

**CHEMICAL ANALYSES OF THE ROOT
BARKS OF *DETARIUM MICROCARPUM*
AND *SCLEROCARYA BIRREA* FOR
ANTIFEEDANT AND ANTIMICROBIAL
PROPERTIES**

BY

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MSC/SCI/18271/98/99

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(A.RICH) HOCHST. FOR ANTIFEEDANT AND
ANTIMICROBIAL PROPERTIES.**

By

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the Award of Master of Science Degree in Organic Chemistry

At

Department of Chemistry
Faculty of Science
Ahmadu Bello University,
Zaria, Nigeria.

May 2004

DECLARATION

I hereby declare that this thesis has been written by me and that it is a record of my research work and have never been presented in whatever form in application for an award of any degree. All quotations and sources of information are indicated and specifically acknowledged by means of references.

.....
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CERTIFICATION

This thesis entitled “Chemical Analyses of the Root barks of *Detarium microcarpum* and *Sclerocarya birrea* for Antifeedant and Antimicrobial Properties by Munirat Modupe Hassan meets the regulations governing the award of the degree of Master of Science in Organic Chemistry of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

To

Mum

For All Your Effort

Thanks.

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ABSTRACT

The extracts of the root barks of *Detarium microcarpum* Guill&Perr and *Sclerocarya birrea* (A.Rich) Hochst. have found traditional use in Nigeria and other African countries for treatment of dysentery, skin diseases and as antifeedant against stored product pests.

These claims have been verified through antifeedant, cytotoxicity and microbial tests. Phytochemical analyses carried out on the extracts of both plants (using solvents of varying polarities) showed the presence of reducing sugars, alkaloids, saponin glycosides, sterols, resins and balsams, flavonoids and tannins. All the extracts (except for the petroleum spirit extract) from the two plants were active against *Bacillus subtilis*, *Escherichia coli*, *Corynebacterium pyogenes*, *Staphylococcus aureus* and *Salmonella typhi*.

The methanol and ethyl acetate extracts from both plants were active in the shrimp lethality bioassay (Cytotoxicity Test). The methanol extracts of the plants had good antifeedant activity. Purification of the methanol fraction of the *S. birrea* extract was carried out to determine the components of the plant. The petroleum spirit extract tested positive for aldehyde and this was oxidized to the acid derivative in order to facilitate its isolation and purification. Infra Red and Nuclear Magnetic Resonance spectroscopic analyses were carried out to determine the possible structures of compounds obtained.

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CHAPTER ONE

1.0. INTRODUCTION

1.1. Pesticides

Pesticides are chemical agents which are intended to prevent, destroy, repel or otherwise decrease the ability of pests to compete with desired organisms such as cultivated crops, animals or humans. Under this broad definition are the insecticides, fungicides, herbicides, molluscicides, nematocides, ovaricides, repellents and antifeedants, etc. They could be synthetic or natural in origin. Some of the synthetic pesticides include carbamates, pirimiphose, permethrin, malathion, decamethrin etc. (Austin, 1984). Many of these compounds are highly potent towards the pests. However, they possess several disadvantages, some of which are lack of selectivity, built-up resistance, toxicity to man and accumulation in the environment [Kroschwitz and Howe-Grant, 1993]. The indiscriminate use of chemical pesticides has given rise to many well-known and serious problems including genetic resistance of pest species, toxic residues in stored products, hazards from handling etc. The problem caused by pesticides and their residues have increased the need for effective biodegradable pesticides with greater selectivity.

The use of synthetic pesticides and the apparent health hazard posed by these pesticides have led to the consideration of biopesticides as a viable

alternative in the control of pests. Biopesticides employ the natural defences that plants and trees have to kill insects. They act by blocking the insect receptor- that is the neurotransmitter that regulates an insect's movement, behaviour and metabolism. (Salihu, 1999).

An area which is receiving considerable attention presently is the isolation and identification of active constituents from plants that have been found to repel or kill some insects, deter feeding and/or distort their life cycles. After isolation and characterization, the aim is to either synthesize their templates for large production or modify the traditional method of processing to make the repellent, antifeedant, insecticidal properties etc. more refined.

1.1.1. Natural Pesticides From Plants

To combat insect attacks, plants have developed a number of protective mechanism such as repellency, feeding deterrency, etc, and a number of natural products have been identified from these plant extracts. These have found use as insecticides, antifeedants, repellents etc. although many of them could not be profitably extracted. However, several of these extracts have provided valuable contact insecticides which possess the advantage that their uses do not appear to result in the emergence of resistant insect strain to the same degree as the application of synthetic insecticides [Cremllyn, 1991]. The major natural products among these are nicotine, derris, pyrethrum, ryania, azadirachtin to mention just a few. Crude extracts of some plants producing these natural products have a long

history of use as fish poisons, insecticides, antifeedants and repellants among many communities.

The bioactive constituent of these plants in most cases do not kill the pests outright, but exert pesticidal effects through deterrence of feeding or inhibition of growth. Inhibition of pest growth pattern and production of phytotoxic symptoms by certain plants is a well established phenomenon of repellents and antifeedants which when present cause such plants to be significantly protected against insect and other predational pests. These deterrents negatively affect the biting, feeding and maintenance of feeding activity by the insects. Toxic action may induce sickness or weakness, inhibit growth, slow maturation, decrease reproduction and promote premature death [Bloszyk et al, 1995]. Plants with these natural pesticidal constituents are termed antifeedants [van Beek, 1993].

1.2. Antifeedants

Antifeedants are substances that are not necessarily food repellants but cancel out the signal to the appropriate organ in the insect to initiate feeding on the host [Cremllyn, 1991]. They are substances in plants which when perceived by the insects or pests reduce or prevent insect feeding. Antifeedants in plant body constitute defence mechanism against insect attack. They are plant substances or constituents which interact with taste receptors of herbivores (insects, pests etc) in such a way that the process of consuming is strongly repressed. In the presence of the antifeedant substance the insect may starve to death while remaining on the host plant, possibly because it makes the host plant distasteful to the insect so

feeding is inhibited. The majority of antifeedants do not directly kill the insect. Antifeedants, unlike insecticides are often selectively toxic towards some insect species, whereas they are not harmful to others and to mammals. They are environmentally friendly because they attack only specific pests without affecting the non-target organisms [Cremllyn, 1991; van Beek, 1993; Paruch, 2000].

Plants with bitter and/or pungent taste have been observed to be typical of tissues containing alkaloids and other classes of toxic compounds such as the sesquiterpenoids, the triterpenoids or limonoids e.g. azadirachtin, and these are known to have some of these antifeedant properties. [Bloszyk et al, 1995]. Some classes of compounds that have been isolated as antifeedants include:

1.2.1. Azadirachtin

One of the most successful antifeedants is a group of tetranortripenoids from the neem tree *Azadirachta indica* [Meliaceae]. The main constituent azadirachtin [I], a naturally occurring insect repellent and antifeedant has provided Indian and South American peasant farmers with a cheap or even free insect pest control technique. It has two main effects on several common insect pests including caterpillars and locust:

- (a) It repels them from eating the leaves or seeds of the plant
- (b) It upsets the cycle of moults by which they grow, slows down their growth and eventually kills them. [Kraus, et al, 1993; Rembold et al, 1987].

Azadirachtin is the most active antifeedant against lepidopterous species known; it is lethal at a concentration of only 10ppm [Ley, 1990]. The azadirachtin is a highly complex compound which has not yet been synthesized though its structure has been elucidated [Kraus et al, 1993; Rembold et al, 1987]. Various products have been formulated from the neem concentrate, the most popular being Margosan-O^[R] [Larson, 1986]. Currently, efforts are being directed towards designing synthetic compounds which can mimic azadirachtin [Ley, 1990].

1.2.2. Lantadenes

Substantial work has been carried out on the utilization of *Lantana camara* [Verbaneaceae] for insecticidal activity. Reports have shown that they contain a wide range of triterpenoids, some of which are hepatotoxic to animals [Barre, 1997]. Some of these are pentacyclic triterpene acids such as Lantadene A and B,

Iceterogenin and dihydrobutandene A [II]. These compounds though with insecticidal activity have also been implicated in lantana poisoning of ruminants [Tripathi et al, 1992].

1.2.3. Quassin

Quassia amara and *Picrasma excelsa* [Simaroubaceae] are plants that contain bitter-tasting seco-triterpenes such as quassin, neoquassin and 18-hydroxyquassin which are used as a bitter tonic, a hop imitate, an anthelmintic and or an insecticide. Quassia extracts prepared from the wood of both trees were widely used against ectoparasites (lice) and flies, the extract were also used as spray solution on farms. The constituent, Quassin [III] acts as a contact, stomach and systemic poison in insects (ectoparasites, lepidopteran larvae, beetles and flies) and nematodes. Quassin seems to be a selective insecticide with no recorded

toxic effects on humans, though if applied in high concentrations, irritation of stomach and vomiting can occur. [Stoll, 1986].

1.2.4. Diterpenes

Tetranorditerpenes have been isolated from the leaves of the plant *Detarium microcarpum* [Caesalpinaceae]. These had antifeedant activity against wood termite *Reticulitermes speratus*. The isolated compound was reported as ent-18-oxo-3, 13-clerodadien-15-oic acid. [IV] [Lajide et al, 1995].

Clerodane diterpenoids and phytoecosteroids with potential insect antifeedants and moulting hormone activities respectively have been isolated from *Ajuga* plants. Some clerodanes were active against larvae of Egyptian cotton

leafworm *Spodoptera littoralis* and it has been observed that the medicinal plant *Ajuga remota* [Labiatae = Lamiaceae] leaves were not attacked by African armyworms. The observation has led to the isolation of three moderately strong antifeedants named ajugarins I – III [V – VI], [Camps et al, 1993]. The lactone, ajugarin I has antifeedant activity against several insects including *Locusta migatoria* [Ley et al, 1990].

1.2.5 Quinolizidine Alkaloids

Another group of potential antifeedants are the quinolizidine alkaloids. These are the constituents of many leguminosae especially in the genera *Lupinus*, *Genista*, *Cytisus*, *Baptisia*, *Thermopsis*, *Sophora* and *Ormosia*. The quinolizidine alkaloids were found to be feeding deterrents for a number of oligo- and polyphagous insects including aphids, moths and butterfly larvae, beetles, grasshoppers, flies, bees and ants, that is, it has a wide spectrum of activity on a wide range of insect order [Wink, 1993]. *Sophora occidentalis* [Dapilioaceae] is reported to have fish poisoning property with nicotine – like action in its root and seed extract [Dalziel, 1934]. Some of these isolated alkaloids include hypanine [VII] and spartein [VIII]. [Wink, 1993].

1.2.6. Harrisonin

Harrisonia abyssinica [Simaroubaceae] is a shrub widely used in East Africa folk remedies as a remedy for bubonic plague, haemorrhoid and snake bite. Phytochemical analysis of the plant led to the isolation of harrisonin [IX] which is known to have antifeedant activity against monophagous army worm *S. exempta* [Nakanishi, 1980].

1.2.7. Warburganal

A constituent Warburganal [X] isolated from the bark of *Warburgia ugandensis* [Canellacea] has been found to exert potent antifeedant effects against *S. exempta* and *S. littoralis*, both insect pests of plants [Kubo et al, 1976].

1.2.8. Guineensine

The powdered dry fruits of *Piper guineense* [Piperaceae] are used as local spice and as an insecticide against bruchid [Ivbijaro et al, 1986]. An insecticide guineensine [XI] has been isolated from the plant [Okogun et al, 1974]. The effectiveness of the essential oil of *P. guineense* as seed dressing for cowpea production has been reported [Olaifa et al, 1986].

1.2.9. (-) Polygodial

This is a less complex antifeedant isolated from the marsh pepper *Polygonium hydropiper* [Piperaceae]. Plants treated with low concentrations of the compound were not colonized by aphids and consequently infection by plant-virus diseases was significantly reduced. Polygodial has been synthesized [Ley,

1985], but unfortunately, the (+) isomer is phytotoxic so the synthetic (racemic) product requires a tedious resolution before it can be used in crop protection. Polygodial [XII] has also been reported to have been isolated from the plant *Warburgia salutaris* [Canellaceae] [Mahlori et al, 1999].

1.2.10. Annonacin

Annona squamosa [Annonaceae] found in tropical America, Asia and Africa contain strong insecticidal, larvicidal, repellent and antifeedant constituents with remarkable activity on aphids, grasshoppers, diamond back-moth, red pumpkin beetle, green bug, etc. [Gaby, 1988; Nayar, 1995].

Chemical investigation of the seeds of *A. squamosa* led to the isolation of novel insecticidal acetogenins, neoannonin [XIII] and annonacin [XIV] [Kawazu et al, 1989]. Alkaloids such as anonaine, ligenamine, anolobine etc and flavonoids, quercetin-3- rutinoside (rutin) have also been isolated from the leaves and root of the plant [Wagner et al, 1980; Sectharaman, 1986].

1.2.11. Sesquiterpenes

The sesquiterpenes Xanthorrhool, have been reported present in the rhizomes of *Curcuma xanthorrhiza* or *Curcuma zedoaria* as having insecticidal activity against the larvae of the vigorous insect pest *Spodoptera littoralis* when applied topically via the larval integument [Nugroho et al, 1996]. Sesquiterpenes have been isolated from *Celastus angulatus* which have been used as natural insecticides for a long time [Liu et al, 1995].

1.3. QUALITIES OF A GOOD ANTIFEEDANT

A good natural insecticide or antifeedant should have the following qualities:

- a. Effectivity - such as in insecticidal activity or insect deterrence.
- b. Low toxicity in vertebrates and beneficial insects.
- c. More than one mode of action to reduce resistance to pest.
- d. Low persistence in plants, soil and water.
- e. Extraction and formulation should be simple, cheap and easy
- f. Plant material should be one that can be easily sourced.

Although not all criteria can be fulfilled in most cases, some of the examples given above come rather close to these demands. For example, azadirachtin, from *Azadirachta indica* (the Neem plant).

1.4. PROJECT OBJECTIVES

It is estimated that 10-35% of all crops are lost either before harvest on the field or during storage. [Cremllyn, 1991].

This project aims to find a solution to the post-harvest loss which occurs due to pest destructive activities on stored grains by sourcing for plants which could be used as antifeedants against agronomic pests.

The plants selected for this work are:

- i. *Detarium microcarpum*. Guill&Perr.
- ii. *Sclerocarya birrea*. (A. Rich) Hochst.

The crude extracts of these plants would undergo the following tests:

- i. Phytochemical screening to identify the constituents present.
- ii. Antibacterial screening to determine its biological activity against bacterially induced ailments.
- iii. Cytotoxicity tests for the lethality bioassay.
- iv. Antifeedant tests to determine its effectiveness or otherwise against insect pests. A reference standard pesticide for stored products, Pirimiphos methyl – 2% Dust, will be used for a comparative antifeedant study with the crude extracts. The rust red flour beetle, *Tribolium casteneum* Hbst. is to be used as the test organism.
- v. Isolation of constituents of the plants, characterization and structural elucidation of these constituents using spectroscopic techniques would also be carried out.

1.5. JUSTIFICATION FOR RESEARCH

One of the developmental priorities of a nation is to grow enough food to support her population. This idea is often not realized because of damages to food crops by pests. The development of insecticide – based technique for protecting crops in small traditional farms in Africa has only been partially successful because of the high cost of synthetic insecticides and erratic supply due to foreign exchange constraints. The harmful effects of some of these synthetic pesticides on non-target organisms especially humans as a result of its persistence calls for great concern.

The need to sustain and increase our capacity for food production in order to meet increasing demand without irreversible damage to ecological and other natural resources cannot be over-emphasized hence the need for this research into the natural antifeedant constituents of plants. The establishment of the antifeedant properties of these plants: *Detarium microcarpum* and *Sclerocarya birrea* by the farmers motivated the interest in order to establish a scientific proof of their claim and the possibility of isolating the active constituent(s) for increased insecticidal/antifeedant activity against stored product pest.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. *Detarium microcarpum* Guill and Perr. [Caesalpinaceae]

2.1.1. Brief Description of General Features of the Family

The family Caesalpinaceae comprises the following genera: Bauhinia, Piliostigma, Zenkerella, Cryptosepalum, Didelotia, Guibourtia, Eurypetalum, Julbernardia, Oxystegina, Cynometra, Hylodendron, Amphimas, Copaigera, Cordyla, Dialium, Detarium, Stemonocdeus, Gossweilerodendron, Distemonanthus, Crudia, Barkiaea, Swartzia, Tamarindus, Monopetalanthus, Daniella, Cassia, Delonix and Parkinsonia.

