded that the aqueous root extract of *M. lucida* exhibited dose dependent potent hypoglycemic effects in both normal and alloxan – induced diabetic mice by oral administration and it is more potent than that observed with chlorpropamide(1 –(p – chlorobenzen-sulphanyl)-3-propylurea). Olajide *et al.,* (1999) also evaluated the hypoglycemic and anti – hyperglycemic activities of methanol extract of *M. lucida* leaves in normal and streptozotocin-diabetic rats. The result showed that the extract, at 400mg/kg demonstrated a significant($P < 0.05$) and dose dependent hypoglycemic activity within 4h after oral administration while the plasma glucose level was brought down to

42.5±0.4mg/100ml as compared to control value of 67.4± 1.2mg/100ml. The extract also produced a significant ($P<0.05$) anti-diabetic effect in hyperglycemic rats having a plasma glucose level of 248.7 ± 5.3mg/100ml as compared with animals treated with 10mg/kg of glibenclamide with a plasma glucose level of 251.5± 5.8mg/100ml from day 3 after oral administration with 400mg/kg of the extract. The result suggested that the leaves of *M. lucida* have a strong glucose lowering property when administered to streptozotocin-treated rats. It was then concluded that, the plasma glucose in normal rats decreased from 67 to 42mg/100ml when given 400mg/kg of a methanol extract of *M. lucida* leaves while the extract also improved streptozotocin diabetic rats as much as glibenclamide did.

2.6.2.5 Gastrointestinal activity

Olajide *et al.,* (1999) studied the effect of the methanol extract of the leaves on the gastric emptying in rats and intestinal motility in mice while they also investigated the effect of the extract on acetylsalicylic acid – induced ulcer in rats. The results showed that the extract promoted gastric emptying time in rats and intestinal motility in mice. Though, the extract did not induce gastric ulceration in rats, however, it failed to protect against acetylsalicylic acid – induced ulcer in rats.

2.6.2.6 Antibacterial activity

Ndukwe *et al.,* (2005) investigated the antibacterial activity of the aqueous extracts from the seventeen selected chewing sticks used in oral hygiene in Nigeria amongst which was the aqueous extract of the root of *M. lucida*, against typed cultures of *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, and Pseudomonas aeruginosa* as compared to a standard

reference compound –chlorocresol. The result showed that *M. lucida* aqueous root extract showed appreciable activity against all classes of bacteria isolates, especially, the Gram positive strains (*S. aureus and B. subtilis*) at a minimum inhibitory concentration <2.5mg/ml. It was suggested that the chewing stick extracts of *M. lucida* is a potential source of agents that can be used in the treatment of oral infections and further studies are required to evaluate its value in this regard. Adomi (2006) also investigated the effect of water and ethanol extracts of the stem bark of two Nigerian medicinal plants amongst which was *M. lucida* on clinical isolates of two Gram - positive and five Gram - negative (*S. aureus, S.typhi, K. pneumonia, P.aeruginosa, E. coli, B. subtilis and Flavobacterium sp.*) bacteria using two standard antibiotics (Chloramphenicol and Ampicilin) as reference drugs. The result showed that the aqueous extract of the stem bark of *M. lucida* at a concentration of between 500-1000mg/ml inhibited the growth of *S. aureus* and *P. aeruginosa* only. The ethanol extract of the stem bark, however gave the best result at MIC dose of between 250-1000mg/ml by inhibiting the growth of *S. aureus, K. pneumonia, E. coli, B. subtilis* and *Flavobacterium sp.*

2.6.2.7 Purgative induction in mice by the methanolic leaf extract

Asuzu *et al.,* (1990) carried out acute toxicity tests of the methanol leaf extract in mice by injecting intraperitonealy the *M. lucida* leaf extract. The result showed that the extract induced purgation in mice from the first hour after oral administration and reached its peak between the third and fourth hour. The LD50 was found to be 2000mg/kg.

2.6.2.8 Anti-spermatogenic activity of the methanol extract of the leaf

Raji *et al.,* (2005) investigated the effect of *M. lucida* methanol leaf extract on male albino rat reproductive functions by treating tw*o* groups of rats with 400mg/kg of the extract for 4 and 13 weeks. The result showed that the methanol leaf extract of *M. lucida* did not cause any changes in body and somatic organ weights, but significantly increased the testis weight ($P < 0.05$). The result further showed that the sperm motility and viability, as well as the epididymal sperm counts of rats treated for 13 weeks were significantly reduced ($P < 0.05$), which could be due to the acetylcholinesterase inhibition and glucose lowering properties of this plant. Fructoseutilization as well as glucose oxidation is an important means by which spermatozoa derive energy for motility. Also, sperm morphological abnormalities and serum testosterone levels were significantly increased($P < 0.05$), which was supported by the various degree of degeneration in the histologic sections of the testes and this suggested that *M. lucida* methanol extract administration for a long period was capable of permeating the blood-testes barriers. There were also various degrees of damage to the seminiferous tubules. The result suggested that the anti-fertility activities exhibited by *M.lucida* in this study could be associated with anthraquinones presented in the extract. It was concluded that the chronic administration of *M. lucida* leaf extract could impair reproductive activities in male albino rats, the reversal of which however occurred after a period of time, hence, the extract of *M.lucida* has reversible anti-spermatogenic properties.

2.6.2.9 Cytotoxicity Effect

Ajaiyeoba *et al.,* (2006) evaluated the *invitro* cytotoxicity of 20 Nigerian medicinal plants collected from Southwest and Middle belt, amongst which was *M. lucida* plant sample by using

the brine shrimp lethality assay. The result showed that, of the 20 plants studied, only two plants extracts were found to be cytotoxic and this included the methanol stem bark extract of *M. lucida* with LD50 of2.6µg/ml.

2.6.13 Hepatotoxicity and nephrotoxicity of the leaf extract in wistar albino rats

Oduola *et al.,* (2010) evaluated hepatotoxicity and nephrotoxicity of ethanol leaf extract of *M. lucida* in albino rats. Acute oral toxicity test was performed to determine the LD50; sub-chronic toxicity study was then carried out by oral administration of different doses of the extract on daily basis to different groups of rats for 42 days. The animals were subsequently sacrificed and liver and kidney function assessed technically using standard techniques. The result of acute oral toxicity (LD50) of *M. lucida* leaf extract was found to be greater than 6400mg/kg body weight as no mortality was recorded in any group of experimental rats. Also, according to Adeneye *et al.*(2008) in an acute oral toxicity study of *M. lucida* leaf extract, it was documented that *M. lucida* leaf extract possessed no lethality in rats at 2000mg/kg body weight. However LD50 of greater than 6400mg/kg is an indication that the extract may be safe for human consumption. It was also established that ingestion of *M. lucida* leaf extracts has no adverse effect on rat's liver and kidney function.

Anofi *et al.,* (2011) evaluated the toxicological effect of ethanol root extracts on rats at 50, 100, 200 and 300mg/kg body weight on haematology, kidney and liver function parameters in Wistar rats for 21days. They reported that the extract did not exhibit any significant ($P<0.05$) effect on red blood cells, hematotoxicity, haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, red cell width coefficient of variation, platelet distribution width and level of total protein, albumen, globulin, sodium,

potassium and calcium at all doses. The extract however, caused a significant reduction in the serum levels of white blood cells, platelets, alkaline phosphate, cholesterol, high density lipoprotein cholesterol and low density lipoprotein cholesterol. At lower dose, the extract increased the aspartate aminotransferase, but at high doses the parameter was significantly reduced. Similarly, the extract at all doses led to significant increase in the body and absolute organ weights of the animals but no effect on the liver, kidney, heart and lungs body weight ratios. They concluded that the alterationsexhibited by the ethanol root extract of *M. lucida*on the haematogical as well as liver and kidney function indices suggested parameter and dose-selective toxicity of the extract.

2.6.2.11 Collaboration of *M. lucida* with other medicinal plants

Malaria: Aqueous extract of stem bark of *Alstonia boonei,* leaves of *Mangifera indica*, fallen dried leaves of *Carica papaya*, stem bark of *Parkia biglobosa* or *Parkia clappertoniana*, leaves of *Morinda lucida*, *Cymbopogon citratus* and leaves of *Cassia podocarpa* (Zac *et al.*, 1986) are useful in the Management of malaria infections causedby *Plasmodium falciparum* and *P. berghei* parasite. Aqueous extract of leaf of *Ocimum gratissimum,* leaf and bark of *Azadirachta indica,* leaf and bark of *Morinda lucida,* bark of *Enantia chloranta* (Agomo *et al.*, 1992) are useful in the Management of malaria infections caused by*Plasmodium yoeli*. Ethanol extracts ofroot bark of *Cryptolepis sanguinolenta,* whole plant of *Euphorbia hirta*, leaves of *Morinda lucida* and whole plant of *phyllantus niruri* (Tona *et al.*, 1999) are useful in the Management of malaria infections caused by*P. falciparum* and *P. berghei* parasites.

***Diabetes*:** Methanol, ethanol and aqueous extracts of *Mangifera indica* leaves, *Vernonia amygdalina* leaves, *Morinda lucida* leaves, *Momordica charantia* fruits and *Ocimum*

gratissimum leaves are useful as anti-diabetic (Lotlikar *et al.*, 1966; Akah *et al.,* 1992; Aguiyi *et al.,* 2000; Bamidele *et al.,* 2002).

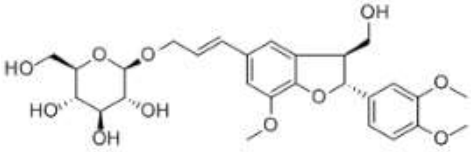
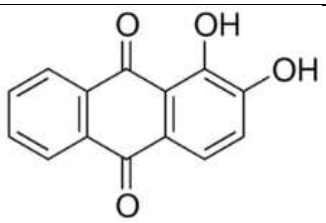
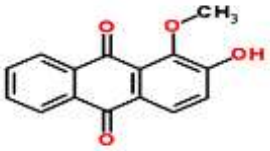
***Orofacial infections*:** Aqueous extract of root of *Vitellaria paradoxa,* stem and twig of *Bridellia ferruginea,* stem of *Garcinia cola,* root of *Terminalia glaucescens,* root of *Morinda lucida* and fruit of *Cnestis ferruginea* (Ndukwe *et al.,* 2005) are used for the treatment of oral infections caused by micro-organisms such as*Staphylococcus aurens, Bacillus subtilis, Escherichia coli, and Pseudomonas aeruginosa.*

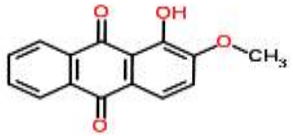
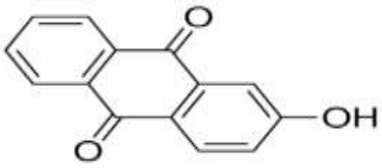
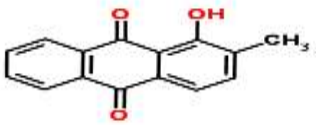
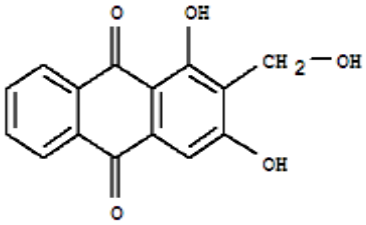
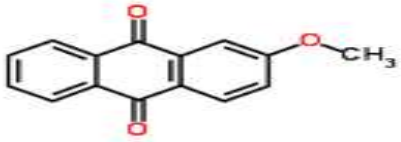
***Cancer*:** Methanol extract of leaves of *Lippia multiflora* and bark of *Morinda lucida*(Ajaiyeoba *et al.,* 2006)are possible combination in the management of cancer due to their *invitro* cytotoxic effect.

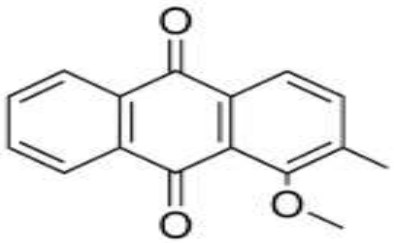
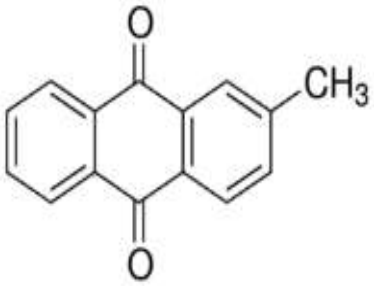
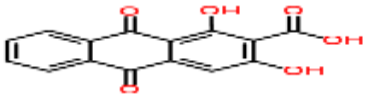
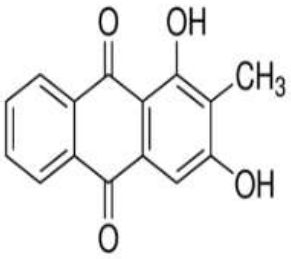
***Bacterial infections*:** Aqueous, ethanol and methanol extracts of stem bark of *Alstonia boonei,* leaves *of Mangifera indica,* leaves *of Psidium guajava* and stem bark of *Morinda lucida* (Adoni *et al.,* 2005), (Akinpelu *et al.,* 2007) combination is useful in the treatment of bacterial infections caused by the Gram +ve and Gram –ve organisms such as*S.aureus, S.typhi, K. pneumonia, P.aeruginosa, E. coli, B. subtilis and Flavobacterium sp.*

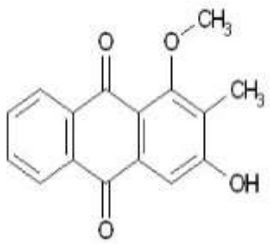
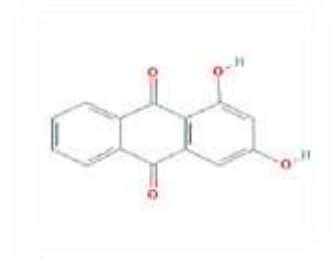
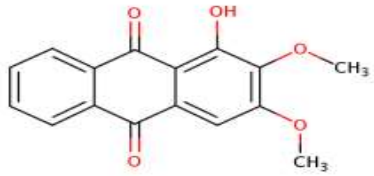
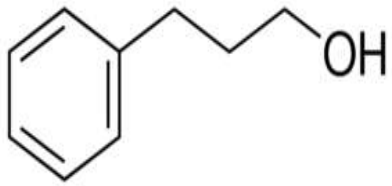
2.7 Compounds Isolated from Previous Research

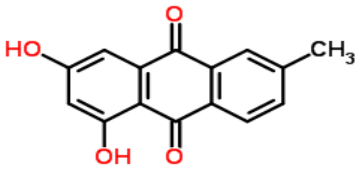
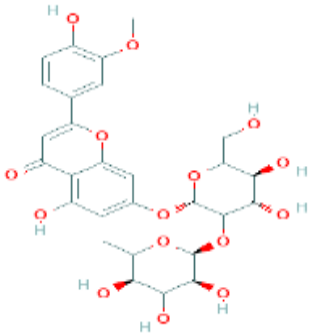
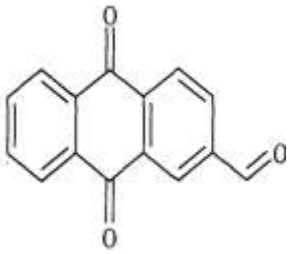
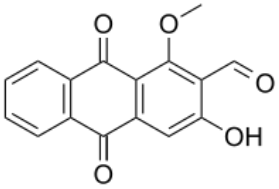
Many species of the *Morinda* genus are rich source of compounds that have been isolated and reported including flavones, flavonols, carbohydrates, iridoid monoterpenes, quinoids and miscellaneous compounds.

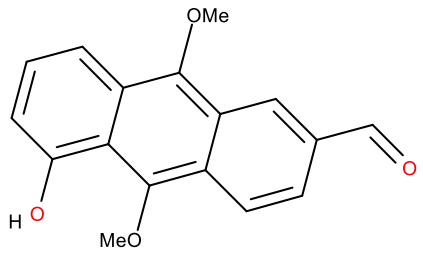
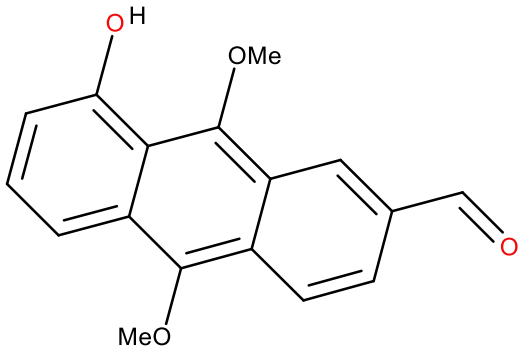
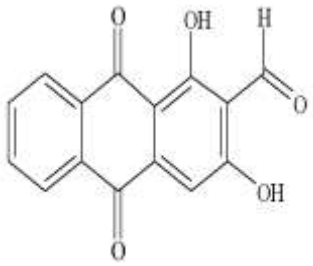
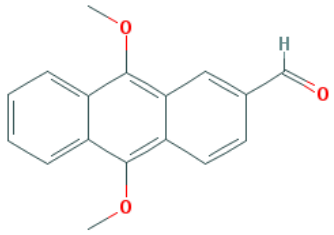
S/N	Name of plant	Parts used	Compound isolated	Structure of the compound isolated	References
1	*Morinda citrifolia*	root-bark	Moridin		Simonsen *et al.*, (1920)
2	*Morinda longiflora*.	root	longifloroside		Paris *et al.*,(1958)
3	*Morinda umbellata*	root stem	alizarin		Burnett *et al.*, (1968)
4	*Morinda umbellate Morinda lucida*	root stem	alizarin-1-methyl ether		Burnett *et al.*, (1968) Adensogan *et al.*, 1973

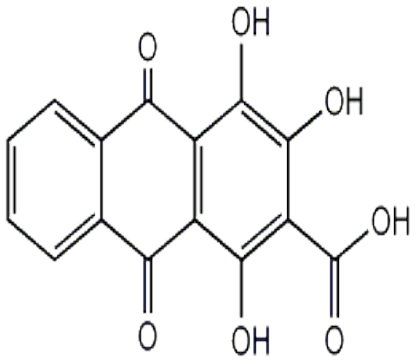
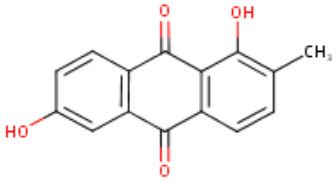
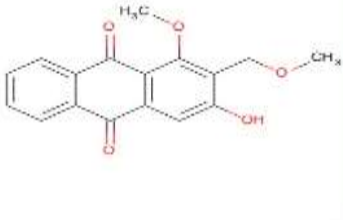
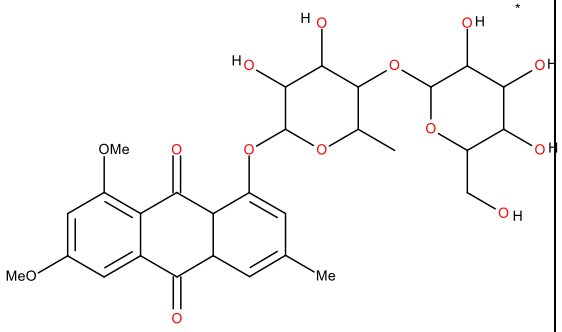
5	*Morinda umbellata*	root stem	alizarin-2-methyl ether		Burnett *et al.*, (1968)
6	*Morinda umbellata*	root stem	2-hydroxyanthraquinone		Burnett *et al.*, (1968)
7	*Morinda umbellate Morinda lucida*	root stem	1-hydroxy-2-methylanthraquinone		Burnett *et al.*, (1968) Adensogan *et al.*, 1973
8	*Morinda umbellata*	root	lucidin		Burnett *et al.*, (1968)
9	*Morinda umbellata*	root stem	2-methoxyanthraquinone		Burnett *et al.*, (1968)

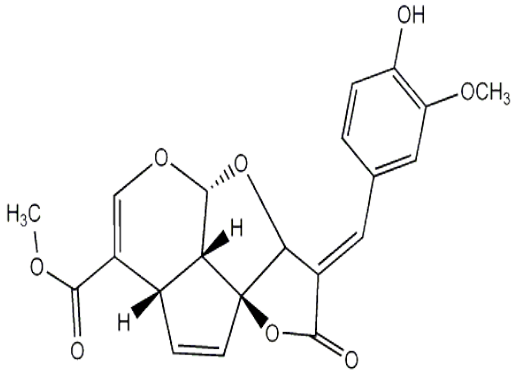
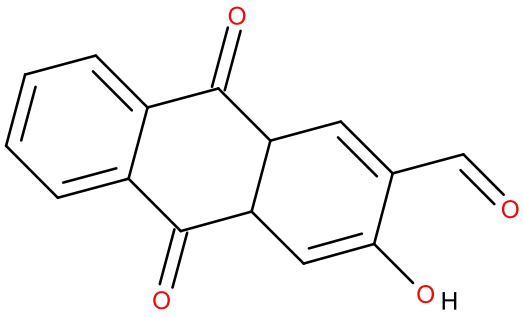
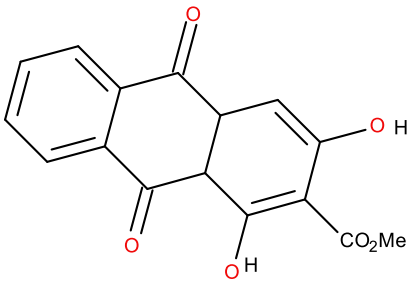
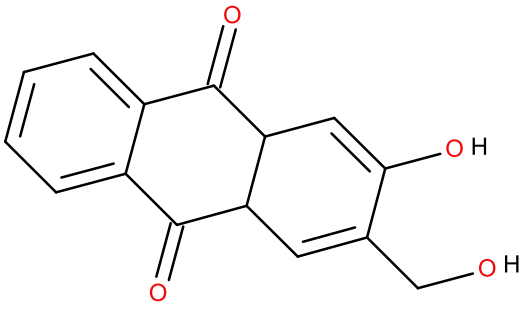
10	*Morinda umbellate* *Morinda lucida*	root stem	1-methoxy-2-methylanthraquinone		Burnett *et al.*, (1968) Adensogan *et al.*, 1973
11	*Morinda umbellata*	root stem	2-methylanthraquinone		Burnett *et al.*, (1968)
12	*Morinda umbellata*	root	munjistin		Burnett *et al.*, (1968)
13	*Morinda umbellate* *Morinda lucida*	root stem	rubiadin		Burnett *et al.*, (1968) Adensogan *et al.*, 1973

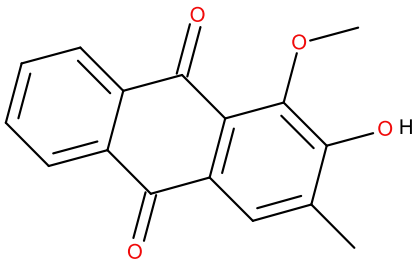
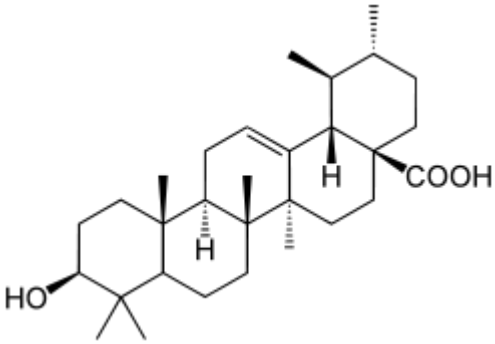
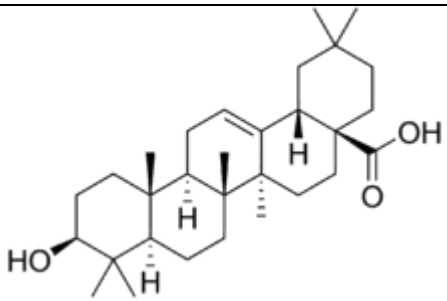
14	*Morinda umbellate* *Morinda lucida*	root stem	rubiadin-1-methyl ether		Burnett *et al.*, (1968) Adensogan *et al.*, 1973
15	*Morinda umbellata*	root stem	xanthopurpurin		Burnett *et al.*, (1968)
16	*Morinda citrifolia*		1-hydroxy-2,3-dimethoxyanthraquinone		Burnett *et al.*, (1968)
17	*Morinda logifolia*	essential oil	3-phenyl-1-propanol		Snyder *et al.*, 1969.

