"PHYTOCHEMICAL INVESTIGATION AND ANTIMICROBIAL ACTIVITY OF *BLIGHIA UNIJUGATA* BAK (SAPINDACEAE)"

BY

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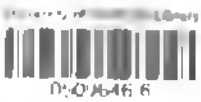

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DECLARATION

This thesis:

a) Is my original work and has not been presented in any other institution for the award of a degree or other qualification.

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b) Has been submitted with our approval as University supervisors.

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LIST OF ABBREVIATIONS AND SYMBOLS

ATCC	American Type Culture Collections
BUCC1	*Blighia unijugata* Chloroform extract Compound 1
BUCC2	*Blighia unijugata* Chloroform extract Compound 2
BUCC3	*Blighia unijugata* Chloroform extract Compound 3
cm	Centimetre
°C	Degrees Celsius
g	Gram
h	Hour
IR	Infrared
KEMRI	Kenya Medical Research Institute
kg	Kilogram
min	Minute
µl	Microlitre
µm	Micrometre
MS	Mass Spectroscopy
NMR	Nuclear Magnetic Resonance
TLC	Thin Layer Chromatography
USA	United States of America
WHO	World Health Organization
%	Percent

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Finally I thank all of those people who unbeknown to me have constantly mentioned my name in prayer or otherwise and prompted the angels of the Lord to strengthen me time and again in my most difficult moments.

DEDICATION

To mum, dad and their children, to Dorcas, Job and Joy for their love, patience and joy!

ABSTRACT

Blighia unijugata Bak (Sapindaceae) is a plant used in traditional medicine for the treatment of various ailments. An infusion of the pounded roots is used to treat fever in East Africa. The roots, pods and leaves of the plant have been used for their hemostatic, anthelminthic and tonic properties. Infections are a major cause of fever and the use of the plant in the treatment of fever may be attributed to phytochemicals with antimicrobial properties. The objectives of this study were to carry out an investigation of the phytochemical constituents as well as to screen for the antibacterial and antifungal activities of the bark of *B. unijugata*.

The plant material was collected from Kiangwachi in Kirinyaga District in November 2007, identified, air-dried, milled and stored in plastic containers. Preliminary phytochemical screening revealed the presence of saponins, tannins and glycosides in the plant. Using a Soxhlet extractor, the milled bark was extracted sequentially with petroleum ether (60-80 °C), chloroform and methanol, each extraction lasting 48 h. Soxhlet extraction was the preferred method of extraction because it is efficient and exhaustive. The chloroform extract was reduced under vacuum and fractionated with the aid of isocratic open column chromatography. Normal-phase silica gel for column chromatography and chloroform were the stationary and mobile phases, respectively. Systematic purification of the fractions thus obtained afforded three compounds which crystallized from a chloroform/ethyl acetate mixture. The purity of these compounds was monitored using thin layer chromatography. With the aid of mass, infrared, nuclear magnetic resonance and ultraviolet/visible spectroscopic data, two of the compounds were identified as the pentacyclic triterpenoids friedelin and epifriedelinol. The structure of the third compound could not be fully elucidated as it was not possible to tell the molecular ion from its crowded mass spectrum.

In vitro antibacterial and antifungal activity of the petroleum ether, chloroform and methanol bark extracts and friedelin was tested using the agar diffusion method. The concentrations used were 50 mg/ml for the extracts and 50 µg/ml for the pure compound. The bacterial strains used in this study were *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*, while the fungi employed included *Cryptococcus neoformans*, *Candida albicans*, *Trichophyton mentagrophytes* and *Microsporum gypseum*. The methanol and chloroform extracts together with the pure compound, friedelin, were active against *S. aureus* with zones of inhibition of 18.0, 22.0 and 10.0 mm, respectively. Gentamicin at a concentration of 10 µg/ml was used as the positive antibacterial control and gave a zone of inhibition of 26.0 mm against *S. aureus*. In the antifungal activity screening, the methanol extract was found to be active against *C. albicans* and *M. gypseum* giving zones of inhibition of 10.0 and 13.0 mm, respectively. The positive antifungal control was fluconazole at a concentration of 25 µg/ml with zones of inhibition of 19.0 and 20.0 mm, respectively, against the two fungal species.

The present study may support the use of this plant in folklore for the treatment of infections where fever is usually present. However, the isolated compounds may not be responsible for the antipyretic activity of the plant. This is due to the fact that the compounds were isolated from a non-polar solvent while only the polar formulations, usually water decoctions and infusions, are used in traditional medicine. Furthermore, the pure compound screened in this study had very low activity. Therefore, the polar extracts – including alcoholic and water extracts – of the plant need to be studied with the aim of isolating more bioactive compounds. Additionally, the plant should be subjected to a wider array of bioassays including antipyretic and antiplasmodial activity testing.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 USE OF PLANTS IN AFRICAN FOLK MEDICINE

Plant-derived products have dominated traditional medicine for thousands of years. The use of natural products with therapeutic properties such as plant, animal and mineral products is reportedly as ancient as human civilization. Plants were an important component of sophisticated traditional medicine systems such as Ayurveda, Unani, as well as Egyptian and Chinese medicine [1, 2, 3]. Some of the earliest recorded plants used for their medicinal properties are *Glycyrrhiza glabra*, *Cupressus sempervirens*, *Papaver somniferum* together with various *Commiphora* and *Cedrus* species. These records exist in ancient scrolls and clay tablets discovered by archaeologists and date as far back as 2600 BC. The use of medicinal plants however is considered to go farther back than these records. Many of the plants used in ancient medicine systems are still in use today for the treatment of ailments ranging from coughs and colds to parasitic infections and inflammation as ingredients of orthodox medicines or herbal preparations [4,5].

Traditional medicine has been practised in one form or another throughout the world. Consequently, different cultures have contributed unique medicinal plants to modern medicine. Current natural product research has rendered credence to the folkloric use of several plants. Besides, some of the plants have been found to possess additional beneficial properties. In Africa, because of the diverse distribution of flora across the continent, communities made use of

locally available plants. For instance, medicinal plants used in the drier northern part of the continent differed significantly from those employed by the communities living in and around the equatorial forest and those living in the southern part of the continent. Well known medicinal plants form the African continent include *Acacia senegal* (Gum Arabic), *Agathosma betulina* (Buchu), *Aloe ferox* (Cape aloes), *Aloe vera* (North African aloe), *Artemisia afra* (African wormwood), *Aspalathus linearis* (Rooibos tea), *Boswellia sacra* (Frankincense), *Catha edulis* (Khat), *Commiphora myrrha* (Myrrh), *Harpagophytum procumbens* (Devil's Claw), *Hibiscus sabdariffa* (Hibiscus, Roselle), *Hypoxis hemerocallidea* (African potato), *Prunus africana* (African Cherry) and *Catharanthus roseus* (Madagascar periwinkle) [5]. Scientific research into the activity of these plants has largely supported their use in traditional medicine.

Acacia senegal is a demulcent emollient gum used internally in inflammation of intestinal mucosa, and externally to cover inflamed surfaces including burns, sore nipples and nodular leprosy. Furthermore, the gum has been used in the treatment of catarrh, colds, coughs, diarrhea, dysentery, gonorrhea, hemorrhage, sore throat, typhoid, urinary tract infections for its antitussive, expectorant, astringent and antimicrobial properties [6,7]. Possible beneficial effects of *A senegal* gum have been demonstrated in patients with chronic renal failure. Orally administered gum lowered serum urea, creatinine, uric acid and phosphorus but increased calcium levels [8]. In a separate study, dietary supplementation with *A. senegal* increased fecal bacterial mass and fecal nitrogen content while lowering serum urea nitrogen in chronic renal failure patients [9].

The leaves of *Agathosma betulina* were traditionally mixed with sheep fat and applied topically or ingested as an infusion to act as an antibiotic protectant. The leaves were chewed or

formulated into 'buchu' and taken to relieve stomach complaints. 'Buchu' was additionally used to treat kidney and urinary tract infections [10]. The essential oil from this plant has been found to possess spasmolytic and weak antimicrobial activity against *Escherichia coli*, *Saccharomyces cerevisiae* and *Staphylococcus aureus* [11].

A decoction of the leaves of *Aloe ferox* was used to wash venereal sores [12]. Using the rat and rabbit models, it has been demonstrated that whole leaf juice of *A ferox* facilitates wound healing. The juice was also reported to inhibit microbial growth [13]. The acetone extract of the plant possesses antimycotic activity [14].

Intravaginally administered fresh leaf juice of *Aloe vera* was used as a contraceptive before or after coitus by the ancient Egyptians [15]. In other regions of the world, the plant has been used for various purposes including the treatment of wounds and burns [16]. Modern clinical use of *A vera* gel began in the 1930s, with reports of successful treatment of X-ray and radiation burns. Studies and case reports provide support for the use of the plant in the treatment of radiation ulcers and stasis ulcers in man and burn and frostbite injuries in animals. The antibacterial and antifungal effect of compounds isolated from *Aloe vera* has been supported in various studies. Most of the healing properties of *A. vera* gel are attributable to the treatment of inflammation. Furthermore, the immunomodulatory properties of the gel polysaccharides have been extensively studied. The antidiabetic and anticancer properties of the plant have been described [17,18,19].

Artemisia afra was used to treat constipation and as an anthelminthic and emetic. The root and aerial parts of this plant have been shown to possess spasmolytic properties. The methanol extract of *A. afra* has been shown to be active against *Staphylococcus aureus*, *Staphylococcus*

epidermidis and *Bacillus subtilis*. The anthelmintic effect of the plant has been demonstrated using *Caenorhabditis elegans* screening [20,21,22]

The medicinal use of *Catha edulis* dates as far back as 1237 AD when an Arabian physician used the plant to treat depression [7]. Khat is however mainly chewed as a recreational and socializing drug The alkaloids cathine and cathinone isolated from the plant have been reported in various studies to possess amphetamine-like effects. Cathinone is more lipophilic than cathine (norpseudoephedrine) and has been demonstrate to have marked euphorigenic and psychostimulant effects in humans [23,24].

Commiphora myrrha was traditionally used to lower fever, relieve pain and inflammation in fractures, treat wounds and snake bites, relieve indigestion and as an aphrodisiac. Compounds isolated from this plant, notably terpenoids from the essential oil possess significant antiseptic, antitumor and anesthetic properties. The resin from *Boswellia sacra* was used to treat mastitis, strengthen teeth, soothe sore eyes and treat emotional and psychological problems. The use of the resin as a purgative and diuretic has also been cited. Incensole acetate, a component of the resin, was found to possess anxiolytic-like and antidepressive like effects in mice. Boswellic acids isolated from the genus *Boswellia* are potent anti-inflammatory agents acting by leukotriene inhibition [25,26,27,28].

For centuries, the dried tubers of *Harpagophytum procumbens* have been used for the treatment of fever, indigestion, malaria, allergies, skin cancer, rheumatism and arthritis [29]. Harpagoside has been isolated from the plant and is responsible for most of the analgesic effect. Several studies have been carried out to assess the efficacy of the plant in managing chronic pain. A study by Wegener and Lupke suggests that the aqueous extract may offer a clinically beneficial

effect in the treatment of arthrosis of the knee or hip [30]. Ganier *et al* report moderate effectiveness of the plant in the treatment of osteoarthritis of the spine, knee and hip and acute exacerbations of chronic non-specific low back pain [31].

In folk medicine, the calyx extracts of *Hibiscus sabdariffa* are used for the treatment of several complaints, including high blood pressure, liver diseases and fever [32]. The antihypertensive effect of the plant has been supported in a study involving Type II diabetic patients with mild hypertension and in patients with essential hypertension [33, 34].

The corm of *Hypoxis hemerocallidea* was widely used in traditional African medicine for the treatment of allergies, ulcers, arthritis, hypercholesterolaemia and infertility [35]. Studies have demonstrated the antinociceptive, anti-inflammatory, antidiabetic, anticonvulsant and hypoglycemic effects of *Hypoxis hemerocallidea* in laboratory models [36,37,38].

The bark and leaves of *Prunus africana* were used to treat fever, gonorrhea, chest and stomach pains, insanity, kidney diseases and many other complaints [39]. Over the last 35 years, the plant has become an over-exploited export product from Africa mainly to European markets for use in the management of benign prostatic hyperplasia. Numerous studies have confirmed the effectiveness of *P. africana* extracts in the treatment of lower urinary symptoms consistent with benign prostatic hyperplasia [40,41,42]. Trade in *P. africana* bark peaked in 1997 when 3225 metric tons were exported. Sales of the bark extracts fetch about $4.36 million annually [43].

A decoction of the roots of *Catharanthus roseus* was taken orally to control blood pressure in folk medicine. The plant was also used for its hypoglycemic effect. Various alkaloids have been isolated from the plant. Ajmalicin is responsible for the hypotensive action of the plant while

catharantin, leurosin, lochnerin, tetrahydroalstonin and vincolin are responsible for the hypoglycemic effects. The plant is currently well known for the anticancer activity of its indolic alkaloids, vincristine and vinblastine [44].

In the mid 19th century, scientists began to isolate active principles from plants. The isolation of quinine from the bark of *Cinchona officinalis* by Caventou and Pelletier was one of the discoveries that sparred greater interest in medicinal plants. By the time of World War 2, morphine, codeine, atropine, hyoscine and digoxin had been isolated [45]. The investigation of natural products as sources of novel human therapeutics reached its peak in the pharmaceutical industry in the period 1970 1980, which resulted in a pharmaceutical landscape heavily influenced by non-synthetic molecules. Out of 877 small-molecule New Chemical Entities (NCEs) introduced between 1981 and 2002, 49 % were natural products, semi-synthetic natural product analogues or synthetic compounds based on natural-product pharmacophores [3].

After the 1980s, there was a decline in natural product chemistry research with a shift to synthetic chemistry attributed to the discovery of high throughput screening against defined molecular targets and advanced bioassay techniques. Advances in synthetic organic chemistry particularly combinatorial chemistry led to the identification of many key chemical molecules that offered more opportunities to develop novel compounds. Many new drugs particularly those now being used to treat infections, infestations, cancers, ulcers, and cardiovascular conditions were produced via this route. Drugs were developed through random screening of thousands of chemicals synthesized as dye-stuffs and other types of chemicals while others resulted from serendipity. Examples of such drugs include sulphonamides, isoniazid, anti-psychotics and anti-histamines [46].