They are mainly trees or shrubs with some of them yielding gum-resin and tannin materials. The leaves are alternate and usually compound. The fruits are variable, often pod-like, flat and papery, containing one or many seeds. [Keay, 1989].

2.1.2. Description of the General Features of the Genera – *Detarium*

A small tropical African genus represented in Nigeria by trees which may be readily recognized by their leaflets [2 – 3cm long] with numerous lateral nerves running to a prominent marginal nerve. The flowers are small, have sepals but no petals, 8 – 10 stamen and a hairy ovary. The fruits are drupe – like with a

distinctive layer of mealy flesh mixed up with numerous fibres surrounding the stone [Keay, 1989].

2.1.3. The Plant – *Detarium microcarpum*. Guill and Perr.

2.1.4. Vernacular names

English – Tallow trees; Ghana – Takyikyiriwa, Twutwiriwa; Sengal – Kpagra, Kpayhga; Nigeria – Hausa:- Taura, Fulani:- Konkehi; Kanuri:- Galapo, Nupe:- Gungorochoi, Tiv:- Agashidam, Etsako:- Aikperlarimi, Ibo:- Ofo, Yoruba:- Ogbogbo, [Irvine, 1961; Bep, 1946; Keay et al 1964; Dalziel 1955].

2.1.5. Location

This is widespread, from Senegal to the Sudan. In Northern Nigeria, it is found in - Sokoto: Jega, Kwarikwara; Kaduna: Zaria, Anchau; Niger: Bida, Kwara, Oke-ode; Kogi: Kabba, Ankpa, Acharare; in Western Nigeria, it is found in Oyo: Ago-Are and in Eastern Nigeria, it is found in Enugu: Nsukka.

2.1.6. General Features

The tree is up to 30ft high with a twisted bole and widely spreading crooked branches. The bark is grey and often reddish beneath. The leaves have a common stalk, sometime with fine hair. The flowers (occur throughout the rainy season) are creamy white, fragrant and closely crowded. The fruits [November – January, May] are more or less circular and disc-shaped, with a dark brown skin, brittle when dry and encloses a sweet greenish fibrous pulp: The fruit usually

contain only one seed. The wood is dark brown and hard. It is a gum-yielding tree [Keay, et al, 1964; Irvine, 1961].

2.1.7. General Uses

The sapwood is white; the heart wood form hard and beautifully marked wood which was sold formerly in England as African mahogany. The wood is used in Liberia for planks and canoe timbers and in Sudan, the wood is used for fences and piles, lasting long under water and also finds use in local carpentry work.

The seeds are also used as necklaces and girdles. The fruits, which are not very fleshy are edible. A sweet meal is sometimes prepared from it, together with the fruits of other plants. The dark purplish – brown and rather oily kernel is also edible; beaten into cakes, it is used as cattle food among the Nupes. The ashes from the fruit are employed in the preparation of snuff. [Irvine, 1961].

2.1.8. Medicinal and other Related Uses

The roots are used medicinally in the Ivory Coast, the bark crushed with other different leaves are applied to buboes and syphilitic chancres. The powdered bark is haemostatic and used to heal deep cuts. A bark decoction is used in Liberia in parturition and is said to have quick effects in cases of retained placenta. A cold infusion of the bark, roots and wood is used in Senegal as a restorative for weakness and anaemia. The bark is poisonous and used for ordeals. The boiled bark is used in Guinea as a lotion for itch. The young boiled shoots are given with

food in S. Leone for fever. A decoction of leaves is used as a wash for itch or drunk or used as an enema for dysentery.

A fragrant gum resin from the bark is used in fumigating garments and huts. The fruit is used for rubbing chronic backache or tuberculosis of the spine. The forest fruits contain an organic acid 'detaric acid' and 0.05% of a bitter principle which is probably the toxic constituent. The bitter, possibly poisonous fruit is used for the treatment of syphilis. The fibre in the fruit – pulp is bitter and is suspected to be poisonous. Despite these effects, the fruits are a reputed remedy for chest diseases in parts of W. Africa. In Northern Nigeria, the fruits, with certain other fruits are used as a tonic and stimulant on a journey.

The seeds are burned to drive away mosquitoes. The young soft seeds are given to people wounded by poisoned arrows in Nigeria and are said to cause vomiting [Irvine, 1961; Bep, 1946].

The quantity of seed oil was low and contained β -carotene, plant sterols, phospholipid and glycolipids. Toxicological studies show absence of gossypol and no detectable mycotoxins. The gum content of the seeds was reported to be high; Linoleic acid was the predominant fatty acid. [Njoku et al, 1999]. Another report on the composition of the seeds gives the crude protein as 12.2 – 23.2%, and fat varied from 4.9 – 12.0%. The level of phytic acid in the raw seeds (192.4 – 215mg/100g) was observed to be lower than the levels found in some commonly consumed pulses in Nigeria [Giame and Wachuku, 1997].

Lajide *et al* (1995) reported feeding deterrent activity in the leaves of the plant using termites, *Reticulitermes speratus* as test organisms. The extracts of the plants have been screened for molluscicidal activity, though plant part was not specified. [Kela *et al*, 1989]. Anticrustacean activity of the stem bark has also reported [Fatope *et al*, 1993].

2.1.9. Other Genus in the Caesalpinaceae Family

In the family, there are other genus that have been reported to have insecticidal and other related uses. The sapwood and heartwood of *Azelia africana* is resistant to both termites and the shipworm (teredo). The *Cassia alata* leaves are insecticidal and anthelmintic and are possibly poisonous to fishes. The leaf infusion is a taenifuge (destroys tapeworm). A decoction of *C. occidentalis* roots, leaves and fruits or the leaves alone is used by the Hausa as a lotion and for fumigation. The wood of *C. sieberiana* is resistant to termites and borers and is suitable for carpentry, implement handles etc. The root is used to kill fishes.

The deo-resin obtained from the tree *Daniellia oliveri* is used as insect deterrent, especially ant attack. An infusion of the root bark (in hot liquid form) is used for vapor baths and fumigation. The strongly scented oily and sticky gum of *D. thurifera* is used as a pomade and perfume for fumigation and to mend pottery.

The wood of *Dialium guineense* is not readily attacked by termites and finds extensive use in various types of wood work.

The sapwood of *Distemonanthus benthamianus* is strong and resistant to termite attack. The wood is also moderately resistant to fungal attack.

The heartwood of *Erythroleum giuneense* does not easily rot and resists insect attack, including that of termites. A strong decoction of the dried bark is used in Nigeria as an arrow poison by the Tivs in Benue State.

The leaves of *Gilbertiodendron splendidum* are sometimes put in hen-houses to kill lice.

The wood of *Tamarindus indica* is resistant to termites and insect attack, hence its use in making household utensils, furniture etc. [Watt and Breyer-Brandwijk, 1962].

2.2. *Sclerocarya birrea* (A. Rich). Hochst [Anacardiaceae].

2.2.1. Brief Description of General Features of the Family

The family Anacardiaceae comprise of the following genera: Rhus, Sorindeia, Mangifera, Anacardium, Haemotostaphis, Trichoscypha, Odina, Spondias, Sclerocarya, Hitzeria and Lanneoma.

They are mainly trees or shrubs, often abounding in a caustic or resinous juice. The leaves are alternate, frequently crowded towards the end of the branches. The flowers are small, regular and unisexual with numerous petals. The fruits are drupaceous with one or many seeds. [Oliver, 1968].

2.2.2. Description of the General Features of the Genera - *Sclerocarya*

The species in these genera are mainly trees with alternate leaves, clustered at the ends of the branches. The sepals and petals on the flowers are many and coloured. The fruit are drupaceous with one seed. The seeds have fleshy and oily cotyledons [Oliver, 1968].

2.2.3. The Plant – *Sclerocarya birrea*. (A.Rich) Hochst.

2.2.4. Vernacular Names

English – Jelly plum, Marula, Maroola plum; Afrikeans – Maroels; Kenya – Didissa, Muua; Swahili – Mngongo; Swazi – Uniganu; South Africa – Marula; Zulu – Fruits: Amaganu, Trees/seeds: Umganu; Nigeria – Hausas: Akushi, bukuru, Danya, Daniya; Fulani: Heri [Keay et al, 1964; Burkill, (1985), SEPASAL, 2002].

2.2.5. Location

This tree is found in the drier Sahel Savannah from Senegal to Niger, West Africa to South Africa, Ethiopia and Uganda. In Nigeria, it is found in: Sokoto, Zamfara, Kano, Bornu, Kaduna and Bauchi States. [Keay et al, 1964].

2.2.6. General Features

The tree is about 40ft high and 2-6ft in girth with a short bole. The bark is grey with reddish trunk, the twigs are grey or sometimes black due to fire outbreak in the forest. The leaves are pinnate with terminal leaflets. The flowering season is usually between December and January. The fruits are usually found between May and June, they are obovoid, thick-skinned and resembling small mangoes.

It could be grown from the seed or by grafting. It is a tannin – producing tree. [Irvine, (1961); Oliver, (1968)].

2.2.7. General Uses

The bark yields a strong fibre, the wood is coarse – grained, soft and grayish and is sometimes used for mortars and bowls.

The foliage is grazed by cattle and camels in Senegal, in drought the leafy branches are cut for fodder. The fruits are eaten in many parts of Tropical Africa, and have a pleasant acid flavour. The fruit juice makes an agreeable drink and is sometimes made into a fermented drink or boiled down to thick black syrup used for sweetening Guinea-corn gruel. It is said that the fruit contains four times as much Vitamin C as an average orange. The kernel is oily and edible. The stone in the fruit is hard, its kernel is of significant nutritional value, because of the high oil content, the nut can be set alight and used as a candle.

The tree is one of the most valued indigenous trees in Africa. The local legend has it that the tree is a strong aphrodisiac so much so that wedding ceremonies are held under its branches, it features prominently in many tribal fertility rites. Expectant mothers of the Venda tribe in South Africa eat the ground bark to determine the sex of their babies – the bark of a tree with male flowers ensures a boy, while the female tree means a girl for the family. The Zulu people believe that a person suffering from measles had to wake up before dawn and without saying a word, walk to the tree and bite its bark, after which the illness would disappear!

The stones also hold significant spiritual value as they are used by Sangomas a tribe in South Africa, as an essential element in bone-throwing ceremonies [Burkill, 1985; Dalziel, 1955; Keay et al, 1964; SEPASAL, 2002].

2.2.8. Medicinal and Other Related Uses

A bark infusion and a mixture containing the juicy inner bark are used by the Fulani of Nigeria and Venda of Southern Africa for constipation and stomach complaints respectively. It is also mixed with natron by the Hausa for use in dysentery. Analgesic action is sought by the Basari of Senegal who chew the bark to compact carious cavities in the teeth to quell toothache. The root and trunk bark or leaves are prescribed as a remedy for snake bites by the Soce of Senegal. This is pounded to form a paste and rubbed to alleviate the swelling, and then a bark decoction is drunk and dressing applied over the area. The bark also finds considerable use as an anti-inflammatory substance (for external use) with fat, for headaches, skin eruptions and for washing scabies. The decoction can be taken internally as a purge, in local veterinary practice; the Fulanis give a decoction of the bark to their stock to increase their appetite. [Burkill, 1985].

The fruits when taken are said to have laxative action [Burkill, 1985]. The fruits of the plant were found to contain high level of ascorbic acid 403.3mg/100g, the mineral contents (Ca, P) were comparable with the average values found in common fruits but the iron contents were about 2-5 times higher than the values for common fruits [Eromosele et al, 1991]. The nuts contain 2 or 3 seeds with oily

and edible kernels. The oil consists of 64% oleic acid, 17% myristic acid and small quantities of several others [Burkill, 1985].

Another report on the physiochemical composition of *Sclerocarya birrea* stated its composition as 11.0% crude oil, 17.2% carbohydrate, 36.70% crude proteins, 3.40% fibre and 0.90% crude saponins. The oil has nine fatty acids of which palmitic, stearic and arachidonic acids are the most dominant [Ogbogbe, 1992].

In Madagascar, the bark has been reported to contain 3.50% tannin. The Transvaal bark collected during the month of October before the appearance of the leaves contains 20.50% tannin. The gum is also said to be rich in tannins. [Watt et al, 1962].

One of the works reported on this plant is on its anticrustacean activity to predict for antitumor, antibacterial and antifungal activities using the stem bark. [Adoum et al, 1997]. The bark is used widely for bacteria related disease by indigenous cultures in Africa; a screening test has reported its MIC values to be between 0.15 to 3.0mg/ml. The inner bark is reported to be the most potent, followed by outer bark and leaf extracts, but the differences were not statistically significant. [Eloff, 2001]. The effect of its leaf extract on calcium signaling in rat cultured skeletal muscle cell has also been investigated. [Belemtougri et al, 2001].

A secretagogue compound (-) – Epicatechin – 3 – galloylester, from the bark of the plant has been reported. [Galvez et al, 1992].

2.2.9. Other Genus in the Anacardiaceae Family

In the family, other genus that have been reported to have medicinal insecticidal and other related uses include the seed kernel oil of *Anacardium occidentale*, which is used medicinally and for preserving wooden floor timbers and books from white ants in the Indian peninsula. It is also used for preserving fishing nets. The shell oil is toxic to mosquito larvae, certain grain weevils and moth larvae. The bark has also been used as a febrifuge (treatment of fever). The tree yields a gum known as cashew gum and acajou gum, which vary from very pale yellow to reddish or dark brown in colour. The mucilage formed from the gum has good adhesive properties and is particularly useful in book binding on account of its insect repellent property. Anacardic acid, $C_{22}H_{32}O_3$; m.pt. $26^{\circ}C$ has been isolated from the fruit; it has shown some activity as a bactericide.

The mango, *Mangifera indica* L., is used as an astringent, in the Indian Peninsula; the powdered plant is used as fumigant against mosquitoes. The plant is also piscicidal. The leaf, bark, stem and both green and half-ripe fruits extracts produced zones of inhibition with *Microcoeus pyogenes*. *Staphylococcus aureus* and *Escherichia coli*; but the same extract has proved negative for both antibiotic and antimalarial tests.

The wood of *Rhus tomentosa* L. has been used for making fencing poles and has the reputation of being indestructible; it is not attacked by insects. The wood is also used in building huts in Basutoland in South Africa.

The wood from *Schimus molle*. L. is also resistant to white ants attack. The fruits of *Sclerocarya caffra* is used for the destruction of ticks, the Zulus regard the fruits as a potent insecticide. [Watt et al, 1962].

2.3. MICRO-ORGANISMS AND THEIR PATHOGENIC ACTIVITIES

The pathogenic micro-organisms used for the microbial tests of the plant extracts include: *Staphylococcus aureus*, *Salmonella typhi*; *Bacillus subtilis*; *Cornybacterium pyogenes* and *Escherichia coli*.

2.3.1. *Staphylococcus aureus*

This is a member of the gram-positive cocci. It is a human pathogen. It is the most common cause of acute pyogenic (pus-producing) infection in man. Most of the *S. aureus* infections occur primarily in individuals who have been compromised by underlying illness or other debilitating conditions. It is pathogenic and causes a wide variety of diseases including skin infections (superficial and deep abscesses) such as boils, carbuncles, pustules, septic fingers and infections of various internal organs: nose, eye, throat, gastro intestinal tract and vagina. From these sites, the infection can spread to deeper tissues causing sinusitis, mastoiditis, bronchitis and pneumonia. It also causes several toxinoses including food poisoning and toxic shock syndrome (TSS). It is resistant to

virtually all useful antibiotics including oxacillin and methicillin (which are derivatives of penicillin) [Boyd and Marr, 1980; Novick et al, 1979].