18	*Morinda umbellata*	root-bark	1, 3-dihydroxy-6-methylanthraquinone		Stoessl *et al*., 1969
19	*Morinda morindoides*	leaves	chrysoeriol 7-neohesperidoside		Harborne *et al*., (1970)
20	*Morinda lucida*	stem	anthraquinone-2-aldehyde		Adensogan *et al*., 1973
21			damnacanthal		

22	*Morinda lucida*	stem	5-hydroxyoruwal		Adensogan *et al.*, 1973
23			8-hydroxyoruwal		
24			nordamnacanthal		
25			oruwal		

26			pseudopurpurin		
27			soranjidiol		
28	*Morinda lucida*		3-hydroxy-1-methoxy-2-(methoxymethyl) anthraquinone		Gonza'le *zet al.*, 1977
29	*Morinda citrifolia*	flower	emodin 6,8-dimethyl ether 1-scillabioside		Tiwari *et al.*, 1977

30	*Morinda lucida*	stem	oruwacin		Adesogan *et al.*, 1979
31	*Morinda lucida*	heartwood	3-hydroxyanthraquinone-2-carboxyldehyde		Demagos *et al.*, 1981
32	*Morinda lucida*	heartwood	1, 3-dihydroxyanthraquinone-2-carboxylic acid		Dossh *et al.*, 1981
33	*Morinda lucida*		2-Hydroxy-3-hydroxymethylanthraquinone		Rath *et al.*, 1995

34	*Morinda lucida*		digitolutein		Adesogan, 1979; Stephens *et al.*, 2008
35	*Morinda lucida*		ursolic acid		Adesogan, 1979; Stephens *et al.*, 2008
36	*Morinda lucida*		oleanolic acid		Adesogan, 1979; Stephens *et al.*, 2008

2.8 Chromatography

Chromatography encompasses a diverse and important group of methods that permit the scientist to separate closely related components of complex mixtures; many of these separations are impossible by other means. In all chromatographic separations the sample is transported in a mobile phase, which may be a gas, a liquid, or a supercritical fluid. This mobile phase is then forced through an immiscible stationary phase, which is fixed in place in a column or on a solid surface. The two phases are chosen so that the components of the sample distribute themselves between the mobile and stationary phase to varying degrees. Some components of the mixture are strongly retained to stationary phase than others and as a consequence of these differences in mobility; sample components are separated (Karger *et al*., 1973; Skoog *et al*., 1998;Cooke and Poole, 2000;Meyer, 2004).

Chromatographic methods can be classified according to the type of mobile phase such as liquid, gas, and super critical fluid chromatography (Skoog *et al*., 1998). The most important one used in this research is liquid chromatography.

2.8.1 Liquid Chromatography

Liquid chromatography (LC) is an analytical chromatographic technique that is useful for separating ions or molecules that are dissolved in a solvent. This separation occurs based on the interactions of the sample with the mobile phase which is liquid and stationary phase which can be either liquid, solid, bonded phase or ion exchange (Karger *et al*., 1973).

2.8.2 Adsorption Chromatography

The principle of adsorption chromatography is known from classical open column and thin-layer chromatography. A relatively polar material with a high specific surface area is used as the stationary phase (silica gel and alumina are the most popular). The mobile phase is relatively non-polar (heptane to tetrahydrofuran). Adsorption chromatography is based on the interaction between the solute molecules and active sites on the stationary phase. This attachment or interaction depends on the polarity of solutes. This techniques proves the statement that "polar like polar" because if the stationary phase is more polar than the mobile phase then high polar compounds in the mixture will tightly bound to the stationary phase whereas less polar compounds will lightly bind to the stationary phase. Less tightly bound compounds will be eluted out by the mobile phase earlier than the tightly bonded ones.

Silica gel is the most frequently used solid-phase in adsorption chromatography. The weakly acidic properties of its surface may be the reason for the chemisorption of alkaloids, especially when neutral nonpolar solvents are used such as chloroform and dichloromethane (Cooke and Poole, 2000).

Normal-phase chromatography is a technique that uses columns packed with polar stationary phases combined with nonpolar or moderately-polar mobile phases to separate the components of mixtures. The rate at which individual solutes migrate through normal –phase chromatography columns is primarily a function of their polarity. Less polar solutes move the fastest and therefore exit the column and are detected first, followed by solutes of increasing polarity which move more slowly. However, polarity can sometimes play a secondary role relative to a solute's ability to experience a specific interaction with active sites on the stationary phase surface. The

importance of these specific solute–stationary phase interactions in normal –phase chromatography gives it some unique advantages over the more widely practiced reversed-phase chromatography technique.

Reversed-phase chromatography utilizes nonpolar stationary phases and aqueous-based polar mobile phases, and the elution order of solutes in a mixture is related to their hydrophobicity, not polarity; more polar solutes move the fastest and appear first, followed by solutes of decreasing polarity. Reversed-phase chromatography is useful for separating mixtures in which components differ in molecular weight and/or water solubility. However, Normal-phase chromatography is preferred for many separation problems, including those in which the water solubility of sample compounds is limited. In addition, normal-phase chromatography is a better technique for separating compounds that differ in the number or character of functional groups and is particularly useful for separating many types of isomers.

2.8.3 Partition Chromatography

Partition chromatography can be subdivided into liquid-liquid and bonded-phase chromatography. The difference in these techniques lies in the method by which the stationary phase is held on the support particles of the packing. With liquid-liquid, a liquid stationary phase is retained on the surface of the packing by physical adsorption (e.g. viscous liquid of hydrocarbons chain) (Skoog *et al*., 1998).

A large number of stationary phases can be produced by careful choice of suitable reaction partners. The most important special case of chemically bonded-phase chromatography is the

reverse-phase method, where the stationary phase is very non-polar and the mobile phase is relatively polar (Karger *et al*.,1973; Skoog *et al*., 1998;Cooke and Poole, 2000; Meyer, 2004).

2.8.4 Column Chromatography

Column chromatography is the most widely and the oldest technique used for separation of organic compounds with the aim of isolating and purifying compounds from 1-100 grams. It is based upon the stationary phase (silica gel is the most popular) held in narrow tube and the sample injected through which the mobile phase is eluted under the force of gravity. The mobile phase is eluted with gradient increase in its polarity. Polar compounds are eluted later than non-polar compounds (Karger *et al*.,1973; Skoog *et al*., 1998;Cooke and Poole, 2000; Meyer, 2004).

2.8.5 Size-exclusion chromatography

Size-exclusion chromatography (SEC) is a chromatographic method in which molecules in solution are separated by their size, and in some cases molecular weight (Paul-Dauphin *et al*., 2007). It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers. Typically, when an aqueous solution is used to transport the sample through the column, the technique is known as gel-filtration chromatography, versus the name Gel permeation chromatography, which is used when an organic solvent is used as a mobile phase. SEC is a widely used polymer characterization method because of its ability to provide good molar mass distribution (MW) results for polymers.

2.8.6 Gel filtration chromatography

Gel filtration separates molecules according to difference in size as they pass through a gel filtration medium packed in a column. Unlike ion exchange or affinity chromatography, molecules do not bind to the chromatography medium so buffer composition does not directly affect resolution (the degree of separation between peaks). Consequently, a significant advantage of gel filtration is that conditions can be varied to suit the type of sample or the requirements for further purification, analysis or storage without altering the separation.

Gel filtration is well suited for biomolecules that may be sensitive to changes in pH, concentration of metal ions or co-factors and harsh environmental conditions. Separations can be performed in the presence of essential ions or co-factors, detergents, urea, guanidine hydrochloride, at high or low ionic strength, at 37^{o}C or in the cold room according to the requirements of the experiment. Purified proteins can be collected in any chosen buffer (Amersham Biosciences AB, 2002).

2.8.7 Gel permeation chromatography

Gel permeation chromatography (GPC) is a type of size exclusion chromatography (SEC) that separates analytes on the basis of size. The technique is often used for the analysis of polymers. As a technique, SEC was first developed in 1955 by Lathe and Ruthven (Lathe, and Ruthven, 1956). The term gel permeation chromatography can be traced back to J.C. Moore of the Dow Chemical Company who investigated the technique in 1964 and the proprietary column technology was licensed to Waters, who subsequently commercialized this technology in 1964 (Moore, 1964). It is often necessary to separate polymers, both to analyze them as well as to

purify the desired product. When characterizing polymers, it is important to consider the polydispersity index (PDI) as well the molecular weight. Polymers can be characterized by a variety of definitions for molecular weight including the number average molecular weight (M_n), the weight average molecular weight (M_w) (see molar mass distribution), the size average molecular weight (M_z), or the viscosity molecular weight (M_v). GPC allows for the determination of PDI as well as M_v and based on other data, the M_n, M_w, and M_z can be determined.

2.8.8 High Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a form of liquid chromatography used to separate compounds that are dissolved in solution under high pressure. HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Compounds are separated by injecting a plug of the sample mixture onto the column. The different components in the mixture pass through the column at different rates due to differences in their distribution behavior between the mobile liquid phase and the stationary phase (Skoog*et al*., 1998; Meyer, 2004).

2.8.8.1 Analytical and Semi-Preparative HPLC

Analytical HPLC is a very efficient instrument used quantitatively for accurate determination of the amount of analyte in a sample. Few micrograms of the analyte could be detected accurately. The method used for analysis must be improved to reach a good separation between analyte peak and sample matrix peaks. The retention time and area under the peaks, comparing with standard, are used for quantitative determination of the analyte (Meyer, 2004).

Semi-preparative and preparative HPLC equipment are used to speed up the efficiency of separation and purification of compounds in amounts varying from 3 mg up to several grams. Special preparative HPLC column, high pressure pump and large sample volume injector are used for efficient separation (Meyer, 2004).

2.8.9 Analytical Thin-Layer Chromatography

Thin-layer chromatography is a technique in which a solute undergoes distribution between two phases, a stationary phase acting through adsorption and a mobile phase in the form of a liquid. The adsorbent is a relatively thin, uniform layer of dry finely powdered material applied to a glass, plastic or metal sheet or plate. Glass plates are most commonly used. Separation may also be achieved on the basis of partition or a combination of partition and adsorption, depending on the particular type of support, its preparation and its use with different solvent (Harborne, 1928; Stahl, 1969). Identification can be effected by observation of spots of identical R_f value and about equal magnitude obtained, respectively, with an unknown and a reference sample chromatographed on the same plate. A visual comparison of the size and intensity of the spots usually serves for semi-quantitative estimation (Funk, 1991).

2.8.10 Preparative Thin-Layer (Planar) Chromatography

Preparative planar (thin-layer) chromatography (PPC) is a liquid chromatographic technique performed with the aim of isolating and purifying compounds, in amounts of 10-100 mg, for structure elucidation and for evaluation of biological activity. PPC is a valuable simple method of sample purification for preparative purposes and isolation. The scope of modifying operating parameters such as the vapor space, development mode and for offline sample application is

enormous in planar chromatography. One of the most important experimental variables in PPC is the vapor space, because the separation process occurs in a three-phase system of stationary, mobile, and vapors phases, all of which interact until equilibrium is reached. In classical PPC the mobile phase migrates by capillary action (Karger *et al*.,1973; Skoog *et al*., 1998;Cooke and Poole, 2000; Meyer, 2004).

2.9 Ultraviolet Spectroscopy

UV spectroscopy is an important tool in analytical chemistry. The other name of UV (Ultra-Violet) spectroscopy is Electronic spectroscopy as it involves the promotion of the electrons from the ground state to the higher energy or excited state. Ultraviolet and visible (UV-Vis) absorption is the measurement of the attenuation of a beam of light after it passes through a sample or after reflection from a sample surface. Absorption measurements can be at a single wavelength or over an extended spectral range.

UV spectroscopy is a type of absorption spectroscopy in which light of ultra-violet region (200-400 nm.) is absorbed by the molecule. Absorption of the ultra-violet radiations results in the excitation of the electrons from the ground state to higher energy state. The energy of the ultra-violet radiation that is absorbed is equal to the energy difference between the ground state and higher energy states.

UV spectroscopy is used for detection of functional groups, detection of impurities, qualitative analysis, quantitative analysis, single compound without chromophore, drugs with chromophoric reagent. It helps to show the relationship between different groups, it is useful to detect the conjugation of the compounds.

UV spectroscopy is more often applied to the study of covalent and non-covalent interactions. Since certain functional groups present in organic molecules absorb light at characteristic wavelengths in the UV-Vis region, this technique is applied qualitatively to identify the presence of these groups in samples, supporting structural information obtained from other spectroscopic methods.

2.10 Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy (NMR) has been the single most important physical method for the determination of molecular structures for more than 30 years. The power of the technique lies in that it not only defines the numbers and types of nuclei present in an organic molecule, but it also describes their individual chemical environments and, more importantly, the way they are interconnected. Driven by its potential to determine the structures of organic compounds, NMR spectroscopy has seen substantial development in the four and a half decades since the first experiments. In particular, the implementation of the pulsed Fourier transforms method and, subsequently, the concept of two-dimensional experiments provided the seeds for vibrant growth. There are currently hundreds of multipulse experiments available to the NMR spectroscopy. The most important experiments are COSY, HMBC, HMQC, NOESY, ROESY, HSQC and DEPT experiments (Lambert and Mazzola, 2000; Hallas, 2004).

The vast majority of nuclei in a solution would belong to the solvent, and most regular solvents are hydrocarbons and would contain NMR-reactive protons. Thus, deuterium (hydrogen-2) is substituted (99%). The most used deuterated solvent is deuterochloroform ($CDCl_3$), although deuterium oxide (D_2O) and deuterated DMSO (DMSO-d_6) are used for hydrophilic analytes and deuterated benzene is also common. The chemical shifts are slightly different in different

solvents, depending on electronic solvation effects. NMR spectra are often calibrated against the known solvent residual proton peak instead of added tetramethylsilane.

2.10.1 One-Dimensional Nuclear Magnetic Resonance Spectroscopy

A type of spectroscopy where the energy states of spin-active nuclei placed in a static magnetic field are interrogated by inducing transitions between the states via radio frequency irradiation. Each experiment consists of a sequence of radio frequency pulses with delay periods in between them, and the spectrum is obtained by plotting chemical shift vs. frequency.

2.10.1.1 Proton Nuclear Magnetic Resonance

Proton nuclear magnetic resonance (proton NMR, hydrogen-1 NMR, or ^{1}H NMR) is the application of nuclear magnetic resonance in NMR spectroscopy with respect to hydrogen-1 nuclei within the molecules of a substance, in order to determine the structure of its molecules(Silverstein *et al.*, 1991). In samples where natural hydrogen (H) is used, practically all the hydrogen consists of the isotope ^{1}H (hydrogen-1; i.e. having a proton for a nucleus). A full ^{1}H atom is called protium. Proton NMR spectra of most organic compounds are characterized by chemical shifts in the range +14 to -4 ppm and by spin-spin coupling between protons. The integration curvefor each proton reflects the abundance of the individual protons. Together with carbon-13 NMR, proton NMR is a powerful tool for molecular structure characterization.

A spectrum of 1H NMR gives the following information: the number of hydrogen atoms present in the compound, which is indicated by the peak integration; the type of correlation between one hydrogen atom and the other, which is indicated by coupling constant (J); the numbers of coupled neighbouring hydrogen atoms, which are indicated by the molecule's splitting pattern;

and the nature or environment of the hydrogen atoms. This is related to the functional groups as indicated by chemical shifts.

2.10.1.2 Carbon-13 Nuclear Magnetic Resonance

Carbon-13 nuclear magnetic resonance (most commonly known as carbon-13 NMR or ^{13}C NMR or sometimes simply referred to as carbon NMR) is the application of nuclear magnetic resonance (NMR) spectroscopy to carbon. It is analogous to proton NMR (^{1}H NMR) and allows the identification of carbon atoms in an organic molecule just as proton NMR identifies hydrogen atoms. As such ^{13}C NMR is an important tool in chemical structure elucidation in organic chemistry. ^{13}C NMR detects only the ^{13}C isotope of carbon, whose natural abundance is only 1.1%, because the main carbon isotope, ^{12}C, is not detectable by NMR since it has zero net spin.

The spectrum of 13C NMR gives the following information: the numbers of carbon atoms inside a molecule; the type of carbon atoms (related to functional groups), which is indicated by the chemical shifts. In 13C NMR spectrum, the peak integration does not directly represent the exact numbers of particular carbon atoms. That is to say, this experiment does not give the information of carbon multiplicity.

2.10.1.3 Distortionless Enhancement by Polarization Transfer (DEPT)

Distortionless enhancement by polarization transfer (DEPT)is a NMR method used for determining the presence of primary, secondary and tertiary carbon atoms (Keeler James, 2010). The DEPT experiment differentiates among CH, CH_2 and CH_3 groups by variation of the selection angle parameter (the tip angle of the final ^{1}H pulse): 135° angle gives all CH and

CH_3 in a phase opposite to CH_2; 90° angle gives only CH groups, the others being suppressed; 45° angle gives all carbons with attached protons (regardless of number) in phase.

Signals from quaternary carbons and other carbons with no attached protons are always absent (due to the lack of attached protons).

The polarization transfer from ^{1}H to ^{13}C has the secondary advantage of increasing the sensitivity over the normal ^{13}C spectrum (which has a modest enhancement from the nuclear overhauser effect (NOE) due to the ^{1}H decoupling).

2.10.2 Two-Dimensional Nuclear Magnetic Resonance Spectroscopy

It is an NMR experiment in which a second frequency dimension is employed to disperse the signals and reveal correlations between interacting nuclei.

2.10.2.1 Correlation Spectroscopy (COSY)

The first and most popular two-dimension NMR experiment is the homonuclear correlation spectroscopy (COSY) sequence, which is used to identify spins which are coupled to each other. It consists of a single RF pulse (p1) followed by the specific evolution time (t1) followed by a second pulse (p2) followed by a measurement period (t2) (Akitt *et al.,* 2000).

The two-dimensional spectrum that results from the COSY experiment shows the frequencies for a single isotope, most commonly hydrogen (^{1}H) along both axes. Techniques have also been devised for generating heteronuclear correlation spectra, in which the two axes correspond to different isotopes, such as ^{13}C and ^{1}H.COSY spectra show two types of peaks. Diagonal peaks have the same frequency coordinate on each axis and appear along the diagonal of the plot;

while cross peaks have different values for each frequency coordinate and appear off the diagonal. Diagonal peaks correspond to the peaks in a 1D-NMR experiment, while the cross peaks indicate couplings between pairs of nuclei (much as multiplet splitting indicates couplings in 1D-NMR) (Akitt *et al.*, 2000).

2.10.2.2 Heteronuclear Multiple-Bond Correlation Spectroscopy (HMBC)

HMBC detects heteronuclear correlations over longer ranges of about 2–4 bonds. The difficulty of detecting multiple-bond correlations is that the HSQC and HMQC sequences contain a specific delay time between pulses which allows detection only of a range around a specific coupling constant. This is not a problem for the single-bond methods since the coupling constants tend to lie in a narrow range, but multiple-bond coupling constants cover a much wider range and cannot all be captured in a single HSQC or HMQC experiment (Keeler, James 2010).

In HMBC, this difficulty is overcome by omitting one of these delays from an HMQC sequence. This increases the range of coupling constants that can be detected, and also reduces signal loss from relaxation. The cost is that this eliminates the possibility of decoupling the spectrum, and introduces phase distortions into the signal. There is a modification of the HMBC method which suppresses one-bond signals, leaving only the multiple-bond signals(Keeler, James 2010).

2.10.2.3 Heteronuclear Multiple-Quantum Correlation Spectroscopy (HMQC)

HMQC is a type of heteronuclear NMR spectroscopy where a second frequency dimension is employed to disperse the signals and reveal correlations between protons and the nuclei to which they are covalently bound. HMQC is selective for direct C-H coupling. This technique provides

a convenient way for the identification of two diastereotopic geminal protons, which are sometimes difficult to be distinguished by COSY. Only in this condition, two correlations to one carbon can be observed in HMQC spectrum.

2.11 Mass Spectrometry

Mass Spectrometry (MS) has proved to be one of the most effective techniques in biomedical research, especially when complex matrixes of biological samples must be analysed. Themain advantages of MS are its high sensitivity, which allows analysis of compounds present in the μg scale, and high specificity, as it is able to separate molecules of the same molecular weight but different atom composition, and sometimes even to differentiate stereoisomeric compounds. Its easy coupling to separation techniques such as liquid and gas chromatography is also an excellent advantage.Sample preparation may also be critical, but that varies from sample to sample; nevertheless, some general guidelines have been reviewed (Prasain *et al.*, 2004).

Mass spectrometry is a fairly routine tool for molecular weight determination and a specialty tool for complex structure identifications. The principle of mass spectrometry (MS) is the separation of ions in a vacuum, using an electrical or magnetic field or a combination of both. The ions may be formed through a variety of ionization methods; fast atom bombardment (FAB), desorption chemical ionization (DCI), chemical ionization (CI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and electron impact ionization (EI). Once a molecule is ionized, the mass spectrometer provided separation of ions according to mass to charge ratio. The chemical structure of the parent ion may be determined from the molecular ion mass and the pattern of the fragment ions recorded. Experienced mass spectrometrists can recognize typical fragment ion patterns (Cooke and Poole, 2000; Lee, 2002).

Ion sources are classified as being hard sources or soft sources. Hard sources (e.g. EI) impart sufficient energy to analyte molecules so that they are left in highly excited energy state. Relaxation then involves rupture of bonds, producing fragment ions that have mass-to-charge (m/z) ratios less than that of the molecular ion. Soft sources (e.g. CI, ESI, and APCI) cause little fragmentation and then the spectrum consists of the molecular ion peak and only a few, if any other peaks (Skoog*et al*., 1998; Cooke and Poole, 2000; Lee, 2002).

2.11.1 Electrospray ionization (ESI)

Electrospray Ionization (ESI) is a technique in which ions are generated by solvent evaporation under a high voltage potential, and can be applied directly, by infusion of the sample with a flow-controlled syringe, or coupled to separation techniques such as LC orcapillary electrophoresis. In both cases, a steady liquid stream enters the system, allowing multiple analyses to be performed over a relatively large period of time. ESI interfaces are mostly coupled to quadrupole mass spectrometers; both are simple and robust equipments,able to produce both positive or negative ions, and their main limitation is the relatively limited m/zrange, usually below 2 kDa.(Prasain *etal*, 2004; De Hoffmann and Stroobant, 2007).ESI use atmospheric pressure and high collision frequency, and thus generate large amounts of ions; as they involve solvent evaporation, the decomposition of the analytes is reduced, and full scans show limited fragmentation. The main disadvantage ofthis technique is that some HPLC solvents interfere with the ionization process, and thus chromatographic separations need to be specifically designed (Prasain *et al*, 2004).

2.11.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS is a technique routinely used in sample analysis that combines liquid chromatography (LC) with mass spectrometry (MS). Extremely sensitive modern MS has helped LC-MS replace several immunoassays. LC-MS has helped improve the efficiency of drug discovery due to its excellent sensitivity and specificity. The technique can be combined with stable isotope dilution for precise and reproducible assays.

Liquid chromatography–mass spectrometry (LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS). Coupled chromatography - MS systems are popular in chemical analysis because the individual capabilities of each technique are enhanced synergistically. While liquid chromatography separates mixtures with multiple components, mass spectrometry provides structural identity of the individual components with high molecular specificity and detection sensitivity.

LC-MS is used for compounds that are thermally unstable. The separation is based on the difference in affinity of samples with stationary phase and the mobile phase. This tandem technique can be used to analyze biochemical, organic, and inorganic compounds commonly found in complex samples of environmental and biological origin. Therefore, LC-MS may be applied in a wide range of sectors including biotechnology, environment monitoring, food processing, pharmaceutical, agrochemical, and cosmetic industries (Chaimbault*et al*., 2014; Dass, 2007).

2.11.3 High Resolution Mass Spectrometry

It is a mass spectrometry in which m/z for each ion is measured to several decimal places (i.e., exact masses are measured, instead of nominal masses). It is particularly useful to differentiate between molecular formulas having the same nominal masses.