However, in spite of the numerous advances made in drug discovery, the use of plants as a source of medicines has persisted. The WHO estimates that in Africa, 80 % of the population still relies on plants and their crude extracts for their primary health care needs [47]. Furthermore, In Europe, North America and other industrialized regions, over 50% of the population have used complementary or alternative medicine at least once. This percentage is highest in Germany where 90% of the population has used a natural remedy at some point in their life. In the USA, US$ 17 billion was spent on traditional remedies in 2000 [47]. Besides, plants especially those with ethnopharmacological uses have been primary sources of leads for drug discovery. Wang and Liu have reviewed research in natural products and report that the global annual growth rate for trade in herbal products stands at 5-15 % with a market value of about US$ 60 billion per annum [48].

1.2 MODERN MEDICINES FROM HERBAL REMEDIES

About 25 % of prescription drugs used in the developed world is derived from plants [3]. Approximately 80 % of 122 plant derived drugs in current use were related to their ethnopharmacological properties [4,49]. The opium poppy (*Papaver somniferum*) has been used by man for a long time to relieve pain and sedate. Chemical substituents isolated from the plant include morphine, codeine, papaverine, noscapine and thebaine. Morphine and codeine have found widespread use in conventional medicine and numerous modifications have been carried out on these compounds to give newer analgesics and opioid antagonists [50].

Plants have been an important source of anticancer drugs. *Podophyllum peltatum* was found to contain podophyllotoxin, the forerunner of the podophyllins such as teniposide and etoposide. Various cytotoxic agents including vincristine, vinblastine and vindesine were first isolated from

Catharanthus roseus. *Taxus brevifolia* has yielded the useful anticancer agent paclitaxel which is active against tongue, brain, breast and endometrial cancers [51] The Chinese tree *Camptotheca acuminata* Decne. (Nyssaceae) contains the natural anticancer alkaloids camptothecin and 10-hydroxycamptothecin which are used to treat gastric, rectal, colon, bladder, liver, and head and neck cancers. Synthetic derivatives such as topotecan and irinotecan were developed to circumvent water solubility problems of the natural alkaloids [52].

The heart medicine, digitalis, from the leaves of the foxglove, *Digitalis purpurea*, has been used to treat heart failure for many centuries. The earliest available scientific publication of the use of digitalis in the treatment of patients with heart problems dates back to 1785 [53]. Initially, whole *D. purpurea* leaves were dried and powdered but later the single chemical digitoxin was isolated from the leaves and used as a medicine. The less toxic digoxin, isolated from *Digitalis lanata*, is now used in the treatment of heart failure [54].

An extract of the bark of various *Cinchona* species had been used since the 16th century for treating fevers in South America. In 1820, the chemical quinine was isolated from the tree barks. Quinine, the forerunner of the antimalarials chloroquine and mefloquine, is still important in the treatment of severe malaria [55]. Among the newer drugs of plant origin used in malaria treatment in the face of widespread resistance to other antimalarials is artemisinin from *Artemisia annua*. Artemisinin, alone or in combination, is currently the recommended first line therapy for uncomplicated malaria [49].

The major anticoagulant drugs used clinically today are derived from veterinary practice in Canada. In the 1920s veterinary surgeons noticed that cattle developed haemorrhage from eating mouldy hay containing sweet clover (*Melilotus officinalis*). Freshly-cut hay was found to contain

sweet smelling coumarins, many of which act as anticoagulants. Dicoumarol was isolated as a result of these observations. Warfarin, a synthetic derivative of the coumarins, was initially used as a rat poison due to its anticoagulant action but an unsuccessful suicide attempt by a US Army recruit showed it to be a less toxic anticoagulant than dicoumarol. Currently, warfarin is the world's most successful anticoagulant [56].

Currently, extensive research on plants with suspected medicinal properties is being conducted by the pharmaceutical industries as well as research institutions. Several new drugs or formulations derived from plants were approved between 2000 and 2005. A new formulation of apomorphine, a derivative of morphine, was approved for the management of Parkinsonism by the Food and Drug Administration in 2004. Different formulations of the drug have been in use in Europe since the 1970s. A derivative of the tropane alkaloids, tiotropium which is structurally related to ipratropium, is approved for bronchospasm treatment. Another notable approved drug currently in the market is arteether, an antimalarial derived from artemisinin [4].

Apart from providing single chemical entities to modern medicine, many plants used in traditional medicine are now formulated into standardized extracts, tablets, capsules or other forms. These formulations are used as medicines or dietary supplements. *Serenoa repens* (saw palmetto) and *Prunus africana* preparations are available for the management of benign prostatic hyperplasia [57,58]. *Allium sativum* (garlic) is marketed mainly for its antihypercholesterolemic effects [59]. In addition, it has been reported to exhibit antihypertensive properties [60]. Ispaghula (*Plantago ovata*) husk is commonly used as a laxative. Studies have demonstrated the beneficial effects of the husk in patients with irritable bowel syndrome and constipation [61]. *Hypericum perforatum* (St. John's wort) is widely used in the management of mild to moderate hypertension and is known to act by inhibiting neuronal reuptake of various neurotransmitters

[62]. Other commercially available preparations include *Panax ginseng*, *Gingko biloba* for dementia and *Echinacea purpura* for upper respiratory tract infections [63].

1.3 THE SIGNIFICANCE OF RESEARCH INTO THE ANTIMICROBIAL ACTIVITY OF PLANTS

Infectious diseases represent a continuous and increasing threat to human health and welfare especially in developing countries. According to WHO estimations, infectious diseases caused 14.7 million deaths each in 2001, accounting for 26% of the total global mortality [64]. AIDS, tuberculosis, and malaria range among the five major obstacles to increased life expectancy [65]. Because of the high burden of infectious disease worldwide, antimicrobials have been reported as the third most profitable class of drugs for pharmaceutical companies, surpassed only by central nervous system and cardiovascular drugs [66].

The emergence of new diseases such as the HIV/AIDS pandemic has reversed the gains made in healthcare towards the end of the 20th century. This pandemic is itself responsible for the reemergence of diseases such as tuberculosis that were on the decline towards the end of the last century. It is estimated that currently about 2 billion people carry the tubercle bacillus. The HIV/AIDS pandemic has brought to the limelight fungal infections that were otherwise a rare occurrence such as oropharyngeal candidiasis and *Pneumocystis jirovecii* pneumonia. The need for effective drugs to combat these diseases cannot be underestimated.

According to Hacker and Klenk, the emergence of microbes resistant to antibiotics is considered to be a most serious threat to humanity [67]. The global problem of antimicrobial resistance is particularly pressing in developing countries, where the infectious disease burden is high and cost constraints prevent the widespread application of newer, more expensive agents [68].

Multidrug resistant microorganisms are now recognized as a threat to optimal care of patients with infection as well as the viability of current healthcare systems [69]. Plants are a promising source of antimicrobial agents to meet these challenges. Although many plants have proved useful medicinally, it is estimated that less than 10 % of the over 500 000 plant species in the world have been scientifically evaluated for activity. To ensure survival in the world of natural selection, plants have experimented with various chemical defenses over thousands of years to ward off predators, pests and infection. This makes plant species an excellent reservoir of bioactive compounds with the potential for application to treat human diseases [70].

1.4 PLANTS WITH ANTIMICROBIAL PROPERTIES

Although none of the antimicrobials currently in the clinical use is of plant origin, various plants are known to possess antimicrobial properties. Microorganisms, especially fungi, are the greatest source of antibiotics. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids which have been found in vitro to have antimicrobial properties. The common herbs tarragon and thyme both contain caffeic acid, which is effective against viruses, bacteria and fungi An anthraquinone from *Cassia italica* was reported to be bacteriostatic for *Bacillus anthracis*, *Corynebacterium pseudodiphthericum*, and *Pseudomonas aeruginosa* and bactericidal for *Pseudomonas pseudomalliae*. Plants of the Ranunculaceae family yield diterpenoid alkaloids which possess antimicrobial properties. Solamargine, a glycoalkaloid from the berries of *Solanum khasianum*, and other alkaloids may be useful against the Human Immunodeficiency Virus (HIV). Various lignans, diterpenes, triterpenes and coumarins isolated from Chinese herbal remedies have been demonstrated to be active against HIV [52,71].

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1.4 PLANTS WITH ANTIMICROBIAL PROPERTIES

Although none of the antimicrobials currently in the clinical use is of plant origin, various plants are known to possess antimicrobial properties. Microorganisms, especially fungi, are the greatest source of antibiotics. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids which have been found in vitro to have antimicrobial properties. The common herbs tarragon and thyme both contain caffeic acid, which is effective against viruses, bacteria and fungi. An anthraquinone from *Cassia italica* was reported to be bacteriostatic for *Bacillus anthracis*, *Corynebacterium pseudodiphthericum*, and *Pseudomonas aeruginosa* and bactericidal for *Pseudomonas pseudomalliae*. Plants of the Ranunculaceae family yield diterpenoid alkaloids which possess antimicrobial properties. Solamargine, a glycoalkaloid from the berries of *Solanum khasianum*, and other alkaloids may be useful against the Human Immunodeficiency Virus (HIV). Various lignans, diterpenes, triterpenes and coumarins isolated from Chinese herbal remedies have been demonstrated to be active against HIV [52,71].

In one study [72], 267 extracts from 100 medicinal Rwandese plants used to treat infections were evaluated for antifungal, antibacterial and antiviral properties. Of the extracts, 45 % were active against *Staphylococcus aureus*, 2 % against *Escherichia coli*, 16 % against *Pseudomonas aeruginosa*, 7 % against *Candida albicans*, 80 % against *Microsporum canis* and 60 % against *Trichophyton mentagrophytes*. When tested for antiviral activity, 12 % showed activity against poliomyelitis, 16 % against coxsackie, 3 % against Semliki forest, 2 % against measles and 8 % against herpes simplex virus. Similarly, in a study by Sindambiwe and coworkers [73], seven plants used by Rwandan traditional healers to treat infections were all found to be active against measles, Semliki forest, and vesicular stomatitis viruses while only two were active against herpes simplex virus type 1. Four plants were active against gram-positive bacteria, two of them against *Mycobacterium fortuitum*. The researchers isolated a virucidal mixture, maesasaponin mixture A, from the methanol extract of *Maesa lanceolata* which was virucidal against herpes simplex types 1 and 2 and vesicular stomatitis viruses.

Deba and coworkers investigated the antifungal and antibacterial activity of essential oil and aqueous extracts of *Bidens pilosa* flowers and leaves. Activity was reported against the bacteria *Micrococcus flavus*, *Bacillus subtillis*, *B. cereus*, *B. pumilis*, *Escherichia coli* and *Pseudomonas ovalis* as well as the fungi *Corticum rolfsii*, *Fusarium solani* and *F. oxysporum*. Antibacterial activity was higher against Gram-negative organisms as compared to the Gram-negative ones [74].

In a study carried out in Ghana on 25 plants used in traditional medicine, 13 plant species were found to inhibit at least one microorganism among methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *Staphylococcus aureus* (MSSA), *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris*. The greatest antimicrobial

activity was seen with the aqueous extract of *Alchornea cordifolia*, a plant traditionally used to treat wounds. This extract in addition was active against all the 21 strains of bacteria used in the study [75].

The methanol extracts of 10 Cameroonian plants were shown to be active against *Helicobacter pylori*, the bacterium responsible for most duodenal, gastric and peptic ulcer disease. Of the ten, *Ageratum conyzoides*, *Scleria striatinux* and *Lycopodium cernua* had the greatest activity [76]. In a similar study in East Africa, the extracts of *Entada abyssinica* (stem bark), *Terminalia spinosa* (young branches), *Harrisonia abyssinica* (roots), *Ximenia caffra* (roots), *Azadirachta indica* (leaves and stem bark) and *Spilanthes mauritiana* (roots and flowers) were evaluated against 12 strains of *Helicobacter pylori*. The most active extracts were those derived from *T. spinosa* with an MIC_{50} of 125 µg/ml and an MIC_{90} of 250 µg/ml [77].

Elsewhere, extracts from 39 different plants belonging to 22 families used to treat infectious diseases in Bunda district, Tanzania, were screened against twelve microorganisms. The highest activity was obtained for the *n*-hexane extract of *Elaeodendron schlechteranum* root bark against the gram positive bacteria *Bacillus cereus* and *Staphylococcus aureus*. Only *Balanites aegyptiaca* stem bark exhibited high antifungal activity against *Candida albicans*. Extracts from *Lannea schweinfurthii*, *Combretum adenogonium*, *Ficus sycomorus* and *Terminalia mollis* showed strong antiviral activity against *Herpes Simplex* Virus type 1 [78].

Desta [79] screened 315 extracts and fractions from 63 Ethiopian plants used in traditional medicine to antimicrobial screening against known strains of *Staphylococcus aureus*, *Salmonella gallinarum*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Candida albicans*. All the plants showed activity against one or more of the

microorganisms. Aqueous extracts from six plants were active against all of the test organisms. The relative susceptibility of the test organisms to the extracts or fractions were in the decreasing order of *S. aureus, P. aeruginosa, C. albicans, S. gallinarum, E. coli, K. pneumoniae* and *P. vulgaris*.

The hydroalcoholic extracts of *Acokanthera schimperi, Calpurnia aurea, Kalanchoe petitiana, Lippia adoensis, Malva parviflora, Olinia rochetiana, Phytolacca dodecandra* and *Verbascum sinaiticum* traditionally used in Ethiopia for the treatment of skin disorders were screened for antimicrobial activity against different strains of bacteria and fungi known to cause skin infections. *Lippia adoensis* and *O. rochetiana* were found to be the most active species against bacterial and fungal strains respectively. Most of the plant species were found to have activity against at least one microbial strain with *S. aureus* and *Trichophyton mentagrophytes* as the most susceptible bacterial and fungal strains respectively [80].