2.3.2. *Salmonella typhi*

This is a gram negative bacilli. *Salmonella typhi* are ubiquitous micro-organisms but are found in the intestinal tract of man and animal and causes a lot of human and animal diseases (it is highly pathogenic to man). Some of these diseases include typhoid fever, gastroenteritis, enteric fever and bacterimia. It also causes food poisoning. Chloramphenicol, various β -lactams or trimethoprim – sulfamethoxazoles are used in treatment of these diseases. [Falkow et al, 1987].

2.3.3. *Bacillus subtilis*

This is a gram-positive bacilli. It is the most commonly encountered member of a large group of aerobic spore – bearing bacilli found in soil, water, dust and air. The organisms are important; their heat-resistant spores may contaminate culture media, injection fluids, etc. or cause spoilage in the food industry. It can also contaminate pharmaceutical products e.g. syrups [Thomas, 1979].

2.3.4. *Cornybacterium pyogenes*

This is gram-positive, and cause a lot of problems both for human and animals. It is responsible for severe infections of pus-forming wounds and burns and is the most important cause of puerperal sepsis (pus formation from a post-

natal infection) and if found in blood, it can cause septicemia. Spread of infection through the tissues may produce cellulitis. [Boyd and Marr, 1980; Thomas, 1979].

2.3.5. *Escherichia coli*

This is a member of the enterobacteriaceae, and is a gram-negative bacilli. It is the typical coliform of the intestinal tract. It is a pathogen particularly of the urinary tract and also in appendicitis, peritonitis, wound infections, blood and cerebrospinal fluid infections and neonatal meningitis (these are extra – intestinal infections). *E. coli* could also result in intestinal infections or diseases such as diarrhea, dysentery and hemorrhagic colitis. Some of the treatments include oral non-absorbable antibiotics such as neomycin or gentamycin, together with maintenance of fluid and electrolyte balance. Ampicillin, tetracycline and trimethprime– sulfame – thoxazole are also used. [Falkow et al, 1987].

CHAPTER THREE

3.0. EXPERIMENTAL

3.1. APPARATUS AND REAGENTS

3.1.1. Apparatus

- i. Soxhlet Extractor
- ii. Heating mantle
- iii. Churchill Cooler
- iv. Water bath
- v. Rotary evaporator
- vi. Oven
- vii. Condensers
- viii. Separatory glass funnel
- ix. Chromatographic columns
- x. Hot plate
- xi. Pipettes: Calibrated and dropping
- xii. Chromatographic plates and tank
- xiii. FTIR spectrophotometer: - ATI Mattson Genesis Series FTIR.
- xiv. NMR –spectrophotometer :- H1-200MHz & C13-50MHz
- xv. Refrigerator
- xvi. Buckner funnel

3.1.2. Reagents

- i. Methanol [GPR---BDH]
- ii. Ethyl acetate [GPR---BDH]
- iii. Petroleum spirit (60-80°C) [GPR---BDH]
- iv. Chloroform [AR---PROLABO]
- v. Diethyl ether [GPR---BDH]
- vi. Molisch Reagent.....1% ~~α~~-naphthol in 80% ethanol.
- vii. H₂SO₄ acid (Conc) [GPR---BDH]; 2.0M....43.50cm³ conc H₂SO₄ in 400ml distilled water.
- viii. HNO₃ acid (-Conc) [GPR---BDH]
- ix. HCl acid (Conc) [AR---BDH]; 2.0M.....100cm³ conc HCl and made up to 500ml with distilled water.
- x. Barfoed's reagent.....13.3g Copper (II) ethanoate and 2cm³ glacial ethanoic and made up to 250ml with distilled water.
- xi. Fehling's solution A.....35g CuSO₄ in 500mls distilled water.
- xii. Fehling's solution B.....60g of NaOH and 173g of sodium potassium tartarate in 500ml of distilled water.
- xiii. 2.0M NaOH solution.....80g of NaOH pellets in distilled water and made up to 1000ml.
- xiv. Iron (II) chloride solution.....5gm FeCl₃ in 100ml ethanol.
- xv. KOH solution ---2M... (56.09gm in distilled water and made up to 500ml); 20%....(20gm in 100ml of distilled water).

- xvi. 2% sodium nitroprusside.....2gm sodium nitropussiate in 100ml water.
- xvii. 2.0M Ammonia solution.....180ml in 1000ml distilled water.
- xviii. Acetic acid (glacial) [AR—BDH]
- xix. Magnesium powder [BDH]
- xx. Mayer's reagent.....1.36gm Mercury (II) chloride in 50cm³ distilled water and 5g potassium iodide and solution made up to 100cm³ in distilled water
- xxi. Dragendorff's reagent.....10.00gm Bismuth nitrate in 20ml KNO₃ and 27.0gm Potassium iodide was prepared in 40mls of distilled water. Combined filtrate of these solutions is made up to 100mls.
- xxii. Copper acetate [GPR---BDH]
- xxiii. Ethanol [GPR---BDH]
- xxiv. Picric acid solution.....1gm picric acid in 25ml distilled water.
- xxv. 1.0M Na₂CO₃ solution.....53gm Na₂CO₃ in 500ml distilled water.
- xxvi. NaHCO₃ solution (saturated)....A saturated solution is made filtered and the filtrate used.
- xxvii. 0.5M Potassium iodide (KI) solution.....83gm of KI in 500ml of distilled water.
- xxviii. CuSO₄ solution – 125gm Copper (II) sulphate crystals in 1000cm³ distilled water.
- xxix. 3, 5-dinitrobenzoic acid [AR- BDH].
- xxx. Olive oil
- xxxi. Iodine & Iodine solution.... 1 gm Iodine: 2gm Potassium (1:2) in 100ml distilled water.

3.2. PLANT SAMPLES COLLECTION AND PREPARATION

The plant samples – that is the root bark of *Detarium microcarpum* Guill&Perr and *Sclerocarya birrea* (A.Rich) Hochst. were collected around the Dakace area in Zaria, Kaduna State. They were collected after the rainy season, between the months of October and November.

These were properly identified at the Herbarium, Department of Biological Science, Ahmadu Bello University, Zaria. The voucher specimen numbers are *D.microcarpum*: -900676 and *S. birrea*: - 1079. The root samples were washed in water to remove soil debris, cut into smaller pieces and air-dried under shade. The dried root bark was ground using a mill at the Federal College of Chemical and Leather Technology (CHELTECH), Zaria.

3.3. EXTRACTION

About 200g of each of the powdered root bark was extracted separately using the Soxhlet extraction method. A successive extraction process was carried out using various solvents – petroleum spirit (60-80°C), ethyl acetate and methanol (in order of increasing polarity). Laboratory and general purpose grade solvents were used but these were redistilled before the extraction. After exhaustive extraction with each solvent, the various extracts were concentrated using the rotary evaporator. The extracts were stored in a desiccator for the tests to be conducted.

Another 200g each of the powdered root sample was macerated in 100cm³ of water for 24 hours and filtered. The filtrate was gradually evaporated to dryness over a water bath and stored in the refrigerator.

The table below gives the weight of extracts and percentage yield obtained per 200g of plant material using successive extraction.

SOLVENTS USED	PLANT SAMPLE – <i>Detarium microcarpum</i>		
For Extraction	Wt of extract (g)	% yield per 200g	Nature of extract obtained
Petroleum spirit	04.31	02.15	A yellow coloured gummy extract
Ethyl acetate	02.06	01.03	A reddish/reddish brown powdered residue.
Methanol	11.42	05.71	A reddish brown coloured extract
Water *	39.60	19.80	A dark brown coloured residue
Sclerocarya birrea			
Petroleum spirit	01.62	00.81	A light yellow oily extract which turns light brown after a while.
Ethyl acetate	02.90	01.45	A reddish brown coloured extract.
Methanol	14.46	07.23	A reddish brown coloured extract
Water *	53.80	26.90	A light brown coloured residue

* From fresh 200g sample

3.3.1. BULK EXTRACTION

Promising results obtained motivated bulk extraction of the plant materials.

About 1.3 kg of the root bark of *D. microcarpum* and 2.0kg of the root bark of *S. birrea* were extracted and yield concentrates recorded in Table 4.0.

3.4. PRELIMINARY PHYTOCHEMICAL SCREENING TESTS

Preliminary phytochemical screening was performed on each of the petroleum spirit, ethyl acetate, methanol and water extracts of the root bark of *D. microcarpum* and *S. birrea* respectively. [Laboratory Manual, I (1986 – 87)].

3.4.1. Tests For Carbohydrates

3.4.1.1. Molisch's Test

5ml of the sample (each extract) was put into a test tube and a few drops of Molisch's reagent (10% α -naphthol solution) added. 1ml of conc. H_2SO_4 was allowed to flow down the side of the inclined tube so that the acid forms a layer beneath the aqueous solution without mixing with it. A red ring would be formed at the interphase with a dull – violet precipitate to indicate presence of carbohydrates.

3.4.1.2. Fehling's Test

5ml of a mixture of equal volumes of Fehlings A and B solutions was added to about 3ml of each extract and boiled for a few minutes. A brick- red precipitate of Copper (I) oxide Cu_2O was taken as an indication of the presence of reducing sugar.

3.4.1.3. Standard Test for Combined Reducing Sugar

3ml of each extract was hydrolysed with 10ml of dilute HCl acid by boiling. These were neutralized with NaOH solution and the Fehlings' test

repeated. A brick-red coloured precipitate indicates the presence of combined reducing sugars.

3.4.2. Tests For Glycosides

3.4.2.1. Hydrolysis of Glycosides – by Dilute Mineral Acid

About 2.5ml of dilute H_2SO_4 acid was added to 5ml of each extract in a test tube and boiled in a water bath for 15 minutes. It was cooled and neutralized with 20% KOH solution. 5ml of a mixture of Fehling's solution A and B was added and boiled. Reducing sugars released as a result of the hydrolysis is shown by a brick red precipitate.

3.4.2.2. Test For Cyanogenetic Glycosides

About 0.5g of the powdered root bark sample for each plant was used for the test. They were placed in separate test tubes and sufficient water to cover the bark added. A moist sodium picrate paper (prepared by dipping picric acid paper in sodium carbonate solution) was suspended in the neck of the tube and trapped by means of a cork. The closed tube was placed in an oven at $45^{\circ}C$ for 1 hour. A brick-red colour observed on the paper strip indicates release of hydrocyanin HCN from the crude plant sample.

3.4.2.3. Test For Saponin Glycosides

(a) The Frothing Test

A small quantity of each extract was shaken with distilled water in a small test tube. Frothing which persisted on warming was taken as evidence for the presence of saponins.

(b) The Olive Oil Test

A small quantity of each extract was shaken with about 5ml of distilled water for 2mins. Two drops of olive oil was added and shaken rigorously.

Frothing occurred and an emulsion was formed with the olive oil as an indication of the presence of saponins.

3.4.2.4. Test For Cardiac Glycosides

(a) Legal Test

A small quantity of each extract was dissolved in pyridine and a few drops of 2% sodium nitroprusside together with a few drops of 20% NaOH solution added. A deep red colour that fades to a brownish colouration is expected to indicate the presence of cardenolides in the extracts.

(b) Libermann Burchardt Test

1ml of anhydrous acetic acid and 1ml chloroform were combined and cooled in an ice bath, one drop conc. H_2SO_4 was carefully added to form a lower layer. The extracts in chloroform were added to the solvent mixture and colour

change observed. A deep pink to greenish pink colour indicates the presence of a steroidal nucleus as the aglycone portion of the cardiac glycosides. [Cannel, 1998].

(c) Kedde Test

8% Methanolic solution of each plant extract was made. 1ml of each extract was mixed with 1ml of 2% solution of 3, 5 – dinitrobenzoic acid in 95% alcohol. The solution was made alkaline with 5% NaOH. A violet colour, which fades through reddish brown to brownish yellow is observed. This indicates the presence of the cardenolide aglycone (free or combined).

3.4.3. Test For Sterols: Salkowski Reaction

1.0 – 2.0mg of the sample was dissolved in 1ml chloroform (CHCl_3) and 1ml conc. H_2SO_4 added, thus forming two layers, with red or yellow coloured interphase. This indicates the presence of sterols and methylated sterols [Cannel, 1998].

3.4.4. Test For Flavonoids: Shinoda Test

A small quantity of magnesium powder and a few drop of conc. HCl were added to an alcoholic solution of each plant extract. The appearance of orange, pink, red to purple colours indicates the presence of flavones, flavanols, the corresponding 2, 3-dihydro derivatives and/or Xanthones.

When zinc is used for the test, a deep red to magenta colour is observed for flavones while the flavanones and flavonols will give a faint pink to magenta colour or no colour change. [Cannel, 1998].

3.4.5. Test For Alkaloids

3.4.5.1. Preparation of Extracts

The extracts were cleaned (to remove non-alkaloidal compounds capable of giving “false positive” results) using the following procedure: To a small quantity of the powdered root bark was added 1% dil. HCl (about 20ml) on a steam bath and heated, this was then filtered. The filtrate was made alkaline with 28% NH₃ solution and then extracted with three 5ml aliquots of CHCl₃. The combined CHCl₃ extracts were concentrated and treated with an equal volume of 1% HCl acid. Each of the combined CHCl₃ extract for each plant was then tested with Mayer’s; Dragendorff’s and Wagner’s Reagents. [Trease and Evans, 1983].

3.4.5.2. Mayer’s Reagent Test

Freshly prepared Mayer’s reagent [Potassium mercuric iodide solution] was added dropwise to each acidified solution of the extracts. Presence of alkaloid was indicated by a white to yellow or creamy white precipitate.

3.4.5.3. Dragendorff’s Reagent Test

An acidified solution of the extract was treated with Dragendorff’s reagent [Bismuth potassium iodide solution]. An orange-red or orange-brownish precipitate was taken as evidence of alkaloids.

3.4.5.4. Wagner's Reagent Test

An acidified solution of the extract was treated with the reagent [Iodine-potassium iodide solution]. A brown precipitate in acidic solution suggests the presence of alkaloids. [Cannel, 1998].

3.4.6. Tests for Resin and Balsams

3.4.6.1. Test For Resin

About 5ml of each extract was treated with equal volume of copper acetate solution, shaken vigorously and allowed to separate. A green colouration observed indicates the presence of resin.

3.4.6.2. Tests For Balsam

About 5ml of each extract was treated with 2-3 drops of alcoholic FeCl_3 solutions. A dark green coloured solution is taken as evidence of balsam in the extracts.

3.4.7. Test For Tannins

A small quantity of each extract was dissolved in distilled water (10ml), boiled and filtered. To the filtrate was added few drops of 1% Iron (II) chloride solution, a blue – black, green or blue-green precipitate was taken as evidence for the presence of tannins.

The results of the phytochemical screening tests on the extracts are summarized in Tables 4.1 and 4.2. The tests were carried out on each plant extract except where protocol/methodology for the test states otherwise.

3.5. ANTIBACTERIAL SCREENING TEST USING THE AGAR-DIFFUSION METHOD

The paper disc-diffusion method [Ericson et al, 1960, Bauer et al, 1966] was used to determine the antibacterial activity of the extracts. Dilution susceptibility testing method was used to determine the minimal concentrations, usually expressed in units of micrograms per millilitre ($\mu\text{g/ml}$) of an antimicrobial agent required to inhibit or kill a micro-organism. Procedure for the determination of antimicrobial inhibitory activity is carried out by either agar – or broth – based methods.

The antibacterial agents were tested using the agar – diffusion technique and the lowest concentration that inhibited visible growth of an organism recorded as the MIC. It is important to note that the actual antimicrobial concentration required to inhibit growth is between the highest tested two fold dilution that inhibited growth and the next – lowest dilutions at which growth was observed.

3.5.1. Isolation of Cultures

Standard strain of *Staphylococcus aureus* NCTC 6571; *Bacillus subtilis* ATCC 11778; *Corynebacterium pyogenes* ATCC 10242; *Salmonella typhi* NCTC 52311 and *Escherichia coli* NCTC 10418 were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital [ABUTH], Zaria. All cultures were checked for purity and maintained in nutrient agar slants.