In assigning mass values to atoms and molecules, we have assumed integral values for isotopic masses. However, accurate measurements show that this is not strictly true. Because the strong nuclear forces that bind the components of an atomic nucleus together vary, the actual mass of a given isotope deviates from its nominal integer by a small but characteristic amount (remember $E = mc^2$). Thus, relative to ^{12}C at 12.0000, the isotopic mass of ^{16}O is 15.9949 amu (not 16) and ^{14}N is 14.0031 amu (not 14).

By designing mass spectrometers that can determine m/z values accurately to four decimal places, it is possible to distinguish different formulas having the same nominal mass. The table below illustrates this important feature, and a double-focusing high-resolution mass spectrometer easily distinguishes ions having these compositions. Mass spectrometry therefore not only provides a specific molecular mass value, but it may also establish the molecular formula of an unknown compound.

Formula	C_6H_{12}	C_5H_8O	$C_4H_8N_2$
Mass	84.0939	84.0575	84.0688

2.11.4 Gas Chromatography–Mass Spectrometry

Gas chromatography–mass spectrometry (GC-MS) is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample (David Sparkman *et al.*, 2011).

In GC-MS, sample mixtures are directly vaporized and enter bonded fused silica columns. Components of the mixture are separated based on their affinity difference with the bonded phase. Separated compounds exit the column and enter the vacuum system of the mass spectrometer. Sample molecules are ionized (EI or CI), and accelerated into a pre calibrated mass analyzer (e.g. Q, Ion Trap, TOF, FTMS etc.). Retention times, molecular masses, and fragmentation patterns are recorded. One of the most important considerations of GC/MS is that the sample(s) must be non thermoabile meaning thermally stable.

Applications of GC-MS include drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples, including that of material samples obtained from planet Mars during probe missions as early as the 1970s. GC-MS can also be used in airport security to detect substances in luggage or on human beings. Additionally, it can identify trace elements in materials that were previously thought to have disintegrated beyond identification. Like liquid chromatography–mass spectrometry, it allows analysis and detection even of tiny amounts of a substance.

2.12 The Immune System

The immune system is a host defense system comprising many biological structures and processes within an organism that protects against disease. To function properly, an immune system must detect a wide variety of agents, known as pathogens, from viruses to parasitic worms, and distinguish them from the organism's own healthy tissue. In many species, the immune system can be classified into subsystems, such as the innate immune system versus the adaptive immune system, or humoral immunity versus cell-mediated immunity. In humans, the blood–brain barrier, blood–cerebrospinal fluid barrier, and similar fluid–brain barriers separate the peripheral immune system from the neuroimmune system, which protects the brain.

Pathogens can rapidly evolve and adapt, and thereby avoid detection and neutralization by the immune system; however, multiple defense mechanisms have also evolved to recognize and neutralize pathogens. Even simple unicellular organisms such as bacteriapossess a rudimentary immune system in the form of enzymes that protect against bacteriophage infections. Other basic immune mechanisms evolved in ancient eukaryotes and remained in their modern descendants, such as plants and invertebrates. These mechanisms include phagocytosis, antimicrobial peptides called defensins, and the complement system. Jawed vertebrates, including humans, have even more sophisticated defense mechanisms (Beck *et al.,* 1996),including the ability to adapt over time to recognize specific pathogens more efficiently. Adaptive (or acquired) immunity creates immunological memory after an initial response to a specific pathogen, leading to an enhanced response to subsequent encounters with that same pathogen. This process of acquired immunity is the basis of vaccination.

Disorders of the immune system can result in autoimmune diseases, inflammatory diseases and cancer (O'Byrne *et al.,* 2001). Immunodeficiency occurs when the immune system

is less active than normal, resulting in recurring and life-threatening infections. In humans, immunodeficiency can either be the result of a genetic diseasesuch as severe combined immunodeficiency, acquired conditions such as HIV/AIDS, or the use of immunosuppressive medication. In contrast, autoimmunity results from a hyperactive immune system attacking normal tissues as if they were foreign organisms. Common autoimmune diseases include Hashimoto's thyroiditis, rheumatoid arthritis, diabetes mellitus type 1, and systemic lupus erythematosus. Immunology covers the study of all aspects of the immune system.

2.12.1 Components of the Immune System

The immune system protects organisms from infection with layered defenses of increasing specificity. In simple terms, physical barriers prevent pathogens such as bacteria and viruses from entering the organism. If a pathogen breaches these barriers, the innate immune system provides an immediate, but non-specific response. Innate immune systems are found in all plants and animals (Litman *etal,* 2005). If pathogens successfully evade the innate response, vertebrates possess a second layer of protection, the adaptive immune system, which is activated by the innate response. Here, the immune system adapts its response during an infection to improve its recognition of the pathogen. This improved response is then retained after the pathogen has been eliminated, in the form of an immunological memory, and allows the adaptive immune system to mount faster and stronger attacks each time this pathogen is encountered (Restifo and Gattinoni, 2013; Kurosaki *et al.,* 2015).

Both innate and adaptive immunity depend on the ability of the immune system to distinguish between self and non-self-molecules. In immunology, self-molecules are those components of an

organism's body that can be distinguished from foreign substances by the immune system (Smith, 1997). Conversely, non-self-molecules are those recognized as foreign molecules. One class of non-self-molecules are called antigens (short for antibody generators) and are defined as substances that bind to immune receptors and elicit an immune response (Alberts *et al.*, 2002).

2.12.2 Innate Immune System

Microorganisms or toxins that successfully enter an organism encounter the cells and mechanisms of the innate immune system. The innate response is usually triggered when microbes are identified by pattern recognition receptors, which recognize components that are conserved among broad groups of microorganisms (Medzhitov, 2007), or when damaged, injured or stressed cells send out alarm signals, many of which (but not all) are recognized by the same receptors as those that recognize pathogens (Matzinger, 2002). Innate immune defenses are non-specific, meaning these systems respond to pathogens in a generic way (Alberts *et al.*, 2002). This system does not confer long-lasting immunity against a pathogen. The innate immune system is the dominant system of host defense in most organisms (Litman *et al.*, 2005).

2.12.2.1 Cells of the Innate Immune System

There are many types of white blood cells or *leukocytes* that work to defend and protect the human body. In order to patrol the entire body, leukocytes travel by way of the circulatory system.

Leukocytes (white blood cells) act like independent, single-celled organisms and are the second arm of the innate immune system (Alberts *et al.*, 2002). The innate leukocytes include the phagocytes (macrophages, neutrophils, and dendritic cells), innate lymphoid cells, mast

cells, eosinophils, basophils, and natural killer cells. These cells identify and eliminate pathogens, either by attacking larger pathogens through contact or by engulfing and then killing microorganisms (Janeway, 2005). Innate cells are also important mediators in lymphoid organ development and the activation of the adaptive immune system(Withers, 2016).

2.12.3. The Adaptive Immune System

The adaptive immune system evolved in early vertebrates and allows for a stronger immune response as well as immunological memory, where each pathogen is "remembered" by a signature antigen (Pancer and Cooper, 2006). The adaptive immune response is antigen-specific and requires the recognition of specific "non-self" antigens during a process called antigen presentation. Antigen specificity allows for the generation of responses that are tailored to specific pathogens or pathogen-infected cells. The ability to mount these tailored responses is maintained in the body by "memory cells". Should a pathogen infect the body more than once, these specific memory cells are used to quickly eliminate it.

2.12.3.1 Cells of the Adaptive Immune System

Unlike the innate immune system, the adaptive immune system relies on fewer types of cells to carry out its tasks: *B cells* and *T cells*.

Both B cells and T cells are lymphocytes that are derived from specific types of stem cells, called multipotent hematopoietic stem cells, in the bone marrow (Janeway, 2005). After they are made in the bone marrow, they need to mature and become activated. Each type of cell follows different paths to their final, mature forms. B cells are involved in the humoral immune response, whereas T cells are involved in cell-mediated immune response.

Both B cells and T cells carry receptor molecules that recognize specific targets. T cells recognize a "non-self" target, such as a pathogen, only after antigens (small fragments of the pathogen) have been processed and presented in combination with a "self" receptor called a major histocompatibility complex (MHC) molecule. There are two major subtypes of T cells: the killer T cell and the helper T cell. In addition there are regulatory T cells which have a role in modulating immune response. Killer T cells only recognize antigens coupled to Class I MHC molecules, while helper T cells and regulatory T cells only recognize antigens coupled to Class II MHC molecules. These two mechanisms of antigen presentation reflect the different roles of the two types of T cell. A third, minor subtype is the γδ T cells that recognize intact antigens that are not bound to MHC receptors (Holtmeier and Kabelitz, 2005).

In contrast, the B cell antigen-specific receptor is an antibody molecule on the B cell surface, and recognizes whole pathogens without any need for antigen processing. Each lineage of B cell expresses a different antibody, so the complete set of B cell antigen receptors represent all the antibodies that the body can manufacture (Janeway, 2005).

2.12.4 Humoral and Cell Mediated Immunity

Immunity refers to the ability of the immune system to defend against infection and disease. There are two types of immunity that the adaptive immune system provides, and they are dependent on the functions of B and T cells, as described above.

Humoral immunity is the aspect of immunity that is mediated by macromolecules found in extracellular fluids such as secreted antibodies, complement proteins, and certain antimicrobial peptides. Humoral immunity is so named because it involves substances found in the humors,

or body fluids. It contrasts with cell-mediated immunity. Its aspects involving antibodies are often called antibody-mediated immunity.

Humoral immunity is immunity from serum antibodies produced by plasma cells. More specifically, someone who has never been exposed to a specific disease can gain humoral immunity through administration of antibodies from someone who has been exposed, and survived the same disease. “Humoral” refers to the bodily fluids where these free-floating serum antibodies bind to antigens and assist with elimination.

Humoral immunity refers to antibody production and the accessory processes that accompany it, including Th2 activation and cytokine production, germinal center formation and isotype switching, affinity maturation and memory cell generation. It also refers to the effector functions of antibodies, which include pathogen and toxin neutralization, classical complement activation, and opsonin promotion of phagocytosis and pathogen elimination (Janeway *et al.*, 2001).

Cell-mediated immunity is an immune response that does not involve antibodies, but rather involves the activation of phagocytes, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen.

Cell-mediated immunity can be acquired through T cells from someone who is immune to the target disease or infection. “Cell-mediated” refers to the fact that the response is carried out by cytotoxic cells. Cell-mediated immunity is directed primarily at microbes that survive in phagocytes and microbes that infect non-phagocytic cells. It is most effective in removing virus-infected cells, but also participates in defending against fungi, protozoans, cancers, and intracellular bacteria. It also plays a major role in transplant rejection.

2.12.5 A summaryof Innate Immunity and Adaptive Immunity

The following chart compares and summarizes all of the important parts of each immune system:

Attribute	**Innate Immunity**	**Adaptive Immunity**
Response Time	Fast: minutes or hours	Slow: days
Specificity	Only specific for molecules and molecular patterns associated with general pathogens or foreign particles	Highly specific! Can discriminate between pathogen vs. non-pathogen structures, and miniscule differences in molecular structures
Major Cell Types	Macrophages, Neutrophils, Natural Killer Cells, Dendritic Cells, Basophils, Eosinophils	T cells, B cells, and other antigen presenting cells
Key Components	Antimicrobial peptides and proteins, such as toxic granules	Antibodies
Self vs. Nonself Discrimination	Innate immunity is based on self vs. nonself discrimination, so it has to be perfect	Not as good as the innate immune system, but still pretty good at determining which is which. Problems in self vs. nonself discrimination result in autoimmune diseases
Immunological Memory	None	Memory used can lead to faster response to recurrent or subsequent infections

Diversity and Customization	Limited: Receptors used are standard and only recognize antigen patterns. No new receptors are made to adapt the immune response	Highly diverse: can be customized by genetic recombination to recognize epitopes and antigenic determinants.

2.12.6 The Cells of the Immune System

a) **T-Cells:** T lymphocytes are usually divided into two major subsets that are functionally and phenotypically (identifiably) different. The T helper subset, also called the CD4+ T cell, is a pertinent coordinator of immune regulation. The main function of the T helper cell is to augment or potentiate immune responses by the secretion of specialized factors that activate other white blood cells to fight off infection. Another important type of T cell is called the T killer/suppressor subset or CD8+ T cell. These cells are important in directly killing certain tumor cells, viral-infected cells and sometimes parasites. The CD8+ T cells are also important in down-regulation of immune responses. Both types of T cells can be found throughout the body. They often depend on the secondary lymphoid organs (the lymph nodes and spleen) as sites where activation occurs, but they are also found in other tissues of the body, most conspicuously the liver, lung, blood, and intestinal and reproductive tracts.

b) **Natural Killer Cells:** Natural killer cells, often referred to as NK cells, are similar to the killer T cell subset (CD8+ T cells). They function as effector cells that directly kill certain tumors such as melanomas, lymphomas and viral-infected cells, most notably herpes and cytomegalovirus-infected cells. NK cells,unlike the CD8+ (killer) T cells, kill their targets without a prior "conference" in the lymphoid organs. However, NK cells that have

been activated by secretions from CD4+ T cells will kill their tumor or viral-infected targets more effectively.

c) **B Cells:** The major function of B lymphocytes is the production of antibodies in response to foreign proteins of bacteria, viruses, and tumor cells. Antibodies are specialized proteins that specifically recognize and bind to one particular protein. Antibody production and binding to a foreign substance or antigen, often is critical as a means of signaling other cells to engulf, kill or remove that substance from the body.

d) **Granulocytes or Polymorphonuclear (PMN) Leukocytes:** Another group of white blood cells is collectively referred to as granulocytes or polymorphonuclear leukocytes (PMNs). Granulocytes are composed of three cell types identified as neutrophils, eosinophils and basophils, based on their staining characteristics with certain dyes. These cells are predominantly important in the removal of bacteria and parasites from the body. They engulf these foreign bodies and degrade them using their powerful enzymes.

e) **Macrophages**: Macrophages are important in the regulation of immune responses. They are often referred to as scavengers or antigen-presenting cells (APC) because they pick up and ingest foreign materials and present these antigens to other cells of the immune system such as T cells and B cells. This is one of the important first steps in the initiation of an immune response. Stimulated macrophages exhibit increased levels of phagocytosis and are also secretory.

f) **Dendritic Cells:** Another cell type, addressed only recently, is the dendritic cell. Dendritic cells, which also originate in the bone marrow, function as antigen presenting cells (APC). In fact, the dendritic cells are more efficient antigen presenting cells than macrophages. These cells are usually found in the structural compartment of the

lymphoid organs such as the thymus, lymph nodes and spleen. However, they are also found in the bloodstream and other tissues of the body. It is believed that they capture antigen or bring it to the lymphoid organs where an immune response is initiated. Unfortunately, one reason we know so little about dendritic cells is that they are extremely hard to isolate, which is often a prerequisite for the study of the functional qualities of specific cell types. Of particular issue here is the recent finding that dendritic cells bind high amount of HIV, and may be a reservoir of virus that is transmitted to CD4+ T cells during an activation event.

2.12.7 Immunomodulation

Immunomodulators are substances that have been shown to modify the immune systems response to a threat upon it.They modulate and potentiate the weapons of the immune system, keeping them in a highly prepared state for any threat it may encounter. With this balancing effect, all subsequent immune responses improve. When the immune system is in this highly prepared state, the invading organisms do not have the time to build up force and strength before the immune system attacks, destroys and/or weakens the invader. Immunomodulation is the process of modifying an immune response in a positive or negative manner by administration of a drug or compound. Many proteins, amino acids, and natural compounds have shown a significant ability to regulate immune responses, including interferon-γ (IFN-γ) (Bach, 1996;Szekeres *et al*, 2001; Hill and Sarvetnick, 2002), steroids (Roberts *et al*, 1996; Abo-Zena and Horwitz, 2002), dimethylglycine (DMG) (Weiss RC., 1992). These are biological or synthetic substances, which can stimulate, suppress or modulate any of the immune systems

including both adaptive and innate arms of the immune response. Clinically, immunomodulators can be classified into the following three categories (Arya and Gupta, 2011).

a) **Immunoadjuvants**: These agents are used for enhancing vaccines efficacy and therefore, could be considered specific immune stimulants. Example in this regard is of Freund's adjuvant. The immunoadjuvants hold the promise of being the true modulators of immune response. It has proposed toexploit them for selecting between cellular and humoral, Th1 (helper T1 cells) and Th2, (helper T2 cells) immunoprotective and immunodestructive, and reagenic (IgE) versus immunoglobin G (IgG) type of immune responses, which poses to be a real challenge to vaccine designers (Agarwal and Singh, 1999).

b) **Immunostimulants:** These agents are inherently non- specific in nature as they envisaged enhancing body resistance against infection. They can act through innate immune response and through adaptive immune response (Billiauand Matthys, 2001). In healthy individuals the immunostimulants are expected to serve as prophylactic and promoter agents i.e. as immunopotentiators by enhancing basic level of immune response, and in the individual with impairment of immune response as immunotherapeutic agents (Susan and Sally,2009).

c) **Immunosuppressants:** These are a structurally and functionally heterogeneous group of drugs, which are often concomitantly administered in combination regimens to treat various types of organ transplant rejection and autoimmune diseases(Susan and Sally, 2009).

2.12.8 Methods for Testing Immunological Factors

The routine process for screening is to extract single ingredient or single distilled fraction from herbal drugs, determine its bioactivity by the classic pharmacological means. The whole animal model is the most classic pharmacological screening model, which is very important at the aspect of medicine evaluation because it can apparently respond to the efficacy, side effect and toxicity of medicines in whole. Although this method is high cost and low efficient, at present it is still a primary way to drug discovery and evaluation. Several *in vitro*, *in vivo* methods of pharmacological screening of medicinal plants having immunomodulatory activity have been listed**(**Vogel, 2002).

***In vitro* methods:**

1. Inhibition of histamine release from mast cells
2. Mitogen induced lymphocyte proliferation
3. Inhibition of T cell proliferation
4. Chemiluminescence in macrophages
5. PFC (plaque forming colony) test *in vitro*
6. Inhibition of dihydro-orotate dehydrogenase

***In vivo* methods:**

1. Spontaneous autoimmune diseases in animals
2. Acute systemic anaphylaxis in rats
3. Anti-anaphylactic activity (Schultz-Dale reaction)
4. Passive cutaneous anaphylaxis
5. Arthus type immediate hypersensitivity

6. Delayed type hypersensitivity

7. Reversed passive arthus reaction

8. Adjuvant arthritis in rats

9. Collagen type II induced arthritis in rats

10. Proteoglycan-induced progressive polyarthritis in mice

11. Experimental autoimmune thyroiditis

12. Coxsackievirus B3-induced myocarditis

13. Porcine cardiac myosin-induced autoimmune myocarditis in rats

14. Experimental allergic encephalomyelitis

15. Acute graft versus host disease (GVHD) in rats

16. Influence on SLE-like disorder in MRL/lpr mice

17. Prevention of experimentally induced myasthenia gravis in rats

18. Glomerulonephritis induced by antibasement membrane antibody in rats

19. Auto-immune uveitis in rats

20. Inhibition of allogenic transplant rejection.

2.12.9 Immunosuppressant Drugs

These drugs have major role in organ transplantation and auto immune diseases. The drugs are:

1. Calcineurin inhibitors (Specific T-cell inhibitors)

Cyclosporine (Ciclosporin): It is a lipophilic cyclic polypeptide composed of 11 amino acids which are L-valine, L-leucine, L-alanine, glycine, 2-aminobutyric acid, 4-methylthreonine, D-alanine, butenyl-methyl-threonine, L-alpha-aminobutryic acid, sarcosine andbutenyl-methyl-L-threonine(Dittmann*et al.*, 1994). The drug is extracted from a soil fungus. It profoundly and

selectively inhibits T lymphocyte proliferation, IL2 and other cytokine production and response to inducer T-cells. Lymphocytes are arrested in G0 to G1 phase. KDT 837 Cyclosporine is used to prevent rejection of kidney, liver and cardiac allogeneic transplanted**(**Spelman *et al*, 2006).Figure 2.3 is the structure of Cyclosporine.

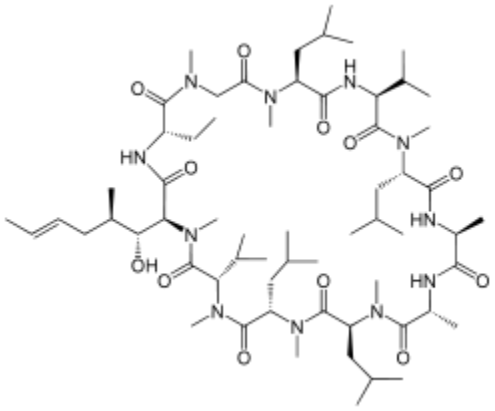


Figure 2.3: Structure of Cyclosporine

Tacrolimus (TAC): It is a newer immunosuppressant, macrolide. TAC exerts its immunosuppressive effect in the same manner as CSA except that it binds to a different immunophillin, FKBP-12 (fk-binding protein). Tacrolimus (figure 2.4) is used in prevention of rejection of liver and kidney and is given with a corticosteroids and antimetabolites**(**Spelman *et al*, 2006).

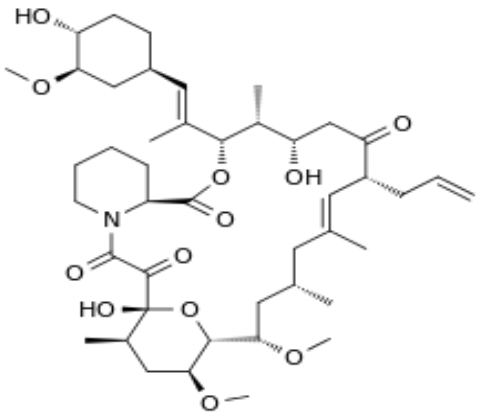


Figure 2.4: Structure of Tacrolimus

2. Antiproliferative drugs (Cytotoxic drugs)

Azathioprine: It is a purine antimetabolite.its selective uptake into immune cells and intracellular conversion to the active metabolite 6-mercaptopurine, which then undergoes further transformation to inhibit de novo purine synthesis and damage to DNA. It is approved for prevention of renal and other graft rejection. Figure 2.5 is the structure ofAzathioprine.

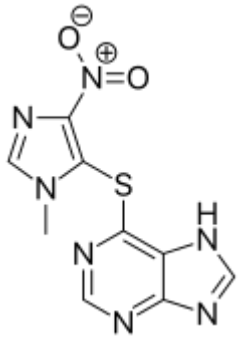


Figure 2.5: Structure of Azathioprine

Cyclophosphamide: This drug has more effect on B cells and humoral immunitycompared to that on t cells and cell- mediated immunity. It is used in bone marrow transplant.

Other drugs of this category are Methotrexate, Chlorambucil,Mycophenolate mofetil (MMF). Figure 2.6 is the structure of Cyclophosphamide.

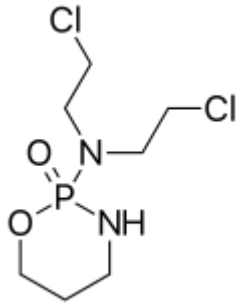


Figure 2.6: Structure of Cyclophosphamide

3. Glucocorticoids

Prednisolone: The steroids are used to suppress acute rejection of solid allograft and in chronic graft versus host disease. The steroids are able to rapidly reduce lymphocyte populations by lysis or redistribution. On entering cells, they bind to the glucocorticoid receptor and the complex passes into the nucleus and regulates the translation of DNA. Figure 2.7 is the structure of Prednisolone.

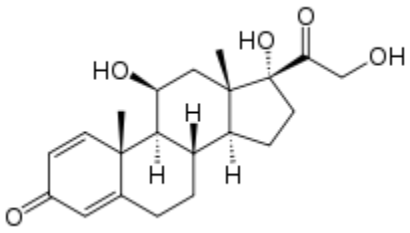


Figure 2.7: Structure of Prednisolone

4. Antibodies

Muromonab CD3 is a murine monoclonal antibody against the CD3 glycoprotein located near to the T cell receptor on helper T cells. It is used for treatment of acute rejection of renal allografts as well as cardiac and hepatic transplantation.

Antithymocyte globin (ATG): It is a polyclonal antibody purified from horse or rats immunized with human thymice lymphocytes. It binds to T-lympocytes and depletes them. It is a potent immunosuppressant used to suppress acute allograft reject episodes.

Other drugs in this category are Rho (D) immuneglobin, Efalizumab

2.12.10 Immunostimulant Drugs

They stimulate the immune system to fight against immunodeficiency (like AIDS), infections and cancers.