In Kenya, a lot of work is going on to identify plants with antimicrobial properties with the aim of isolating the bioactive compounds responsible. Research on the antimicrobial activity of *Erythrina burttii* found the bark chloroform extract to be active against fungi and gram-negative bacteria. Flavanoids were reported as the compounds responsible for the activity [81]. In a study of the aqueous, hexane and methanol extracts of 12 plant species traditionally used in Kenya for treatment of infections and inflammatory conditions, the plants were found to be active against gram positive bacteria with nine plant species showing some activity against *Staphylococcus aureus*. The highest activity was found in the methanol extracts of *Maytenus senegalensis, Plectranthus barbatus, Zanthoxylum chalybeum, Zanthoxylum usambarense* and hexane extracts of *Spiranthes mauritianum* [82]. *Hymenodictyon parvifolium*, a plant used traditionally to treat skin infections, dysentery and venereal sores has been found active against *Bacillus subtilis*,

Pseudomonas aeruginosa, *Escherichia coli*, *Staphylococcus aureus*, *Trychophyton mentagrophytes*, *T. interdigitale*, *Microsporum gypseum*, *Epidermophyton flocosum* and *Candida albicans* [83]. Rhoda and Houghton found the methanol extracts and high concentrations of the dichloromethane extract of *Newtonia hildebrandtii* to possess antifungal and antibacterial activity [84]. In unpublished findings, Njogu has reported the activity of *Girardinia divesifolia* methanol, ethyl acetate, chloroform and petroleum ether extracts against *Bacillus pumilus*, *Staphylococcus aureus* and *Escherichia coli*. The ethyl acetate extract was found active against the fungi *Aspergillus niger*, *Candida albicans* and *Saccharomyces cerevisiae*. One of the compounds isolated from the petroleum ether extract, 7-hydroxysitosterol, exhibited activity against *Bacillus pumilus* [85].

1.5 PRINCIPLES OF PHYTOCHEMICAL INVESTIGATION

The major objective of phytochemical studies is the separation, purification and identification of the bioactive chemical components in a plant. Bioactivity may be attributed to a single chemical entity or a fraction. Fractionation is, consequently, key to phytochemical study. The first mechanism of fractionation involves the use of solvents. The plant material may be extracted using solvents of increasing polarity such as petroleum ether, chloroform, methanol and ethyl acetate to separate nonpolar components such as lipids and sterols from the more polar ones. An alternative to sequential extraction is extracting with a polar solvent and partitioning with nonpolar solvents to separate components depending on their polarity. The next and more effective fractionation method is the use of chromatography. Thin layer chromatography, gas chromatography, high performance liquid chromatography and open column chromatography have all been employed. The choice of chromatographic method will depend on the nature of the

sample and the quantities available. The fractions obtained may be tested for activity or worked up to isolate pure chemical compounds, a process that may involve further fractionation [86].

Efficient coupling of fractionation and bioassay can help trace the activity of a complex mixture to one or a few constituents in a mixture. The bioassays are usually carried out using microorganisms, cell cultures, cellular systems such as enzymes, isolated organs, insects or *in vivo* in whole animals. The generally accepted trend is to carry out bioactivity-guided fractionation where fractions or isolated components are tested for activity to eventually obtain active compounds [3]. An example of such a procedure is bioautography where the extract is first subjected to thin layer chromatography to achieve separation of its components. The developed chromatographic plate is subsequently covered with a thin layer of a gar inoculated with a suitable microorganism and the antimicrobial activity of the separated components is detected using an appropriate spray reagent. An alternative approach is the use of fractionation-driven bioassays where the isolated phytochemicals are identified, the known components are ignored and the unknown components subjected to bioassays [86].

Structure elucidation of isolated compounds has been made simpler with the development of state-of-the-art spectroscopic techniques such as infrared (IR) spectroscopy, ultraviolet (UV) spectroscopy, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (MS). Comparison of spectral data with an authentic material or with literature data will usually confirm the structure. For crystalline samples, X-ray crystallography is useful. This method is especially useful in the case of complex terpenoids since it provides both structure and stereochemistry in one operation. With these new techniques structures of as low as sub-milligram quantities of small molecules can be elucidated. Besides, these techniques have greatly accelerated the process of structure elucidation [87]

1.6 LITERATURE REVIEW

1.6.1 The Genus *Blighia*.

Blighia unijugata Bak. is a dicotyledon of the family Sapindaceae. This family comprises about 150 genera and 2000 species. Members of the Sapindaceae family are trees, shrubs or tendrillate climbers characterized by alternate leaves usually without stipules. The flowers are mostly dioecious. The genus *Blighia* comprises four species including *B. sapida*, *B. unijugata* and *B. welwitschii*. These plants all bear a thee-lobed fruit

Blighia sapida K. Koenig has been used for its medicinal properties in different parts of the world. In Brazil an aqueous extract of the seeds is used as a vermifuge. The Cubans have been reported to use the fruits to treat fever and dysentery. The tree bark mixed with spices and the crushed new foliage are used to relieve pain and headache respectively by people in the Ivory Coast. The leaves of the plant are used in West Africa as a poultice on ulcers [88,89].

The chemical constituents of *B. sapida* have been studied extensively following early suspicion that consumption of the fruit causes hypoglycemia. Hypoglycin A, a non-proteinogenic amino acid has been demonstrated to be responsible for the hypoglycemic effect of the fruit. Hypoglycin B, a dipeptide isolated from the seeds of *B. sapida* [90,91] also possesses hypoglycemic activity. Other compounds isolated from the plant are blighinone (a quinone from the fruits), vomifoliol (from the leaves and stems) and 2'-carboxypropylglycine (a non-proteinogenic amino acid from the fruits) [92]. Penders and Delaude have isolated and determined the structure of an acetylated saponin, 3-O-[beta-D-Glc p-(1--)3)-alpha-L-Rha p-(1--)2)-alpha-L-Ara p]-28-O-[beta-D-Glc p-(1--)6)-beta-D-Glc p]hederagenin from *B. welwitschii* [93].

1.6.2 *Blighia unijugata* Bak.

Figure 1: A middle-aged *Blighia unijugata* tree (in the foreground)

Blighia unijugata Bak. is a tree that grows to a height of between 5 and 30 metres. It has a smooth brown-grey back which may have warty excrescences. The plant is mostly found in moist or dry as well as riverine forests. It is known by various names in different communities: mkivule or mwakamwatu (Swahili), mpwakupwaka (Digo), muikoni (Kikuyu), shiarambatsa (Luhya), oggon-ackak (Luo) and muthiama (Meru) [94].

Kokwaro has reported that an infusion of the pounded roots of *B. unijugata* is taken twice daily in East Africa to treat fever [95]. The roots, pods and leaves of the plant have been used for their hemostatic, anthelmintic and tonic properties [7]. The molluscidal activity of the fruits of *B. unijugata* against *Bulinus globosus* and *Bu. truncatus* has been reported [96]. Fowden *et al.*

isolated *trans*-2-(2-carboxymethylcyclopropyl)glycine and other amino acids from the seeds of *B unijugata* [97].

1.7 RATIONALE OF THE PROJECT

There has been a resurgence of interest in recent years in alternative therapies and the therapeutic use of natural products especially those derived from plants. Several reasons have been suggested for this. Firstly, conventional medicines have been alleged to be inefficient owing to their side effects and ineffective therapy for instance in resistant strains of pathogenic microbes. Secondly, a large part of the world has no access to conventional drugs due to poverty. It is also suggested that plants, being natural products are not as harmful as the pure chemicals used in orthodox medicines [50].

The threat of new and reemerging diseases such as human immunodeficiency virus and H5N1 influenza as well as drug resistant tuberculosis and malaria has been recognized as a serious challenge to human health [98]. Drug development is considered one of the important fields of science to tackle this problem [99]. Many diseases that afflict the poor especially in Africa have been neglected in mainstream research and there is need to explore possibilities of developing new and effective molecules useful against these ailments [99].

Ethnobotany is an important starting point for research into medicinal plants [100,101]. Many plants used in traditional medicine have found their way into modern medicine, their properties being confirmed in various scientific studies. The chemical components responsible for the pharmacological effects of many plants have been isolated. *Blighia unijugata* as well as other *Blighia* spp notably *B sapida* have been used traditionally as medicinal plants. No phytochemical and antimicrobial investigations have been reported on the bark *B. unijugata* Bak.

The aim of the proposed study is to isolate and identify the phytochemicals in the plant and test the plant extracts and isolated compounds for antimicrobial activity.

1.7.1 Hypothesis

This research project was based on the hypothesis that the stem bark of *Blighia unijugata* possesses antimicrobial activity due to one or more of its polar chemical principles to which its use in traditional medicine may be attributed.

1.7.2 General objective

To carry out a phytochemical study and investigation of the antimicrobial activity of *Blighia unijugata* Bak.

1.7.3 Specific objectives

1. To obtain extracts of *Blighia unijugata* using petroleum ether, chloroform and methanol.
2. To isolate and establish the chemical nature and/or structures of phytochemical constituents of *B. unijugata* bark.
3. To determine the antibacterial and antifungal activity of *B. unijugata* extracts and isolated compounds.

CHAPTER 2: EXPERIMENTAL

2.1 SOLVENTS, MATERIALS, REAGENTS AND EQUIPMENT

2.1.1 Solvents

General purpose reagents including petroleum ether (60-80 °C), chloroform, methanol, acetone and ethyl acetate (Sigma Aldrich, GmbH, Seelze, Germany) were used. The solvents were distilled in glass apparatus before use

2.1.2 Materials

Whatman filter paper No. 1 (Whatman International Ltd, Maidstone, England) was used in all filtration procedures. Thin layer chromatography aluminium plates precoated with normal phase silica gel 60 GF_{254} (Sigma Aldrich, GmbH, Seelze, Germany) were used for qualitative screening of extracts and in following the purification process of the isolated compounds. Column chromatography was carried out using normal phase silica gel of particle size 32-63 µm (Sigma Aldrich, GmbH, Seelze, Germany).

Kenya Medical Research Institute (KEMRI) Microbiology section kindly donated microorganisms used for antifungal and antibacterial activity screening. *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29737) were employed in antibacterial studies while *Cryptococcus neoformans* (ATCC 66031), *Candida albicans* (ATCC 90028), *Trichophyton mentagrophytes* (clinical isolate) and *Microsporum gypseum* (clinical isolate) were used to screen for antifungal activity. Gentamicin and fluconazole (Troje Medical GmbH, Hamburg, Germany were used as reference antibacterial and antifungal standards, respectively.

Bioautography was carried out using glass plates measuring 20 cm x 20 cm coated with normal phase silica gel 60 GF_{254} for thin layer chromatography. The indicator microorganisms were the bacterium *Bacillus pumilus* (NC 08241) and the fungus *Saccharomyces cerevisiae* (ATCC 9763), both obtained from the Microbiology Laboratory of the Drug Analysis and Research Unit, Department of Pharmaceutical Chemistry, School of Pharmacy, University of Nairobi.

Mueller Hinton Agar (Oxoid Ltd. Basingstoke, Hampshire, England) was used to subculture bacterial strains while Sabouraud's Dextrose Agar (Oxoid Ltd, Basingstoke, Hampshire, England) was used for the fungal strains. Tryptone Soy Agar (Oxoid Ltd, Basingstoke, Hampshire, England) was the growth medium for *S. cerevisiae*.

2.1.3 Equipment

Extraction was carried out using a Soxhlet apparatus (Quickfit, Birmingham, UK) equipped with an electrothermal isomantle (Isopad Isomantle, Weston-super-mare, Avon, England). Extracts were reduced to dryness using a Heidolph VV2000® rotary vacuum evaporator (Heidolph Electro, GmbH, KG, Kelheim, Germany) connected to a Polyscience cooler (Polyscience, Niles, USA) and an N820.3FT Laboport diaphragm vacuum pump (KNF Neuberger GmbH, Freiburg, Germany®).

The uncorrected melting points of the isolated compounds were determined using an SMP10 melting point apparatus (Barloworld Scientific Limited, Stone, Staffordshire, United Kingdom). Fractionation was carried out using open glass chromatographic columns with internal diameter 2 cm connected to a Superfrac™ automatic fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden). Ultraviolet visualization of developed TLC plates was performed on a Min UVIS® ultraviolet light lamp (Desaga GmbH, Heidelberg, Germany). Infrared spectroscopy was

carried out on an IRPRESTIGE-21 FTIR 8400S Fourier transform infrared spectrophotometer (Shimadzu Corporation, Kyoto, Japan). A BUV 12200 UV/visible spectrophotometer (Perkin Elmer Corp., Norwalk, CT, USA) was used to obtain UV/visible spectra. A JEOL GCmatell mass spectrophotometer (Jeol, Peabody, MA, USA) was used to obtain mass spectra in the direct probe inlet electron impact mode at 70 eV. Nuclear magnetic resonance spectra were obtained using a 200 MHz YH200 Varian NMR spectrophotometer (Varian Inc., Palo Alto, CA, USA) supported by Mercury VxWorks software.

2.1.4 Reagents

Vanillin spray reagent was prepared using vanillin (BDH Chemicals Ltd., Poole, England) while iodine vapor was obtained from iodine resublimed general reagent (Merck, Damstadt, Germany). Methylthiazolyl tetrazolium bromide (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used as a microbial viability indicator in bioautography.

2.2 METHODS

2.2.1 Collection and preparation of plant material

The roots, stem bark and leaves of *Blighia unijugata* were collected from Kiangwachi in Kirinyaga district. The plant was identified at the Herbarium of the School of Biological Sciences, University of Nairobi. A voucher specimen, number SOP/DSO/2007/01, was deposited at the School of Pharmacy herbarium. Plant material was air-dried for three weeks, milled and stored in labeled plastic containers.

2.2.2 Extraction

About 1 kg of the milled bark in a muslin bag was introduced into a Soxhlet extractor and extracted sequentially using 5 litres of petroleum ether (60-80 °C), chloroform and methanol, each extraction lasting for 48 h. Soxhlet extraction was the preferred method of extraction because it is efficient and exhaustive. The extracts obtained were reduced using a vacuum rotary evaporator to dryness to afford yields of 0.33 %, 0.35 % and 13.8 % from the petroleum ether, chloroform and methanol extracts respectively

2.2.3 Preparation of reagents

2.2.3.1 Reagents for phytochemical screening

Dragendorff's reagent. About 0.21 g of basic bismuth nitrate was dissolved in a mixture of 2.5 ml acetic acid and 10 ml water to produce solution A. Solution B was prepared by dissolving 2 g of potassium iodide in 5 ml of water. A 1 ml aliquot of each of solutions A and B was added to 4 ml acetic acid and made to 20 ml with water.

Mayer's reagent Approximately 0.27 g of mercuric acetate was dissolved in 12 ml water. At the same time 1 g of potassium iodide was dissolved in 2 ml of water. The two solutions were mixed and made to 20 ml with water.