3.5.2. Preparation and Storage of Media

In the paper-disc method adopted for the tests, solutions of varying concentration from $1.0 - 5.0 \times 10^4 \mu\text{g/ml}$ were prepared from each extract using pure extruding solvents.

Petridishes were washed and sterilized in autoclave at about 120°C for 25 minutes and allowed to equilibrate to $48^\circ\text{C} - 50^\circ\text{C}$ before use. They were labelled indicating the concentrations, test organisms and type of extracts.

Mueller – Hinton nutrient agar medium was used as the growth medium for the microbes. The nutrient agar medium was prepared by dissolving 38g of nutrient agar in 1000cm^3 of distilled water, heated to dissolve and autoclaved at 121°C for 15 minutes. It was allowed to cool and about 20ml was poured into sterilized Petri dishes, covered and allowed to solidify after a few minutes. This afforded what is known as plates of Mueller – Hinton sensitivity Agar [Oxoid]. These oxoid plates were then seeded with the test micro-organisms by the spread-plate technique and were then left for half hour to dry. [Felmghan and Stokes, 1972].

Sterile paper discs were soaked in the prepared solutions of the extracts with varying concentrations. They were dried at 50°C . The dried paper discs were then planted (with enough spacing to avoid overlapping of the expected inhibition zones) on the nutrient agar plates seeded with the test organisms. These were used to determine the zones of inhibition and minimum inhibitory concentration (MIC).

A control experiment was also set up using only the extruding/extracting solvent for each of the test organisms. The plates were incubated for 24 hours at 37°C, after which they were inspected for the zones of inhibition of growth. The observed zones of growth inhibition were measured and recorded in millimeters of their diametrical sections. The results are given in Tables 4.3 and 4.4 and photographs of the plates are shown in Appendix I.

3.6. CYTOTOXICITY TEST

The method used for the cytotoxicity test was the Brine shrimp lethality assay. This method utilizes the lethality of brine shrimp nauplii as an indication of toxicological activity.

The test was carried out on the methanol and ethyl acetate extracts of each plant because from the phytochemical screening tests, most of the constituents identified in the water and methanol extracts were similar, hence the test was conducted only on the methanol extracts of both plants. The pet spirit extracts had shown no inhibition against the micro organisms, thus were not screened.

3.6.1. Materials

- a. Small tank with perforated dividing dam to hatch shrimp eggs:-
[Hatching chamber].
- b. *Artemia salina* (Artemia saline leach or Brine shrimp eggs).
- c. Micro pipettes (0.50ml, 1.0ml)
- d. Test-tubes (10mm x 5mm) [15 per extract plus one control]
- e. Calibrated pipettes (1.0ml, 2.0ml)

- f. Sea water (from Bar beach, Lagos)
- g. Dimethylsulphoxide [DMSO].

3.6.2. Procedure

Sea water was put into the hatching chamber and shrimp eggs were added at the extreme end before the dam. This was allowed to stand for 48 hours to hatch and mature as nauplii at room temperature but near a light source so as to attract the shrimp larvae through the perforation in the dam. After 48 hours, the phototropic nauplii (shrimp larvae) were ready for use. (Laboratory Manual II)

The methanol and ethyl acetate extracts of both *D. microcarpum* [DTMe], [DTEa] and *S. birrea* [SBMe],[SBEa] were dissolved in Dimethylsulphoxide [DMSO]. 30mg of each extract was dissolved in 3ml of the appropriate solvent and used as stock solution. The following concentrations were prepared for each extract from the stock solution by serial dilution: 1000 g/ml , 500 g/ml , 250 g/ml , 125 g/ml and 62.5 g/ml . Three test-tubes were prepared for each concentration, hence a total of 15 test-tubes for each sample and one test tube containing only sea water for control].

10 shrimp larvae were collected from the illuminated side of the tank with micropipette and successively transferred into each test tube and the control test tube. Sea water was added to each test tube to make up a total volume of 5ml. The test tubes were allowed to stand for 24 hours under illumination after which the number of survivors were counted. From the survivor, the number of the deaths at each concentration and control were recorded.

3.6.3. Determination of LC₅₀.

The recorded percentage death for the extracts was determined at 95% confidence interval using the method of linear regression analysis described by Saunders and Fleming (1971).

Results of the brine shrimp lethality test are given in Table 4.5.

3.7. ANTIFEEDANT TESTS

The antifeedant activity of the two plants were also evaluated against stored – product insect pests. The petroleum ether and methanolic crude extracts were used in this evaluation.

3.7.1. Preparation of Extracts

Pure extruding solvents for each of the plant extract was used as a vehicle and varying concentrations of 1.0% w/v, 5.0%w/v and 10%w/v were prepared.

3.7.2. Insect Culture

A culture of *Tribolium casteneum*, rust red flour beetle (a stored product pest in maize) was used for this experiment. The culture was obtained from Biological Sciences Department, Ahmadu Bello Univerisity, Zaria. The insects were raised in wheat powder under laboratory conditions at $30 \pm 3^{\circ}\text{C}$.

3.7.3. Procedure

A modified standard method [Bloszyk et al, 1995] was adopted for the deterrent test. Wafer discs of wheat flour were used as the testing food. The discs, about 1.0cm diameter and of variable weights (25 – 30mg) were prepared

and allowed to equilibrate to the test environment moisture conditions for a minimum of two weeks before the feeding trial commenced. The extracts (E) were applied to the discs using a dropping pipette. Another set of discs received only an equal amount of pure solvents to serve as a control [C]. The solvents were evaporated completely from the wafer discs by equilibrating the wafer discs in the test environment for three (3) days prior to the experiment. After equilibration, the wafers were placed in sets of three tubes with:

Tube 1 [CC] containing two control discs.

Tube 2 [CE] containing one disc with extract and one control disc.

Tube 3 [EE] containing two extract discs.

This was repeated for the varying concentrations prepared from the pet. spirit and methanolic extracts for each plant.

A standard reference; a storage pesticide Pirimiphos methyl-2% dust was also set up under the same experimental condition. Methanol was used as the extruding solvent.

For each set of experiment, fifteen [15] unfed, adult *Tribolium castaneum* were introduced into each tube and the weight loss from each disc in the tubes (representing the amount consumed) after 12days was taken. This was used to calculate the antifeedant index (T). The experiment was replicated five times, and observations were made on the conditions and activity or otherwise of the insect towards the wafer during these periods. For each set of values, the mean \bar{x} , mean

deviation D, variance σ^2 and standard deviation σ were calculated to obtain the antifeedant index T.

The data generated from the tests are tabulated in Table 4.6.

3.8. FUNCTIONAL GROUP TEST OF THE *Sclerocarya birrea* Pet. Spirit extract. [SBPe].

Preliminary investigation of functional groups on the *Sclerocarya birrea* [SBPe] oil indicated the presence of an aldehyde (by the Silver mirror test). For this test, about 3ml of silver nitrate solution was put into a clean test tube, a drop of dilute NaOH solution was added, followed by ammonia solution dropwisely until the brown precipitate of silver oxide dissolved. To this solution was added a little quantity of the SBPe oil.

The test-tube was warmed in a beaker of boiling water for about 5 minutes. The presence of the alkanal (aldehydic) functional group is indicated by the silver deposit on the wall of the test-tube.

3.8.1. Oxidation of The Aldehyde Functional Group.

About 8.50g of the *Sclerocarya birrea* pet. spirit extract, SBPe oil was put in a round bottom flask. This was treated with saturated solution of sodium dichromate in concentrated H_2SO_4 and stirred. The reaction was left to stand for about 2 hours; cooling was by immersion of the flask in an ice-bath because the reaction was exothermic.

The reaction mixture was allowed to attain room temperature gradually, after which about 50mls of diethyl ether was used to extract the organic matter. The ethereal layer was separated and the mixture was further washed with another 50mls of diethyl ether.

The ethereal extracts were combined, washed with saturated NaHCO_3 to remove the acid content. The lower aqueous layer which contains the acid salt was acidified with conc. HCl and re-extracted into diethyl ether. The diethyl ether solution was washed with distilled water, dried over anhydrous Magnesium sulphate (MgSO_4). The ether was removed by heating over a steam bath. A white crystalline solid, weighing 1.58g was obtained as the acid derivative, SBPeA.

3.9. PURIFICATION AND CHARACTERIZATION OF THE *Sclerocarya birrea* methanol extract. [SBMe].

3.9.1. Acid Hydrolysis of SBMe Extract

About 125gm of the crude *Sclerocarya birrea* methanolic extract [SMBe] was hydrolyzed with 400ml 2M HCl acid over a water bath for 45mins. The solution was stirred at regular intervals. The hydrolysis is to breakdown the glycoside molecule into its sugar and aglycone component.

3.9.1.1. Test for Reducing Sugar

About 5ml of the hydrolyzed SBMe extract was put into a test tube and equal volumes of Fehling's A and B (about 5ml) added. This was boiled for about 5mins. A brick red precipitate indicates the presence of reducing sugars.

3.9.1.2. Extraction of SBMe Hydrolysate

The residue obtained from the hydrolysis weighed about 48.16g. This was extracted exhaustively with chloroform by sohxlet extraction.

1.47g of a reddish brown chloroform extract was obtained from the extract, after concentration on the rotary evaporator. The IR-spectrum of this extract was run and reported as Appendix VII.

3.9.2. Functional Group Tests.

Tests for Alkaloids, cardiac glycosides and phenolic compounds.

3.9.2.1. Alkaloid Test

The Wagner reagent [Iodine – Potassium iodide solution] test was used. The sample was spotted on micro TLC plates coated with alumina and developed using the Methanol: Chloroform [1:2] solvent mixture. This was sprayed with a manual spray-pump. A reddish brown (or brown) colour observed is taken as a positive result for the presence of alkaloids. [Cannel, 1998].

3.9.2.2. Test for Cardiac Glycosides: Salkowski Test

A small quantity of the hydrolysate was dissolved in 2ml chloroform. Concentrated sulphuric acid (H₂SO₄) was carefully added to form a lower layer. A

reddish-brown colour at the interface indicates the presence of a steroidal ring (i.e. aglycone portion of the cardiac glycoside). [Cannel, 1998].

3.9.2.3. Test for Phenols

A small quantity of the hydrolysate was stirred with 2mls of distilled water and FeCl_3 reagent added to the filtrate. A blue-black, green or blue-green precipitate was taken as evidence for the presence of condensed tannins [Trease and Evans, 1989]. A brown precipitate indicates the presence of polyphenols [Cannel, 1998]. The result is tabulated in Table 4.8.

3.9.3. Chromatographic Separations

The Thin-Layer [TLC] and Column [CC] chromatographic techniques were adopted for the purification process.

3.9.3.1. Preparation of Plates

a. Preparation of Thin-Layer Chromatographic [TLC] Plates

Plates [20 x 5.0cm] of thickness 0.50mm were used for qualitative analytical purposes to enable the determination of the appropriate solvent mixture required to separate the components of the given extracts. The support used was glass plates and the adsorbent Alumina [Aluminium oxide with 15% CaSO₄].

A slurry was made by stirring 30g Alumina with 40cm³ of distilled water. This was shaken to give uniform slurry and was spread at 250cm thickness on glass plates which had been cleaned with cotton wool soaked in acetone. The plates were allowed to dry for about 2 hours and used.

b. Preparation of Preparative TLC Plates

For the preparative TLC, Larger plates (20 x 20cm) of 0.75mm thickness were used. An equal weight/volume ratio of 320g Alumina (with 15% CaSO₄) and 320mls distilled water was mixed and shaken. This was allowed to stand for a few minutes and shaken again and spread on the plates. The freshly coated plates were then stored horizontally for 15 hours at room temperature before use.

For spotting the TLC plates, capillary tubes were used, and for the preparative TLC plates, a micropipette was used. Ascending development method, and in some cases, multiple development was used to enhance proper separation.

For developing the chromatogram on the TLC plates, various solvent mixtures were tried. Methanol: Chloroform in the ratio of 1:2 gave the best

separation. The spots/bands were observed under uv-flourescent lamp (366nm and 254nm). The 254nm wavelength was used predominantly. The column chromatography was conducted on the basis of this mobile phase.

3.9.3.2. Purification of the SBMe Hydrolysate by Column Chromatography

1.02g of the SBMe hydrolysate was purified using a column packed with alumina. The extract was introduced into the column and developed using the mobile phase – Methanol: Chloroform [1:2]. Fractions of 20ml were collected and analyzed by TLC and similar fractions pooled. The combined fractions were concentrated at reduced pressure and dried. Preparative TLC was carried out on fractions requiring further purification.

Table 4.9. gives the main fractions obtained and the R_f values.

CHAPTER THREE

4.0. EXPERIMENTAL

3.1. APPARATUS AND REAGENTS

3.1.1. Apparatus

- xvii. Soxhlet Extractor
- xviii. Heating mantle
- xix. Churchill Cooler
- xx. Water bath
- xxi. Rotary evaporator
- xxii. Oven
- xxiii. Condensers
- xxiv. Separatory glass funnel
- xxv. Chromatographic columns
- xxvi. Hot plate
- xxvii. Pipettes: Calibrated and dropping
- xxviii. Chromatographic plates and tank
- xxix. FTIR spectrophotometer: - ATI Mattson Genesis Series FTIR.
- xxx. NMR –spectrophotometer :- H1-200MHz & C13-50MHz
- xxxi. Refrigerator
- xxxii. Buckner funnel

3.1.2. Reagents

- xxxii. Methanol [GPR---BDH]
- xxxiii. Ethyl acetate [GPR---BDH]
- xxxiv. Petroleum spirit (60-80°C) [GPR---BDH]
- xxxv. Chloroform [AR---PROLABO]
- xxxvi. Diethyl ether [GPR---BDH]
- xxxvii. Molisch Reagent.....1% α -naphthol in 80% ethanol.
- xxxviii. H_2SO_4 acid (Conc) [GPR---BDH]; 2.0M....43.50cm³ conc H_2SO_4 in 400ml distilled water.
- xxxix. HNO_3 acid (-Conc) [GPR---BDH]
- xl. HCl acid (Conc) [AR---BDH]; 2.0M.....100cm³ conc HCl and made up to 500ml with distilled water.
- xli. Barfoed's reagent.....13.3g Copper (II) ethanoate and 2cm³ glacial ethanoic and made up to 250ml with distilled water.
- xlii. Fehling's solution A.....35g CuSO_4 in 500mls distilled water.
- xliii. Fehling's solution B.....60g of NaOH and 173g of sodium potassium tartarate in 500ml of distilled water.
- xliv. 2.0M NaOH solution.....80g of NaOH pellets in distilled water and made up to 1000ml.
- xlvi. Iron (II) chloride solution.....5gm FeCl_3 in 100ml ethanol.
- xlvi. KOH solution ---2M... (56.09gm in distilled water and made up to 500ml); 20%....(20gm in 100ml of distilled water).

- xlvi. 2% sodium nitroprusside.....2gm sodium nitropussiate in 100ml water.
- xlviii. 2.0M Ammonia solution.....180ml in 1000ml distilled water.
- xliv. Acetic acid (glacial) [AR—BDH]
 - l. Magnesium powder [BDH]
 - li. Mayer's reagent.....1.36gm Mercury (II) chloride in 50cm³ distilled water and 5g potassium iodide and solution made up to 100cm³ in distilled water
 - lii. Dragendorff's reagent.....10.00gm Bismuth nitrate in 20ml KNO₃ and 27.0gm Potassium iodide was prepared in 40mls of distilled water. Combined filtrate of these solutions is made up to 100mls.
 - liii. Copper acetate [GPR---BDH]
 - liv. Ethanol [GPR---BDH]
 - lv. Picric acid solution.....1gm picric acid in 25ml distilled water.
 - lvi. 1.0M Na₂CO₃ solution.....53gm Na₂CO₃ in 500ml distilled water.
 - lvii. NaHCO₃ solution (saturated)....A saturated solution is made filtered and the filtrate used.
 - lviii. 0.5M Potassium iodide (KI) solution.....83gm of KI in 500ml of distilled water.
 - lix. CuSO₄ solution – 125gm Copper (II) sulphate crystals in 1000cm³ distilled water.
 - lx. 3, 5-dinitrobenzoic acid [AR- BDH].
 - lxi. Olive oil
 - lxii. Iodine & Iodine solution.... 1 gm Iodine: 2gm Potassium (1:2) in 100ml distilled water.