1. Levamisole: An anthelmintic drug that also restores functions of B lymphocytes, T lymphocytes, monocytes and macrophages. Hence it has been used in colon cancer along with 5-FU. Figure 2.8 is the structure of Levamisole.

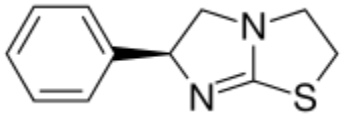


Figure 2.8: Structure of Levamisole

2. Thalidomide: Different effects of this old drug have been utilized in conditions such as:

- Erythema nodosum leprosum: Anti-inflammatory effect
- Multiple myeloma: Anti-angiogenesis
- Rheumatoid arthritis: Anti TNF effect.
- BCG**:** Used in carcinoma bladder.
- Recombinant cytokines
- Interferons: In tumors and chronic hepatitis B and C
- Interleukin 2 (aldeslukin): has been used in renal cell carcinoma and melanoma

Figure 2.9 is the structure ofThalidomide.

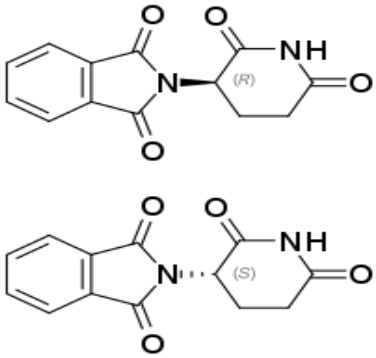


Figure 2.9: Structure of (R) and (S) Thalidomide

2.12.11 Autoimmunity

Overactive immune responses comprise the other end of immune dysfunction, particularly the autoimmune disorders. Here, the immune system fails to properly distinguish between self and non-self, and attacks part of the body. Under normal circumstances, many T cells and antibodies react with “self” peptides (Miller, 1993). One of the functions of specialized cells (located in the thymus and bone marrow) is to present young lymphocytes with self-antigens produced throughout the body and to eliminate those cells that recognize self-antigens, preventing autoimmunity (Sproul *et al*, 2000).

2.12.12 Hypersensitivity

Hypersensitivity is an immune response that damages the body's own tissues. They are divided into four classes (Type I – IV) based on the mechanisms involved and the time course of the hypersensitive reaction. Type I hypersensitivity is an immediate or anaphylactic reaction, often associated with allergy. Symptoms can range from mild discomfort to death. Type I hypersensitivity is mediated by IgE, which triggers degranulation of mast cells and basophils when cross-linked by antigen. Type II hypersensitivity occurs when antibodies bind to antigens on the patient's own cells, marking them for destruction. This is also called antibody-dependent (or cytotoxic) hypersensitivity, and is mediated by IgG and IgM antibodies.Immune complexes (aggregations of antigens, complement proteins, and IgG and IgM antibodies) deposited in various tissues trigger Type III hypersensitivity reactions (Ghaffar, 2006). Type IV hypersensitivity (also known as cell-mediated or delayed type hypersensitivity) usually takes between two and three days to develop. Type IV reactions are involved in many autoimmune and

infectious diseases, but may also involve contact dermatitis(poison ivy). These reactions are mediated by T cells, monocytes, and macrophages (Ghaffar, 2006).

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Plant Collection

The fresh leaves of *M. lucida* were collected from Nsukka in the month of February, 2015. They were authenticated by a plant taxonomist, Mr Alfred O. Ozioko, of the Bioresources Development and Conservation Programme (BDCP) Centre, Nsukka, Nigeria.

3.1.2 Chemicals and Reagents

Analytical grade of methanol, n-hexane, ethyl acetate, dichloromethane, butanol (Sigma Aldrich) were used. Other chemicals used were silica gel. Aluminum pre-coated (silica gel 60 $F_{254,}$ layer thickness 0.2mm) (Merck) plates used for thin layer chromatography (TLC) were obtained from Germany, silica gel 60, 200−400 μm mesh size (Merck) and Sephadex LH-20, 25−100 μm mesh size (Merck). Deuterated methanol and Dimethyl sulphoxide (Uvasol, Merck) were used for NMR measurements, Methanol LiChroSolv HPLC (Merck), nano-pure water (distilled and heavy metals free water) obtained by passing distilled water through nano- and ion exchange filter cells (Barnstead, France) was used for HPLC measurements. All laboratory reagents were freshly prepared and freshly distilled water was used when required.

3.1.3 Equipment

These include Freezer (Haier Thermocool, China); Gentalab Hot-Air Oven (Model: MINO/50); Single channel micropipette (ELGA); Glass ware; Rotary evaporator; RE300 (Stuart,

Barloworld Scientific Ltd, UK); Globe shaped separatory funnel, Mechanical grinder, Weighing balance (Ohaus, China) and a Retort stand; Whatman No.1 filter paper (Whatman International Ltd. England); chromatographic column, test tube racks, syringes and needle, glass rod.

Analytical HPLC Machine; Pump: P580A LPG (Dionex), Autosampler: ASI-100T (injection volume =20 µl) (Dionex), Detector: UVD 340S (photoiode array detector) (Dionex), Column oven; STH 585 (Dionex), Column Eurospher 100-CI8, (5 µm; 125 mm× 4 mm) (Knauer), Pre-column: vertex column, Eurospher 100-5 CI8 (5-4 MM) (Knauer), Software: Chromeleon (v. 6.30),

For Semi preparative HPLC; Pump: L-7100 and Chromaster 5110, Merck/Hitachi, Detector: UV-L7400 and Chromaster 5410 (photodiode array detector), Merck/Hitachi, Column: Eurospher 100-C18, [10μm; 300 mm × 8 mm] (Knauer), Pre-column: Eurospher 100- C18 [10µm; 30 x 8 mm] (Knauer), Printer: Chromato-Integrator D-2000, Merck/Hitachi

For NMR; ^{1}H spectra were recorded at 300^{o} K on Bruker DPX 300, ARX 400, 500 or AVANCE DMX 600 MHz NMR spectrometers. All 1D and 2D spectra were obtained using the standard Bruker software.

3.1.4 Experimental Animals

Albino mice (Swiss) of either sex (20 – 30 g) were used. The animals were fed with standard pellet diet and water freely. They were maintained under standard environment condition employed. They were housed under standard conditions such as temperature 22 ± 5 ^{o}C, 12 h of light/dark cycle.

3.2 METHODS

3.2.1 Preparation of Plant material

The leaf of *M. lucida* were air-dried at room temperature (25 – 27^{o}C), protected from sunlight. They were pulverized into powdered form using a mechanical grinder and were used for extraction and analysis.

3.2.2 Extraction

The leaf powder(1 kg) was extracted for 24h by cold maceration in 5.0 L methanol with intermittent shaking. The maceration process was then repeated two more times (a total of 72 h and 15 L of methanol) for maximal extraction. The methanol extract was then collected and concentrated almost to dryness (MeOH fraction) under vacuum at 40^{o}C using rotary evaporator. It was dried in Gentalab Hot-Air Oven at 40^{o}C for 7 days. The dried concentrated methanol leafextract collected was 100 g.

3.2.3 Liquid-Liquid fractionation of the Crude Extract

The crude methanol extract of *M. lucida* wasfractionated using liquid – liquid fractionation with n-hexane, ethyl acetate, and butanol (in increasing order of polarity). The crude extract of 50 g was suspended in 200ml of distilled water in a 1 L separating funnel and partitioned with 500ml n-hexane. The n – hexane was collected and the method repeatedagain (500 L x 2) until all the hexane components was collected. The aqueous fraction was further extracted as was done for hexane with ethyl acetate and butanol in succession. A rotary evaporator, RE300 was used to concentrate the fractions at 45^{o}C± 5^{o}C. The fractions were dried in Gentalab Hot-Air Oven at

40ºC for 7 days. The fractions obtained were stored at 4ºC in a refrigerator until required for analysis.

3.2.3.1 Vacuum Liquid Chromatographic Separation of n-Hexane, Ethyl acetate and Butanol Fractions Using Silica Gel.

Admixture: The n-hexane fraction of *M. lucida* (6970 mg) was dissolved in n-hexane and silica gel (200-400, mesh) was added to the mixture to make slurry to obtain uniform mixing.

The ethyl acetate fraction of *M. lucida* (5000 mg) was dissolved in ethyl acetate and silica gel (200-400, mesh) was added to the mixture to make slurry to obtain uniform mixing.

The butanol fraction of *M. lucida* (5000 mg) was dissolved in butanol and silica gel (200-400 mesh) was added to make slurry to obtain uniform mixing.

Column Packing: The glass column (diameter 6 cm×50 cm height) was packed with dry silica gel (200-400) to the bed height of 15cm.To the top of the column, admixture was loaded and column was eluted gradually with increasing order of polarity of solvents from non- polar to highly polar solvents. Using 500ml of the solvents (hexane: ethylacetate; dichloromethane: methanol), the ethylacetate fraction of the leaf was separated using vacuum liquid chromatographic method. The gradient system used for hexane: ethylacetate are 100:0, 90:10 (500 ml x 2), 80:20 (500 ml x 2), 70:30 (500 ml x 3),60:40 (500 ml x 2), 50:50, 40:60, 20:80 (500 ml x 3), 0:100while for dichloromethane: methanol, 100:0, 90:10 (500 ml x 2), 70:30, 50:50, 30:70, 10:90, 0:100 Each system was collected separately in a flask (though improvised with Buchner flask) (Appendix A). A total of 22VLC fractions (EC1 – EC22) of ethylacetate fraction were obtained.

3.2.3.2 Sephadex LH-20 Separations of Ethyl Acetate and Butanol Fraction

- **Preparation of Sephadex Gel**

Sephadex LH-20 of 100 g was dispersed in 200 mL of methanol and the mixture sonicated for 30 min. The slurry was transferred into a column (50 × 2 cm, length× internal diameter) and the gel is allowed to stabilize for about 5 h before use (Appendix B).

- **Preparation of Sample**

About 303.7 mg and 222 mg, of sub fractions of ethyl acetate fraction EC7 and EC13 respectively, were reconstituted in 10 ml of methanol and the solution sonicated for 5 min and then centrifuged × 10000 for 10 min to remove undissolved solid particles. The supernatant was pipetted and used for the separation studies.

- **Gel Chromatography of the Supernatant**

The supernatant was introduced into the Sephadex LH-20 column adjusted to the flow rate of approximately 1.1 mL/min. About 47 and 40 eluate of 10 mL aliquot each of EC7 and EC13 respectively were collected with the aid of a test tube and monitored with TLC on silica gel G_{254} developed with hexane: ethylacetate, 6:1, 2:8 respectively. Eluates with the same R_f values were combined and concentrated with rotary evaporator to obtain 5 and 4 subfractions of EC7(EC71-EC74) and EC13 (EC131 – EC134) respectively. Subfractions weighing 230 mg and 185 mg of EC73 and EC133respectively were further used for analysis.

3.2.3.3 Thin Layer Chromatography

The eluate in each test tube were applied with the help of a fine bore glass capillary tube on the entire length of aluminium pre-coated silica gel TLC plates as stationary phase of size 6.5 x 10 cm. The solvent system hexane in ethylacetate (6:4 for EC73 and 2:8 for EC133) as the mobile phase wereused for the development of chromatograms of the respective fractions.

- **Visualization of the TLC plates:**

The plates were examined under UV at 254 and 365 nm. The spots were marked with a pencil (Apendix B). The value for each compound as evident from colored spots under UV was calculated as the R_f value (retention factor) for that compound:

R_f value = Distance traveled by the compound / Distance traveled by the solvent front.

3.2.3.4 Analytical High Pressure Liquid Chromatography (HPLC)

The EC73 and EC133 fractions and the isolated compounds were subjected to analytical HPLC. Each of the dried fractions was reconstituted with 2 mL of HPLC grade methanol. 100 µL of the dissolved samples were each transferred into HPLC vials containing 500 µL of HPLC grade methanol. HPLC analysis was carried on the samples with a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany). Detection was at 235, 254, 280 and 340 nm. The separation column (125 cm × 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent.

3.2.3.5 Semi-preparative HPLC

Semi-preparative HPLC was used for the purification of the isolated compounds from complex and nearly pure fractions. Each injection was concentrated to 3 mg/ml and the maximum injection volume was 1 ml. The flow rate was set to 5 ml/min.

3.2.4 Spectroscopic Methods for Structural Elucidation.

3.2.4.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

The ^{1}H NMR spectra were recorded at 300^{o}K on Avans 600 NMR spectrometers (Bruker). The 1Dspectrumwas obtained using the standard Bruker software. The sample was dissolved in a deuterated dimethyl sulphooxide (DMSO-d6), the choice of which is dependent on the solubility of the sample. TMS was used as internal standard reference signal. The observed chemical shifts (δ) were recorded in ppm and the coupling constants (*J*) were recorded in Hz.

All NMR spectra were measured at the Institut für Anorganische Chemie und Strukturchemie, Heinrich-Heine Universität, Düsseldorf with a Bruker ARX-300, and 600, by Dr. Peter Tommes and co-workers.

3.2.5 Assessment of Immunomodulatory Activity of the Extracts of *M.lucida*

The ability of the crude extracts and the fractionsto affect a number of immune functions was assessed in normal white (albino) mice. This included the ability of the extracts to influence total WBCs and differential WBCs. The ability of extracts to reverse leucopenia induced by cyclophosphamide treatment in albino mice was also investigated.

3.2.5.1 Suppression of the Immune System of the Experimental Animals (Induction of Leucopenia)

- Leucopenia was induced by treating the mice (30 mg/kg body weight) intra-peritoneally (Thatte *et al*., 1987) for three days with Cyclophosphamide.

a). Curative Group

Animals were divided into seven groups of five animals per group. All the groups were immunosuppressed using Cyclophosphamide (30 mg/kg,i.p.) administered in single doses for three days. The crude extract was dissolved in distilled water and doses administered were 50, 100 and 200 mg/kg body weight. The crude extract was administered to animals for 14 consecutive days. The groups are as follows;

Group I (Normal control group) received 10ml/kg distil water.

Group II (Positive control group) received standard drug (immunostimulant) 100mg/kg of Levamisole.

Group III, IV and V were given dose of 50mg/kg, 100mg/kg and 200 mg/kg crude extract of *M. lucida* respectively, p.o., daily for 14 days.

Group VI and VII were given dose of 200mg/kg each of ethyl acetate and butanol fractions respectively.

Blood samples were collected from retro orbital plexus on the 14th day of the experiment. Determination of total and differential white blood cells was carried out**.**

b). Bleeding of the Mice and Blood Sample Collection

Blood samples used in this study were collected by retro orbital puncture. Blood samples were collected in clean EDTA bottles in the following order for the curative experiment:-

i. Before suppression (basal blood sample i.e. before treatment),

ii. After suppression with cyclophosphamide for 3 days

iii. After fourteen (14) days treatment with crude extracts, ethylacetate and butanol fractions of the *M. lucida*.

c). Evaluation of the Curative Effect of the Crude Extracts, Ethylacetate and Butanol Fractions of *M. lucida* on the Total White Blood Cell Counts in Cyclophosphamide Treated Mice

Total white blood cell (TWBC) counts of the blood of the mice were determined by the method described by Cheesbrough (2006) using Neubauer haemocytometer.The diluting fluid (Turk's solution)**,** 0.38 ml (380 µl) was measured into a small container or tube and 0.02 ml (20 µl) of well-mixed EDTA anticoagulated blood sample added. The counting chamber was assembled making sure that the central grid areas of the chamber and the special haemocytometer cover glass were completely clean and dry. The chamber surface was moistened on each side of the

grid areas to help the cover glass adhere to the chamber. The cover glass was slide into position over the grid areas and pressed down on each side until rainbow colours (Newton's rings) were seen. The diluted blood sample was then re-mixed and with a capillary (or Pasteur pipette) held at an angle of about 45^{o}C; one of the grids of the chamber was filled with the sample while taking care not to overfill the area. The chamber was left undisturbed for 2 min to allow time for the white cells to settle. The underside of the chamber was dried and placed on a microscopic stage. Using the 10 objective with the condenser iris closed sufficientlyto give good contrast, the rulings of the chamber and white cells were focused until they appear as small black dots. The cells in the four large corner squares of the chamber marked W1, W2, W3, and W4 were counted. Total white blood cell counts were conducted before suppression with cyclophosphamide, after suppression, after fourteen days treatment of animals with the various crude extracts and fractions. The negative control groups were given distilled water while the positive control groups received the standard drug- NONI. The crude extracts were administered orally.

d). Evaluation of the Curative Effect of Crude Extracts, Ethylacetate and Butanol Fractions of *M. lucida* on the Differential White Blood Cell Counts In Cyclophosphamide Treated Mice

Differential white blood cell (DWBC) count was done as described by Cheesbrough (2006). Thin blood smears were made from mice blood before suppression with cyclophosphamide (Basal blood), after suppression, at the end of fourteen days treatment with the crude extracts, ethylacetate and butanol fractions. The thin blood smears were fixed with absolute methanol, stained with Leishman's stain and the stain washed off with tap water and the slides left to dry.

The cells were then observed under the compound microscope using oil immersion objective. White blood cells were identified, counted and recorded. The results were given as mean of the percentage together with the standard error of mean. The absolute number of each white cell type were also calculated by multiplying the number of each cell counted (expressed as a decimal fraction) by the total WBC count.

3.2.5.2 Antigen preparation

Fresh blood was collected from jacular head of a healthy sheep and mixed with sterile Alsever's solution(2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride) (1:1). The blood was washed 3 times with pyrogen-free phosphate buffered saline (pH 7.2) by centrifuging at 1500 rpm for 10 min each. They were then kept under refrigeration for use in the immunization and challenge study.

3.2.5.3 Determination of humoral antibody response to SRBC (hemagglutination antibody titer test)

Mice in test Groups III, IV, V, VI and VII were immunized by injecting 0.2 mL of SRBCs intraperitoneally (i.p.) on the 9th day of the experiment. Administration of *M.lucida* methanol leaf extract continued for another 5 days until day 14 and blood samples were collected by cardiac puncture. Blood was centrifuged at 1500 rpm to get serum. Antibody titers were then determined by the hemagglutination technique as described by Bin-Hafeez et al. (2001). Serial two-fold dilutions of serum were made with phosphate buffered saline (PBS) in micro titer plates of 96-well capacity and SRBC (50 µL of 1% SRBC prepared in normal saline) added to each of these dilutions. The hemagglutination plates were then incubated at 37 °C for 18 h and then

examined for hemagglutination. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the hemagglutination antibody titer (HA units/µL).

% Inhibition = 100- (a/b x 100/1)

Where a = mean rank of control group and

b = mean rank of treated group

3.2.5.4 Determination of Delayed Type Hypersensitivity Response (DTHR)

The delayed type hypersensitivity (DTH) response was determined using the method of Raisuddin *et al.* (1991). Groups III, IV, V, VI and VII mice were primed by subcutaneously injecting 0.1 mL of suspension (10%) containing 1×10^9 SRBC into the right hind footpad. The administration of *M. lucida* methanol leaf extracts was continued until the 14th day. On the 14th day, the animals were challenged by subcutaneously injecting 0.1 mL of 1×10^8 SRBCs into the left hind footpad of the mice. The extent of delayed-type hypersensitivity (DTH) response in the mice was determined by measuring the footpad thickness after 24 h of challenge using vernier calipers. The difference in the thickness of the right hind paw before and after was then used as a measure of DTH reaction and was expressed as a mean percent increment in thickness/edema.

$$\% \text{ Edema} = \frac{\text{Mean final reading-Mean initial reading}}{\text{Mean initial reading}} \times \frac{100}{1}$$

3.2.6 Statistical analysis

Graph Pad prism 3 software and MS Excel were used for statistical analysis of data. All the results were expressed as mean± standard error of mean (S.E.M.), analyzed for one way analysis

of variance (ANOVA) and post hoc Tukey-Kramer multiple comparison tests. Differences between groups were considered significant at $p<0.05$ levels.

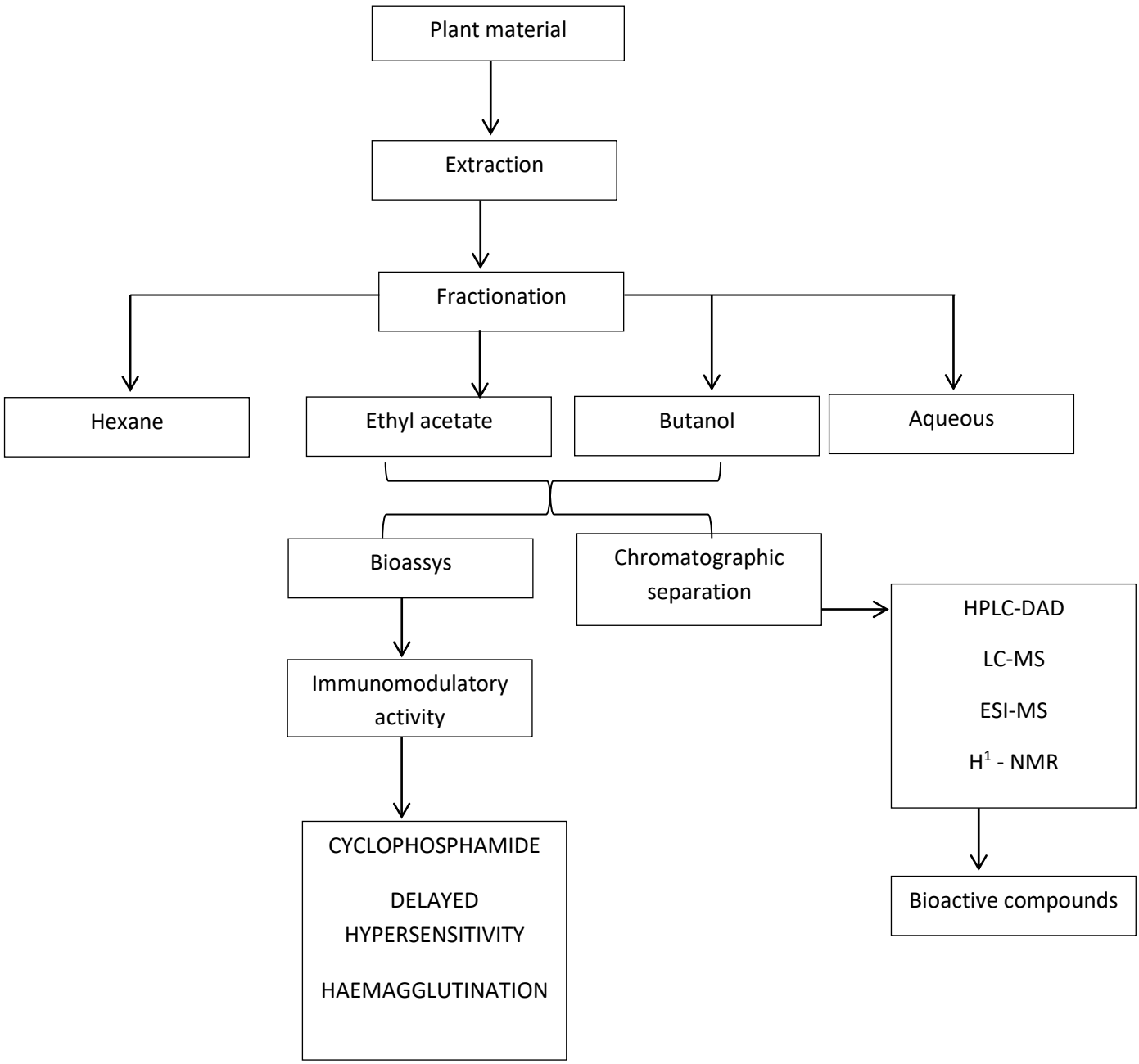


Figure 3.1: Scheme of experimentations employed in this study for the plant

CHAPTER FOUR

RESULT AND DISCUSSION

4.1 RESULTS

4.1.1 Extraction

The yields of the extract and fractions of *M. lucida*leaf are shown in Table 4.1

Table 4.1: Extract, fractions and yield of extraction process of *M. lucida*leaf

Extract / Fractions	**Yield (% w/w)**
MLE[a]	10
HLF[b]	13.94
EALF[b]	17.08
BLF[b]	31.24
WLF[b]	25.25

MLE = Methanol leaf extract

HLF = Hexane leaf fraction

EALF = Ethyl acetate leaf fraction

BLF = Butanol leaf fraction

WLF = Water leaf fraction

4.1.2 Isolated compounds and their structures.

COMPOUND 1 (MOLUCIDIN)

Table 4.2: Structural Information of Compound 1(Molucidin)

Synonym	(3aS,4aR,4a 1 S,7aS,9aS,3E)-methyl 3-(4-hydroxy- 3-methoxybenzylidene)-2-oxo-2,3,3a,4a,4a 1 ,7a-hexahydro-1,4,5-trioxadicyclopenta[a,hi]indene- 7-carboxylate.
Sample Code	ESCP 73-3
Appearance	Colourless or silver crystal
Molecular Formula	C_{21} H_{18} O_8
Formula Weight	398.36
Calculated Exact Mass	398.10017g/mol
Retention Time	25.197 min
Absorption Maximum	343.8nm

Compound 1 (ECSP 73-3) was isolated from ethyl acetate extract of *M. lucida*. It showed a retention time of 25.197min in HPLC analysis and UV absorption maxima at 240.4nmcharacter of a cinnamyl substitutive. The H-NMR showed a singlet at δ 3.92 (3'-OCH_3) and δ 3.76 (14-$COOCH_3$) due to methoxy group; there is a doublet of a triplet at δ 4.04 (H=5). In addition, the presence of a 1, 3, 4-trisubstituted aromatic ring with a typical ABX coupling pattern (δ7.55 [d, *J*=2.0, H-2'], 6.90 [d, *J*= 8.0 Hz, H-5'], and 7.28 [dd, *J*=8.0, 2.0 Hz, H-6']) in the ^{1}HNMRspectrum which represents a cinnamyl substitutive as speculated from UV (Abe *et al*,2002). Analysis of other HNMR signals showed that this moiety has a link to an iridoid nucleus. The NMR data and UV as well correlated with that of molucidin.