Keckle's reagent A volume of 5 ml of 3 % ethanolic 3,5-dinitrobenzoic acid with 5 ml of 2M NaOH.

2.2.3.2 Reagents for TLC visualization

About 10 g of iodine resublimed general reagent placed at the bottom of a tightly closed glass chromatographic tank was allowed to sublime and saturate the tank with iodine vapor. A 1 %

w/v vanillin solution was prepared by dissolving about 0.5 g of vanillin in 50 ml of concentrated H_2SO_4.

2.2.3.3 Reagent for bioautography activity indication

A 2.5 mg/ml aqueous solution of methylthiazolyl tetrazolium bromide was prepared by dissolving 5 mg of methylthiazolyl tetrazolium bromide in 20 ml of distilled water.

2.2.4 Phytochemical tests [102]

These tests were conducted on both the roots and bark of *Blighia unijugata* Bak.

2.2.4.1 Tests for cardiac glycosides

Approximately 1 g of powdered plant material in 10 ml of 70 % alcohol was heated in a water bath at 70 °C for 2 min and filtered whilst hot. A 10 ml volume of water and 5 drops of a strong lead subacetate solution were added to the filtrate. To this solution, 10 % H_2SO_4 was added dropwise until no further precipitation was observed. The mixture was filtered and the filtrate extracted twice with 5 ml portions of chloroform. The chloroform extracts were combined, washed with 1 ml of water and filtered. The filtrate was divided into equal portions and evaporated to dryness on two petri dishes. The resulting dried extracts were subjected to the Kedde and Keller-Kiliani tests.

Kedde Test. Two drops of Kedde's reagent were added to one of the dried extracts obtained above. A purple color indicates the presence of cardiac glycosides with an aglycone containing an unsaturated lactone ring.

Keller-Kiliani test. The other extract was dissolved in 0.4 ml of glacial acetic acid containing trace amounts of ferric chloride with gentle shaking. This was followed with the careful addition

of 0.5 ml of concentrated H_2SO_4 down the side of the tube. A reddish-brown color at the interphase gradually turning blue-green indicates the presence of deoxysugars.

2.2.4.2 Tests for saponins

The first test was carried out by adding water to about 0.5 g of powdered plant material in a test tube and shaking. Persistent frothing indicates the presence of saponins.

In the second test, 0.5 g of powdered plant material in 20 ml of water was heated in a water bath at 70 °C for 5 min. The extract was filtered. Two 2 ml aliquots of 1.8 % w/v NaCl solution were introduced into two test tubes. To either test tube were added 2 ml of water and extract respectively. A drop of blood was then added to each test tube. The tubes were gently inverted to mix the contents. The occurrence of hemolysis in the tube containing the extract but not in the second tube indicates the presence of saponins.

2.2.4.3 Tests for anthracene glycosides

Borntrager test: Approximately 0.5 g of the powdered root and bark was boiled in 5 ml of dilute H_2SO_4 for 5 min, filtered while hot and cooled. The filtrate was shaken with an equal volume of CCl_4 to extract. The organic layer was separated and shaken with a few drops of dilute NH_4OH. A rose pink to red color in the ammoniacal layer indicates the presence of oxidized anthracene glycosides.

Modified Borntrager test: This test was carried out in exactly the same manner as the Borntrager test with the addition of a few drops of 5 % $FeCl_3$ when shaking the organic layer with dilute NH_4OH. A rose pink to red color in the ammoniacal layer indicates the presence of reduced anthracene glycosides.

2.2.4.4 Tests for tannins

About 1 g of the powdered material was boiled 10 ml of water for 5 min and filtered. To two 2 ml aliquots of the filtrate were added a few drops of $FeCl_3$ solution and 1 ml of lead subacetate respectively. The appearance of a brown-green precipitate with $FeCl_3$ and a creamy brown precipitate with lead subacetate indicates the presence of tannins.

2.2.4.5 Test for alkaloids

About 1 g of the powdered material was mixed with 5 ml of dilute H_2SO_4 for 5 min, warmed for 2 min in a water bath and filtered. Dilute ammonia was used to alkalinize the filtrate before extracting with 2 ml of chloroform. The residue left after evaporating the chloroform was dissolved in 2 ml of 10 % H_2SO_4 and placed in two petri dishes. A drop of Mayer's reagent was added to one dish. A white to puff precipitate indicates the presence of alkaloids. A drop of Dragendorff's reagent was added to the other petri dish. An orange-red precipitate suggests the presence of alkaloids.

2.2.5 Investigation of antimicrobial activity of *Blighia unijugata*

2.2.5.1 Screening for antibacterial and antifungal activities

The procedure followed for antimicrobial activity screening has been described elsewhere [103]. The agar diffusion assay method was used. Solutions of the extracts, isolates, and antimicrobial reference standards (gentamicin and fluconazole) were prepared in dimethylsulfoxide (DMSO). Dimethylsulfoxide was used as the negative control.

Test microorganisms were subcultured for 18 h to obtain freshly growing strains and suspended in 5 ml of sterile distilled water. Mueller Hinton agar was prepared by introducing 36 g of agar in

one litre of distilled water, boiling to dissolve and sterilizing by autoclaving at 121 °C for 15 min. Sabouraud's dextrose agar and tryptone soy agar were prepared similarly but at concentrations of 30 g/l and 40 g/l, respectively. After sterilization, the media was cooled to 50 °C. The microorganism suspension was inoculated in the cooled growth media such that the resulting agar contained 10^6 colony forming units/ml prior to introduction into petri dishes to give a uniform thickness of 3 mm. Further cooling was allowed to occur.

Cylindrical wells of diameter 6 mm were punched into the set agar using a cork borer and 50 µl aliquots of the test (50 mg/ml for the extracts and 50 µg/ml for the pure compound) and control solutions (10 µg/ml for gentamicin and 25 µg/ml for fluconazole) were introduced into the wells using micropipettes. The concentrations were selected empirically to give optimal zones of inhibition. After introduction of samples and standards, prediffusion was allowed to take place for one hour before incubating the petri dishes with the fungi and bacteria at 23 °C and 37 °C, respectively. Incubation lasted 18 h for the bacteria and 30 h for the fungi. The diameters of the resulting zones of inhibition were recorded.

2.2.5.2 Bioautography

The petroleum ether, chloroform and methanol extracts were dissolved in DMSO to obtain concentration of 20 mg/ml. Clean glass plates were coated with silica gel for TLC, activated and spotted with 100 µl of each of the extracts. Development was carried out using chloroform:methanol (95:5). Standardized inocula of *Bacillus pumilus* and *Saccharomyces cerevisiae* in Tryptone Soy Agar and Sabouraud's Dextrose Agar respectively were uniformly spread on the developed TLC plates to a thickness of 1 mm. After a prediffusion period of 1 h,

the plates layered with *S. cerevisiae* and *B. pumilus* were incubated at 23 °C and 37 °C, respectively. Incubation lasted 18 h for the bacteria and 30 h for the fungi.

Subsequently, the plates were sprayed with an aqueous methylthiazolyl tetrazolium bromide solution and incubated for a further 4 h. Viable microorganisms reduce the dye to a purple, pink or red colored formazan while dead microorganisms appear colorless on the colored background.

CHAPTER 3: FRACTIONATION AND ISOLATION OF PHYTOCHEMICALS

3.1 FRACTIONATION

A slurry of 70 g silica gel in chloroform was prepared and packed in a glass column of internal diameter 2 mm. About 4 g of the dried chloroform extract was then dissolved in chloroform and introduced into the column and eluted isocratically with chloroform. The eluent was collected in fractions of about 5 ml each in test tubes with the aid of an automated fraction collector. A total of 120 fractions were collected. The TLC profile of the fractions was determined by spotting aliquots from every fifth to seventh tube on a TLC plate, developing the plate in a 95:5 % v/v chloroform/methanol mobile phase system and visualizing using short and long UV light, iodine and vanillin spray reagent. Fractions with a similar profile were pooled to yield six merged fractions and left to dry at room temperature.

3.2 ISOLATION AND PURIFICATION OF COMPOUNDS

3.2.1 Isolation of BUCC1

Compound BUCC1 was isolated from the second merged fraction of the chloroform extract column. Upon drying, the merged fraction formed long needlelike crystals. The crystals were cleaned using chilled ethyl acetate to remove pigments. It was observed that these crystals were slightly soluble in the cleaning solvent. The final cleaning phase was accomplished using petroleum ether. The resultant white needles were recrystallized from an ethyl acetate/chloroform. Purity was monitored by TLC using a 95:5 % v/v $CHCl_3$:MeOH mobile phase system. The BUCC1 spot appeared faint yellow in iodine and was strongly fluorescent

quenching under UV light at both 254 and 366 nm. After the plate was sprayed with vanillin reagent and incubated at 105 °C for 10 minutes, the spot stained dark purple in vanillin and had an RF value of 0.80 in 95:5 % v/v chloroform/methanol. The crystals were readily soluble in chloroform.

3.2.2 Isolation of BUCC2

Compound BUCC2 was isolated from merged fraction 3 of the chloroform extract column. The fraction dried at room temperature to form platy crystals. These were cleaned using chilled ethyl acetate to afford colorless plates. These were recrystallized from a chloroform/ethyl acetate mixture. Purity was monitored by TLC using a 95:5 % v/v chloroform/methanol mobile phase system. The BUCC2 spot stained faint yellow in iodine but did not show any fluorescent quenching at both 254 and 366 nm. The compound had an RF value of 0.70 in 95:5 % v/v chloroform/methanol.

3.2.3 Isolation of BUCC3

Merged fraction 5 of the chloroform extract column on drying at room temperature afforded a dark green powder. This was cleaned with chilled ethyl acetate to remove pigments. The resulting yellowish powder was redissolved in chloroform and left to crystallize at room temperature. After two weeks, large white cubic crystals formed. Further recrystallization was carried out to purify these crystals and the process was monitored by TLC. Thin layer chromatography showed the crystals to be strongly fluorescence quenching at 254 nm, and to stain dark purple with vanillin. The spot did not stain in iodine.

Table 1 gives the yields obtained for the three isolated compounds.

Table 1: Yields of isolated compounds

Compound	Yield per kg of material (mg)	Yield (%)
BUCC1	12.2	0.00122
BUCC2	10.8	0.00108
BUCC3	25.6	0.00256

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Results of Phytochemical Tests

The results of phytochemical screening are shown in Table 2. The plant was found to contain saponins, glycosides, tannins, deoxy-sugars and tannins. Some of these components have been shown to be bioactive [71].

Table 2: Results of Phytochemical Screening

Phytochemical class	Root	Stem bark
Saponins	-	+
Glycosides	+	+
2 Deoxy-sugars	+	+
Anthracene glycosides	+	-
Tannins	+	+

4.2 Structure Elucidation

Structure elucidation of the isolated compounds was attempted using the spectroscopic data and physical characteristics of the isolated compounds.

4.2.1 BUCC1

Melting point: 261-262 °C (literature 262-263 °C [104])

The following spectral data was obtained for compound BUCC1:

UV λ_{max} nm: Broad band

IR ν_{max} (KBr) cm^{-1}: 2968 (CH_3, C-H str), 2866 (CH_2, C-H str), 1714 (C=O str), 1458 (CH_3, C-H bend)

MS m/z (rel. int.): 426 (M^+, 79), 411 (18), 341 (10), 302 (40), 273 (59), 246 (37), 232 (35), 218 (46), 205 (54), 191 (31), 179 (45), 163 (44), 161 (27), 149 (28), 137 (42), 134 (32), 125 (80), 123 (82), 107 (45), 95 (98), 81 (74), 69 (100), 55 (65), 43 (23).

H-NMR ($CDCl_3$, 100 MHz) δ: 2.357 (m, H-2), 2.264 (q, H-4), 0.867 (d, H-23), 0.721 (s, H-24), 0.857 (s, H-25), 0.997 (s, H-26), 1.047 (s, H-27), 1.177 (s, H-28), 0.950 (s, H-29), 0.975 (s, H-30).

^{13}C NMR ($CDCl_3$, 100 MHz) δ: 22.3 (C-1), 41.5 (C-2), 213.3 (C-3), 58.2 (C-4), 42.2 (C-5), 41.3 (C-6), 18.2 (C-7), 53.1 (C-8), 37.4 (C-9), 59.4 (C-10), 35.6 (C-11), 30.5 (C-12), 39.7 (C-13), 38.3 (C-14), 32.4 (C-15), 36.0 (C-16), 30.0 (C-17), 42.7 (C-18), 35.3 (C-19), 28.2 (C-20), 32.7 (C-21), 39.2 (C-22),7.0 (C-23), 14.8 (C-24), 17.8 (C-25), 20.1 (C-26), 18.0 (C-27), 32.0 (C-28), 35.1 (C-29), 31.9(C-30).

The electron impact mass spectrometry (EIMS) of compound BUCC1 gave a molecular ion (M^+) with a mass-to-charge ratio (m/z) of 426 and a relative intensity of 79.2 %. This is consistent with the molecular formula $C_{30}H_{50}O$. The loss of a methyl group gives an ion at m/z 411. The ion with an m/z value is obtained by two bond fission between C-13 and C-18 and between C-14 and C-15 followed by loss of a proton. Two bond fission between C-12 and C-13 and between C-8 and C-14 gives the ion with m/z 205. Ions with m/z values below 137 are generally formed through multi-step fissions. The MS data obtained is in agreement with literature. The mass spectrum of the compound gives several peaks some of which are illustrated in the proposed fragmentation pattern based on work by Hirota *et al.* [105]. Table 3 is a comparison of the major fragments obtained for BUCC1 and those given in literature for friedelin [106].

The ^{13}C NMR spectrum has 32 resonance lines corresponding to the 30 carbon atoms of friedelin. The extra two lines are explained by the fact that C-29 and C-30 may be rotating and changing orientations repeatedly and the instrument therefore detects two resonances per atom. The resonance lines assigned to C-29 and C-30 for this reason are the pairs 34.9, 35.1 and 31.7, 31.9 respectively. Distortionless Enhancement by Polarization Transfer (DEPT) shows the compound to have 8 methyl, 11 methylene and 4 methine carbon atoms. The total number of protonated carbon atoms is 23.