3.2. PLANT SAMPLES COLLECTION AND PREPARATION

The plant samples – that is the root bark of *Detarium microcarpum* Guill&Perr and *Sclerocarya birrea* (A.Rich) Hochst. were collected around the Dakace area in Zaria, Kaduna State. They were collected after the rainy season, between the months of October and November.

These were properly identified at the Herbarium, Department of Biological Science, Ahmadu Bello University, Zaria. The voucher specimen numbers are *D.microcarpum*: -900676 and *S. birrea*: - 1079. The root samples were washed in water to remove soil debris, cut into smaller pieces and air-dried under shade. The dried root bark was ground using a mill at the Federal College of Chemical and Leather Technology (CHELTECH), Zaria.

3.4. EXTRACTION

About 200g of each of the powdered root bark was extracted separately using the Soxhlet extraction method. A successive extraction process was carried out using various solvents – petroleum spirit (60-80°C), ethyl acetate and methanol (in order of increasing polarity). Laboratory and general purpose grade solvents were used but these were redistilled before the extraction. After exhaustive extraction with each solvent, the various extracts were concentrated using the rotary evaporator. The extracts were stored in a desiccator for the tests to be conducted.

Another 200g each of the powdered root sample was macerated in 100cm³ of water for 24 hours and filtered. The filtrate was gradually evaporated to dryness over a water bath and stored in the refrigerator.

The table below gives the weight of extracts and percentage yield obtained per 200g of plant material using successive extraction.

SOLVENTS USED	PLANT SAMPLE – <i>Detarium microcarpum</i>		
For Extraction	Wt of extract (g)	% yield per 200g	Nature of extract obtained
Petroleum spirit	04.31	02.15	A yellow coloured gummy extract
Ethyl acetate	02.06	01.03	A reddish/reddish brown powdered residue.
Methanol	11.42	05.71	A reddish brown coloured extract
Water *	39.60	19.80	A dark brown coloured residue
Sclerocarya birrea			
Petroleum spirit	01.62	00.81	A light yellow oily extract which turns light brown after a while.
Ethyl acetate	02.90	01.45	A reddish brown coloured extract.
Methanol	14.46	07.23	A reddish brown coloured extract
Water *	53.80	26.90	A light brown coloured residue

* From fresh 200g sample

3.4.1. BULK EXTRACTION

Promising results obtained motivated bulk extraction of the plant materials.

About 1.3 kg of the root bark of *D. microcarpum* and 2.0kg of the root bark of *S. birrea* were extracted and yield concentrates recorded in Table 4.0.

3.4. PRELIMINARY PHYTOCHEMICAL SCREENING TESTS

Preliminary phytochemical screening was performed on each of the petroleum spirit, ethyl acetate, methanol and water extracts of the root bark of *D. microcarpum* and *S. birrea* respectively. [Laboratory Manual, I (1986 – 87)].

3.4.2. Tests For Carbohydrates

3.4.1.1. Molisch's Test

5ml of the sample (each extract) was put into a test tube and a few drops of Molisch's reagent (10% α -naphthol solution) added. 1ml of conc. H_2SO_4 was allowed to flow down the side of the inclined tube so that the acid forms a layer beneath the aqueous solution without mixing with it. A red ring would be formed at the interphase with a dull – violet precipitate to indicate presence of carbohydrates.

3.4.1.2. Fehling's Test

5ml of a mixture of equal volumes of Fehlings A and B solutions was added to about 3ml of each extract and boiled for a few minutes. A brick- red precipitate of Copper (I) oxide Cu_2O was taken as an indication of the presence of reducing sugar.

3.4.1.3. Standard Test for Combined Reducing Sugar

3ml of each extract was hydrolysed with 10ml of dilute HCl acid by boiling. These were neutralized with NaOH solution and the Fehlings' test

repeated. A brick-red coloured precipitate indicates the presence of combined reducing sugars.

3.4.2. Tests For Glycosides

3.4.2.2. Hydrolysis of Glycosides – by Dilute Mineral Acid

About 2.5ml of dilute H_2SO_4 acid was added to 5ml of each extract in a test tube and boiled in a water bath for 15 minutes. It was cooled and neutralized with 20% KOH solution. 5ml of a mixture of Fehling's solution A and B was added and boiled. Reducing sugars released as a result of the hydrolysis is shown by a brick red precipitate.

3.4.2.2. Test For Cyanogenetic Glycosides

About 0.5g of the powdered root bark sample for each plant was used for the test. They were placed in separate test tubes and sufficient water to cover the bark added. A moist sodium picrate paper (prepared by dipping picric acid paper in sodium carbonate solution) was suspended in the neck of the tube and trapped by means of a cork. The closed tube was placed in an oven at $45^{\circ}C$ for 1 hour. A brick-red colour observed on the paper strip indicates release of hydrocyanin HCN from the crude plant sample.

3.4.2.3. Test For Saponin Glycosides

(c) The Frothing Test

A small quantity of each extract was shaken with distilled water in a small test tube. Frothing which persisted on warming was taken as evidence for the presence of saponins.

(d) The Olive Oil Test

A small quantity of each extract was shaken with about 5ml of distilled water for 2mins. Two drops of olive oil was added and shaken rigorously.

Frothing occurred and an emulsion was formed with the olive oil as an indication of the presence of saponins.

3.4.2.4. Test For Cardiac Glycosides

(b) Legal Test

A small quantity of each extract was dissolved in pyridine and a few drops of 2% sodium nitroprusside together with a few drops of 20% NaOH solution added. A deep red colour that fades to a brownish colouration is expected to indicate the presence of cardenolides in the extracts.

(b) Libermann Burchardt Test

1ml of anhydrous acetic acid and 1ml chloroform were combined and cooled in an ice bath, one drop conc. H_2SO_4 was carefully added to form a lower layer. The extracts in chloroform were added to the solvent mixture and colour

change observed. A deep pink to greenish pink colour indicates the presence of a steroidal nucleus as the aglycone portion of the cardiac glycosides. [Cannel, 1998].

(c) Kedde Test

8% Methanolic solution of each plant extract was made. 1ml of each extract was mixed with 1ml of 2% solution of 3, 5 – dinitrobenzoic acid in 95% alcohol. The solution was made alkaline with 5% NaOH. A violet colour, which fades through reddish brown to brownish yellow is observed. This indicates the presence of the cardenolide aglycone (free or combined).

3.4.3. Test For Sterols: Salkowski Reaction

1.0 – 2.0mg of the sample was dissolved in 1ml chloroform (CHCl_3) and 1ml conc. H_2SO_4 added, thus forming two layers, with red or yellow coloured interphase. This indicates the presence of sterols and methylated sterols [Cannel, 1998].

3.4.4. Test For Flavonoids: Shinoda Test

A small quantity of magnesium powder and a few drop of conc. HCl were added to an alcoholic solution of each plant extract. The appearance of orange, pink, red to purple colours indicates the presence of flavones, flavanols, the corresponding 2, 3-dihydro derivatives and/or Xanthones.

When zinc is used for the test, a deep red to magenta colour is observed for flavones while the flavanones and flavonols will give a faint pink to magenta colour or no colour change. [Cannel, 1998].

3.4.5. Test For Alkaloids

3.4.5.1. Preparation of Extracts

The extracts were cleaned (to remove non-alkaloidal compounds capable of giving “false positive” results) using the following procedure: To a small quantity of the powdered root bark was added 1% dil. HCl (about 20ml) on a steam bath and heated, this was then filtered. The filtrate was made alkaline with 28% NH₃ solution and then extracted with three 5ml aliquots of CHCl₃. The combined CHCl₃ extracts were concentrated and treated with an equal volume of 1% HCl acid. Each of the combined CHCl₃ extract for each plant was then tested with Mayer’s; Dragendorff’s and Wagner’s Reagents. [Trease and Evans, 1983].

3.4.5.2. Mayer’s Reagent Test

Freshly prepared Mayer’s reagent [Potassium mercuric iodide solution] was added dropwise to each acidified solution of the extracts. Presence of alkaloid was indicated by a white to yellow or creamy white precipitate.

3.4.5.3. Dragendorff’s Reagent Test

An acidified solution of the extract was treated with Dragendorff’s reagent [Bismuth potassium iodide solution]. An orange-red or orange-brownish precipitate was taken as evidence of alkaloids.

3.4.5.4. Wagner's Reagent Test

An acidified solution of the extract was treated with the reagent [Iodine-potassium iodide solution]. A brown precipitate in acidic solution suggests the presence of alkaloids. [Cannel, 1998].

3.4.6. Tests for Resin and Balsams

3.4.6.1. Test For Resin

About 5ml of each extract was treated with equal volume of copper acetate solution, shaken vigorously and allowed to separate. A green colouration observed indicates the presence of resin.

3.4.6.2. Tests For Balsam

About 5ml of each extract was treated with 2-3 drops of alcoholic FeCl_3 solutions. A dark green coloured solution is taken as evidence of balsam in the extracts.

3.4.7. Test For Tannins

A small quantity of each extract was dissolved in distilled water (10ml), boiled and filtered. To the filtrate was added few drops of 1% Iron (II) chloride solution, a blue – black, green or blue-green precipitate was taken as evidence for the presence of tannins.

The results of the phytochemical screening tests on the extracts are summarized in Tables 4.1 and 4.2. The tests were carried out on each plant extract except where protocol/methodology for the test states otherwise.

3.5. ANTIBACTERIAL SCREENING TEST USING THE AGAR-DIFFUSION METHOD

The paper disc-diffusion method [Ericson et al, 1960, Bauer et al, 1966] was used to determine the antibacterial activity of the extracts. Dilution susceptibility testing method was used to determine the minimal concentrations, usually expressed in units of micrograms per millilitre ($\mu\text{g/ml}$) of an antimicrobial agent required to inhibit or kill a micro-organism. Procedure for the determination of antimicrobial inhibitory activity is carried out by either agar – or broth – based methods.

The antibacterial agents were tested using the agar – diffusion technique and the lowest concentration that inhibited visible growth of an organism recorded as the MIC. It is important to note that the actual antimicrobial concentration required to inhibit growth is between the highest tested two fold dilution that inhibited growth and the next – lowest dilutions at which growth was observed.

3.5.1. Isolation of Cultures

Standard strain of *Staphylococcus aureus* NCTC 6571; *Bacillus subtilis* ATCC 11778; *Corynebacterium pyogenes* ATCC 10242; *Salmonella typhi* NCTC 52311 and *Escherichia coli* NCTC 10418 were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital [ABUTH], Zaria. All cultures were checked for purity and maintained in nutrient agar slants.

3.5.2. Preparation and Storage of Media

In the paper-disc method adopted for the tests, solutions of varying concentration from $1.0 - 5.0 \times 10^4 \mu\text{g/ml}$ were prepared from each extract using pure extruding solvents.

Petridishes were washed and sterilized in autoclave at about 120°C for 25 minutes and allowed to equilibrate to $48^\circ\text{C} - 50^\circ\text{C}$ before use. They were labelled indicating the concentrations, test organisms and type of extracts.

Mueller – Hinton nutrient agar medium was used as the growth medium for the microbes. The nutrient agar medium was prepared by dissolving 38g of nutrient agar in 1000cm^3 of distilled water, heated to dissolve and autoclaved at 121°C for 15 minutes. It was allowed to cool and about 20ml was poured into sterilized Petri dishes, covered and allowed to solidify after a few minutes. This afforded what is known as plates of Mueller – Hinton sensitivity Agar [Oxoid]. These oxoid plates were then seeded with the test micro-organisms by the spread-plate technique and were then left for half hour to dry. [Felmghan and Stokes, 1972].

Sterile paper discs were soaked in the prepared solutions of the extracts with varying concentrations. They were dried at 50°C . The dried paper discs were then planted (with enough spacing to avoid overlapping of the expected inhibition zones) on the nutrient agar plates seeded with the test organisms. These were used to determine the zones of inhibition and minimum inhibitory concentration (MIC).

A control experiment was also set up using only the extruding/extracting solvent for each of the test organisms. The plates were incubated for 24 hours at 37°C, after which they were inspected for the zones of inhibition of growth. The observed zones of growth inhibition were measured and recorded in millimeters of their diametrical sections. The results are given in Tables 4.3 and 4.4 and photographs of the plates are shown in Appendix I.

3.6. CYTOTOXICITY TEST

The method used for the cytotoxicity test was the Brine shrimp lethality assay. This method utilizes the lethality of brine shrimp nauplii as an indication of toxicological activity.

The test was carried out on the methanol and ethyl acetate extracts of each plant because from the phytochemical screening tests, most of the constituents identified in the water and methanol extracts were similar, hence the test was conducted only on the methanol extracts of both plants. The pet spirit extracts had shown no inhibition against the micro organisms, thus were not screened.

3.6.1. Materials

- h. Small tank with perforated dividing dam to hatch shrimp eggs:-
[Hatching chamber].
- i. *Artemia salina* (Artemia saline leach or Brine shrimp eggs).
- j. Micro pipettes (0.50ml, 1.0ml)
- k. Test-tubes (10mm x 5mm) [15 per extract plus one control]
- l. Calibrated pipettes (1.0ml, 2.0ml)

- m. Sea water (from Bar beach, Lagos)
- n. Dimethylsulphoxide [DMSO].

3.6.2. Procedure

Sea water was put into the hatching chamber and shrimp eggs were added at the extreme end before the dam. This was allowed to stand for 48 hours to hatch and mature as nauplii at room temperature but near a light source so as to attract the shrimp larvae through the perforation in the dam. After 48 hours, the phototropic nauplii (shrimp larvae) were ready for use. (Laboratory Manual II)

The methanol and ethyl acetate extracts of both *D. microcarpum* [DTMe], [DTEa] and *S. birrea* [SBMe],[SBEa] were dissolved in Dimethylsulphoxide [DMSO]. 30mg of each extract was dissolved in 3ml of the appropriate solvent and used as stock solution. The following concentrations were prepared for each extract from the stock solution by serial dilution: 1000 g/ml , 500 g/ml , 250 g/ml , 125 g/ml and 62.5 g/ml . Three test-tubes were prepared for each concentration, hence a total of 15 test-tubes for each sample and one test tube containing only sea water for control].

10 shrimp larvae were collected from the illuminated side of the tank with micropipette and successively transferred into each test tube and the control test tube. Sea water was added to each test tube to make up a total volume of 5ml. The test tubes were allowed to stand for 24 hours under illumination after which the number of survivors were counted. From the survivor, the number of the deaths at each concentration and control were recorded.

3.6.3. Determination of LC₅₀.

The recorded percentage death for the extracts was determined at 95% confidence interval using the method of linear regression analysis described by Saunders and Fleming (1971).

Results of the brine shrimp lethality test are given in Table 4.5.

3.7. ANTIFEEDANT TESTS

The antifeedant activity of the two plants were also evaluated against stored – product insect pests. The petroleum ether and methanolic crude extracts were used in this evaluation.

3.7.1. Preparation of Extracts

Pure extruding solvents for each of the plant extract was used as a vehicle and varying concentrations of 1.0% w/v, 5.0%w/v and 10%w/v were prepared.

3.7.2. Insect Culture

A culture of *Tribolium casteneum*, rust red flour beetle (a stored product pest in maize) was used for this experiment. The culture was obtained from Biological Sciences Department, Ahmadu Bello Univerisity, Zaria. The insects were raised in wheat powder under laboratory conditions at $30 \pm 3^{\circ}\text{C}$.