As previously reported (Suzuki *et al*,2015) the structure of molucidin has been elucidated by HR-ESI-MS experiment, NMR data and comprehensive analysis of the HMQC, HMBC, H-H COSY, and NOESY spectra permitted complete assignments of its proton and carbon signals. The general name of molucidin is (3aS,4aR,4a 1 S,7aS,9aS,3E)-methyl 3-(4-hydroxy- 3-methoxybenzylidene)-2-oxo-2,3,3a,4a,4a 1 ,7a-hexahydro-1,4,5-trioxadicyclopenta[a,hi]indene-7-carboxylate. It is interesting that molucidin is (-)-oruwacin, which is the enantiomer of oruwacin isolated also from *M. lucida* previously (Umar *et al*, 2013).

ECSP 73-3(MOLUCIDIN)

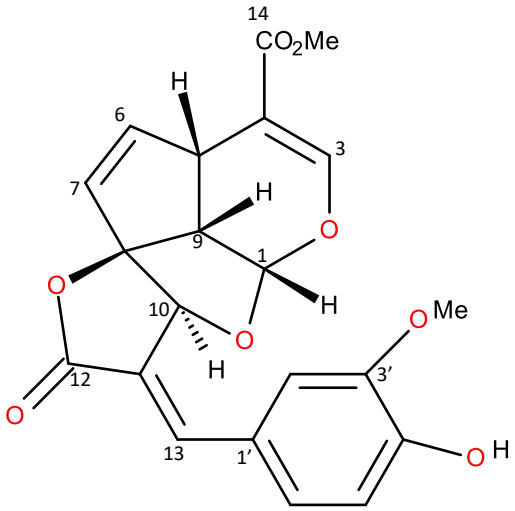


Figure 4.1.The Chemical structure of Molucidin

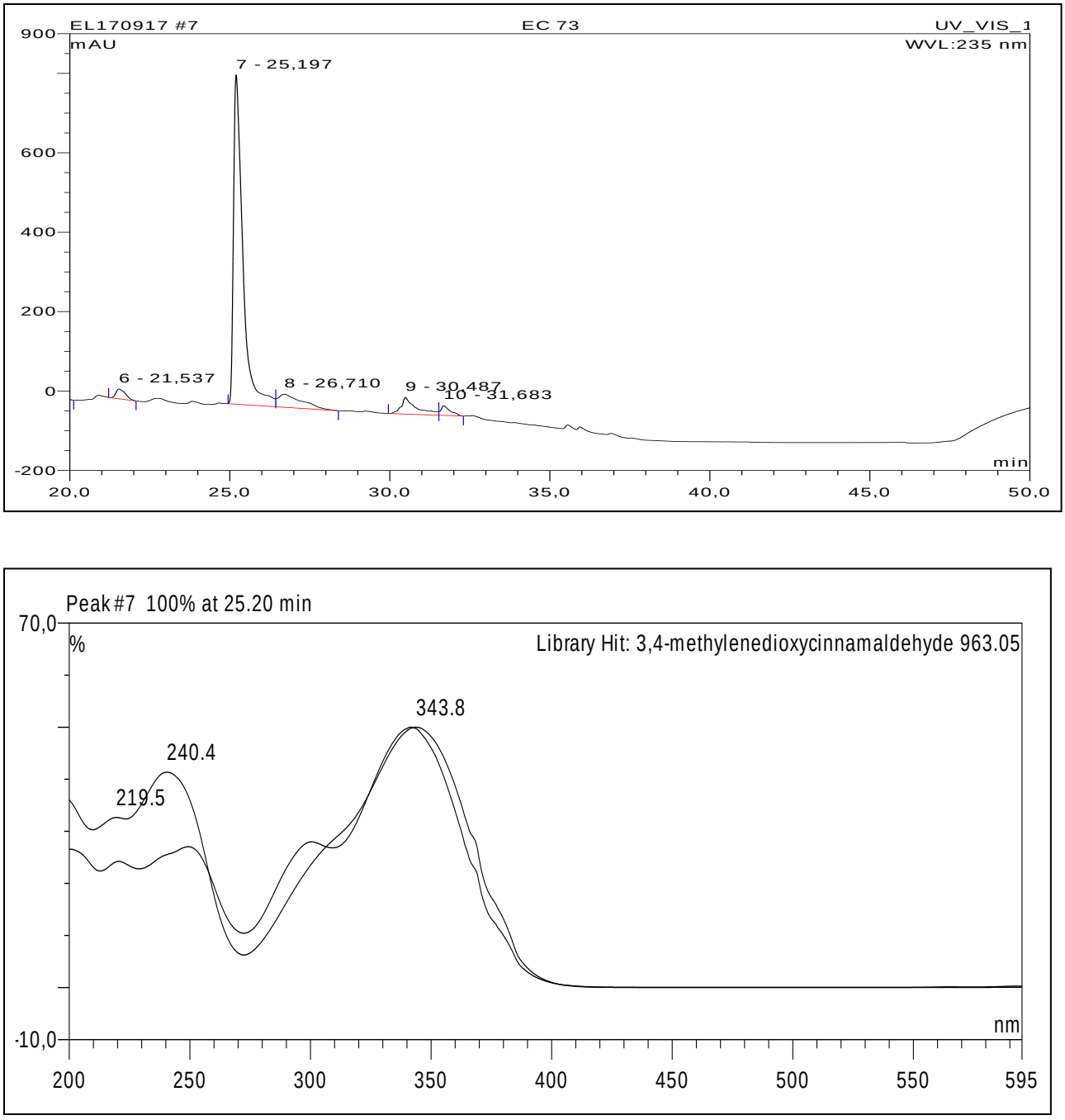


Figure 4.2: HPLC Chromatogram and UV spectrum of Molucidin

1H NMR (300 MHz, Methanol-d_4) δ 7.75 (d, J = 1.3 Hz, 1H), 7.55 (d, J = 2.1 Hz, 1H), 7.51 (s, 1H), 7.29 (dd, J = 8.3, 2.0 Hz, 1H), 6.91 (d, J = 8.3 Hz, 1H), 6.03 (dd, J = 5.5, 2.2 Hz, 1H), 5.75 (d, J = 2.2 Hz, 1H), 5.73 (dd, J = 4.0, 1.3 Hz, 3H), 5.31 (s, 1H), 4.05 (dt, J = 9.5, 2.2 Hz, 1H), 3.94 (s, 3H), 3.78 (s, 3H), 3.62 (dd, J = 10.4, 5.9 Hz, 1H).

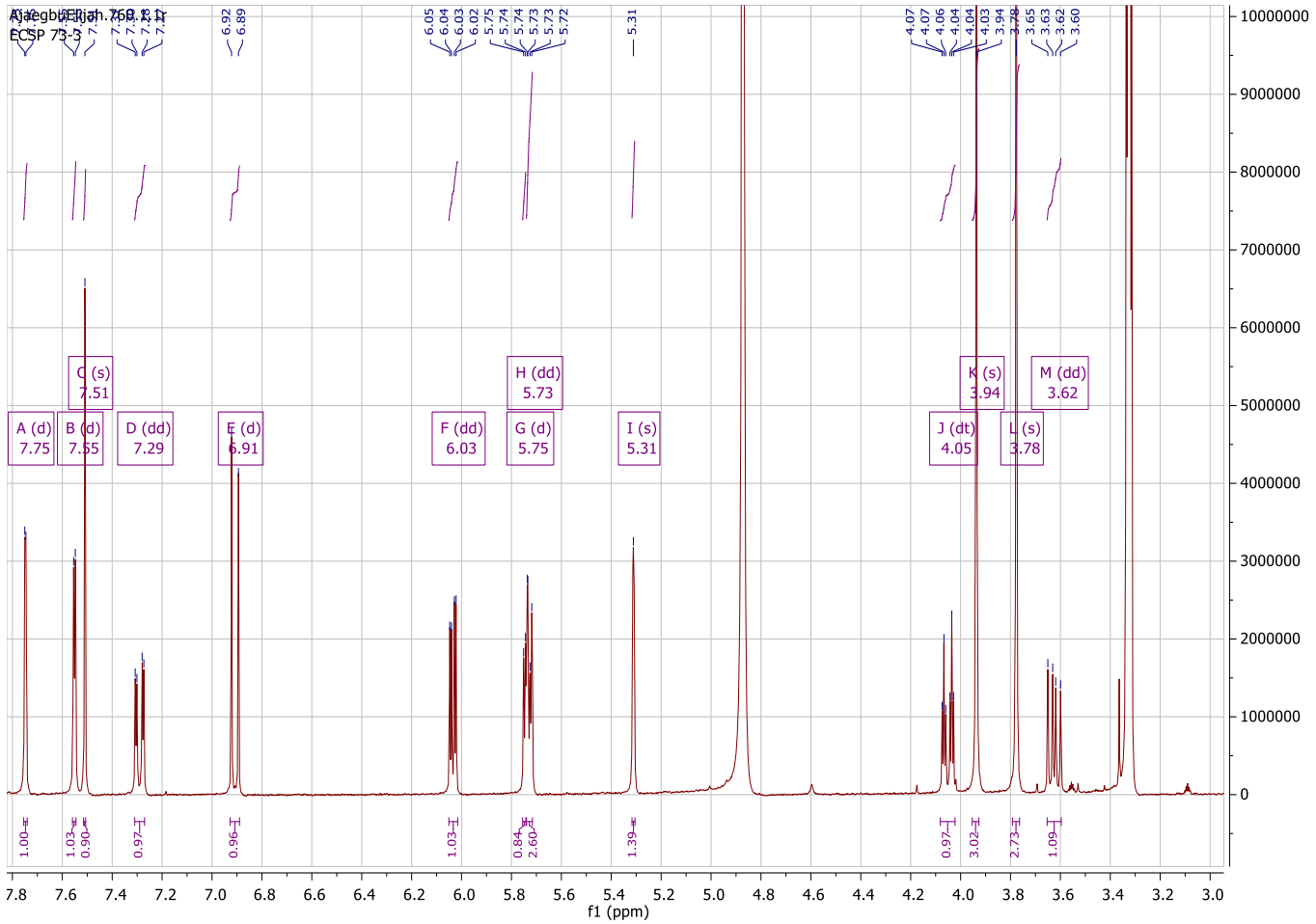


Figure 4.3: ^{1}H-NMR spectrum of Compund 1 (Molucidin)

Table 4.3: Comparison of the ^{1}H NMR data of Compund 1(Molucidin)

	Compound 1 (300 MHz, Methanol-d_4)			Reference Compound(Molucidin) (400 MHz,$CDCl_3$) (kwofie et al, 2016)		
Position	δ H	mult	J (Hz)	δ H	mult	J (Hz)
1	5.75 d	d, 1H		5.64 d	d, 1H	6.0
2’	7.55 d	d, 1H	2.1	7.43 d	d, 1H	2.0
3	7.51 s	s, 1H		7.46 s	s, 1H	
3’ –OCH_3	3.94 s	s, 3H		3.96 s	s, 3H	
5	4.05 dt	dt, 1H	9.5, 2.2	4.05 dt	dt, 1H	10.0, 2.0
5’	6.91 d	d, 1H	8.3	6.99 d	d, 1H	8.0
6	6.03 dd	dd, 1H	5.5, 2.1	6.03 dd	dd, 1H	6.4, 2.0
6’	7.29 dd	dd, 1H	8.3, 2.0	7.26 dd	dd, 1H	8.0, 2.0
7	5.73 dd	dd, 1H		5.63 dd	dd, 1H	6.4, 2.4
9	3.62 dd	dd, 1H	9.5, 5.8	3.58 dd	dd, 1H	10.0, 6.0
10	5.31 s	s, 1H		5.22 s	s, 1H	
13	7.75 s	s, 1H	1.3	7.78 s	s, 1H	
14-$COOCH_3$	3.78 s	s, 3H		3.78 s	s, 3H	

COMPOUND 2 (DESMETHYL MOLUCIDINE)

Table 4.4: Structural Information of Compound 2 (Desmethyl Molucidin)

Synonym	(3aS,4aR,4a 1 S,7aS,9aS,3E)-methyl 3-(4-hydroxy- 3-methoxybenzylidene)-2-oxo-2,3,3a,4a,4a 1 ,7a-hexahydro-1,4,5-trioxadicyclopenta[a,hi]indene- 7-carboxylic acid
Sample Code	ESCP 133-2
Appearance	Brown crystal
Molecular Formula	C_{20} H_{17} O_8
Formula Weight	385
Calculated Exact Mass	385.0923g/mol
Retention Time	23.187 min
Absorption Maximum	370. 3 nm

Compound 2 (ECSP 133-2) was isolated from ethyl acetate extract of *Morinda lucida*. It showed a retention time of 23.187min in HPLC analysis and UV absorption maxima at 222.4 nm.

The HNMR spectrum of 2 is similar to that of 1. There is the presence of a 1, 3, 4-trisubstituted aromatic ring with a typical ABX coupling pattern (δ7.55 [d, *J*=2.0, H-2'], 6.90 [d, *J*= 8.0 Hz, H-5'], and 7.28 [dd, *J*=8.0, 2.0 Hz, H-6']) in the 1HNMRspectrum which links to the iridoid nucleus (Abe *et al*,2002). The major difference was the absence of the second methoxy signal at 14. The observation suggested that compound 2 is a denoting derivative of compound 1. Compound 2 was then elucidated as ECSP 133-3. Note that the lower retention time of compound 2 (23.187 min) as compared to compound 1 (25.197 min) further suggested that 2 is more polar and has area of carboxylic acid peculiarity as opposed to the methyl ester of molucidin. The name of the compound is Desmethyl molucidin.

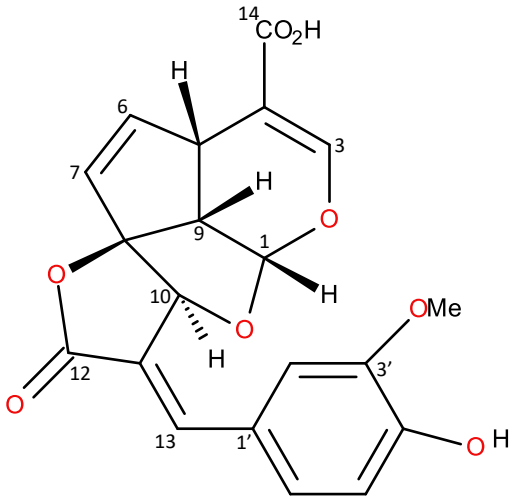
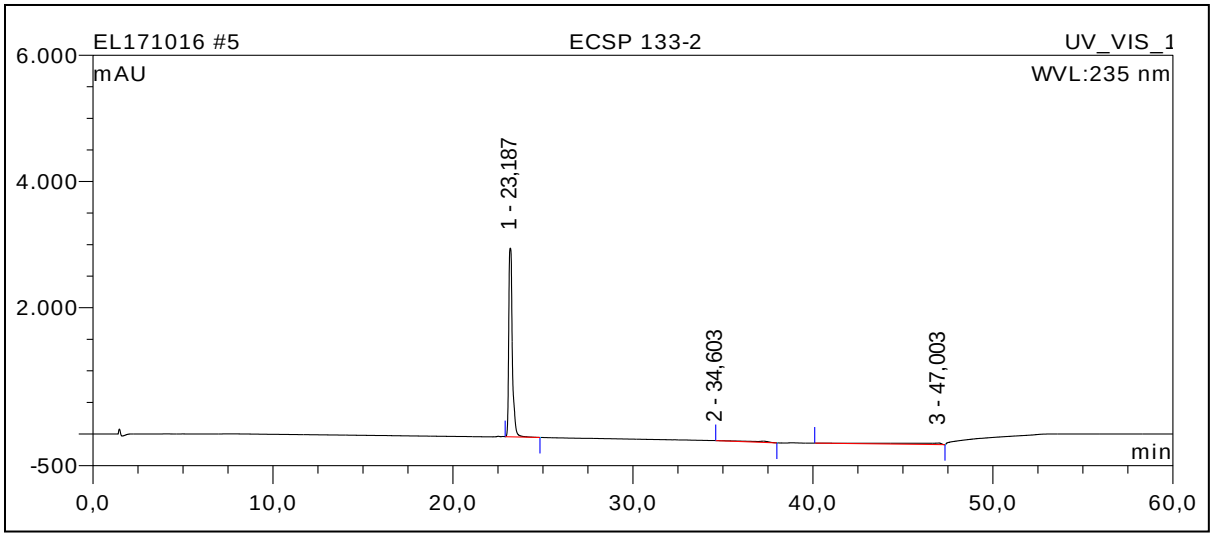


Figure 4.4: The Chemical Structure of Desmethyl Molucidin



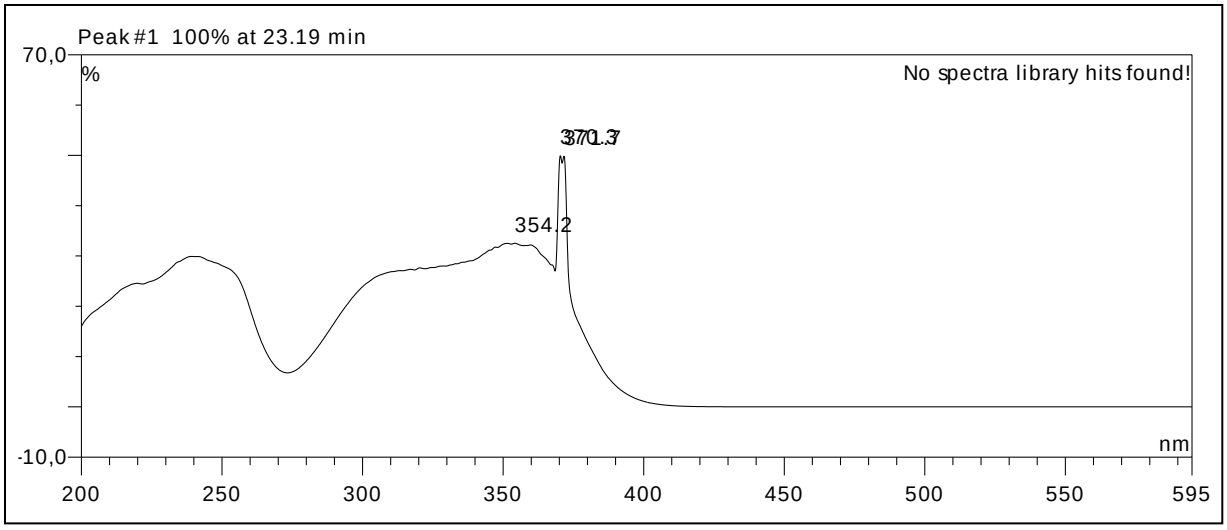


Figure 4.5: HPLC Chromatogram and UV spectrum of Desmethyl Molucidin

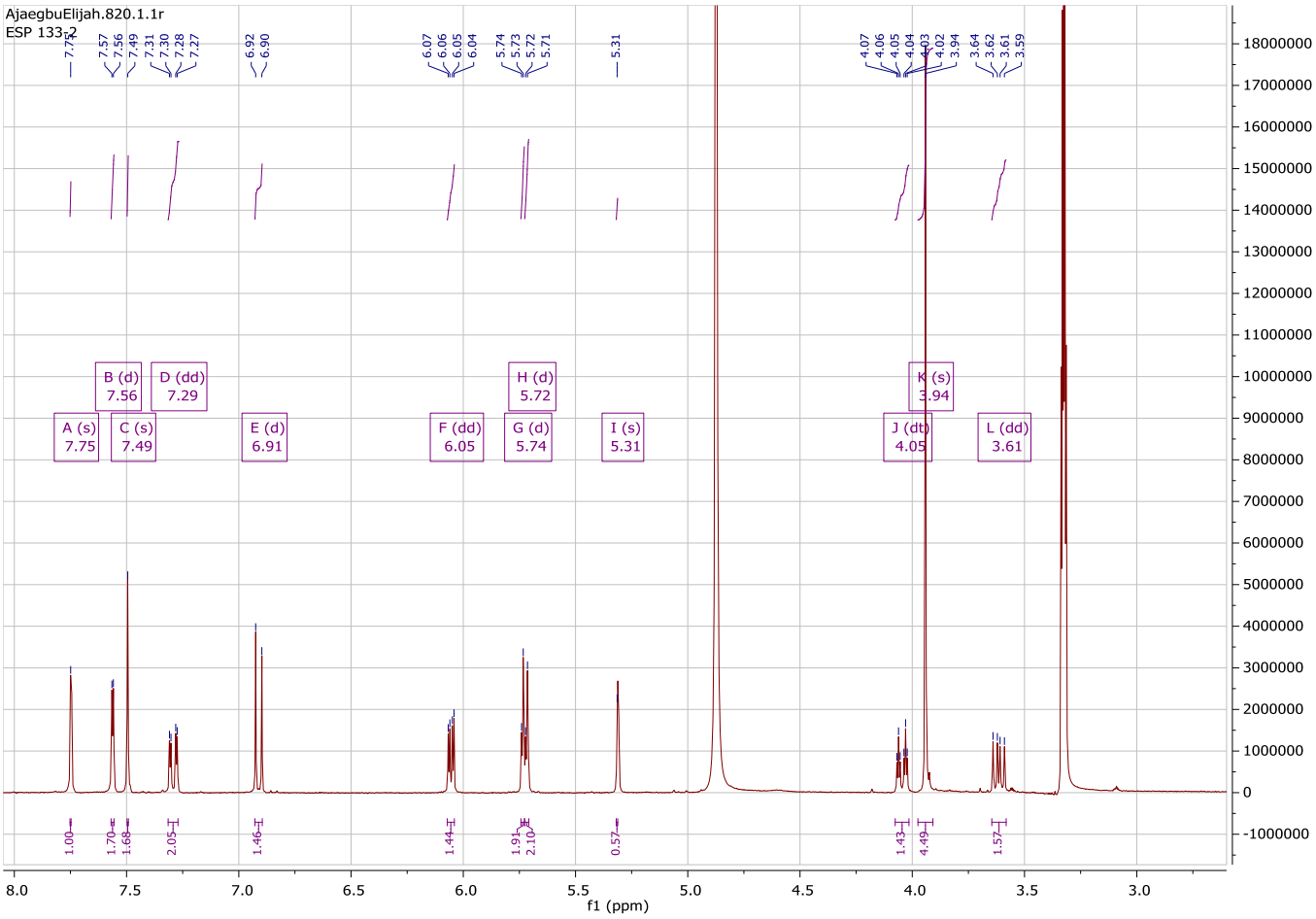


Figure 4.6: ^{1}H-NMR spectrum of Compound 2 (Desmethyl Molucidin)

Table 4.5: Comparison of the ^{1}H NMR data of Compound 2

	Compound 2 (300 MHz, Methanol-d_4)			Reference Compound (Desmethyl Molucidin)(400 MHz,$CDCl_3$) (kwofie et al, 2016)		
Position	δ H	Mult	J (Hz)	δ H	Mult	J (Hz)
1	5.74	d, 1H	5.6	5.68 d	d, 1H	5.6
2’	7.55	d, 1H	2.0	7.49 d	d, 1H	2.0
3	7.48	s, 1H		7.50 s	s, 1H	
3’ –OCH_3	3.93	s, 3H		3.95 s	s, 3H	
5	4.03	dt, 1H	9.4, 2.1	4.05 dt	dt, 1H	10.0, 2.0
5’	6.90	d, 1H	8.3	6.92 d	d, 1H	8.0
6	6.04	dd, 1H	5.5, 2.2	6.06 dd	dd, 1H	6.4, 2.0
6’	7.28	dd, 1H	8.2, 2.0	7.25 dd	dd, 1H	8.0, 2.0
7	5.72	dd, 1H	5.6, 2.4	5.67 dd	dd, 1H	6.4, 2.4
9	3.60	dd, 1H	9.4, 5.8	3.60 dd	dd, 1H	10.0, 6.0
10	5.31	s, 1H		5.28 s	s, 1H	
13	7.73	s, 1H	1.3	7.75 s	s, 1H	

4.1.3 Identification of Compounds Present In the Crude Extract and Fractions of *M. Lucida* by High Performance Liquid Chromatography (HPLC).