The most downfield resonance line at 213.3 is characteristic of highly magnetically deshielded atoms and is assigned to the carbonyl at C-3. C-4 is deshielded by the carbonyl at C-3 and has a δ value of 58.2. Two methane carbon atoms, C-8 and C-10, resonate at relatively high fields because of their proximity to the deshielding carbonyl group and the absence of attached methyl groups which would have an inductive shielding effect on these atoms. The methyl carbons have the lowest delta values. The NMR spectrum of BUCC1 was compared with literature values for friedelin in Table 4 and found to correlate well [106].

In the H-NMR spectrum, the protons attached to the carbon atoms adjacent to the carbonyl have the most downfield shifts at δ 2.357 and 2.264 for H-2 and H-4, respectively. The highly shielded methyl protons resonate upfield and are assigned their shifts in comparison with literature. The C-24 methyl protons are the most shielded at δ 0.721.

The UV absorption spectrum of this compound is a low intensity broad band from about 220 nm to 350 nm which is characteristic of ketones. Infrared spectroscopy confirms the presence of a ketone carbonyl at 1714 cm^{-1} and of methyl groups at 2968 cm^{-1}. Using the spectroscopic data it was possible to identify BUCC1 as friedelin.

Table 3: Comparison of major m/z (rel. int.) values for BUCC1 and friedelin

BUCC1	Friedelin	BUCC1	Friedelin
426 (79)	426 (18)	218 (46)	218 (28)
411 (18)	411 (10)	205 (54)	205 (40)
341 (10)	341 (5)	125 (80)	125 (73)
302 (40)	302 (18)	95 (98)	95 (94)
273 (59)	273 (36)	69 (100)	69 (100)

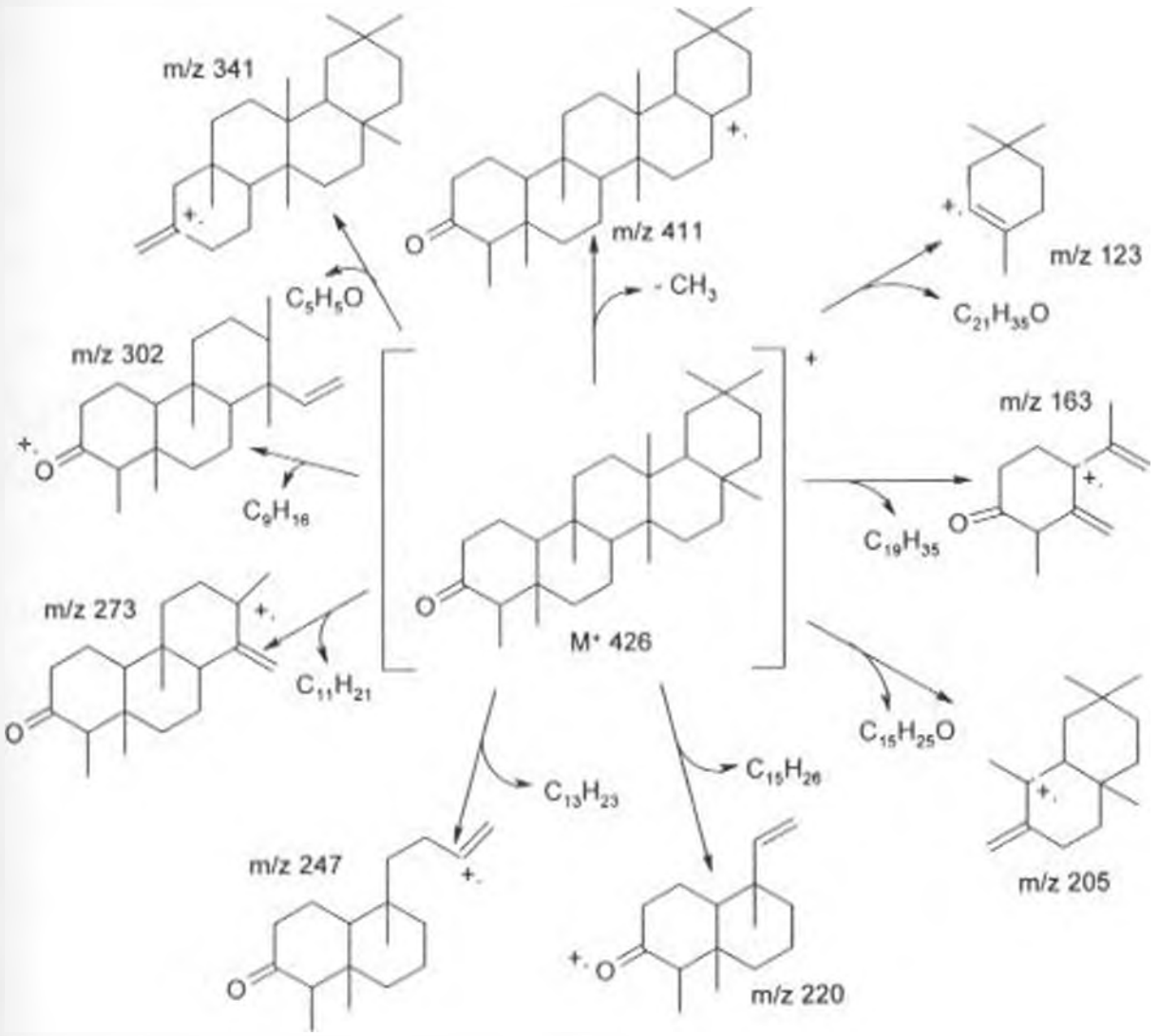


Figure 2: Proposed fragmentation pattern of BUCC1

Table 4: A comparison of ^{13}C NMR data for friedelin and BUCC1 [107]

Atom	Delta value		Atom	Delta value	
	BUCC1	Friedelin		BUCC1	Friedelin
C-1	22.3	22.3	C-16	36.0	36.0
C-2	41.5	41.5	C-17	30.0	30.0
C-3	213.3	213.2	C-18	42.7	42.8
C-4	58.2	58.2	C-19	35.3	35.3
C-5	42.2	42.1	C-20	28.2	28.1
C-6	41.3	41.3	C-21	32.7	32.7
C-7	18.2	18.2	C-22	39.2	39.2
C-8	53.1	53.1	C-23	7.0	6.8
C-9	37.4	37.4	C-24	14.8	14.6
C-10	59.4	59.4	C-25	17.8	17.9
C-11	35.6	35.6	C-26	20.1	20.2
C-12	30.5	30.5	C-27	18.0	18.6
C-13	39.7	39.7	C-28	32.0	32.1
C-14	38.3	38.3	C-29	35.1	35.0
C-15	32.4	32.4	C-30	31.9	31.8

4.2.2 BUCC2

Melting point: 279-282 °C (literature 280-283 °C [108])

Compound BUCC2 had the following spectral data

IR ν_{max} (KBr) cm^{-1}: 3476 (O-H str), 2933 (CH_3, C-H str), 2870 (CH_2, C-H str), 1458 (CH_3, C-H bend)

MS m/z (rel. int): 428 (M^+, 34), 413 (26), 395 (10), 275 (40), 234 (30), 231 (32), 220 (38), 205 (35), 191 (25), 179 (28), 177 (39), 165 (77), 161 (15), 149 (48), 137 (38), 135 (34), 125 (67), 123 (63), 121 (57), 119 (17), 109 (80), 107 (47), 96 (77), 95 (100), 93 (37), 81 (66), 79 (20), 69 (81), 67 (36), 57 (20), 55 (52), 43 (21), 41 (29).

H-NMR ($CDCl_3$, 100 MHz) δ: 3.674 (br d, H-3), 0.899 (br m, H-23, H-24, H-29), 0.830 (s, H-25), 0.967 (br m, H-26, H-28), 0.949 (s, H-27), 1.134 (s, H-30).

^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 16.0 (C-1), 35.6 (C-2), 72.8 (C-3), 49.2 (C-4), 37.2 (C-5), 41.9 (C-6), 17.7 (C-7), 53.3 (C-8), 38.0 (C-9), 61.5 (C-10), 35.4 (C-11), 30.8 (C-12), 39.8 (C-13), 38.5 (C-14), 33.0 (C-15), 36.2 (C-16), 30.2 (C-17), 43.0 (C-18), 35.9 (C-19), 28.2 (C-20), 32.5 (C-21), 39.3 (C-22), 11.9 (C-23), 16.4 (C-24), 18.3 (C-25), 18.7 (C-26), 20.2 (C-27), 32.2 (C-28), 35.2 (C-29), 32.0 (C-30).

The EIMS spectrum of this compound gave a molecular ion (M^+) with a mass-to-charge ratio (m/z) of 428 and a relative intensity of 34.3 % consistent with the molecular formula $C_{30}H_{52}O$. The peak at m/z 413 (M-15) corresponds to the loss of a methyl group while a peak at m/z 395 (M-33) corresponds to the loss of a methyl group together with a water molecule. The rest of the

pattern is typical for the friedelane skeleton [105]. The proposed fragmentation pattern is given in figure 3.

The ^{13}C NMR spectrum has 30 resonance lines corresponding to 30 carbon atoms. The most downfield signal at δ 72.8 is assigned to C-3 which bears the hydroxyl group. C-4, C-8 and C-10 are the next most deshielded by their proximity to the hydroxyl group and have relatively high δ values. The rest of the resonance lines were assigned to the other carbon atoms by comparison with literature as shown in Table 5 [108]. The H-NMR spectrum was not well resolved and most of the peaks appear as broad multiplets. However, the alcoholic proton attached to C-3 has a resolved peak at δ 3.674.

The compound does not absorb UV light to any extent confirming the absence of a conjugated system. The IR spectrum of the compound confirms the presence of a hydroxyl group due to the stretch at 3476 cm^{-1}. The rest of the spectrum is relatively similar to that of friedelin with the major difference being the absence of a carbonyl band. Using the spectroscopic data, it was possible to identify compound BUCC2 as friedelinol.

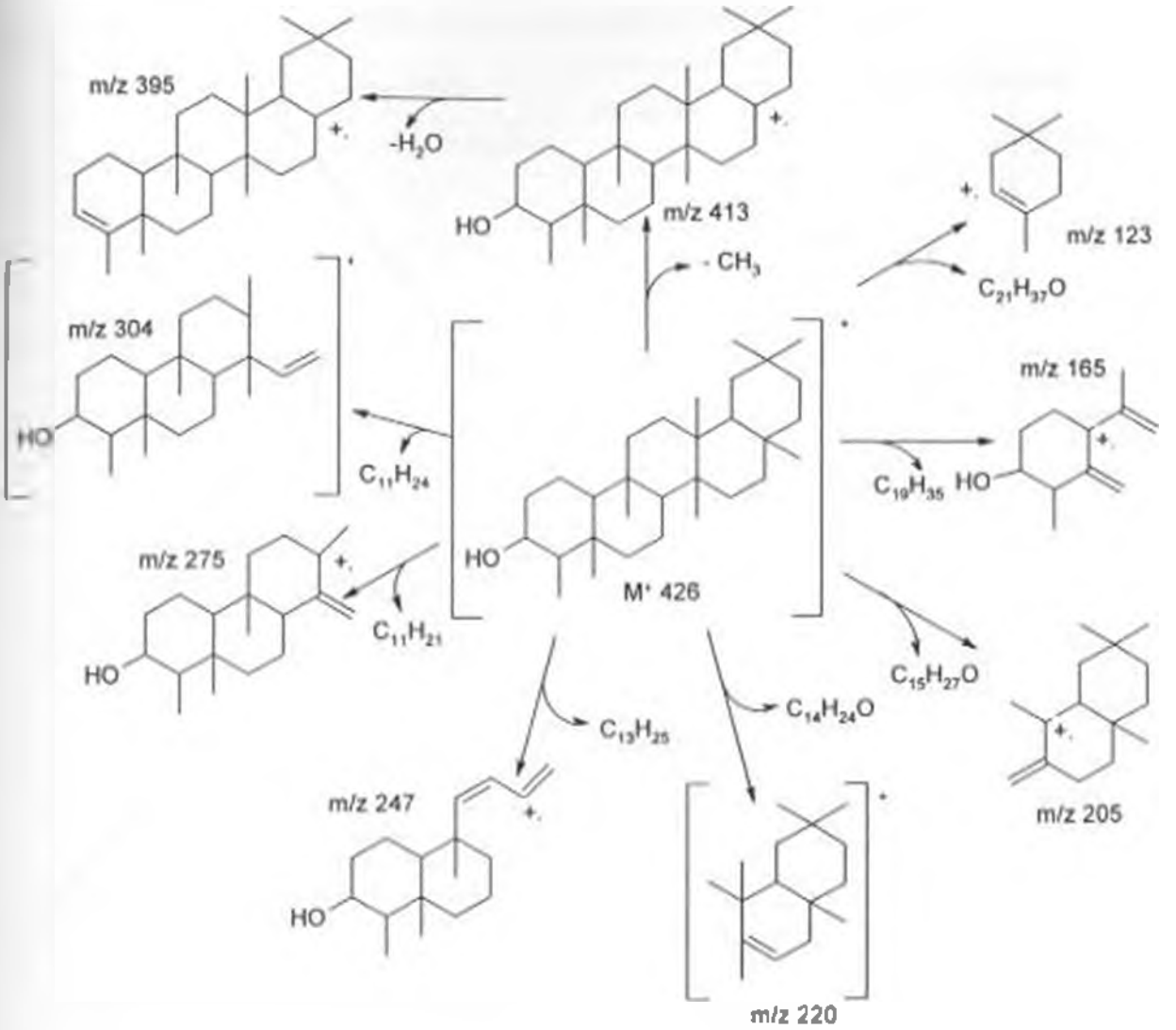
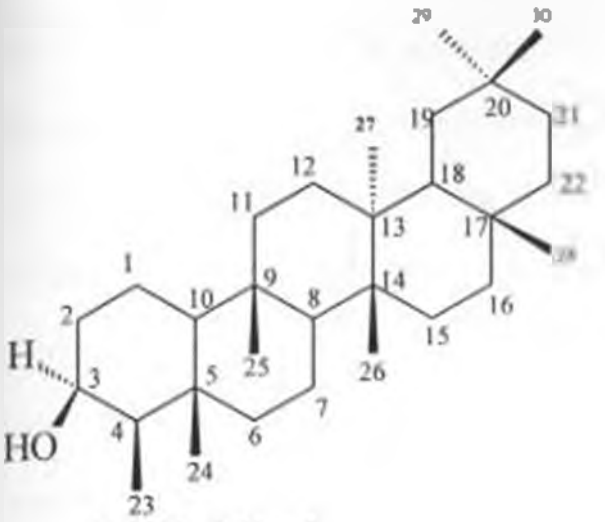