3.7.3. Procedure

A modified standard method [Bloszyk et al, 1995] was adopted for the deterrent test. Wafer discs of wheat flour were used as the testing food. The discs, about 1.0cm diameter and of variable weights (25 – 30mg) were prepared

and allowed to equilibrate to the test environment moisture conditions for a minimum of two weeks before the feeding trial commenced. The extracts (E) were applied to the discs using a dropping pipette. Another set of discs received only an equal amount of pure solvents to serve as a control [C]. The solvents were evaporated completely from the wafer discs by equilibrating the wafer discs in the test environment for three (3) days prior to the experiment. After equilibration, the wafers were placed in sets of three tubes with:

Tube 1 [CC] containing two control discs.

Tube 2 [CE] containing one disc with extract and one control disc.

Tube 3 [EE] containing two extract discs.

This was repeated for the varying concentrations prepared from the pet. spirit and methanolic extracts for each plant.

A standard reference; a storage pesticide Pirimiphos methyl-2% dust was also set up under the same experimental condition. Methanol was used as the extruding solvent.

For each set of experiment, fifteen [15] unfed, adult *Tribolium castaneum* were introduced into each tube and the weight loss from each disc in the tubes (representing the amount consumed) after 12days was taken. This was used to calculate the antifeedant index (T). The experiment was replicated five times, and observations were made on the conditions and activity or otherwise of the insect towards the wafer during these periods. For each set of values, the mean \bar{x} , mean

deviation D, variance σ^2 and standard deviation σ were calculated to obtain the antifeedant index T.

The data generated from the tests are tabulated in Table 4.6.

3.8. FUNCTIONAL GROUP TEST OF THE *Sclerocarya birrea* Pet. Spirit extract. [SBPe].

Preliminary investigation of functional groups on the *Sclerocarya birrea* [SBPe] oil indicated the presence of an aldehyde (by the Silver mirror test). For this test, about 3ml of silver nitrate solution was put into a clean test tube, a drop of dilute NaOH solution was added, followed by ammonia solution dropwisely until the brown precipitate of silver oxide dissolved. To this solution was added a little quantity of the SBPe oil.

The test-tube was warmed in a beaker of boiling water for about 5 minutes. The presence of the alkanal (aldehydic) functional group is indicated by the silver deposit on the wall of the test-tube.

3.8.1. Oxidation of The Aldehyde Functional Group.

About 8.50g of the *Sclerocarya birrea* pet. spirit extract, SBPe oil was put in a round bottom flask. This was treated with saturated solution of sodium dichromate in concentrated H_2SO_4 and stirred. The reaction was left to stand for about 2 hours; cooling was by immersion of the flask in an ice-bath because the reaction was exothermic.

The reaction mixture was allowed to attain room temperature gradually, after which about 50mls of diethyl ether was used to extract the organic matter. The ethereal layer was separated and the mixture was further washed with another 50mls of diethyl ether.

The ethereal extracts were combined, washed with saturated NaHCO_3 to remove the acid content. The lower aqueous layer which contains the acid salt was acidified with conc. HCl and re-extracted into diethyl ether. The diethyl ether solution was washed with distilled water, dried over anhydrous Magnesium sulphate (MgSO_4). The ether was removed by heating over a steam bath. A white crystalline solid, weighing 1.58g was obtained as the acid derivative, SBPeA.

3.10. PURIFICATION AND CHARACTERIZATION OF THE *Sclerocarya birrea* methanol extract. [SBMe].

3.9.1. Acid Hydrolysis of SBMe Extract

About 125gm of the crude *Sclerocarya birrea* methanolic extract [SMBe] was hydrolyzed with 400ml 2M HCl acid over a water bath for 45mins. The solution was stirred at regular intervals. The hydrolysis is to breakdown the glycoside molecule into its sugar and aglycone component.

3.9.1.1. Test for Reducing Sugar

About 5ml of the hydrolyzed SBMe extract was put into a test tube and equal volumes of Fehling's A and B (about 5ml) added. This was boiled for about 5mins. A brick red precipitate indicates the presence of reducing sugars.

3.9.1.2. Extraction of SBMe Hydrolysate

The residue obtained from the hydrolysis weighed about 48.16g. This was extracted exhaustively with chloroform by sohxlet extraction.

1.47g of a reddish brown chloroform extract was obtained from the extract, after concentration on the rotary evaporator. The IR-spectrum of this extract was run and reported as Appendix VII.

3.9.2. Functional Group Tests.

Tests for Alkaloids, cardiac glycosides and phenolic compounds.

3.9.2.1. Alkaloid Test

The Wagner reagent [Iodine – Potassium iodide solution] test was used. The sample was spotted on micro TLC plates coated with alumina and developed using the Methanol: Chloroform [1:2] solvent mixture. This was sprayed with a manual spray-pump. A reddish brown (or brown) colour observed is taken as a positive result for the presence of alkaloids. [Cannel, 1998].

3.9.2.2. Test for Cardiac Glycosides: Salkowski Test

A small quantity of the hydrolysate was dissolved in 2ml chloroform. Concentrated sulphuric acid (H₂SO₄) was carefully added to form a lower layer. A

reddish-brown colour at the interface indicates the presence of a steroidal ring (i.e. aglycone portion of the cardiac glycoside). [Cannel, 1998].

3.9.2.3. Test for Phenols

A small quantity of the hydrolysate was stirred with 2mls of distilled water and FeCl_3 reagent added to the filtrate. A blue-black, green or blue-green precipitate was taken as evidence for the presence of condensed tannins [Trease and Evans, 1989]. A brown precipitate indicates the presence of polyphenols [Cannel, 1998]. The result is tabulated in Table 4.8.

3.9.3. Chromatographic Separations

The Thin-Layer [TLC] and Column [CC] chromatographic techniques were adopted for the purification process.

3.9.3.1. Preparation of Plates

a. Preparation of Thin-Layer Chromatographic [TLC] Plates

Plates [20 x 5.0cm] of thickness 0.50mm were used for qualitative analytical purposes to enable the determination of the appropriate solvent mixture required to separate the components of the given extracts. The support used was glass plates and the adsorbent Alumina [Aluminium oxide with 15% CaSO₄].

A slurry was made by stirring 30g Alumina with 40cm³ of distilled water. This was shaken to give uniform slurry and was spread at 250cm thickness on glass plates which had been cleaned with cotton wool soaked in acetone. The plates were allowed to dry for about 2 hours and used.

b. Preparation of Preparative TLC Plates

For the preparative TLC, Larger plates (20 x 20cm) of 0.75mm thickness were used. An equal weight/volume ratio of 320g Alumina (with 15% CaSO₄) and 320mls distilled water was mixed and shaken. This was allowed to stand for a few minutes and shaken again and spread on the plates. The freshly coated plates were then stored horizontally for 15 hours at room temperature before use.

For spotting the TLC plates, capillary tubes were used, and for the preparative TLC plates, a micropipette was used. Ascending development method, and in some cases, multiple development was used to enhance proper separation.

For developing the chromatogram on the TLC plates, various solvent mixtures were tried. Methanol: Chloroform in the ratio of 1:2 gave the best

separation. The spots/bands were observed under uv-flourescent lamp (366nm and 254nm). The 254nm wavelength was used predominantly. The column chromatography was conducted on the basis of this mobile phase.

3.9.3.2. Purification of the SBMe Hydrolysate by Column Chromatography

1.02g of the SBMe hydrolysate was purified using a column packed with alumina. The extract was introduced into the column and developed using the mobile phase – Methanol: Chloroform [1:2]. Fractions of 20ml were collected and analyzed by TLC and similar fractions pooled. The combined fractions were concentrated at reduced pressure and dried. Preparative TLC was carried out on fractions requiring further purification.

Table 4.9. gives the main fractions obtained and the R_f values.

CHAPTER FOUR

4.0. RESULTS AND DISCUSSION

4.1. BULK EXTRACTION

Table 4.0. gives the results of extracts (wt in grams and % yield) obtained from the bulk extraction of plant material of the *D. microcarpum* and *S. birrea*.

Solvent	Wt of Extracts (gms) from 1.3kg of <i>D. microcarpum</i> plant sample	% yield	Wt of Extracts (gms) from 2.0kg of <i>S. birrea</i> plant sample	% yield
Pet. spirit	28.04	2.16	8.69	0.0043
Ethyl acetate	13.38	1.03	10.15	0.0051
Methanol	74.20	5.71	144.69	0.0723

4.2. PRELIMINARY PHYTOCHEMICAL SCREENING

The phytochemical screening was carried out to test for the presence of carbohydrates, sterols, tannins, resins and balsams, cardiac glycosides, saponin glycosides, alkaloids and flavonoids.

4.2.1. *Detarium microcarpum*

The results of the phytochemical screening test performed on the crude extracts of the root bark of *D. microcarpum* showed that the water extract contained carbohydrates (reducing sugar), saponin glycosides, cardiac glycosides,

tannins and alkaloids. The methanol extract contained tannins, cardiac glycosides, saponins, flavonoids, alkaloids and sterols. The ethyl acetate extract contained cardiac glycosides, sterols and flavonoids. The petroleum spirit extract was positive only for resins. The result of the phytochemical screening test is summarized in Table 4.1.

Table 4.1. **Phytochemical Tests of Crude Extracts of *D. microcarpum***

	TEST	OBSERVATION	INFERENCE	STATUS
A	Carbohydrate Tests			
i	Molisch Tests. Pet. Spirit extracts. Ethyl acetate extract	No red ring observed at the interphase	Carbohydrate absent	-ve
	Methanol extract	No red ring observed at the interphase, the solution turned reddish brown.	Carbohydrate absent	-ve
	Water extract	A red ring at the interphase with a violet coloured precipitate observed.	Carbohydrate present	+ve
		A red ring at the interphase with a violet coloured precipitate observed	Carbohydrate present	+ve
ii	Fehling's and Combined Reducing sugar Test. Pet. Spirit extract	An oily layer above the blue coloured solution of the Fehling's solution observed.	Reducing sugar absent	-ve
	Ethyl acetate extract	An orangish coloured precipitate observed	Reducing sugar absent	-ve
	Methanol extract	A brick-red precipitate of copper (II) oxide observed.	Reducing sugar present	+ve
	Water extract	A brick red precipitate of copper (II) oxide observed.	Reducing sugar present	+ve
B	Test For Glycosides			
i.	Hydrolysis of Glycosides Pet. Spirit extract.	General Tests An oily layer above colourless solution observed.	Glycosides absent	-ve
	Ethyl acetate extract	A brick-red precipitate observed	Glycosides present	+ve
	Methanol extract	A brick-red precipitate observed	Glycosides present	+ve
	Water extract	A brick-red precipitate observed	Glycosides present	+ve
ii	Cyanogenetic glycosides Pet-spirit extract Ethyl acetate extract Methanol extract Water extract	Not used Not used Not used No brick-red colour on the paper strip	- - - Cyanogenetic glycosides	- - - -ve

			absent.	
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	TEST	OBSERVATION	INFERENCE	STATUS
iii I	Saponin glycosides Frothing Test Pet-spirit extract	No frothing observed, solution was immiscible	Saponin glycosides absent	-ve
	Ethyl acetate extract	No frothing observed.	Saponin glycoside absent	-ve
	Methanol extract	Frothing observed, this persisted on warming	Saponin glycosides present	+ve
	Water extract	Frothing observed this persisted on warming.	Saponin glycoside present	+ve
II	The Olive oil Test Pet. Spirit extract	No frothing or emulsion formed.	Saponins absent	-ve
	Ethyl acetate extract	No frothing or emulsion formed	Saponins absent	-ve
	Methanol extract	Frothing occurred and an emulsion was formed with the olive oil	Saponins present	+ve
	Water extract	Frothing occurred and an emulsion was formed with the olive oil	Saponins present	+ve
iii I	Cardiac glycosides Legal Test Pet. Spirit extract	A red precipitate observed	Cardenolides absent	-ve
	Ethyl acetate extract	A deep red coloured solution observed.	Cardenolides likely present	+ve
	Methanol extract	A deep red coloured solution which turns reddish brown observed	Cardenolides present	+ve
	Water extract	A deep red coloured solution which turns reddish brown observed.	Cardenolides present.	+ve
II	Lieberman Burchardt Test. Pet. Spirit extract	A light green coloured mixture observed.	Steroidal nucleus absent	-ve
	Ethyl acetate extract	A reddish coloured solution observed.	Steroidal nucleus absent	-ve

	Methanol extract	A deep purple colour observed.	Steroidal nucleus present	+ve
	Water extract	A deep purple colour observed.	Steroidal nucleus present	+ve
III	Kedde Test			
	Pet. Spirit extract	A brownish coloured solution observed	Cardenolide aglycone absent	-ve
	Ethyl acetate extract	A reddish brown coloured solution observed.	Cardenolide aglycone absent	-ve
	Methanol extract	A deep purple coloured solution obtained.	Cardenolide aglycone likely present	+ve
	Water extract	A deep purple to red coloured solution observed.	Cardenolide aglycone likely present	+ve
C	Test for sterols			
	Chloroform extract	A yellow coloured interphase observed.	Presence of sterols indicated	+ve
D	Flavonoid Test			
	Pet-spirit extract	Not used	-	-
	Ethyl acetate extract	Not used	-	-
	Methanol extract	The appearance of a reddish purple (magenta) colour indicates the presence of flavanones	Flavanones present	+ve
	Water extract	Not used	-	-
E.	Alkaloid Test			
i	Mayer's Reagent Test	A brownish cloudy precipitate observed.	Alkaloid absent	-ve
ii	Dragendorff's Reagent test	An orange-red precipitate observed.	Alkaloid present	+ve
iii	Wagner's Test.	A reddish-brown precipitate observed.	Alkaloid present	+ve

F	Resin and Balsam Test				
	i	Test For Resin			
		Pet-spirit extract	A green coloured cloudy solution observed.	Resin present	+ve
		Ethyl acetate extract	A reddish brown solution obtained.	Resin absent	-ve
		Methanol extract	A reddish brown solution obtained	Resin absent	-ve
	Water extract	A pale brown solution obtained	Resin absent	-ve	
ii	Test For Balsam				
		Pet-spirit extract	A light yellow to brown coloured solution obtained	Balsam absent	-ve
		Ethyl acetate extract	A reddish brown solution obtained	Balsam absent	-ve
		Methanol extract	A dirty greenish blue or blue black curdy precipitate observed.	Balsam present	+ve
	Water extract		Balsam present	+ve	
G	Test for Tannins				
	Pet. Spirit extract	A yellow coloured solution observed	Tannin absent	-ve	
	Ethyl acetate extract	A brown precipitate observed	Tannin absent	-ve	
	Methanol extract	A blue-black precipitate observed.	Tannins present	+ve	
	Water extract	A blue-black precipitate observed.	Tannins present	+ve	

Generally, from the results, the root bark of *D. microcarpum* can be said to contain alkaloids, cardiac glycosides, saponin glycosides, sterols, tannins,

flavonoids, resin and balsams. These classes of compounds have been reported to be responsible for many of the pharmacological properties attributed to plants; [Fatope et al, 1993] have reported antimicrobial activities in the stem bark of *D. microcarpum*.

4.2.2. *Sclerocarya birrea*

For the *S. birrea* extracts, the water extract, contained carbohydrates (free and combined reducing sugars), glycosides, saponins and tannins. The methanol extract contained flavones, sterols, saponins cardiac glycosides and carbohydrates. The ethyl acetate extract contained tannins, sterols and cardiac glycosides. The pet-spirit extract contained sterols and cardiac glycosides but the test could not be considered conclusive.

The result of the phytochemical screening test is summarized in Table 4.2.