4.1.3.1 Bioactive Compounds Identified from Ethyl acetate and Butanol Fractions of *M.lucida* leaves.

Some compounds were identified in the promising extracts of *M. lucida*by HPLC analysis. HPLC analysis of the ethyl acetate extract revealed the following secondary metabolites; Luteolin, Kaempferol, Quercetin, Cinnamic acid, Hyperoside, Cyclopenol.

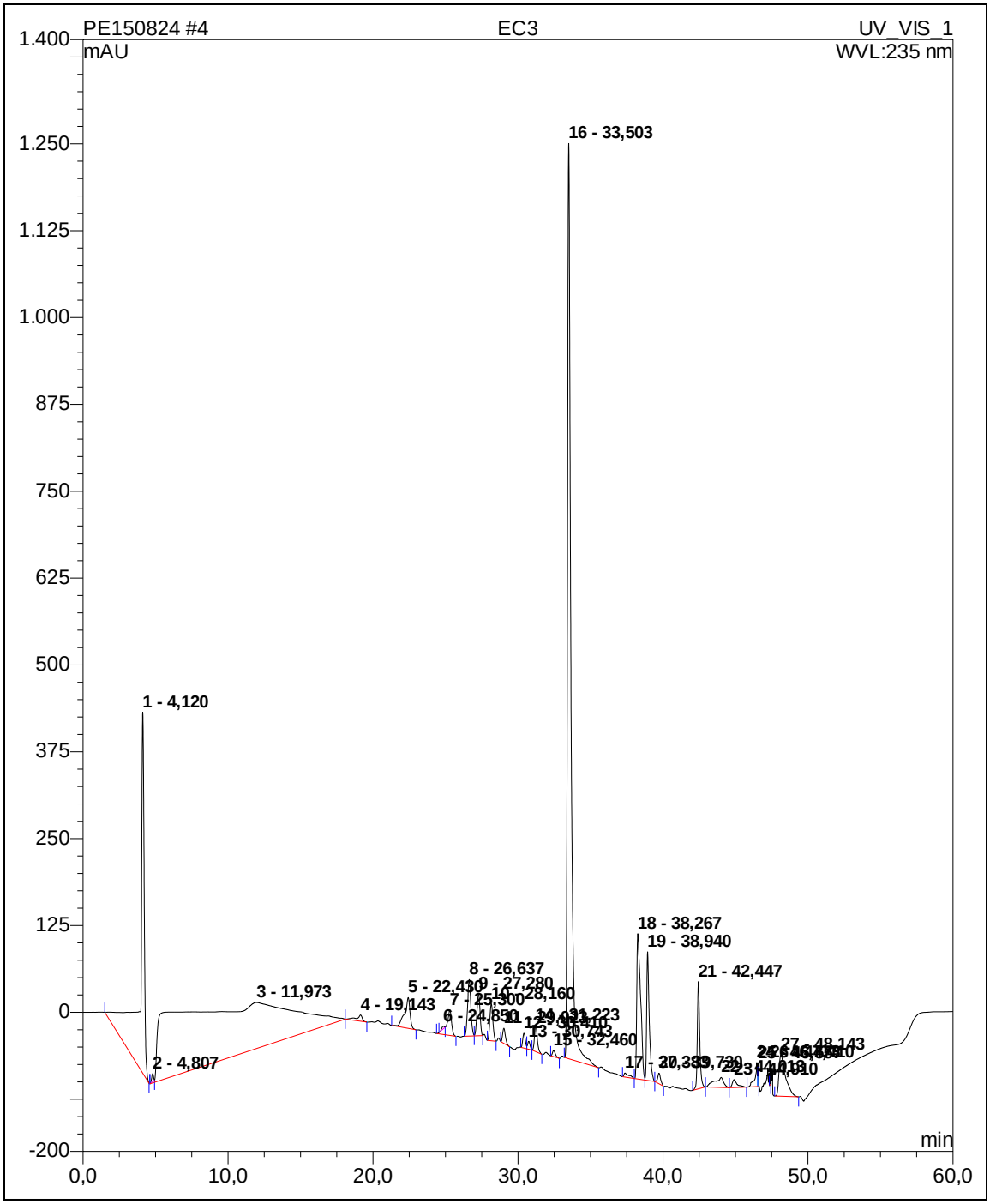


Figure 4.7: HPLC Chromatogram of ethyl acetate fractions of *M. lucida*.

Quercetin

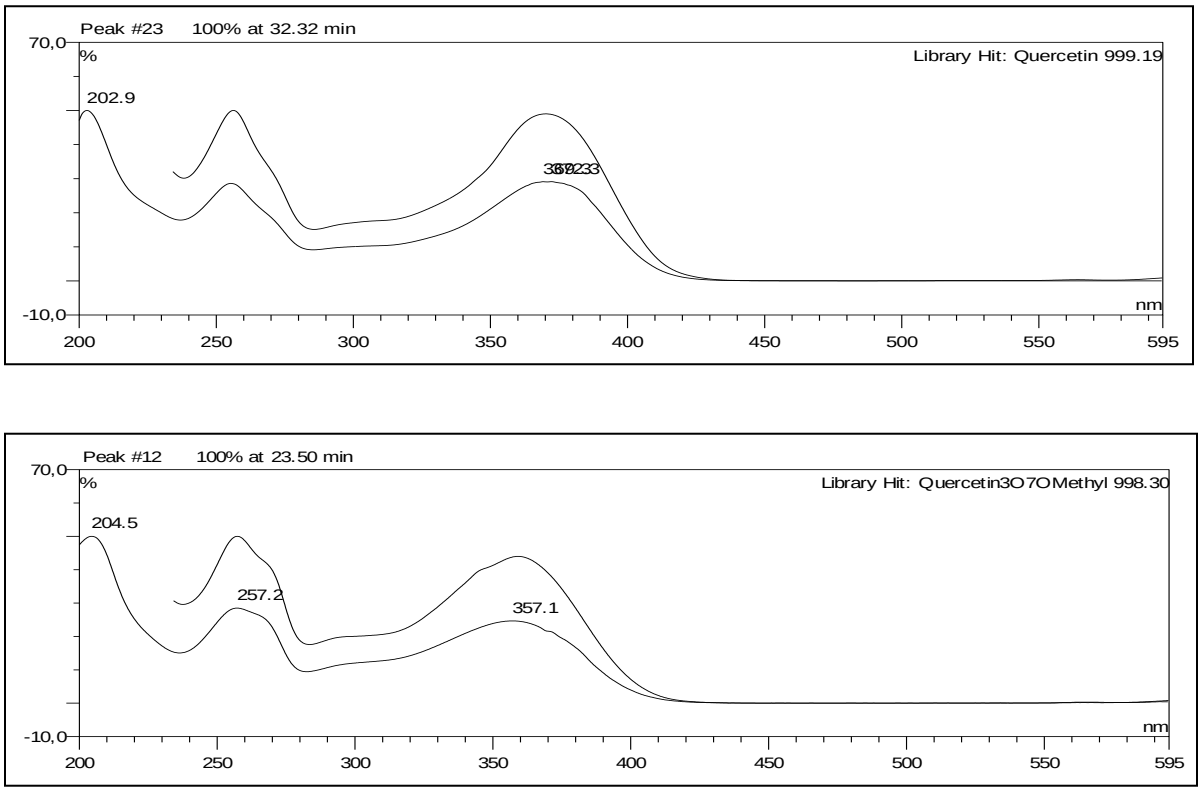


Figure 4.8: UV Spectra of Quercetin isolated from both ethyl acetate and butanol of *M. lucida*.

Quercetin was identified from both ethyl acetate extract of *M. lucida*. It showed a retention time of 32.32min in HPLC analysis and UV absorption maxima at 370.7nm.

Hyperoside

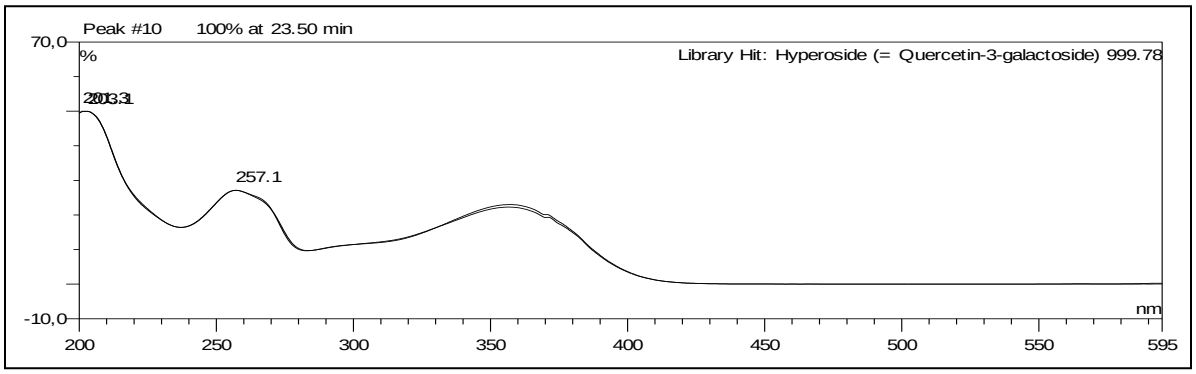


Figure 4.9: UV Spectra of Hyperoside isolated from butanol fraction of *M. lucida*.

Hyperoside was identified from butanol extract of *M. lucida*. It showed a retention time of 23.5min in HPLC analysis and UV absorption maxima at 203 and 257.1nm.

Kaempferol

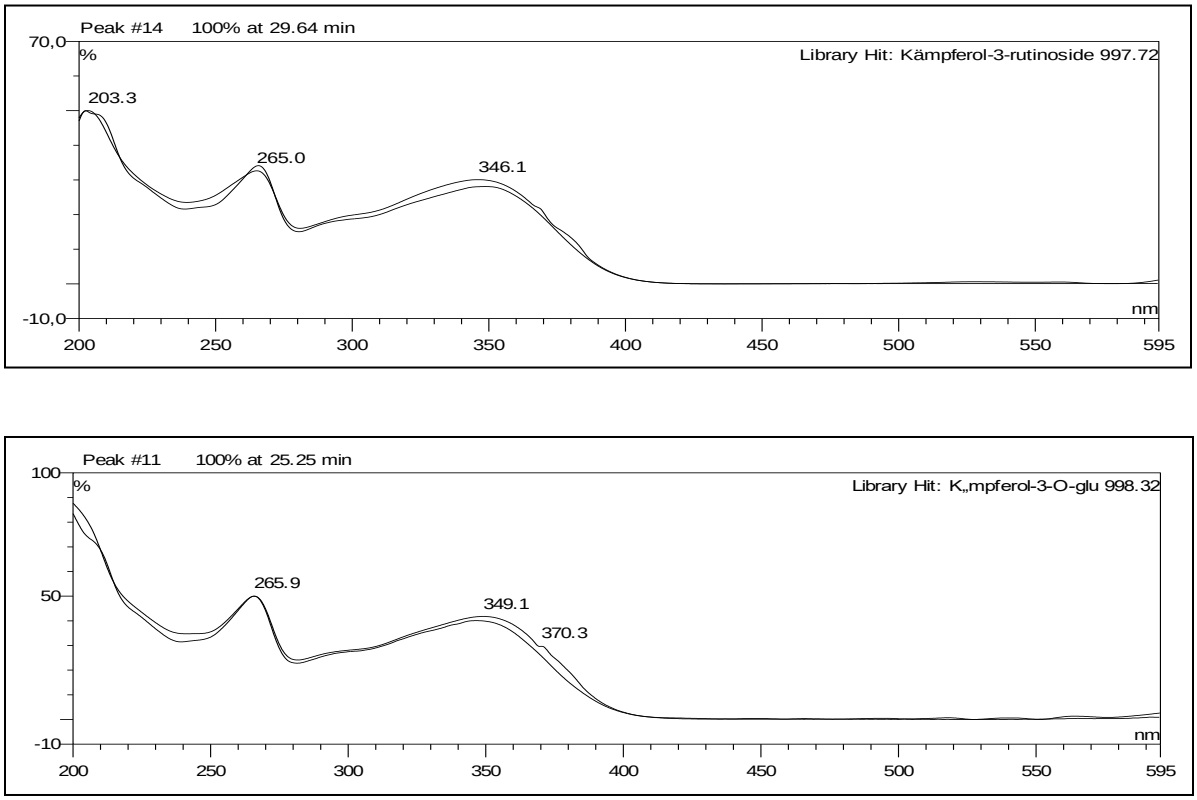


Figure 4.10: UV Spectra of Kaempferol isolated from both ethyl acetate and butanol fraction of *M. lucida*.

Kaempferol-3-rutinoside was identified from ethyl acetate extract of *M. lucida*. It showed a retention time of 29.64min in HPLC analysis and UV absorption maxima at 203.3nm, 266nm and 346.1nm. (Figure 4.10a)

Kaempferol-3-O-glucoside was identified from butanol extract of *M. lucida*. It showed a retention time of 25.25min in HPLC analysis and UV absorption maxima at 265.9, 349.1 and 370.3nm. (Figure 4.10b)

Luteolin

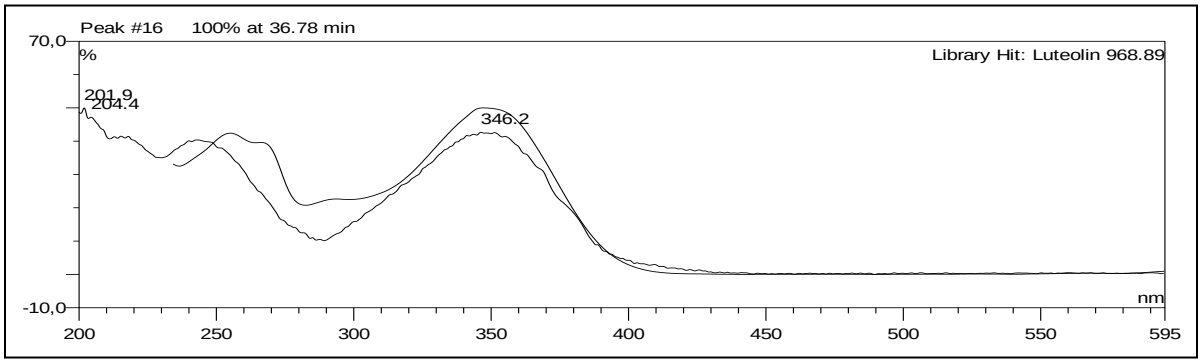


Figure 4.11: UV Spectra of Luteolin isolated from ethyl acetate fraction of *M. lucida*.

Luteolin was identified from ethyl acetate extract of *M. lucida*. It showed a retention time of 36.78min in HPLC analysis and UV absorption maxima at 204.4, 242.3 and 346.2nm. (Figure 4.11)

Cinnamic acid

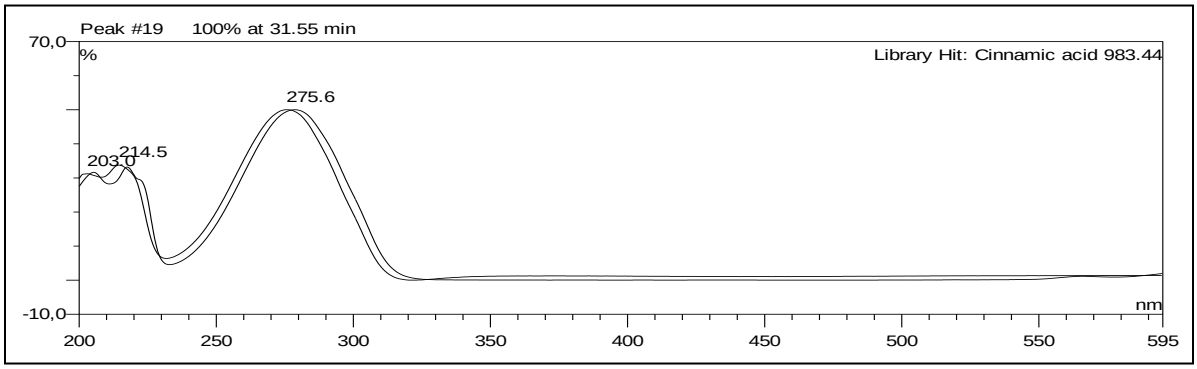


Figure 4.12: UV Spectraof Cinnamic acid isolated from ethyl acetate fraction of *M. lucida* through HPLC study.

Cinnamic acid was identified from ethyl acetate extract of *M. lucida*. It showed a retention time of 31.56min in HPLC analysis and UV absorption maxima at 214.5nm and 275.6nm. (Figure 4.12)

Cyclopenol

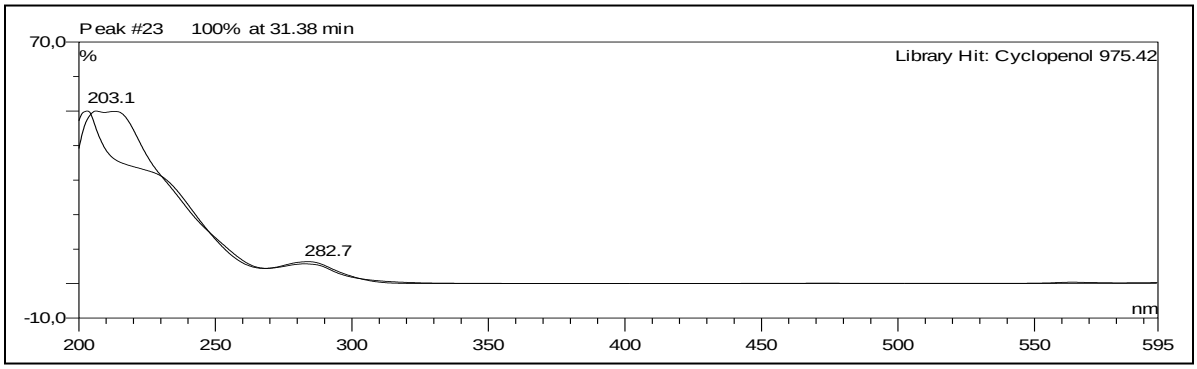


Figure 4.13: UV Spectra of Cyclopenol isolated from both ethyl acetate and butanol fraction of *M. lucida* through HPLC study.

Cyclopenol was identified from both ethyl acetate and butanol extract of *M. lucida*. It showed a retention time of 31.38min in HPLC analysis and UV absorption maxima at 203.1nm and 282.7nm. (Figure 4.13)

4.1.4. Cyclophosphamide-induced myelosuppression and Total Leukocyte Counts

There was a significant reduction in total white blood cell count for all groups when treated with only cyclophosphamide for 3 days. There was a significant increase in the total WBC count for all groups when distilled water, levamisole, crude extract where fed to the mice for 14 days. Figure 4.14 shows that the group that received 100mg/kg of levamisole and 50mg/kg of *M.lucida* leaf extract showed significant elevation in the total WBC counts in relation to other groups with the highest counts observed in Group III which has the 50 mg/kg of crude methanol extract. The ethyl acetate fraction showed an increase in the total WBC count than the butanol fraction after the treatment. There was a significant restoration of the total WBC count as shown in Figure 4.14.

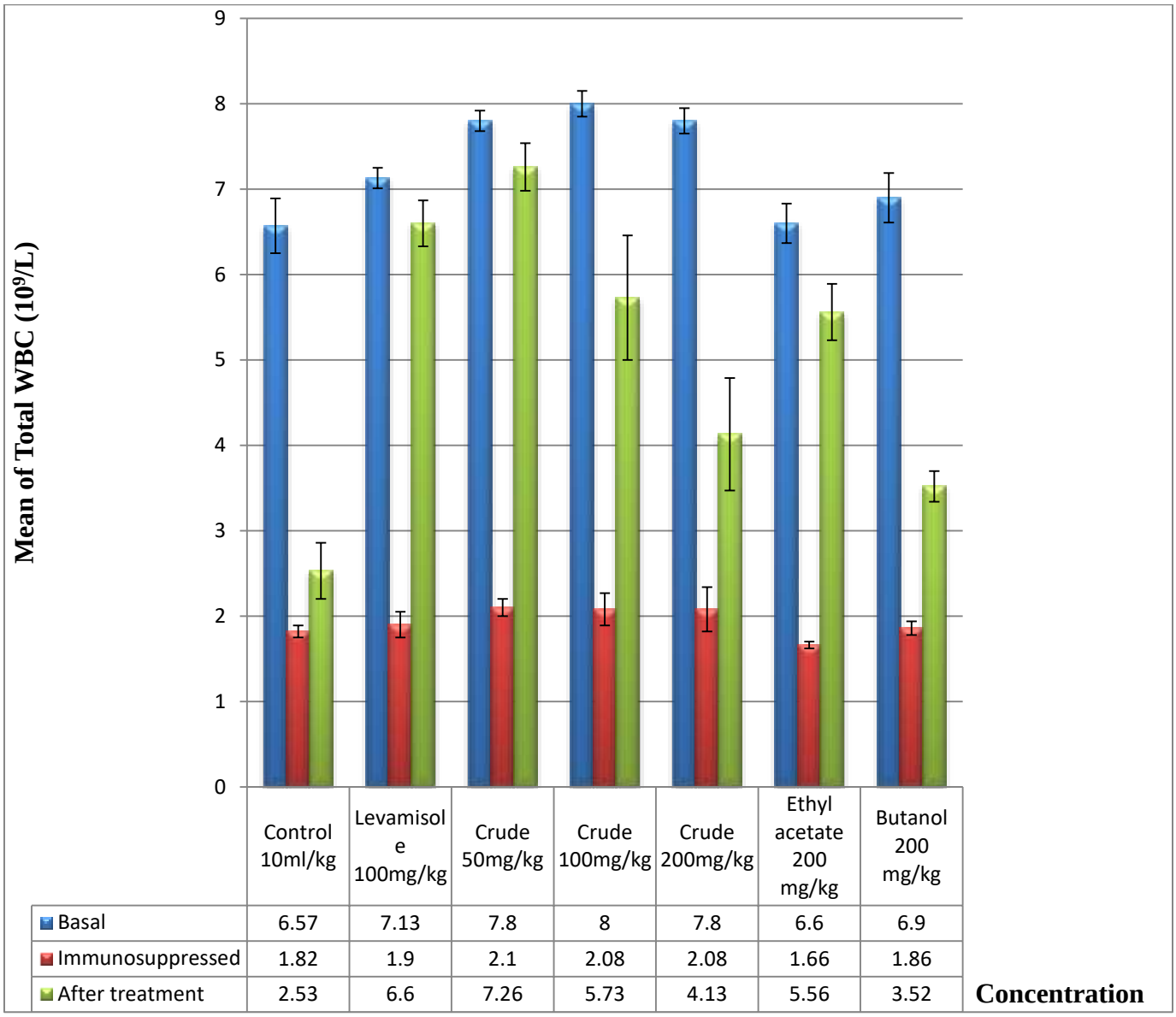


Figure 4.14: Mean Total White Blood Cell (WBC) Count

4.1.4.2 Differential Leukocyte Counts

There was a significant ($P < 0.05$) decrease in Neutrophils (N), Lymphocytes (L), Eosinophils (E) and Monocytes (M) in mice treated with cyclophosphamide as compared to basal samples.There was also a significant increase in the Neutrophils (N), Lymphocytes (L), Eosinophils (E) and Monocytes (M) when distilled water, levamisole, crude extract where fed to the mice for 14 days as compared to when they received immunosuppressive drug cyclophosphamide as shown in figure 4.15 – 4.18.