Figure 3: Proposed fragmentation pattern of BUCC2

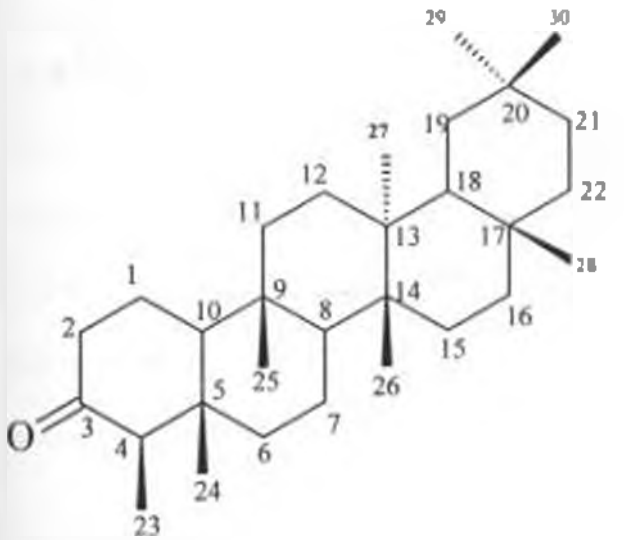
Table 5: A comparison of ^{13}C NMR data for epifriedelinol and BUCC2 [108]

Atom	Chemical Shift		Atom	Chemical Shift	
	BUCC2	Epifriedelinol		BUCC2	Epifriedelinol
C-1	16.0	16.2	C-16	36.2	36.5
C-2	35.6	35.7	C-17	30.2	30.0
C-3	72.8	73.1	C-18	43.0	43.2
C-4	49.2	49.6	C-19	35.9	35.9
C-5	37.2	37.5	C-20	28.2	28.7
C-6	41.9	42.1	C-21	32.5	32.7
C-7	17.7	17.9	C-22	39.3	39.7
C-8	53.3	53.6	C-23	11.9	12.0
C-9	38.0	38.2	C-24	16.4	16.8
C-10	61.5	61.8	C-25	18.3	18.6
C-11	35.4	35.6	C-26	18.7	19.0
C-12	30.8	31.0	C-27	20.2	20.5
C-13	38.5	38.7	C-28	32.2	32.5
C-14	39.8	40.0	C-29	35.2	35.4
C-15	33.0	33.2	C-30	32.0	32.2

Epimerization occurs in the molecule $C_{30}H_{52}O$ because the hydroxyl group can take two different conformations. The resulting two isomers are 3α-friedelinol (friedelinol) and 3β-friedelinol (epifriedelinol). The melting points of the two epimers differ by over 10 °C with epifriedelinol having the lower melting point [108]. Thus with the aid of the melting point the epimer isolated was identified as 3β-friedelinol or epifriedelinol.



Epifriedelinol



Friedelin

Figure 4: Chemical structures of epifriedelinol and friedelin showing the numbering of carbon atoms

4.3 Results of antimicrobial studies

The results of antimicrobial screening are shown in Tables 6 and 7. The methanol and chloroform extracts and friedelin exhibited activity against *Staphylococcus aureus* with zones of inhibition of 18.0, 22.0 and 10.0 mm, respectively. No activity was recorded against the other bacterial strains tested. From these findings it is evident that the chloroform extract had greater activity compared to the methanol extract. The extracts were tested at the same concentration. No comparison can be made with friedelin although it is worth noting that the compound displayed some activity at the tested concentration.

Since friedelin was isolated from this extract, it can be concluded that part of the antimicrobial activity is due to the isolated compound. Other chemicals in the extract may be responsible for the high activity seen in this extract. The activity of the methanol extract is due to polar components of the plant including the phenols and polyphenols which are known to be potent antimicrobial agents [71]. The concentration of friedelin used in the study is quite high suggesting the compound has very low antimicrobial activity. Other studies have demonstrated that friedelin possesses antibacterial properties and the compound has been isolated from plants that have antimicrobial activity [109]. Elsewhere, both friedelin and epifriedelinol have been reported to possess concentration dependent antibacterial activity [110].

The methanol extract displayed antifungal activity against *Candida albicans* and *Microsporum gypseum* with zones of inhibition of 10.0 and 13.0 mm, respectively. The zones of inhibition observed for the two fungal species were nearly identical. This can be attributed to the high concentration of polar components in this extracts that have high antimicrobial activity [71].

Kuiate *et al.* isolated and tested friedelin against the dermatophytes *Microsporum audouinii*, *Trichophyton soudanense* and *T. mentagrophytes* and recorded MICs of more than 100, 50 and 25 µg/ml for the three organisms respectively [111]. In the present study, the MIC was not determined.

Elsewhere the antimalarial activity of the friedelanes has been reported [112]. In this study, Ngouamegne and coworkers tested friedelin and six other compounds isolated from *Endodesmia calophylloides* against the multi-drug resistant *Plasdmodium falciparum* W2 strain. The IC_{50} range for the tested compounds was 7.2 to 18 µM with friedelin giving the lowest value. This finding led the researchers to conclude that friedelane derivatives might be interesting sources for antimalarial leads.

Table 6: Antibacterial activity of *Blighia unijugata* bark extracts and compound BUCC1 against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Test solution	Diameters of zones of inhibition (mm)		
	E. coli	*Ps. aeruginosa*	*S. aureus*
BuPEb (50 mg/ml)	NI	NI	NI
BuCb (50 mg/ml)	NI	NI	22.0
BuMb (50 mg/ml)	NI	NI	18.0
Friedelin (50 µg/ml)	NI	NI	10.0
Gentamicin (10 µg/ml)	25.0	20.0	26.0

BuPEb (petroleum ether extract), BuCb (chloroform extract), BuMb (Methanol extract).
NI = No inhibition observed (Zone diameter 6.1±0.2 mm)

Table 7: Antifungal activity of *Blighia unijugata* bark extracts and compound BUCC1 against *Cryptococcus neoformans*, *Candida albicans*, *Trychophyton mentagrophytes* and *Microsporum gypseum*.

Test solution	Diameters of zones of inhibition (mm)			
	Cr. neoformans	*C. albicans*	*T. mentagrophytes*	*M. gypseum*
BuPEb (50 mg/ml)	NI	NI	NI	NI
BuCb (50 mg/ml)	NI	NI	NI	NI
BuMb (50 mg/ml)	NI	10.0	NI	13.0
Friedelin (50 µg /ml)	NI	NI	NI	NI
Fluconazole (25 µg/ml)	18.1	19.4	18.3	20.1

BuPEb (petroleum ether extract), BuCb (chloroform extract), BuMb (Methanol extract).
NI = No inhibition observed (Zone diameter 6.1±0.2 mm)

CONCLUSION AND RECOMMENDATIONS

The present study was carried out with the aim of isolating phytochemicals from *Blighia unijugata* Bak and testing the plant extracts and isolated compounds for antimicrobial activity. Preliminary phytochemical screening found the plant to contain saponins, glycosides, deoxy-sugars and tannins.

The chloroform extract was fractionated using open column chromatography on normal phase silica gel to yield three crystalline compounds. Two of the compounds were identified using a combination of spectroscopic techniques as the triterpenoids friedelin and epifriedelinol. The structure of the third compound is yet to be elucidated. The spectroscopic techniques used included nuclear magnetic resonance, infra red and mass spectroscopy. Although these compounds are present in many plant species, this study reports their isolation for the first time from *Blighia unijugata*.

In the antimicrobial screening using the agar diffusion method, the chloroform and methanol extracts as well as the isolated compound friedelin were found to be active against *Staphylococcus aureus*. Friedelin has been shown in other studies to possess antibacterial, antifungal and antiplasmodial activity. Therefore, the findings of this study may justify the use of this plant in traditional medicine.

No attempt was made at this stage of the study to fractionate and isolate compounds from the polar extracts of the plant namely the methanol, ethyl acetate and aqueous extracts. Owing to the fact that aqueous formulations are used in traditional medicine, it is highly recommended that further work be focused on polar extracts with the aim of isolating more bioactive constituents.

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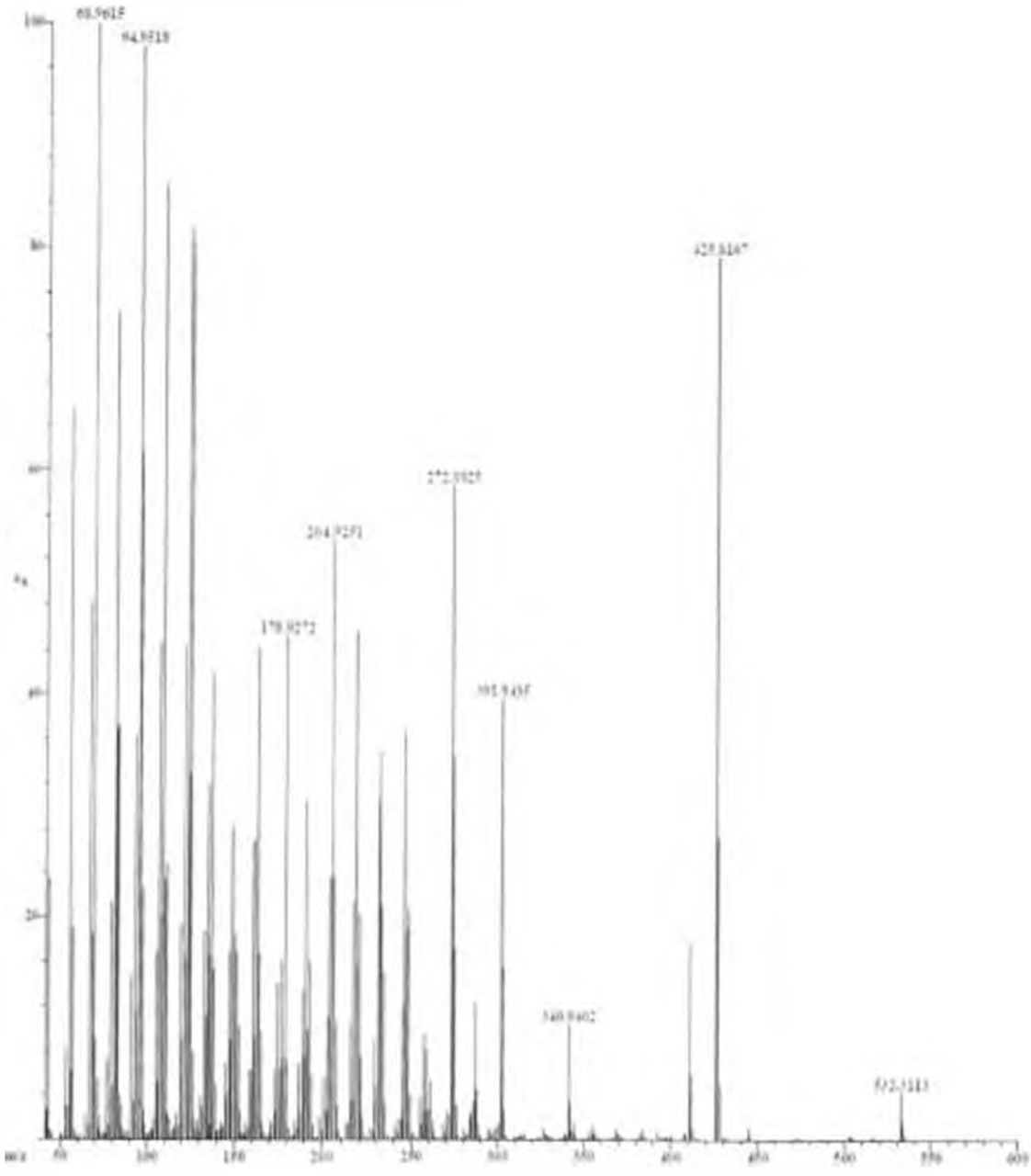
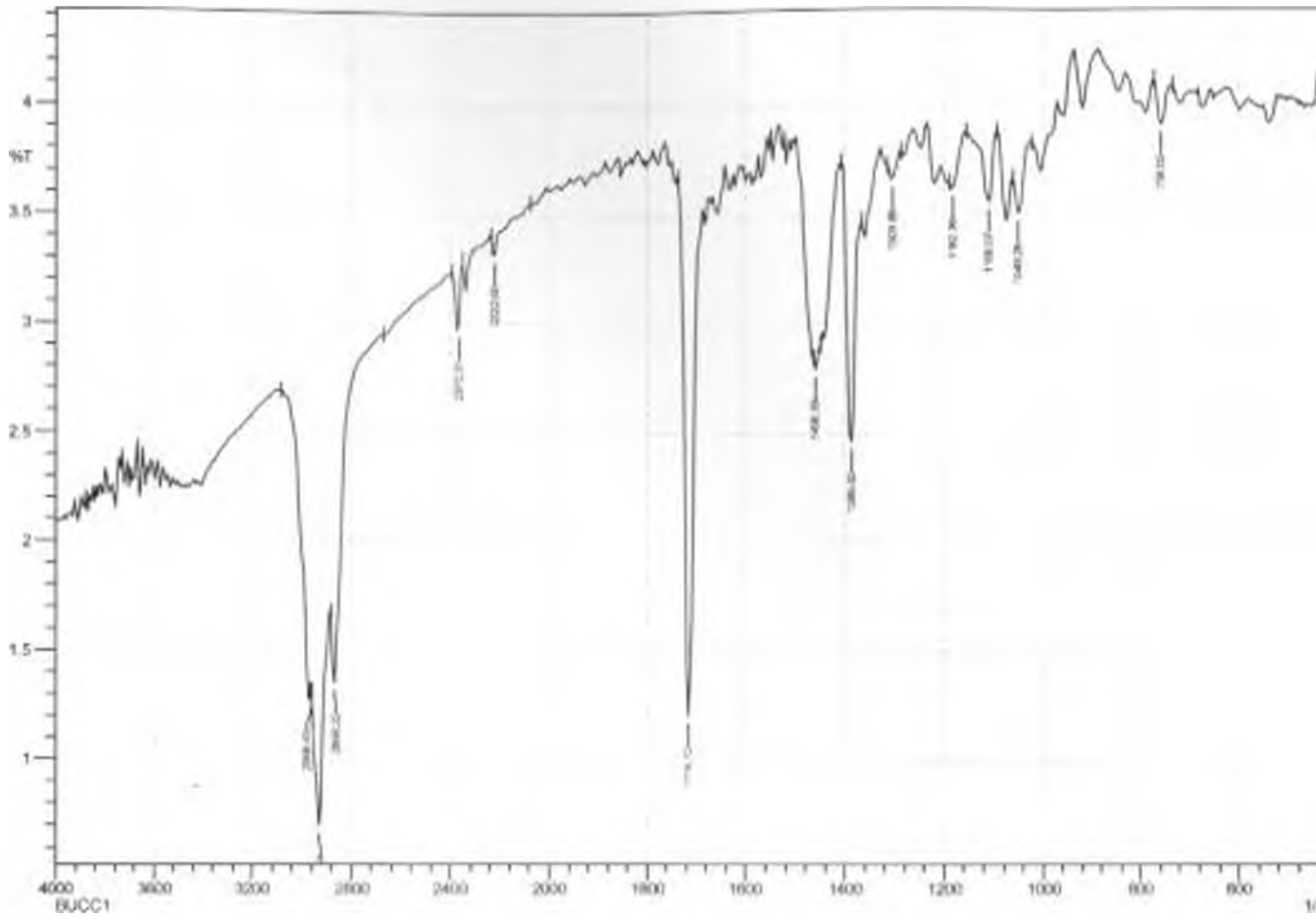
APPENDIX 1: Mass Spectrum of BUCC1 at 70 eV Ionizing Energy