Table 4.2. **Phytochemical Tests of Crude extracts of *S. birrea*.**

	TEST	OBSERVATION	INFERENCE	STATUS
A i	Carbohydrate Tests			
	Molisch Tests			
	Pet-spirit extract	No red ring observed at the interphase	Carbohydrate absent	-ve
	Ethyl acetate extract	No red ring observed at the interphase, the solution just turned reddish brown.	Carbohydrate absent	-ve
	Methanol extract	A red ring at the interphase with a violet precipitate observed.	Carbohydrate present	+ve
	Water extract	A red ring at the interphase with a violet precipitate observed.	Carbohydrate present	+ve

ii	Fehling's and Combined Reducing Sugar test. Pet-spirit extract Ethyl acetate extract Methanol extract Water extract	A light brown oily layer above the blue coloured solution observed. An orange-red coloured solution observed. A brick-red precipitate observed. A brick red precipitate observed	Reducing sugar absent Reducing sugar absent Reducing present Reducing sugar present	-ve -ve +ve +ve
B I	Tests for Glycosides General tests- Hydrolysis of glycosides. Pet-spirit extract Ethyl acetate extract Methanol extract Water extract	An oily layer observed above the colourless solution A brick red precipitate observed A brick red precipitate A brick red precipitate	Glycosides absent Glycosides present Glycosides present Glycosides present	-ve +ve +ve +ve
ii	Cyanogenetic glycosides Pet-spirit extract Ethyl acetate extract Methanol extract Water extract	Not used Not used Not used No brick red colour on the paper strip	- - - Cyanogenetic glycoside absent	- - - -ve
iii I	Saponin glycosides Frothing Test Pet-spirit extract Ethyl acetate extract Methanol extract Water extract	No frothing occurred solution was immiscible. No frothing observed Frothing observed, persisted after warming Frothing observed, persisted after warming.	Saponins absent Saponins absent Saponins present Saponins present	-ve -ve +ve +ve

II	The Olive oil Test Pet-spirit extract Ethyl acetate extract Methanol extract Water extract	No frothing or emulsion formed No frothing or emulsion formed Frothing occurred and an emulsion was formed with the olive oil. Frothing occurred and an emulsion formed	Saponin absent Saponins absent Saponins present Saponins present	-ve -ve +ve +ve
iv I	Cardiac glycosides Legal Tests Pet-spirit extract Ethyl acetate extract Methanol extract Water extract	A brown coloured solution obtained A red coloured solution observed A red coloured solution observed A reddish brown precipitate formed	Cardenolide absent " " "	-ve -ve -ve -ve
II	Lieberman Burchardt Test. Pet-spirit extract Ethyl acetate extract Methanol extract Water extract	A light brown solution obtained A light red coloured solution observed. A light red coloured solution observed. A dirty brown coloured precipitate observed	Absence of steroidal nucleus indicated	-ve -ve -ve -ve
III	Kedde Test Pet-spirit extract Ethyl acetate extract Methanol extract Water extract	A light red coloured solution that turns brown after a period of time observed. A light purple coloured solution observed which after a period of time fades through reddish brown to a brownish yellow or light brown colour.	Cardenolide aglycone likely present. Presence of Cardenolides indicated.	+ve +ve +ve +ve
C	Test for Sterols Chloroform extract was used.	A yellow coloured interphase observed.	Presence of sterols indicated	+ve

D	Flavonoid Test Pet-spirit extract Ethyl acetate extract Methanol extract Water extract	Not used Not used The appearance of a red coloured solution indicates the presence of flavones Not used	- - Flavones present -	- - +ve -
E	Alkaloid Tests			
i	Mayer's Reagent Test	A light yellow coloured solution observed	Alkaloid absent	-ve
ii	Dragendorff's Reagent Test	An orange-brown coloured solution observed.	Alkaloid present	+ve
iii	Wagner's Reagent Test	An orange-brown coloured solution observed.	Alkaloid present	+ve
F	Resin and Balsam Test			
i	Test for Resin Pet-spirit extract Ethyl acetate extract Methanol extract Water extract	A light brown coloured solution obtained. A reddish brown solution obtained. A pale brown solution obtained	Resin absent Resin absent Resin absent	-ve -ve -ve -ve
ii	Test for Balsam Pet-spirit extract Ethyl acetate extract Methanol extract Water extract	A light brown coloured solution obtained A deep purple to black coloured solution obtained A deep purple to black coloured solution obtained. A dirty brown coloured precipitate formed.	Balsam absent Balsam absent Balsam absent Balsam absent	-ve -ve -ve -ve
G	Test for Tannins			
	Pet-spirit extract	A brown coloured solution observed	Tannins absent	-ve
	Ethyl acetate extract	A brown precipitate observed.	Tannins absent	-ve
	Methanol extract	A blue-black curdy precipitate formed.	Tannins present	+ve
	Water extract	A blue-black curdy precipitate formed.	Tannins present	-ve

The root bark of *S. birrea* contained cardiac glycosides, tannins, saponins, flavanones and sterols. These classes of compounds could be attributed to the pharmacological/medicinal uses attributed to the plants by the locals. [Burkill, 1985; Adoum et al, 1997] reported that extracts from the stem bark of *S. birrea* had shown some activity against bacterial microorganisms.

4.3. ANTIBACTERIAL SCREENING TESTS

4.3.1. *Detarium microcarpum*

The crude petroleum spirit, ethyl acetate, methanol and water extracts obtained from the root bark of the plant, *D. microcarpum* were screened for antimicrobial activities using the agar-diffusion method. The results are shown in Table 4.3 (a) – (b); and the photographs of the plates are shown in Appendix I.

**Table 4.3.(a): Inhibition of Bacterial Growth of the Root bark Extract of *D. microcarpum*:
Determination of Zone of Inhibition (mm).**

Extracts	Pet. Spirit				Ethyl acetate				Methanol				Water			
Concentrations x 10 ³ in g/ml	1.0	2.0	3.0	4.0	1.0	2.0	3.0	4.0	1.0	2.0	3.0	4.0	1.0	2.0	3.0	4.0
Test Organisms	Zone of Inhibition (mm)															
<i>S. aureus</i>	0	0	0	0	29	31	33	35	33	34	36	37	35	37	39	45
<i>B. subtilis</i>	0	0	0	0	25	28	31	36	26	29	30	35	27	29	32	36
<i>C. pyogenes</i>	0	0	0	0	32	35	36	37	32	36	37	39	35	38	41	46
<i>S. typhi</i>	0	0	0	0	27	29	31	33	33	34	38	39	30	34	38	42
<i>E. coli</i>	0	0	0	0	14	16	20	22	12	14	19	21	15	17	19	21

Table 4.3(b): **Determination of Minimum Inhibition Concentration (MIC) of *D. microcarpum* extracts.**

Test Organisms	Concentrations of extract in µg/ml																			
	Petroleum Spirit					Ethyl acetate					Methanol					Water				
	4.0	3.0	2.0	1.0	9.0	4.0	3.0	2.0	1.0	9.0	4.0	3.0	2.0	1.0	9.0	4.0	3.0	2.0	1.0	9.0
	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	10 ³	10 ³	10 ³	10 ³	10 ⁴	10 ³	10 ³	10 ³	10 ³	10 ⁴	10 ³	10 ³	10 ³	10 ³	10 ⁴	10 ³	10 ³	10 ³	10 ³	10 ⁴
<i>S. aureus</i>	-	-	-	-	-	+	+	+	+	+0	+	+	+	+0	-	+	+	+	+	+0
<i>B. subtilis</i>	-	-	-	-	-	+	+	+	+0	-	+	+	+0	-	-	+	+	+	+	+0
<i>C. pyogenes</i>	-	-	-	-	-	+	+	+	+	+0	+	+	+0	+0	-	+	+	+	+	+0
<i>S. typhi</i>	-	-	-	-	-	+	+	+	+	+0	+	+	+0	-	-	+	+	+	+	+0
<i>E. coli</i>	-	-	-	-	-	+	+	+0	-	-	+	+0	-	-	-	+	+	+	+	+0

KEY

+ - Inhibition

- - No Inhibition

+0 - Minimum Inhibition Concentration (MIC)

Table 4.3(a) tabulates the zone of inhibitions of bacterial growth at various concentrations for the four solvent used for extraction processes of the plant material. The water, methanol and ethyl acetate extracts showed considerable amount of inhibition against *S. aureus*, *B. subtilis*, *C. pyogenes* and *S. typhi*. These microorganisms are the causative agents for several illnesses and disease for which the plant is traditionally reported to remedy such as skin eruptions dysentery etc. [Irvine, 1961; Oliver, 1946]. The extracts have also shown strong inhibition against *B. subtilis*, a micro-organism found in the soil which could also cause disruption in plant growth. [Thomas, 1979]. The pet. spirit extract showed no inhibition at all. It was observed that the extract was gummy and sticky in nature and did not diffuse into the agar medium. Table 4.3(b) shows the minimum inhibitory concentration (MIC) of the extracts on the isolates. The MIC for the

ethyl acetate extract was recorded at $9.0 \times 10^4 \mu\text{g/ml}$ for *S. aureus*, *C.pyogenes* and *S. typhi*. The methanol extract had MIC value at $1.0 \times 10^3 \mu\text{g/ml}$ for *S. aureus* and *C. pyogenes*; while for *B. subtilis* and *S. typhi*, the MIC value was recorded at $2.0 \times 10^3 \mu\text{g/ml}$. The water extract had MIC value of $9.0 \times 10^4 \mu\text{g/ml}$ for the organisms, *S. aureus*, *B. subtilis* and *S. typhi* and $1.0 \times 10^3 \mu\text{g/ml}$ for *E. coli* and *C.pyogenes*.

4.3.2. *Sclerocarya birrea*

The crude petroleum spirit, ethyl acetate, methanolic and water extracts were screened for antimicrobial activities in a similar manner.

The results are shown in Tables 4.4. (b)

Table 4.4. (a): **Inhibition of Bacterial Growth of the Root bark Extracts of *S. birrea*: Determination of Zone of Inhibition (mm).**

Extracts	Pet. Spirit				Ethyl acetate				Methanol				Water			
	1.0	2.0	3.0	4.0	1.0	2.0	3.0	4.0	1.0	2.0	3.0	4.0	1.0	2.0	3.0	4.0
Concentrations $\times 10^3$ in g/ml																
Test Organisms	Zone of Inhibition (mm)															
<i>S. aureus</i>	0	0	0	0	0	5	10	15	0	9	14	20	0	0	8	15
<i>B. subtilis</i>	0	0	0	0	0	9	14	20	0	7	13	16	0	0	6	13
<i>C. pyogenes</i>	0	0	0	0	0	4	11	16	0	8	12	18	0	5	10	14
<i>S. typhi</i>	0	0	0	0	0	0	5	14	0	0	8	14	0	0	6	13
<i>E. coli</i>	0	0	0	0	0	0	6	9	0	0	5	10	0	0	8	16

Table 4.4. (b): **Determination of Minimum Inhibition Concentration (MIC) of *Sclerocarya birrea* extracts.**

Extracts	Petroleum spirit					Ethyl acetate					Methanol					Water				
	Concentrations $\times 10^4$ in $\mu\text{g/ml}$																			
	5.0	4.0	3.0	2.0	1.0	5.0	4.0	3.0	2.0	1.0	5.0	4.0	3.0	2.0	1.0	5.0	4.0	3.0	2.0	1.0
Test Organisms																				
<i>S. aureus</i>	-	-	-	-	-	+	+	+	+0	-	+	+	+	+0	-	+	+	+0	-	-
<i>B. subtilis</i>	-	-	-	-	-	+	+	+	+0	-	+	+	+	+0	-	+	+	+0	-	-
<i>C. pyogenes</i>	-	-	-	-	-	+	+	+0	+0	-	+	+	+	+0	-	+	+	+	+0	-
<i>S. typhi</i>	-	-	-	-	-	+	+	+0	-	-	+	+	+0	-	-	+	+	+0	-	-
<i>E. coli</i>	-	-	-	-	-	+	+	+0	-	-	+	+	+0	-	-	+	+	+0	-	-

KEY

+ - Inhibition

- - No inhibition

+0 - Minimum Inhibition Concentration (MIC)

Table 4.4. (a) tabulates the zone of inhibitions of bacterial growth at various concentrations for the four solvent used the plant extractions. The ethyl acetate, methanol and water extracts showed considerable amount of inhibition against *S.aureus*, *B. subtilis*, *C. pyogenes*, *S. typhi* and *E. coli*. Though it was observed that the inhibition zones were of lower values and inhibitions were mostly at concentrations above $1.0 \times 10^3 \mu\text{g/ml}$. The plant is used locally for the treatment of wounds which could be infected by micro-organisms such as *C. pyogenes* and for

stomach and intestinal complaints due to micro-organisms such as *E. coli* and this result really provides a scientific basis for the folk medicinal properties of the plant.

Inhibition was minimal (<2.0mm) in the pet. spirit extract for most of the micro-organisms test.

Table 4.4. (b) shows the minimum inhibitory concentration of the extracts on the isolates. The MIC for the ethyl acetate was recorded at recorded at $2.0 \times 10^4 \mu\text{g/ml}$ for *S. aureus* and *B. subtilis*, while *C. pyogenes*, *S. typhi* and *E. coli* had MIC value of $3.0 \times 10^4 \mu\text{g/ml}$. The methanol extract's MIC value was for *S. aureus*, *B. subtilis* and *C. pyogenes*; while the water extract at this value only or *C. pyogenes*. The water extract had MIC of $3.0 \times 10^4 \mu\text{g/ml}$ for most of the micro-organisms used in the test.

Appendix I shows the pictures of the some of the petri-dishes with the zones of inhibition observed for some of the *D. microcarpum* extracts.

4.4 CYTOTOXICITY TEST

This is a simple bioassay used in the detection of plant constituents with a variety of pharmacological activities. It has the advantage of being rapid, inexpensive and simple.

The level of toxicity, which is its activity, is indicated by the number or percentage of dead shrimp larvae after 24 hours in the extract solution. Tables 4.5

(a) and (b) show the results of the brine shrimp lethality test for the methanolic and ethyl acetate extracts for *D. microcarpum* and *S. birrea* at varying concentrations.

Table 4.5. (a): **Result of brine shrimp larvae test for crude *D. microcarpum* extracts.**

PLANT	<i>Detarium microcarpum</i> crude extracts											
Concentrations (µg/ml)	1000		500		250		125		62.5		Control	
EXTRACTS	ME	EA	ME	EA	ME	EA	ME	EA	ME	EA	Sea water	
Total no. of shrimps	30	30	30	30	30	30	30	30	30	30	10	
No. of survivor	00	00	05	06	08	08	09	11	14	16	10	
No. of death	30	30	25	24	22	22	21	19	16	14	00	
% Mortality	100	100	83.33	80	73.33	73.33	70.00	63.33	43.33	46.67	-	
	LC ₅₀ ME ⇒ 91.20 ± 8.32											
	LC ₅₀ EA ⇒ 93.33 ± 15.14											

Table 4.5. (b): **Result of Brine Shrimp Larvae Test for Crude *S. birrea* extracts.**

PLANT	<i>Sclerocarya birrea</i> crude extracts											
Concentrations (µg/ml)	1000		500		250		125		62.5		Control	
EXTRACTS	ME	EA	ME	EA	ME	EA	ME	EA	ME	EA	Sea water	
Total no. of shrimps	30	30	30	30	30	30	30	30	30	30	10	
No. of survivor	00	05	02	10	12	15	17	21	22	23	10	
No. of death	30	25	28	20	18	15	13	09	08	07	0	
% Mortality	100	83.33	93.33	62.22	60	50	43.33	30	26.67	23.33	-	
	LC ₅₀ ME ⇒ 134.90 ± 3.98											
	LC ₅₀ EA ⇒ 257.04 ± 36.31											

KEY:

ME - Methanol extracts for each plant

EA - Ethyl acetate extract for each plant.

For each plant, it was observed that in the methanolic and ethyl acetate extracts, the number of death or % mortality decreased with decreasing concentrations of the extracts from 1000 μ g/ml to 62.5 μ g/ml.

The petroleum spirit extracts were not screened because results obtained from the antimicrobial and antifeedant test had indicated low activity in these extracts. The preliminary phytochemical screening had shown that most of the plant constituents were in the methanol and ethyl acetate extracts.

The regression line method was used in determining the LC₅₀ values as reported in Appendix II.