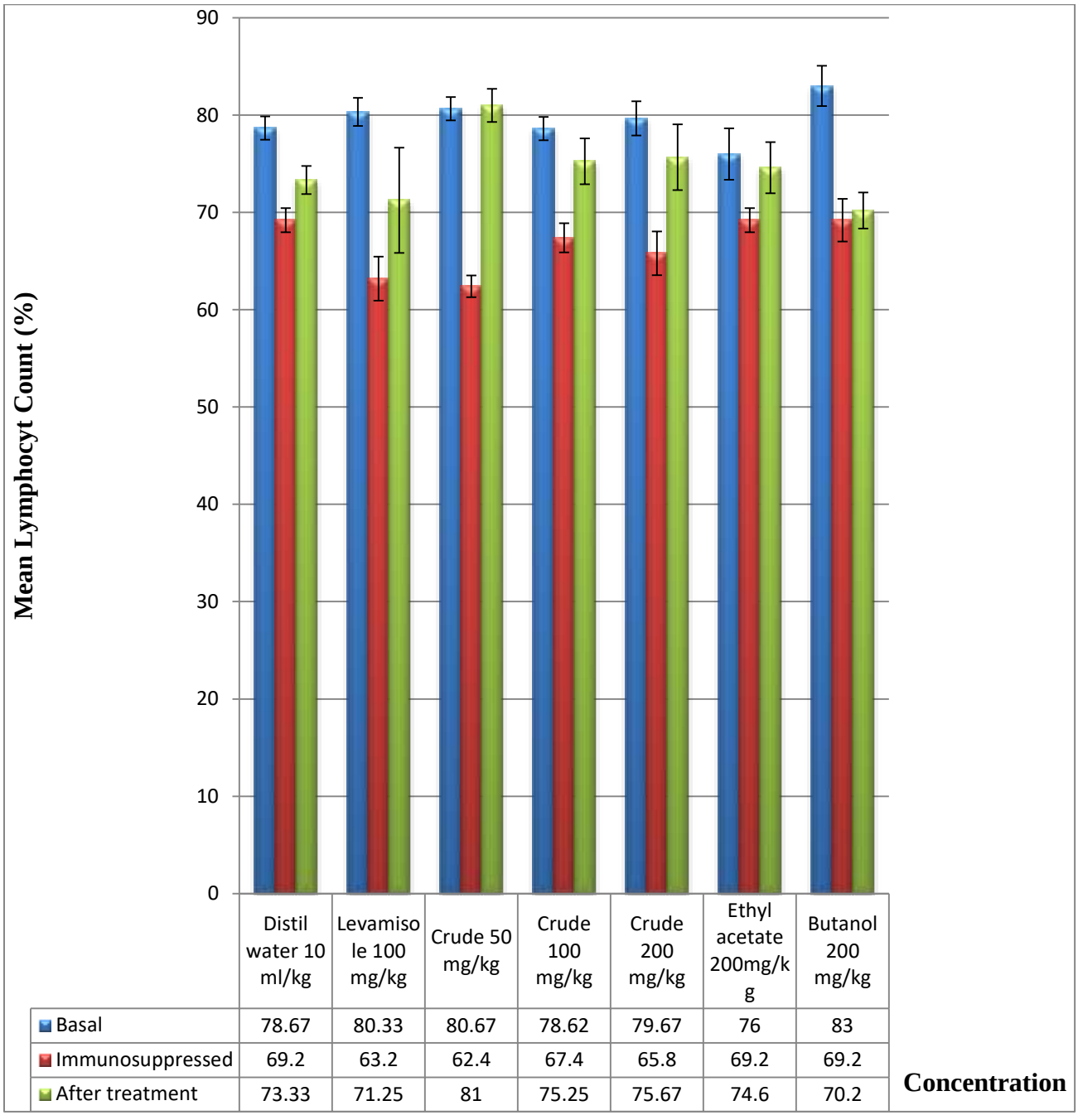


Figure 4.15: Mean Lymphocyte Count

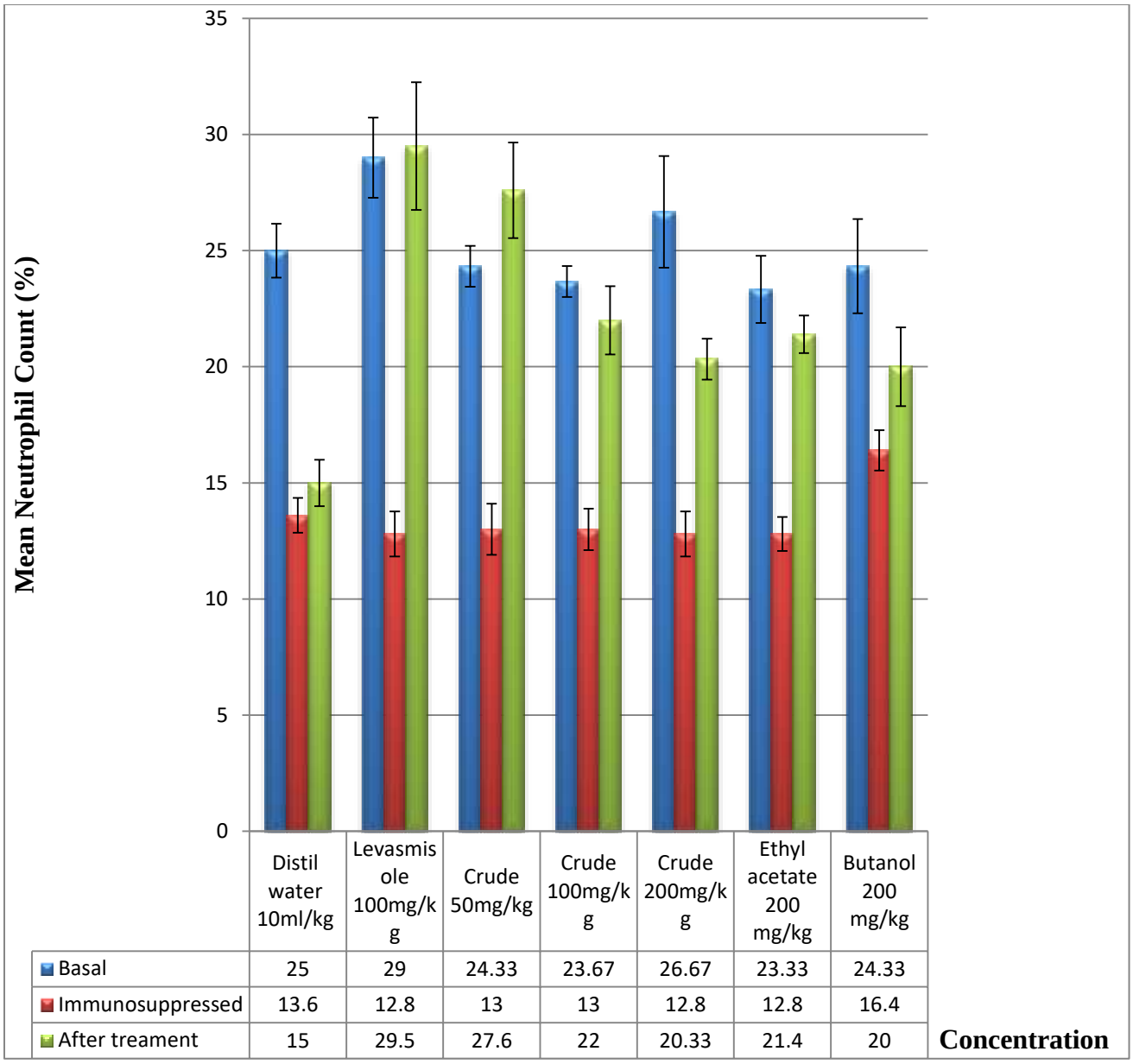


Figure 4.16: Mean Neutrophil Count

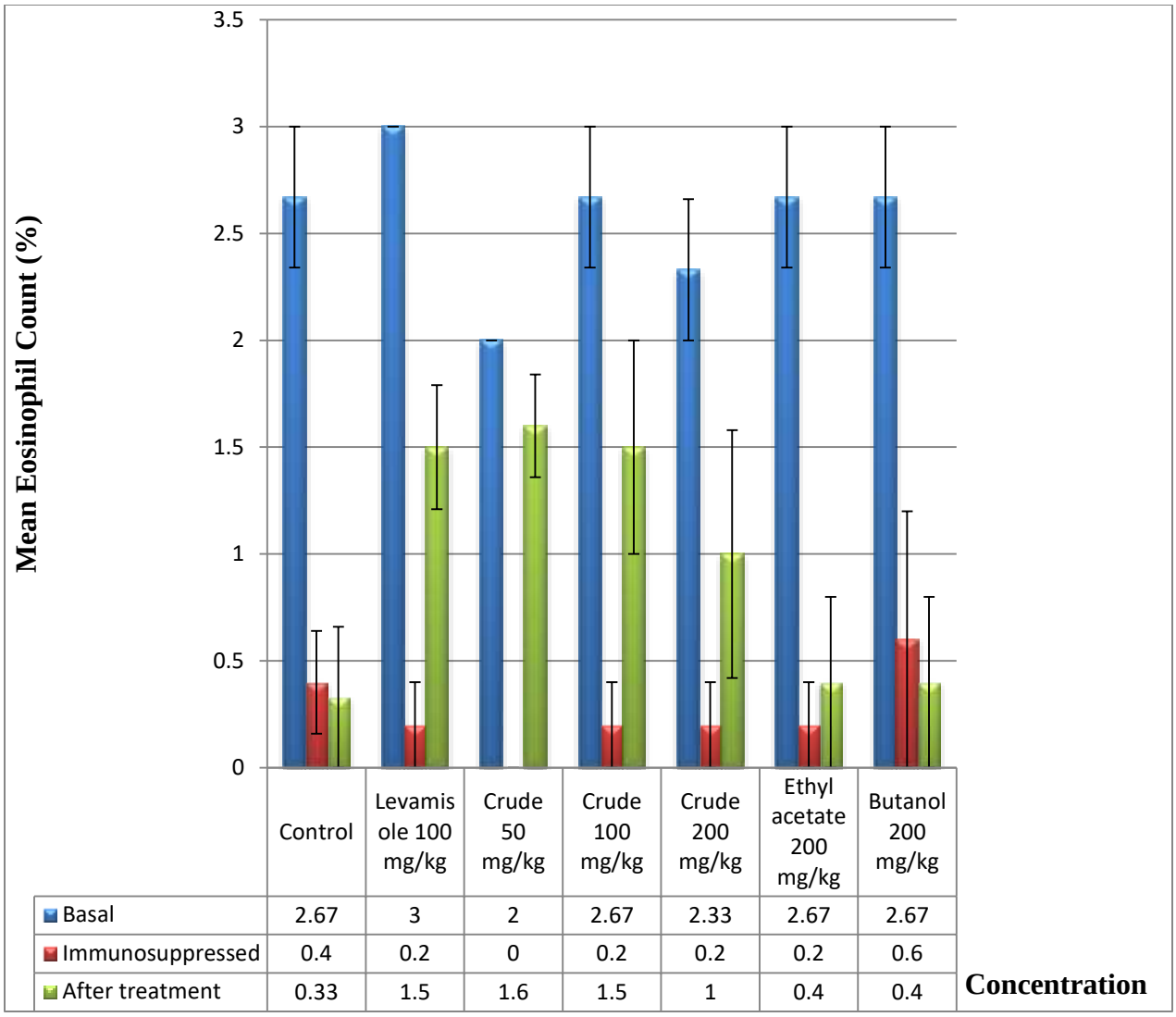


Figure 4.17: Mean Eosinophil Count

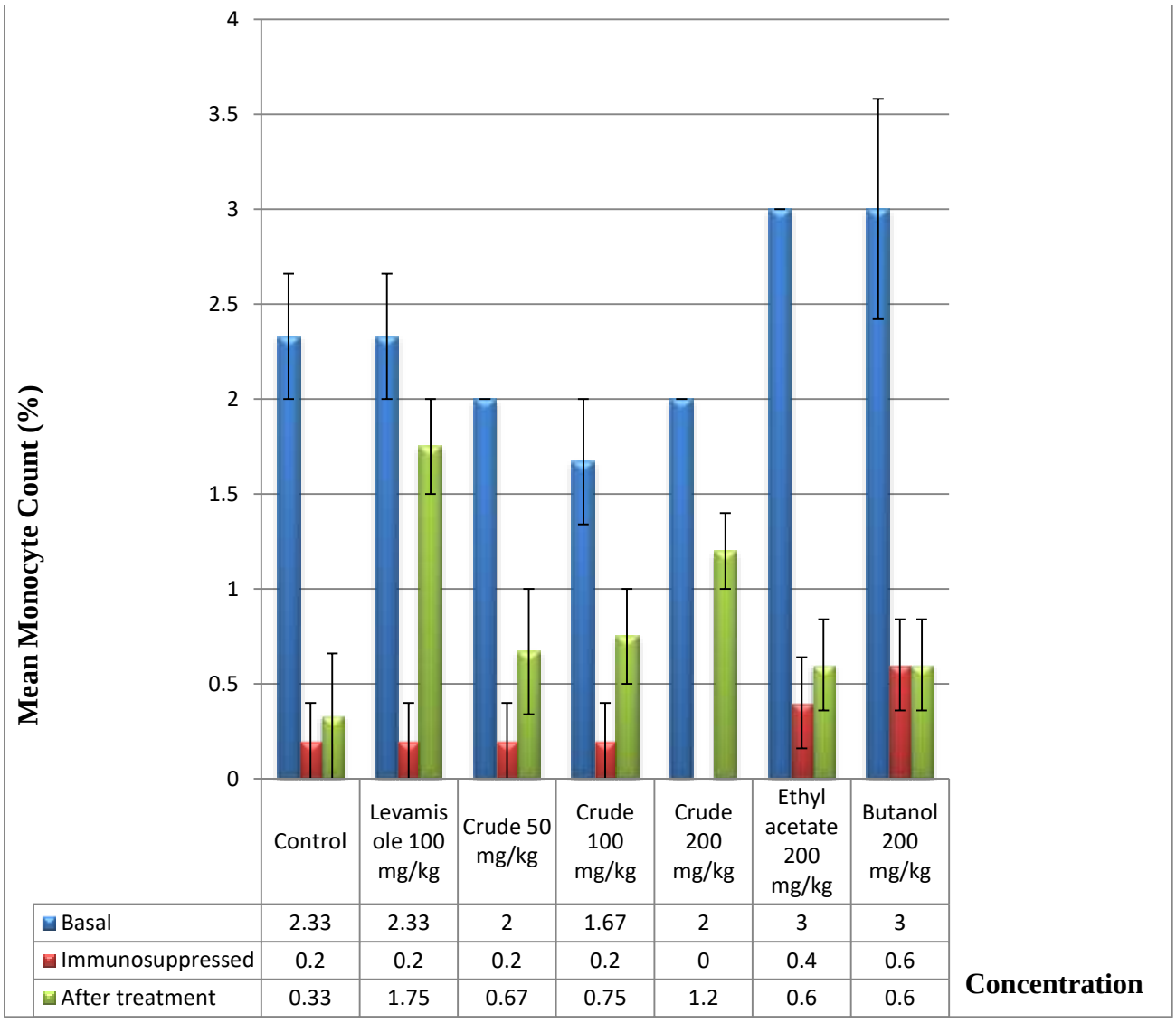


Figure 4.18: Mean Monocyte Count

4.1.4.3 Haemagglutination titer test

In Haemagglutination titer test, agglutination was compared with control and standard drug, Group III, Group IV and GroupV treated with crude extract of *M. lucida* 50 mg/kg, 100 mg/kg, 200 mg/kg. Group III, Group IV and Group V shows agglutination titre in X:25, X:50 dilutions, X: 12.5, X: 25, X: 50 dilutions, X:12.5, X:25, X:50 dilutions respectively likewise Group VI and Group-VII treated with 200 mg/kg ethyl acetate and 200 mg/kg butanol fractions shows agglutination titre in X:6.25, X:12.5 dilutions, and X: 12.5, X:50 dilutions respectively Table 4.6 shows the result of the comparison of the groups to control and standard group.

Table 4.6: Haemagglutination titer test for crude extract,ethyl acetate and butanol fraction of *M. lucida*

Group	Treatment	Dose	Antibody titre Mean ± SEM(n=5)	% inhibition
I	Control	10ml/kg	41.7 ± 8.33	
II	Levamisole	100mg/kg	8.3 ± 2.08	80.10
III	Crude	50mg/kg	33.3 ± 8.33 *	20.14
IV	Crude	100mg/kg	16.7 ± 4.17 *	59.95
V	Crude	200mg/kg	12.5 ± 0.00 *	70.02
VI	Ethylacetate	200mg/kg	10.2 ± 2.08	75.01
VII	Butanol	200mgkg	16.7 ± 4.17 *	59.95

Values are expressed as Mean ± SEM, (n=5). All the groups were compared with control group and standard group. Significant values are expressed as (*= $p<0.05$), ns- non significant.

4.1.4.4 Effect of Delayed hypersensitivity response

The result indicates that there was significant increase in the foot paw thickness at all the doses of crude methanolic extract of treated group after 24 h when compared against 0 h, likewise the ethyl acetate fraction and butanol fraction. All the results as shown in Appendix J are in terms of mean difference in the foot paw thickness when compared to before treatment. There was also no significant difference of the doses against control in figure 4.19.

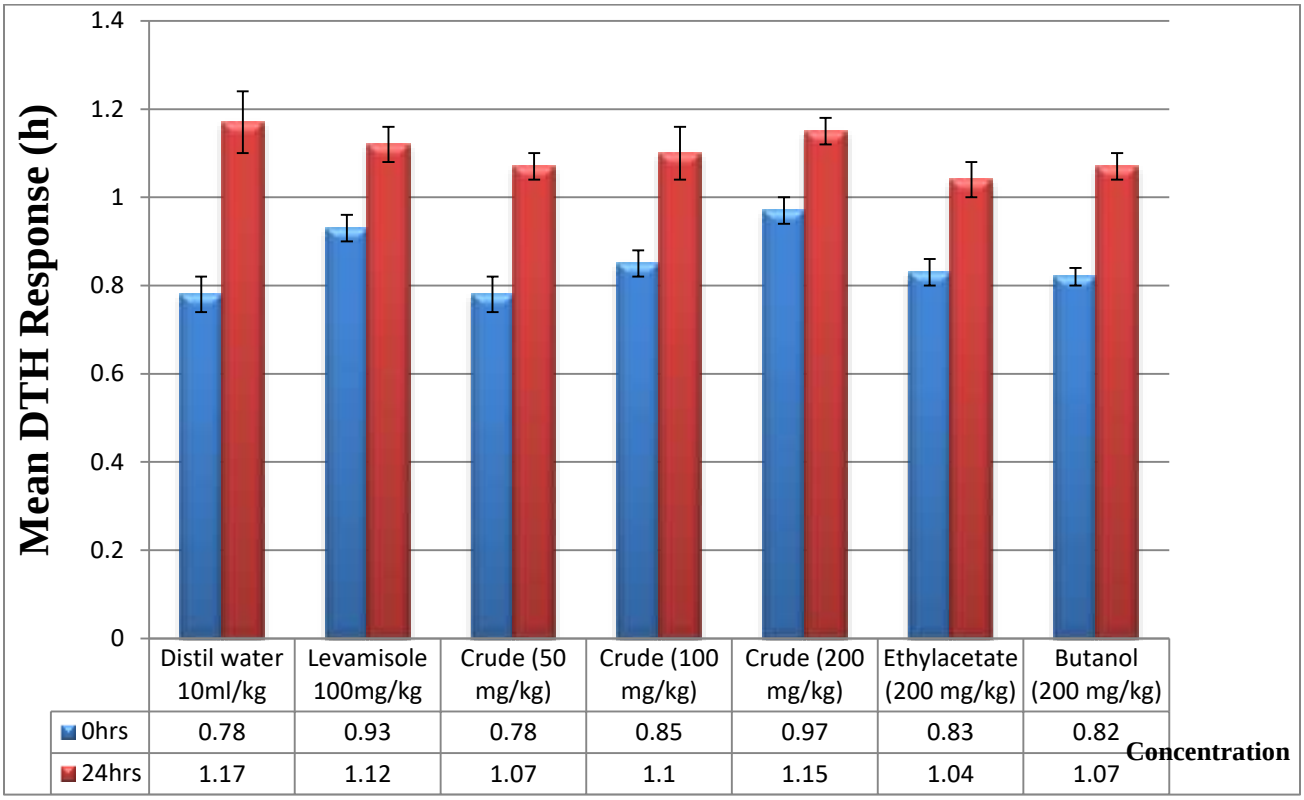


Figure 4.19: DTH response for crude extract, ethyl acetate and butanol fraction of *M. lucida*

Table 4.7: Percentage inhibiton of DTH responsefor crude extract, ethyl acetate and butanol fraction of *M. lucida*

Group	Treatment	Dose	% inhibition
I	Control	10ml/kg	
II	Levamisole	100mg/kg	51.28
III	Crude	50mg/kg	25.64
IV	Crude	100mg/kg	35.90
V	Crude	200mg/kg	53.85
VI	Ethylacetate	200mg/kg	46.15
VII	Butanol	200mgkg	35.90

4.2 DISCUSSION

4.2.1. Isolated Compounds

Molucidin and Desmethyl Molucidinhave tetracyclic iridoid skeletons, and their absolute configurations were previously determined (1*R*,5*S*,8*S*,9*S*,10*S*) (Karasawa *et al*,2015). ESP133-2 has a carboxylic acid while Molucidin has a methyl ester functional group.

The two tetracyclic iridoid compounds, Molucidin and Desmethyl Molucidin, have previously been identified from *M. lucida* leaf and their anti-trypanosoma, anti-leishmania (Amoa-Bosompem *et al*, 2016) and anti-malaria activities were determined using in-vitro and in-vivo methods (Kwofie *et al*, 2016). Their structure-activity relationship analysis shows that they shared an aryl propanone moiety as well as similar aromatic rings (Suzuki *et al*, 2015). The anti-bacterial activity of Molucidin and Desmethyl Molucidinwere also determined (Antwi *et al*, 2017), owing to the reports of anti-protozoan compounds having anti-bacterial activity (Leite *et al*,2005). The compounds showed activity against only gram-positive bacteria.

4.2.2. Bioactive Compounds Identified from Ethyl acetate and Butanol Fractions of *M.lucida* leaves.

Quercetin is a type of flavonoid antioxidant that's found in plant foods, including leafy greens, tomatoes, berries and broccoli.

Quercetin plays an important part in fighting free radical damage, the effects of aging and inflammation. Quercetin and other flavonoids are "anti-viral, anti-microbial, anti-inflammatory and anti-allergic agents" with potential to be expressed positively in different cell types in both

animals and humans (Chirumbolo, 2010). Flavonoid polyphenols are most beneficial for down-regulating or suppressing inflammatory pathways and functions. Quercetin is considered the most diffused and known nature-derived flavonol there is, showing strong effects on immunity and inflammation caused by leukocytes and other intracellular signals.

Research shows that anti-inflammatory foods containing quercetin can help manage a number of inflammatory health problems, including heart disease and blood vessel problems, allergies, infections, chronic fatigue, and symptoms related to autoimmune disorders like arthritis. How exactly do flavonoids like quercetin do so much to benefit us?