File: 8123 BUCC 1 Date Run: 09.23.2008 (Time Run: 10.32:57)
Sample:
Instrument: JEOL GC mateII
Inlet: Direct Probe Ionization mode: EI-

Scan: 148 R.T.: 1.74
Base: m/z 69; 90.4% TIC : 43702328 Ions: 501

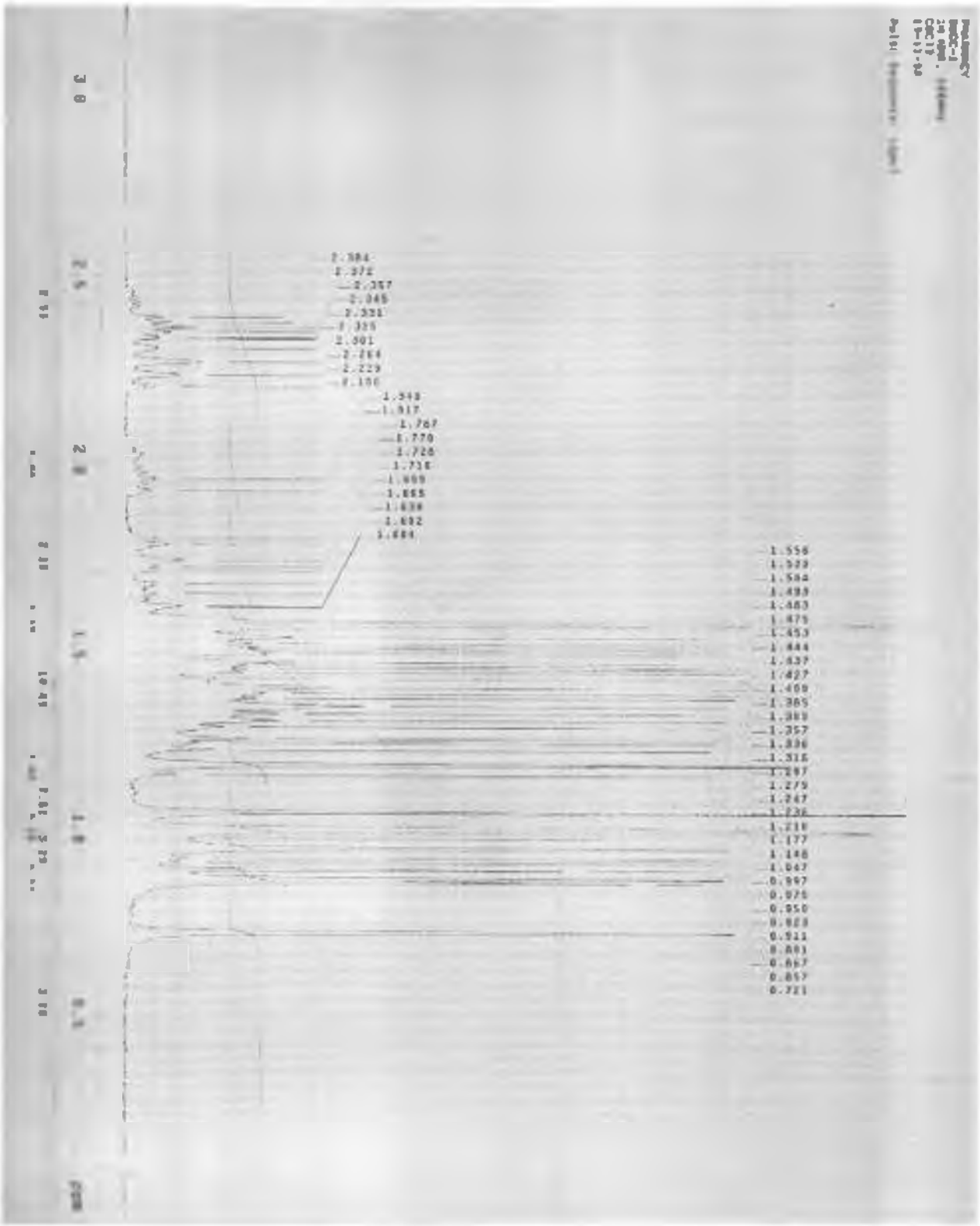


APPENDIX 2: Infrared Spectrum of BUCC1

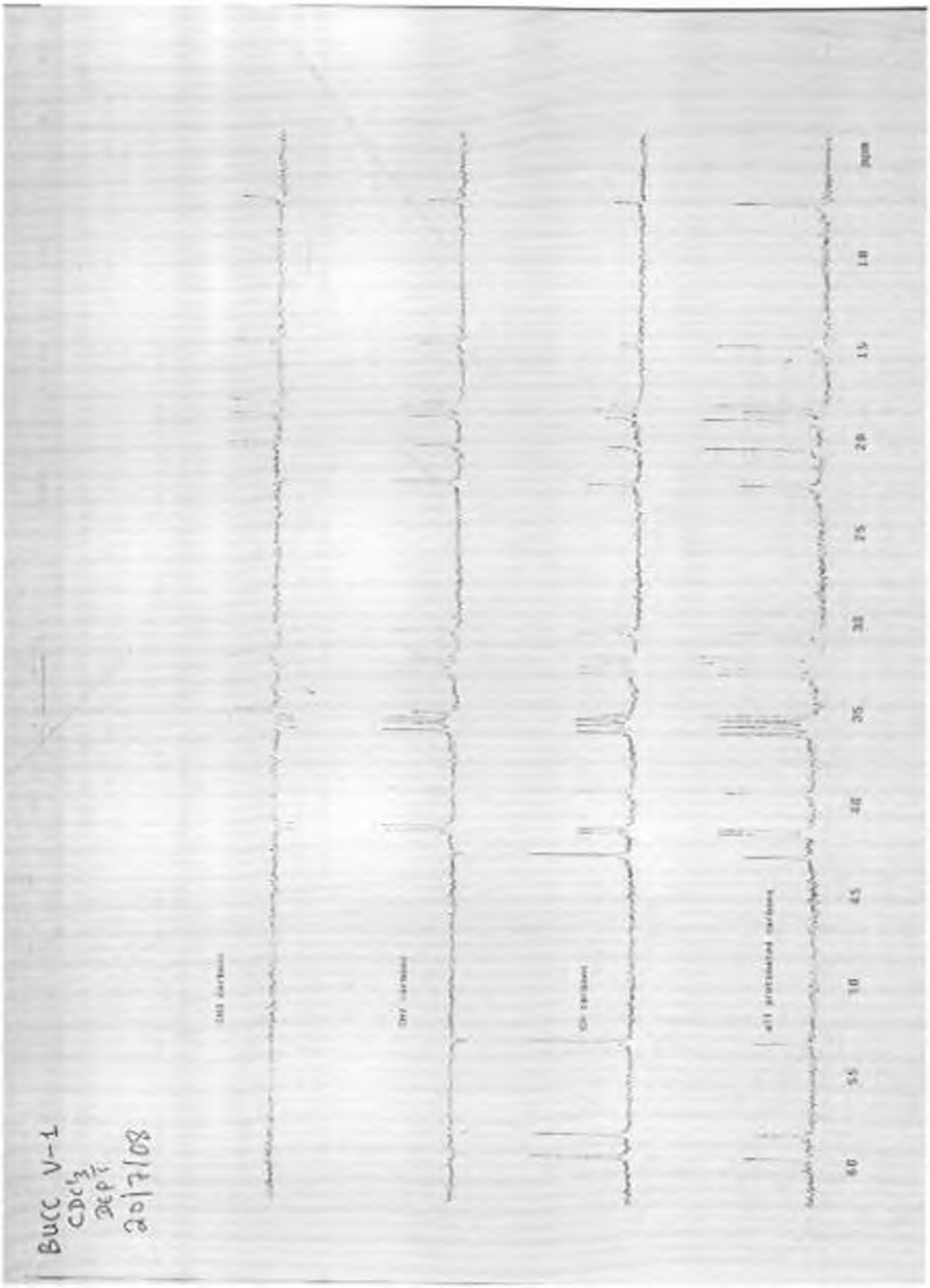


APPENDIX 3: Carbon-13 NMR Spectrum of BUCC1

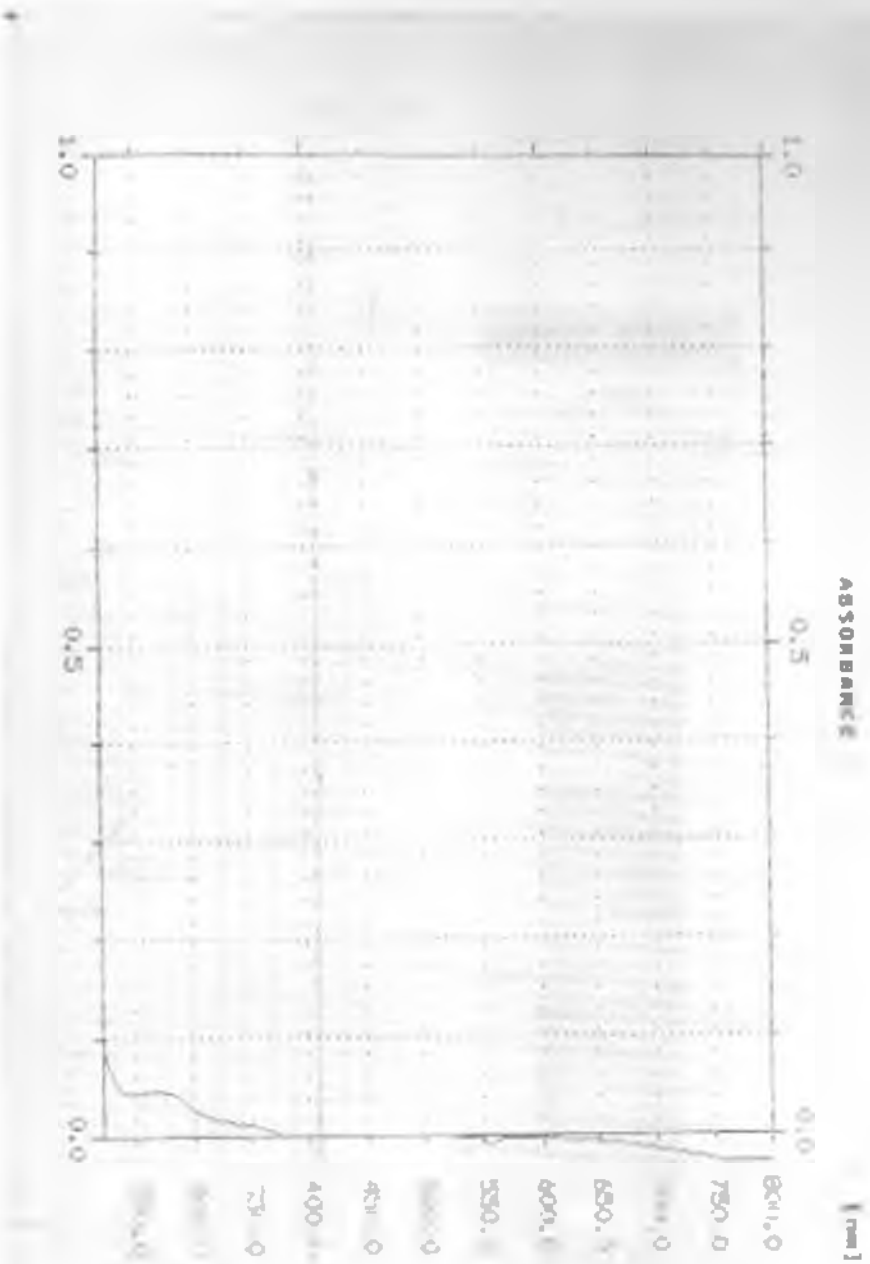
APPENDIX 4: Proton NMR Spectrum of BUCC1