It all comes down to food's ability to be "scavenge free radicals." As a major bioflavonoid in our diets, quercetin (a type of "polyphenolic antioxidant") helps slow the aging progress because it lessens the effects of oxidative stress on the body (Yao *et al,* 2004). Oxidative stress takes place in all of us but is increased by things like a poor diet, high levels of stress, a lack of sleep and exposure to chemical toxins.

Quercetin (figure 4.20) plays a role in regulating the immune system's response to outside stressors through cell signaling pathways called kinases and phosphatases, two types of enzyme and membrane proteins needed for proper cellular function.

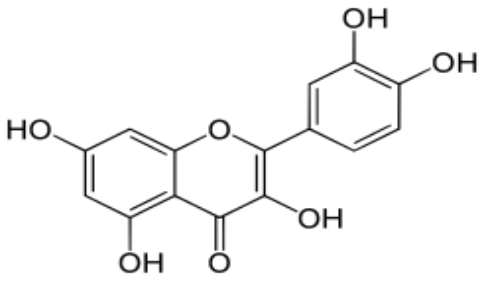


Figure 4.20: Structure of Quercetin

Keampferol (figure 4.21 and figure 4.22) is a strong antioxidant and helps to prevent oxidative damage of our cells, lipids and DNA. Kaempferol seems to prevent arteriosclerosis by inhibiting the oxidation of low density lipoprotein and the formation of platelets in the blood. Studies have also confirmed that kaempferol acts as a cancer chemopreventive agent, which means that it inhibits the formation of cancer cells (Okoye *et al,* 2015).

An in-vitro study by Jan Kowalski *et al* (2005) showed that kaempferol inhibits monocyte chemoattractant protein (MCP-1). MCP-1 plays a role in the initial steps of atherosclerotic plaque formation.

The flavonoids kaempferol and quercetin seems to act synergistically in reducing cell proliferation of cancer cells, meaning that the combined treatments with quercetin and kaempferol are more effective than the additive effects of each flavonoid. This was a conclusion from a study by Ackland *et al* (2005).

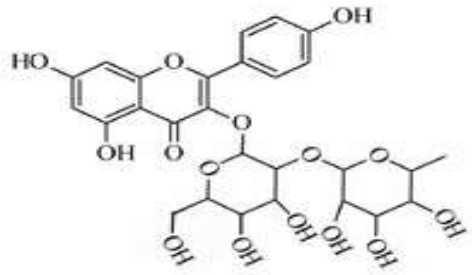


Figure 4.21: Structure of Kaempferol 3-O-rutinoside

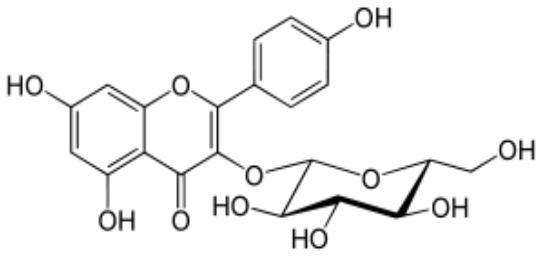


Figure 4.22: Structure of Kaempferol 3-O-glucoside

Hyperoside (figure 4.23) is a 3-*O*-galactoside of quercetin. It can have a protective antioxidant effect on cultured PC12 cells (Liu *et al*, 2005). Like various other flavonoids, hyperoside has been found to possess antagonist activity at the κ-opioid receptor(Katavic *et al*, 2007).

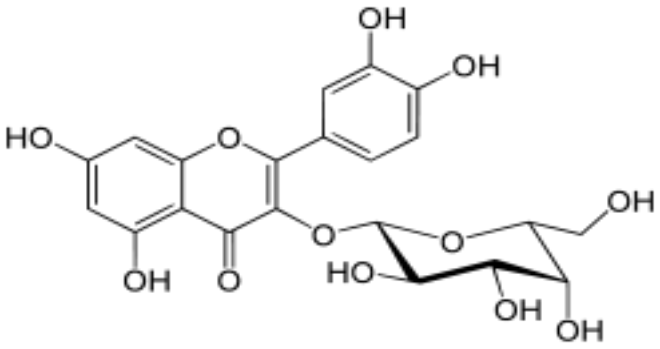


Figure 4.23: Structure of Hyperoside

Luteolin (figure 4.24) is a chemical that is classified as a citrus bioflavonoid. It is a yellow crystal in pure form, which is typical for flavonoids. Luteolin has many uses as a health supplement due to its antioxidant and anti-inflammatory properties. These properties allow luteolin to scavenge reactive compounds containing oxygen and nitrogen, which can cause cellular damage. Additional biological effects of luteolin include the activation of the dopamine transporter.

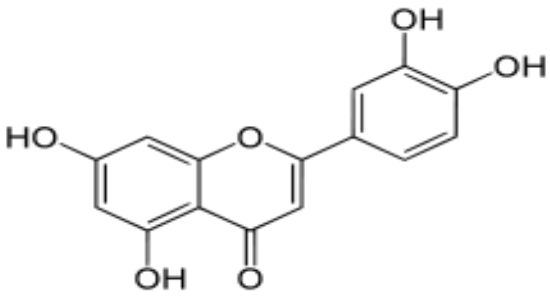


Figure 4.24: Structure of Luteolin

Cinnamic acid**(**figure 4.25) is an odorless white crystalline acid that has only been recently studied for its potential in cancer prevention. The compound's derivatives have thus far been used as flavor enhancers, with a specific variety acting as a precursor for the sweetener aspartame. Despite aspartame's negative health effects on the body, cinnamic acid is in itself quite healthy.

The lack of data on cinnamic acid makes identifying the body systems that it supports difficult. In broad strokes, cinnamic acid helps the gastrointestinal system as it allegedly helps balance insulin levels. This makes it a potential candidate in diabetes management.

Due to its touted benefit on cancer prevention, it can be hypothesized that cinnamic acid supports the immune system as well.

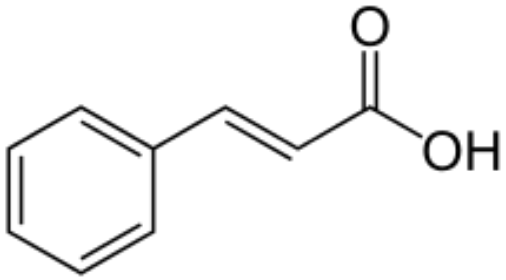


Figure 4.25: Structure of Cinnamic acid

Cyclopenol (figure 4.26) is one of benzodiazepine alkaloids, and an intermediate in the biosynthesis of the viridicatol. It possesses antimicrobial properties and they are phytotoxic.

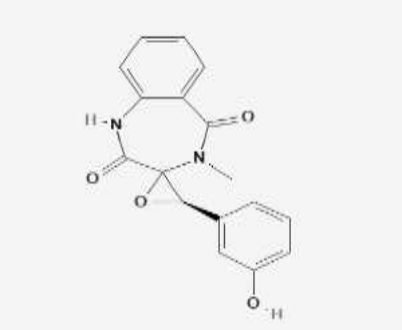


Figure 4.26: Structure ofCyclopenol

4.2.3 Immunomodulatory activity of *M. lucida*

Immunomodulation is any process which can alter the immune system by interfering with its functions. If it results in an enhancement of immune reactions it is termed immunostimulatory which primarily implies stimulation of specific and non-specific systems, i.e. granulocytes, macrophages, complement, certain T-lymphocytes and different effector substances. Immunosuppression is mainly to reduce the immune resistance against infections and stress which may occur as a result of environmental or chemotherapeutic factor (Makare *et al*, 2001). The immune responses through stimulation or suppression may help in maintaining a disease-free state. Agents that activate host defense mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy.The immune system is the defense mechanism of the body and it helps to protect it from foreign bodies and infection thus playing a part in homeostasis of the body. Modulation of the immune system by way of stimulation or suppression helps in maintaining a disease-free state within an individual.

Immunomodulators have therefore been used globally to control disease conditions. The study explored the immunomodulatory activity of the methanol, ethylaetate and butanol leaf extract of *M. lucida* by evaluating its effect on total white blood count, differential blood count, delayed hypersensitivity (DTH) reactions and hemagglutination antibody titres.

4.2.3.1. Effect on Total White Blood Counts

The results obtained in the present study indicate that *M. lucida* leaf extract is a potent Immunostimulant, stimulating specific and non-specific immune mechanisms.

Cyclophosphamide at the dose of 30 mg/kg caused a significant reduction in total WBC count in mice as compared to basal group. The rise in the total WBC count lowered by cyclophosphamide was observed after treatment with *M. lucida*leafextract.The crude methanol extract at 50 mg/kg gave a better restoration than 100 and 200 mg/kg but the ethyl acetate fraction at 200 mg/kg showed a good restoration when compared crude extract and butanol fraction.This could be the result of the compounds presenct in the ethyl acetate fraction which reacted positively on the immune system of the mice. The active compound Molucidin and Desmethyl molucidin have been shown to possess pharmacological activities. The bioactive compounds detected from ethyl acetate show that the flavonoids, qucertin and kaemferol play a very important role in regulating the immune system resonse.

The observed increment in WBC counts could have been due to the presence of different nutritional elements in the *M. lucida* methanol leaf extract and the observed effect was similar to previous studies done elsewhere (Ngozi *et al,* 2016). Methanol as a solvent has been reported to extract most of the compounds both polar and nonpolar compounds in the plant materials such as leaves, roots, fruits, and stems (Bart *et al*,2011).

Effect on Differential Leukocyte Counts

There was a significant decrease of Neutrophils lymphocytes, eosinophils and monocytes in animals treated with cyclophosphamide as compared to basal group. Cyclophosphamide showed that in mice lymphocytes decrease due to immunotoxic effect as well as decreases in the activity of lymphoid cells especially the CD4+ lymphocytes. Eosinophils attack parasites and phagocyte antigen-antibody complexes. Monocytes are the precursors of macrophages. They are larger blood cells, which after attaining maturity in the bone marrow, enter the blood circulation where they stay for 24-36 h. Then they migrate into the connective tissue, where they become macrophages and move within the tissues. In the presence of an inflammation site, monocytes quickly migrate from the blood vessel and start an intense phagocytory activity. The role of these cells is not solely in phagocytosis because they have also an intense secretory activity. They produce substances which have defensive functions such as lysozyme, interferons and other substances which modulate the functionality of other cells. Macrophages cooperate in the immune defense. *M. lucida*leaf extract showed significant increase and restoration of lymphocytes, Neutrophils, eosinophils and monocytes. Thecrude extract at 50 mg/kg showed very good restoration of the differential white blood count likewise the ethyl acetate fraction. This could be attributed to the bioactive compounds present in the plant.

Effect on hemagglutination antibody titer

A hemagglutination test was performed to determine the effect of methanol leaf extract of *M. lucida* on the humoral immune response. Humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into plasma cells that secretes antibodies. Antibodies thus function as the effectors of the humoral response by binding to the

antigens and neutralizing them or facilitating their elimination by cross linking to form clusters that are then ingested by phagocytic cells. The study results demonstrated that methanol leaf extract of *M. lucida* had a stimulatory effect on the humoral immune response. This was evidenced by the mean hemagglutination antibody titer to SRBC that showed a dose-dependent increment for the mice dosed with *M. lucida* leaf extract as compared to the control and standard drug. Immunoglobulins and antigen-binding fragments are essential in the humoral immune responses that are products of amino acid chains and glycoproteins (Bender and Meyers, 2006), most of which are present in *M. lucida* leaf extract. Compounds such as fatty acids, zinc, vitamin C, vitamin B6, vitamin B12, manganese, and selenium are also essential for the maturation of the B-lymphocytes in the bone marrow (Janeway*et al*, 2001). Results from this study therefore demonstrated that the *M. lucida* leaf extract contains compounds like quecertin, kaemferol, hyperoside, luteolin that can stimulate the production of antibodies in an immunocompromised animal. This may justify the common usage of the leaf as an immune stimulant.

Effect on Delayed hypersensitivity (DTH) reaction

The DTH reaction is a type IV cell-mediated immune response according to the Coombs and Gell (1975) classification of hypersensitivity reactions. The test provides a functional in-vivo assessment of the cell-mediated immunity. It is often used as a skin test which capitalizes on intradermal inoculation of an antigen. It is therefore used to assess the skin response following intradermal inoculation of the antigen which is dependent on antigen specific memory T-cells and the observed results were due to the recruitment of mononuclear cells and neutrophils. Activation of the T cells leads to the release of lymphokines which causes the activation and accumulation of macrophages, increases vascular permeability, induces vasodilatation and produces inflammation (Janeway*et al*, 2001; Goronzy and Weyand, 2007). It also produces a

boost in phagocytic activity and increases the concentration of lytic enzymes for more effective killing of microorganisms (Janeway*et al,* 2001). This results in the net increase in the thickness of the foot pad in previously immunized animals. This increment in footpad thickness of the Wistar albino mice that were subjected to *M.lucida* extracts in this study could be attributed to the ability of the extract to activate lympohcytes and their accessory cell types leading to enhancement in the production of antibodies in the previously immunosuppressed animals thereby increasing cell-mediated immunity. This was in line with other finding from previous studies done using the same plant (Nworu *et al*, 2012). *M. lucida* has been found to contain vitamins A, E, and K (Abu *et al*, 2016). These compounds stimulate the immune system by enhancing T-cell proliferation, increasing cytokine production and synthesis of immunoglobulins (Rodrigo *et al*, 2008) all of which are important in the inflammatory response that was seen as an increment in the foot pad thickness. Amino acids also present in *M. lucida* leaf extract are also important in the formation of immunoglobulins and major histocompatibility complexes which are essential in the mediation of the DTH reaction. Trace elements are also essential for the proliferation of the T-cells and Langerhan cells and the activity of the lytic enzymes which are important components of the DTH reaction to antigen. The results of the study therefore showed that the methanol leaf extract, ethyl acetate fraction and butanol fraction of *M. lucida* can be used to boost the immune system as there was an increment in paw size in response to antigen.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

Though lots of researches have been carried out on this plant, the immunomodulatory activities with respect to the chemical constituents was carried out in this research.In this study, Morinda lucida leaf was extracted with methanol. The methanol extract was further fractionated using hexane, ethyl acetate and butanol. The crude extract, ethyl acetate and butanol fractions were screened for immunomodulatory activity using the cyclophosphamide induced myelosuppression, total blood count and differential leukocyte count, delayed hypersensitivity and haemagglutination titre.

The ethyl acetate fractions of the methanol extract was purified and some compounds isolated.From the leaf of *M. lucida,* two compounds, compound 1(Molucidin) and compound 2 (Desmethyl Molucidin) were isolated.Furthermore, it shows the presence of bioactive compounds like Luteolin, Kaempferol, Quercetin, Hypersoide, Cyclopenol and Cinnamic acid.

From the results obtained in the present study, it could be concluded that *M. lucida* leaf is a potent immunostimulant, stimulating specific and non-specific immune mechanisms. It may be due to the presence of various phytoconstituents present in *M. lucida* like phenolics, flavonoids, tannins, and alkaloids, which are already reported to possess immunomodulatory activity.

The present study also revealed that ethyl acetate fraction and butanol fraction of *M. lucida*leaves have immunostimulants activity which strongly affected the immune system. However, the mechanism of action could be unfolded only after detailed investigations whereby the fractions modulate the immune system as the extract contains compounds which had immunomodulatory activity.

The ethyl acetate fraction of *M.lucida* leaf showed immunomodulatory activity, which may be attributed to the two major compounds isolated, molucidin and desmethyl molucidin. These two compounds may represent "lead" molecules for further development of novel immunomodulatory drugs.

5.2 RECOMMENDATIONS

- Further work should be done on the *invitro* study of the ethyl acetate fraction and butanol fraction of *M. lucida* leaves.
- Further studies should be done on the *invivo* immune enhancing studies of the fractions of *M. lucida* plant parts.
- Purification of the other fractions using gel chromatography; column chromatography and identification of the bioactive compounds present
- Structural elucidation of bioactive compounds from *M. lucida* plant parts

5.3 CONTRIBUTIONS TO KNOWLEDGE

- This research work presentsa scientific study on the immunomodulatory properties of active constituents of ethyl acetate fraction and butanol fraction *M. lucida* leave.
- Isolation and identification of the pure bioactive compounds which has being present in the species from pharmacological, microbiological, etc. screening carried out before now by some researchers using HPLC-DAD.
- Validation of the identification of those pure isolated bioactive compounds using state of the art NMR, MS and HPLC-UV techniques which has been isolated before now from the plant.

- Immune enhancing screening of the isolated and identified pure bioactive compounds using at least three models.

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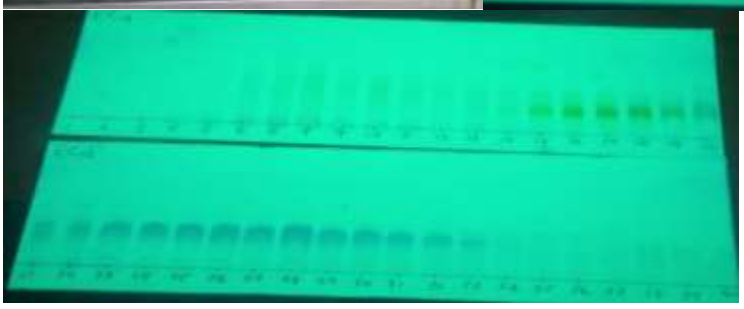
APPENDIX






APPENDIX A: VACCUM LIQUID CHROMATOGRAPHY PROCEDURE



APPENDIX B: GEL CHROMATOGRAPHY PROCEDURE

APPENDIX C: LIQUID-LIQUID FRACTIONATION

APPENDIX D: IMMUNOMODULATORY TEST PROCEDURE

APPENDIX E:TABLE OF MEAN TOTAL WHITE BLOOD CELL (WBC) COUNT (10^9/L)

Group	Treatment	Dose	Basal	Immunosuppressed	After treatment
I	Control	10ml/kg	6.57 ± 0.32	1.82 ± 0.07	2.53 ± 0.33
II	Levamisole	100mg/kg	7.13 ± 0.12	1.90 ± 0.15	6.6 ± 0.27
III	Crude	50mg/kg	7.8 ± 0.12	2.1 ± 0.10	7.26 ± 0.28
IV	Crude	100mg/kg	8.0 ± 0.15	2.08 ± 0.19	5.73 ± 0.73
V	Crude	200mg/kg	7.8 ± 0.15	2.08 ± 0.26	4.13 ± 0.66*
VI	Ethyl acetate fraction	200 mg/kg	6.6 ± 0.23	1.66 ± 0.04	5.56 ± 0.33*
VII	Butanol fraction	200 mg/kg	6.9 ± 0.29	1.66 ± 0.08	3.52 ± 0.18

Values are expressed as Mean ± SEM, (n=5). All the post treatment groups were compared with basal group and immunosuppressed group. Significant values are expressed as (*= $p<0.05$)

APPENDIX F: TABLE OF MEAN LYMPHOCYTE COUNT (%)

Group	Treatment	Dose	Basal	Immunosuppressed	After treatment
I	Control	10ml/kg	78.67 ± 1.20	69.2 ± 1.24	73.33 ± 1.45
II	Levamisole	100mg/kg	80.33 ± 1.45	63.2 ± 2.27	71.25 ± 5.41*
III	Crude	50mg/kg	80.67 ± 1.2	62.4 ± 1.12	81.00 ± 1.70
IV	Crude	100mg/kg	78.67 ± 1.76	67.4 ± 1.50	75.25 ± 2.36*
V	Crude	200mg/kg	79.67 ± 1.20	65.8 ± 2.25	75.67 ± 3.38*
VI	Ethyl acetate fraction	200 mg/kg	76 ± 2.65	69.2 ± 1.24	74.6 ± 2.62
VII	Butanol fraction	200 mg/kg	83 ± 2.08	69.2 ± 2.20	70.2 ± 1.86

Values are expressed as Mean ± SEM, (n=5). All the post treatment groups were compared with basal group and immunosuppressed group. Significant values are expressed as (*= $p<0.05$).

APPENDIX G: TABLE OF MEAN NEUTROPHIL COUNT (%)

Group	Treatment	Dose	Basal	Immunosuppressed	After treatment
I	Control	10ml/kg	25.00 ± 1.16	13.60 ± 0.75	15.00 ± 1.00
II	Levamisole	100mg/kg	29.00 ± 1.73	12.8 ± 0.97	29.5 ± 2.75
III	Crude	50mg/kg	24.33 ± 0.88	13.00 ± 1.10	27.6 ± 2.06
IV	Crude	100mg/kg	23.67 ± 0.67	13.00 ± 0.89	22.00 ± 1.47
V	Crude	200mg/kg	26.67 ± 2.40	12.8 ± 0.97	20.33 ± 0.88*
VI	Ethyl acetate fraction	200 mg/kg	23.33 ± 1.45	12.8 ± 0.73	21.4 ± 0.81
VII	Butanol fraction	200 mg/kg	24.33 ± 2.03	16.4 ± 0.87	20.0± 1.70

Values are expressed as Mean ± SEM, (n=5). All the post treatment groups were compared with basal group and immunosuppressed group. Significant values are expressed as (*= $p<0.05$).

APPENDIX H: TABLE OF MEAN EOSINOPHIL COUNT (%)

Group	Treatment	Dose	Basal	Immunosuppressed	After treatment
I	Control	10ml/kg	2.67 ± 0.33	0.40 ± 0.24	0.33 ± 0.33*
II	Levamisole	100mg/kg	3.00 ± 0.00	0.20 ± 0.20	1.50 ± 0.29
III	Crude	50mg/kg	2.00 ± 0.00	0.00 ± 0.00	1.60 ± 0.24
IV	Crude	100mg/kg	2.67 ± 0.33	0.20 ± 0.20	1.50 ± 0.50 *
V	Crude	200mg/kg	2.33 ± 0.33	0.20 ± 0.20	1.00 ± 0.58
VI	Ethyl acetate fraction	200 mg/kg	2.67 ± 0.33	0.2 ± 0.2	0.4 ± 0.24
VII	Butanol fraction	200 mg/kg	2.67 ± 0.33	0.6 ± 0.24	0.4 ± 0.24

Values are expressed as Mean ± SEM, (n=5). All the post treatment groups were compared with basal group and immunosuppressed group. Significant values are expressed as (*= $p<0.05$).

APPENDIX I:TABLE OF MEAN MONOCYTE COUNT (%)

Group	Treatment	Dose	Basal	Immunosuppressed	After treatment
I	Control	10ml/kg	2.33 ± 0.33	0.20 ± 0.20	0.33 ± 0.33*
II	Levamisole	100mg/kg	2.33 ± 0.33	0.20 ± 0.20	1.75 ± 0.25
III	Crude	50mg/kg	2.00 ± 0.00	0.00 ± 0.00	1.20 ± 0.20
IV	Crude	100mg/kg	1.67 ± 0.33	0.20 ± 0.20	0.75 ± 0.25*
V	Crude	200mg/kg	2.00 ± 0.00	0.20 ± 0.20	0.67 ± 0.33
VI	Ethyl acetate fraction	200 mg/kg	3.00± 0.00	0.4 ± 0.24	0.60 ± 0.24
VII	Butanol fraction	200 mg/kg	3.0 ± 0.58	0.6 ± 0.24	0.60 ± 0.24

Values are expressed as Mean ± SEM, (n=5). All the post treatment groups were compared with basal group and immunosuppressed group. Significant values are expressed as (*= $p<0.05$).

APPENDIX J:TABLE OF DTH RESPONSE FOR CRUDE, ETHYL ACETATE AND BUTANOL EXTRACT OF *M. LUCIDA*

Group	Treatment	Dose	DTH response Mean ± SEM(n=5)	
			0hrs	24hrs
I	Control	10ml/kg	0.78 ± 0.04	1.17 ± 0.07
II	Levamisole	100mg/kg	0.93 ± 0.03	1.12 ± 0.04
III	Crude	50mg/kg	0.78 ± 0.04	1.07 ± 0.03
IV	Crude	100mg/kg	0.85 ± 0.03	1.10 ± 0.06
V	Crude	200mg/kg	0.97 ± 0.03	1.15 ± 0.03
VI	Ethylacetate	200mg/kg	0.83 ± 0.03	1.04 ± 0.04
VII	Butanol	200mgkg	0.82 ± 0.02	1.07 ± 0.03

Values are expressed as Mean ± SEM, (n=5). All the groups were compared with control group.

Significant values are expressed as (*= $p<0.05$).