APPENDIX 5: DEPT Spectrum of BUCC1



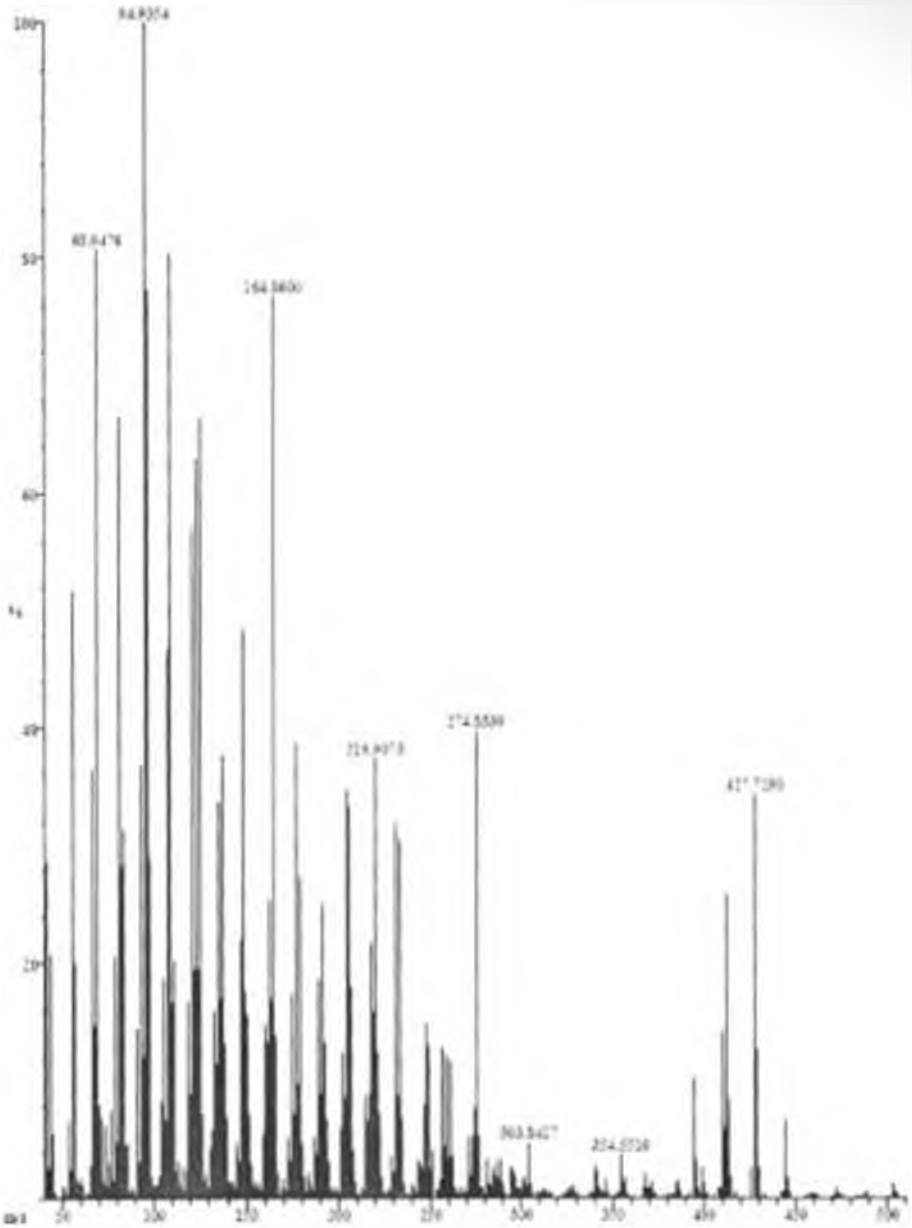
APPENDIX 6: UV/VISIBLE Spectrum of BUCC1



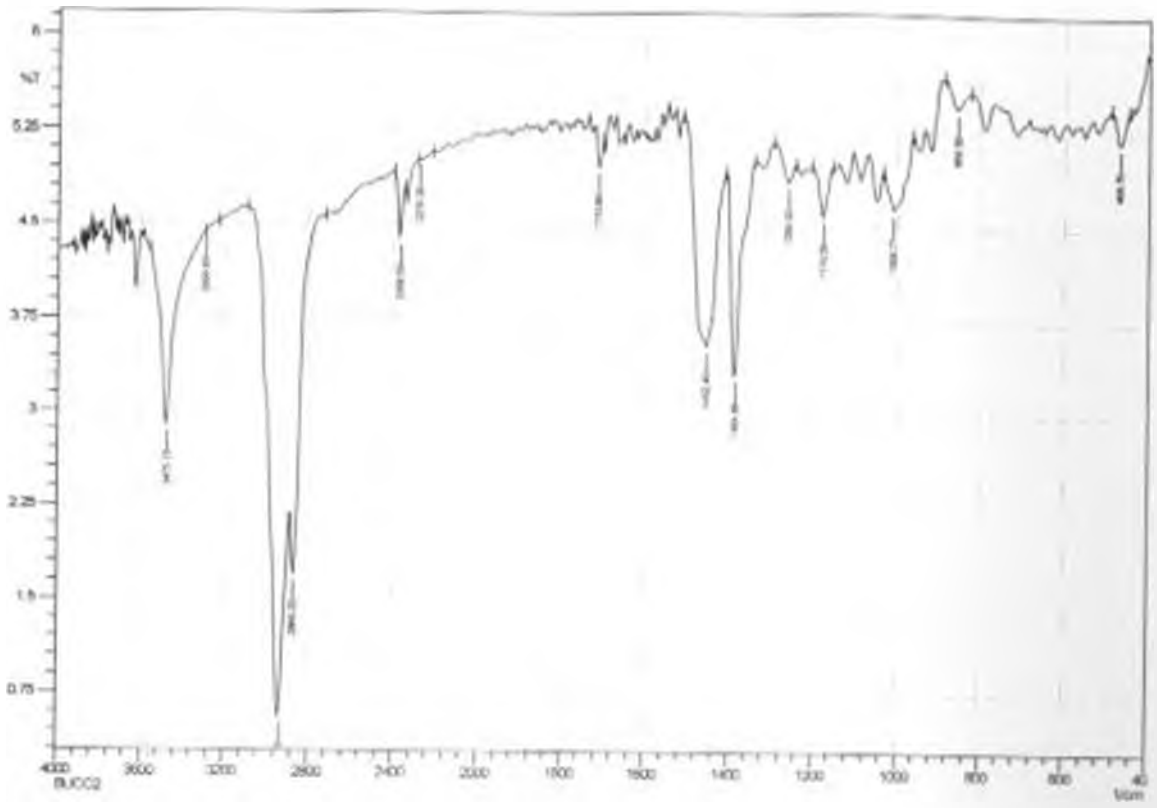
APPENDIX 7: Mass Spectrum of BUCC2 at 70 eV Ionizing Energy

File: 8123-BUCC 2(2) Date Run: [illegible] (Time Run: 10:42:01)
Sample:
Instrument: JEOL GCmate II
Inlet: Direct Probe Ionization mode: EI-

Scan: 160 R.T.: 1.8"
Base: m/z 95; 1499 eFS TIC: 1209216

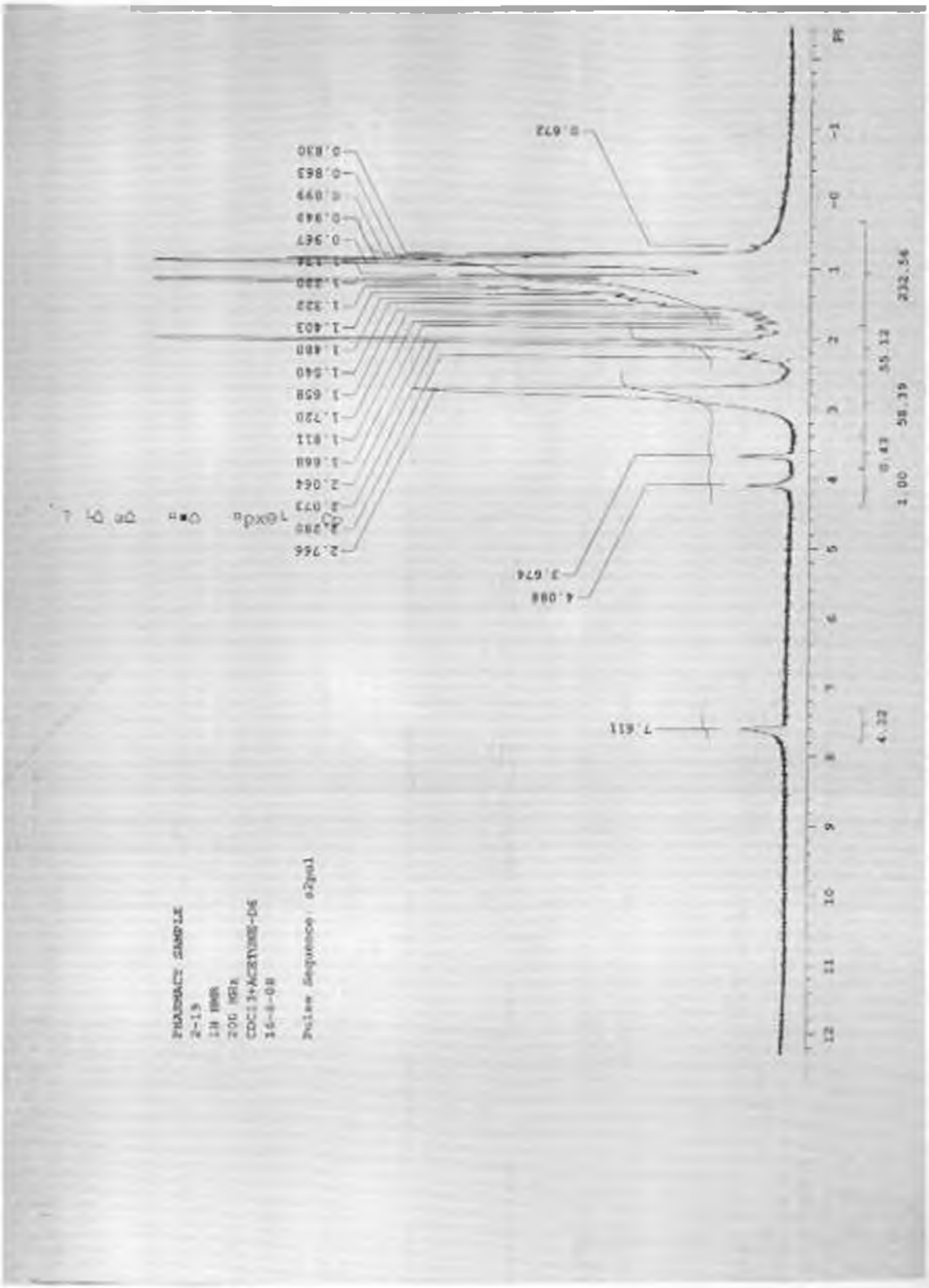


APPENDIX 8: Infrared Spectrum of BUCC2

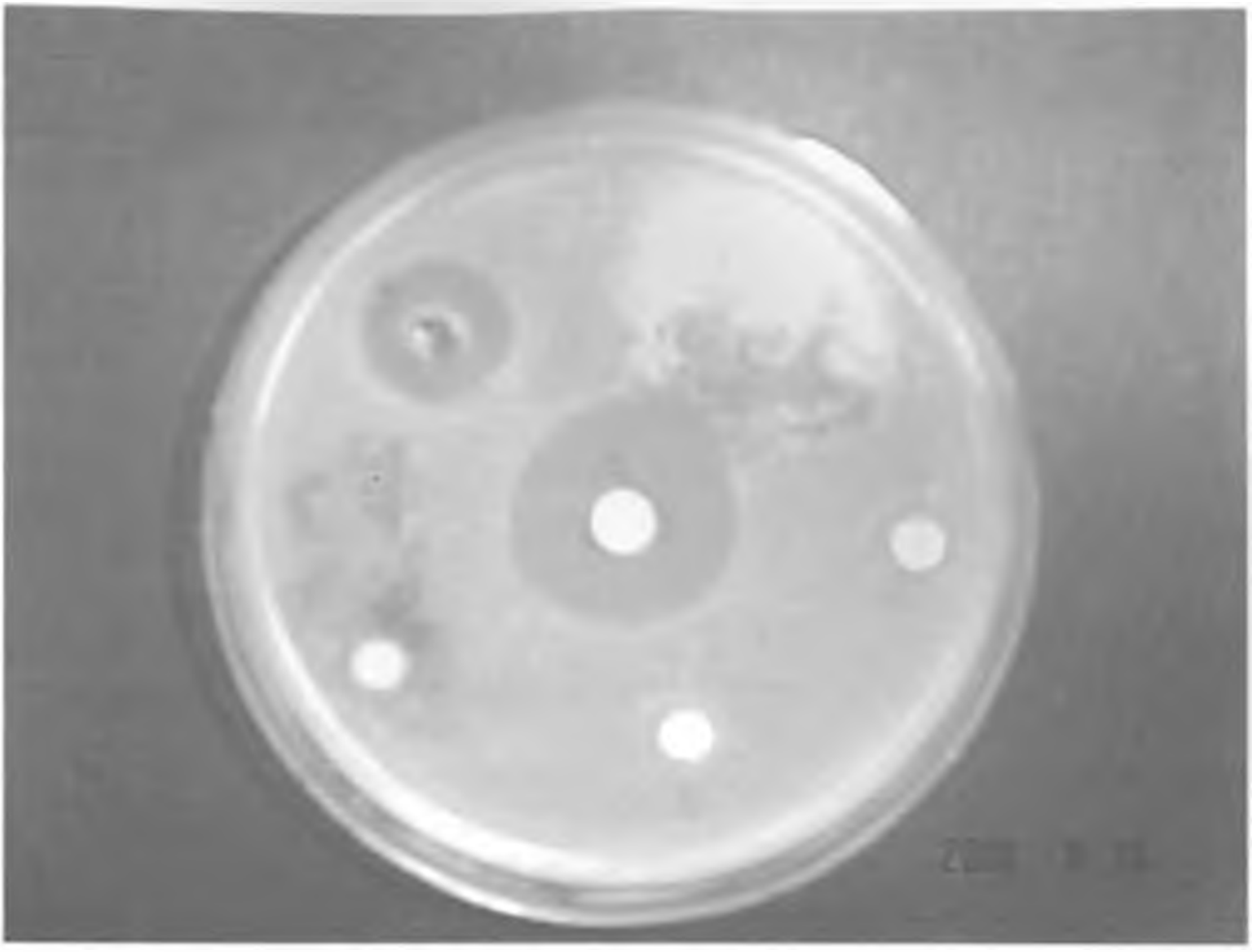


APPENDIX 9: Carbon-13 NMR Spectrum of BUCC2

APPENDIX 10: Proton NMR Spectrum of BUCC1



APPENDIX 11: Anti-Staphylococcal activity of *Blighia unijugata* extracts and friedelin



Key: The tested solutions from the bottom clockwise: Negative control, Petroleum ether extract, methanol extract, chloroform extract, friedelin

APPENDIX 12 TLC Images of Purified BUCC1 and BUCC2 and Their Profile in the Mother Liquor