From the LC₅₀ values, the methanol extract from *D. microcarpum* is the most potent of the extracts, though all the extracts can be said to be potent or active since the LC₅₀ values were less than 1000 μ g/ml. Extracts are said to display toxicity in the brine shrimp assay when LC₅₀ is less than 1000 μ g/ml, as such all the crude extracts showed significant toxicity since the values are less than 1000 μ g/ml. This cytotoxicity test results is an indication that the root bark of *D. microcarpum* and *S. birrea* possess some pharmacologically active antimicrobial and/or antifeedant constituents. This result is in agreement with the antimicrobial screening tests where the extracts were observed to have inhibited the growth of the microorganisms. This could also be attributed to the presence of chemical constituents such as cardiac glycosides, tannins sterols, etc. in the plant extracts. For example, it could be suggested that the tannin content of the plant *S. birrea* is

responsible for its claimed efficacy in the treatment of wounds and burns that could have been infected by micro-organisms such as *Staphylococcus aureus*. For the plant *D. microcarpum*, traditionally, the bark is said to be poisonous and used for ordeals (an ancient form of trial to ascertain judgment) and this has been proven scientifically by the toxicity test because the crude extracts had a significant toxicity value.

The brine shrimp lethality test was carried out on the ethyl acetate and methanol extracts for the each plant because these were the ones had shown good antimicrobial activities. Moreover, most of the phytochemical tests conducted indicated that many of the constituents present in the plants are in the methanol, ethyl acetate and water extracts.

4.5. ANTIFEEDANT TESTS

The antifeedant index T is a co-efficient providing a measure of the unpalatableness of a substance and this is reported on the antifeedant scale. This scale infers that T values from:

200	→	151	means	Excellent antifeedants
150	→	101	means	Good antifeedants
100	→	051	means	Relatively good/Medium antifeedants
050	→	001	means	Neutral substances
Negative values			means	Attractants

These are values indicating the antifeedant quality/traits of a substance.

The formula for the calculation is given as:

$T = A + R$ where:

$A = CC - EE$ from each tube where

CC is the average weight of wafer discs in each CC tube [Control Tubes]

EE is the average weight of wafer discs in each EE tube [Extract Tubes]

And $R = C - E$ where $C - E$ is the averaged weight difference between the wafer discs in each combined CE tubes

CC – EE are the “no choice option” tubes, in which the insects fed basically on either the treated or untreated wafer since that is what is available to them.

CE tubes are the “choice option” tubes, in which the insect have a combination of a treated and an untreated wafer disc in the tube and can make a choice in feeding.

Table 4.6 gives a summarized data of results obtained from the various antifeedant tests.

Table 4.6 Result of the Antifeedant Tests.

Treatments at varying concentrations	Food Consumed * (% Wt of wafers)	
<i>Detarium microcarpum</i>	Petroleum spirit extract	Methanol extracts
1.0% w/v		
Control – Control [CC] tubes	72.29 ± 7.61	67.01 ± 2.23
Control – Extract [Control] CE tubes	68.55 ± 5.13	75.07 ± 9.16
Control – Extract [Extract] CE tubes	33.07 ± 11.05	21.96 ± 8.21
Extract – Extract [EE] tubes	47.28 ± 6.35	22.32 ± 7.46
T value	60.49	97.80
5.0% w/v		
Control – Control [CC] tubes	63.78 ± 3.90	64.18 ± 1.46
Control – Extract [Control] CE tubes	65.96 ± 3.46	65.36 ± 2.41
Control – Extract [Extract] CE tubes	07.69 ± 4.76	03.96 ± 3.99
Extract – Extract [EE] tubes	07.77 ± 3.02	05.21 ± 2.53
T value	114.28	120.37
10.0% w/v		
Control – Control [CC] tubes	67.70 ± 4.43	70.63 ± 1.64
Control – Extract [Control] CE tubes	69.45 ± 1.89	69.49 ± 2.78
Control – Extract [Extract] CE tubes	07.86 ± 5.68	02.87 ± 1.35
Extract – Extract [EE] tubes	13.75 ± 9.40	02.41 ± 0.76
T value	115.54	135.24
<i>Sclerocarya birrea</i>		
1.0% w/v		
Control – Control [CC] tubes	63.47 ± 1.74	64.10 ± 2.27
Control – Extract [Control] CE tubes	64.42 ± 1.60	68.49 ± 3.19
Control – Extract [Extract] CE tubes	37.75 ± 4.96	27.87 ± 2.83
Extract – Extract [EE] tubes	32.30 ± 4.83	23.91 ± 5.68
T value	57.84	80.81
5.0% w/v		
Control – Control [CC] tubes	65.57 ± 3.13	65.42 ± 2.39
Control – Extract [Control] CE tubes	68.76 ± 5.04	64.90 ± 1.38
Control – Extract [Extract] CE tubes	38.10 ± 9.01	13.28 ± 0.69
Extract – Extract [EE] tubes	29.24 ± 5.43	14.31 ± 1.05
T value	66.99	102.73
10.0% w/v		
Control – Control [CC] tubes	66.54 ± 5.27	66.63 ± 4.12
Control – Extract [Control] CE tubes	66.87 ± 3.18	67.94 ± 1.77
Control – Extract [Extract] CE tubes	26.89 ± 5.23	06.33 ± 1.82
Extract – Extract [EE] tubes	21.16 ± 5.78	04.53 ± 0.69
T value	85.36	123.71

Pirimiphos methyl – 2% Dust		
0.1% w/v		
Control – Control [CC] tubes	-	63.63 ± 2.66
Control – Extract [Control] CE tubes	-	63.75 ± 2.07
Control – Extract [Extract] CE tubes	-	16.24 ± 2.36
Extract – Extract [EE] tubes	-	18.80 ± 5.21
T value		92.34
0.5% w/v		
Control – Control [CC] tubes	-	68.04 ± 1.52
Control – Extract [Control] CE tubes	-	40.00 ± 11.05
Control – Extract [Extract] CE tubes	-	02.50 ± 1.26
Extract – Extract [EE] tubes	-	02.72 ± 0.33
T value		102.76
1.0% w/v		
Control – Control [CC] tubes	-	63.30 ± 3.09
Control – Extract [Control] CE tubes	-	57.91 ± 6.53
Control – Extract [Extract] CE tubes	-	03.77 ± 1.68
Extract – Extract [EE] tubes	-	03.24 ± 0.67
T value		114.20

*% values represent mean ± S.D from five different observations.

For the antifeedant tests, only the methanol and petroleum spirit extracts of each plant were screened because wafers treated with the water extracts became mouldy and were not easy to handle. Since the preliminary phytochemical screening had shown that the ethyl acetate and methanol extract of each plant contained almost the same class of compounds, only the methanol extracts of each plant was screened for antifeedant activity.

For the standard reference, Pirimiphos methyl -2% Dust, it dissolved only in methanol, hence the antifeedant test was conducted using methanol as the extruding solvent. Water was also a suitable solvent but unsuitable for treatment using the wafer discs in this experiment.

Antifeedant tests results calculated based on the antifeedant index T, (Abubakar et al, 2001) is reported in Appendix III.

4.5.1. Observations

i. *Detarium microcarpum*

In the 1.0% w/v petroleum spirit extract test, the insects seemed to be able to survive as no death was recorded and the insect were still able to feed substantially on the treated wafer discs. For the 5% w/v concentrations, an average of three insects per set of 15 insects were recorded dead during the period of the trials and for the 10% w/v concentration, about 7-8 insect died by the end of each experimental set-up; most of them got stuck to the surface of the wafer discs. This was due to the sticky nature of the wafer after treatment with the petroleum spirit extract. Though, the antifeedant index rate makes it a good antifeedant, but the application of a sticky substance on surfaces of stored food crops would not only be inappropriate but hazardous.

The methanol extract could be termed a good antifeedant material for stored product pests. Concentrations as low as 1.0% w/v showed relatively good activity with an antifeedant index of 97.80. An average of two insects was recorded dead in the 1.0% w/v tubes, between three and four in 5.0% w/v tubes and up to 10 or more in some of the 10.0% w/v EE tubes. It was also observed that there was a higher level of inactivity among the insects in the EE tubes compared to those in the CC tubes due to their inability to feed. The insect in CE tubes kept mostly to the C-wafer discs and abstained from feeding on the E – wafer discs.

ii. *Sclerocarya birrea*

Antifeedant activity was observed to be very low in the petroleum spirit extracts. Mortality rate of the insects was also very low. The insects did not seem disturbed by the extract; they thrived well under the experimental set-up. An average of two to three insects were recorded dead, but it was mostly in the EE tubes. With an antifeedant index of 85.36 at 10% w/v concentration, the pet. Spirit extract cannot be regarded as having a strong antifeedant trait.

Antifeedant activity was observed to be more prominent on the methanol extract compared to the petroleum spirit extract. At 1.0% w/v, an antifeedant index of 80.81 was obtained, though mortality rates were lower, in the 1.0% w/v, only one or two insects were recorded dead, about four to five insects in the 5.0% and 10.0% w/v concentrates were recorded respectively, though at 10.0%w/v, the insects were inactive especially in the EE tubes. Generally the antifeedant index obtained indicates that the methanol extracts contain a constituent which is responsible for suppressing their feeding habit.

iii. **Pirimiphos methyl-2% Dust**

Inhibition of feeding was observed in all concentrates, in 0.1% w/v concentrate, an average of five insects was recorded dead in the CE tubes, while about eight were recorded in the EE tubes. Most of the insect in the 0.5% and 1.0% w/v EE tubes were dead before the end of each experimental set-up. Attempts at using 5.0 and 10.0% w/v lead to the death of all the insects within hours and minutes (respectively) of setting up the experiment.

The antifeedant constituent of the standard reference material not only hinders the insect from feeding but killed them outright on contact with the treated wafer especially at very high concentrations. Even in the 1.0%w/v CE tube, it was observed that the feeding deterrence was high compared to that observed in the 0.1%w/v and 0.5%w/v because the insects seemed to have been hindered by the presence of the treated wafer and feeding was inhibited and it was observed that the insect kept away from the area where the treated wafers were in the tube.

4.5.2 Relative Comparison

Table 4.7: **Relative Comparison of Plant Crude extracts with Reference Standard.**

%w/v Concentrations	EXTRACTS AND THE % VALUES				
	Reference Standard	DTPe	DTMe	SBPe	SBMe
0.1	92.34	<50	<50	<50	<50
0.5	102.76	<50	<50	<50	<50
1.0	114.20	60.49	97.80	57.84	80.81
5.0	>150	114.28	120.37	66.99	102.73
10.0	>200	115.54	135.24	85.36	123.71

It was observed that at 10.0%w/v, crude extracts from both plants especially the methanol extracts showed good activity in comparison with 1.0% w/v concentrate used for the reference standard, commercial Pirimiphos methyl-2% Dust; below this, the values were too low or of less significance in determining feeding deterrence activity. At concentrations high than 10% w/v, it can be extrapolated that antifeedant activity will increase, as the crude extracts would have higher concentrations of the active constituents.

4.6. OXIDATION REACTION OF THE *S. birrea* PETROLEUM SPIRIT EXTRACT [SBPe]

4.6.1. Spectroscopic Analysis of the SBPe-oil.

Infra-red spectral analysis of *Sclerocarya birrea* petroleum spirit extract was carried out. The SBPe oil has absorptions in the IR spectrum at frequencies 2913cm^{-1} (C – Hstr) and 1730cm^{-1} (C=Ostr for ald). This indicates the presence of an alkanal functional group which was further confirmed by the silver mirror test for carbonyl compounds. The IR spectrum of the sample is reported as Appendix V.

Though, results from the antifeedant activity and antimicrobial tests showed low and no activity respectively, biological activity have been reported in acidic components obtained from plant extracts [Fatope et al, 2000]. This was the reason for the chemical reaction undertaken to obtain the acid derivative, to determine if it could initiate antimicrobial activity or increase that observed in the antifeedant tests.

4.6.2. Spectroscopic Analysis of Acid derivative from Oxidative Process

The acid derivative [SBPeA] had absorption in the IR spectral region (using Nujol mull) at frequencies $3000 - 250\text{cm}^{-1}$ (C-H alkane and a broad O-Hstr), 2852cm^{-1} (C-Hstr), and 1699cm^{-1} (C = Ostr). The solid had a melting point between $107-110^{\circ}\text{C}$. The melting point of comparable acids is azelaic acid

(nonanedioic acid) m.pt 98-102° C and undecanedioic acid (1,9-nonanedicarboxylic acid) m.pt 108-110° C. (Pouchert and Campbell, 1974). From the NMR spectrum, the acid was deduced to be undecanedioic acid. The ¹H-NMR spectrum had signals at δ = 1.3ppm (d), 2.2ppm (m) which are typical of methylene protons [---CH₂---] which occur between 0.9-2.3ppm. The broad signal at 11.8ppm is due to the signal corresponding to the hydroxyl group in the carboxyl group [COOH] which usually falls off peak (above 10.0ppm). From the ¹H-NMR, the compound is likely to be a saturated compound as there are no signals between 4.0-6.0ppm [C=C] or 6.0-8.0ppm [aromatic protons].

From the C-13 NMR, signals at 24.63ppm to 33.78ppm are due to the symmetrical but non-equivalent methylene carbons which fall in the range of 0-80ppm. The low field signal at 176.60ppm is attributed to the quaternary carbon in the carboxyl group which usually falls in the range 166-181ppm. (Kemp, 1991). These signals are assigned thus:

This compares with the standard C-13 NMR for undecanedioic acid (ALDRICH) with signals at:

The lowest value 24.63ppm is assigned to the carbon closest to the carboxyl carbon atom and this is due to the influence of the functional group which causes a decrease in chemical shift. The varying values observed for the other symmetrical methylene carbons increased with decreasing influence of the carboxyl functional group on the carbons.

From the proton-proton correlation spectrum (homonuclear COSY), the proton signal at $\delta = 2.2$ ppm corresponds to the proton on the carboxyl group which occurs as a triplet. The spectrum is labeled as Appendix VI (d). The standard 60MHz proton-NMR spectrum of undecanedioic acid (Pouchert and Campbell, 1974) also showed a relative comparison with the ¹H-NMR spectrum for the acid derivative. This is labeled VI (e).

4.7. PURIFICATION OF *S. birrea* METHANOL EXTRACT HYDROLYSATE SBMeH.

4.7.1. Functionality Test

Table 4.8: **Result of Functional Group Tests**

	TESTS	OBSERVATION	INFERENCE
i	Alkaloid Test:- Wagner Reagent Test	A reddish brown (or brown) colouration was observed on the sprayed plate. This faded slightly after a period of time	It is likely, alkaloids might be present, since initial phytochemical screening had indicated its presence.
ii	Test for Cardiac Glycosides	A reddish brown colour (ring) was observed at the interphase	This indicates the presence of a steroidal ring (i.e. aglycone portion of the cardiac glycoside).
iii	Test For Phenols	A blue-black precipitate was observed.	This indicated the presence of condensed tannins.

The methanol extract hydrolysate SBMeH may be a phenolic compound. From literature, it was reported that *S. birrea* is a tannin – producing tree [Irvine, 1961; Oliver, 1968; Watt et al, 1962] and this could be attributed to the presence of condensed tannins in the hydrolysate. Initial IR-analysis conducted on the neat SBMeH extract showed absorption frequencies of a strong O-H band at 3446cm^{-1} , a C-Ostr at 1460cm^{-1} , C=Ostr at 1708cm^{-1} and a C-Hstr at 2853cm^{-1} . The IR spectrum is labeled Appendix VII. The presence of the steroidal ring is an indication that the cures for various pharmacological activity claimed by the traditional users can be established [Burkill, 1985]. Though the spray test indicated presence of alkaloid, no further work was done to ascertain the alkaloid.

4.7.2. Chromatographic Separation of *S. birrea* Methanolic Hydrolysate

Column and Preparatory Thin-Layer Chromatographic techniques were used to purify the *S. birrea* methanol hydrolysate. The extract was loaded on a column and pooled fractions further purified using the prep TLC method. Table 4.9 gives the main fractions obtained and their R_f values.

Table 4.9.: R_f Values of Purified Constituents from *S. birrea* Methanolic

Hydrolysate.

Fractions	Approximate R_f Values
1	0.22
2	0.52
3	0.62
4	0.94

4.7.3. Spectroscopic Analysis and Physical Constants

The spectroscopic analysis carried out on the methanol hydrolysate was not successful; no significant or meaningful deductions could be obtained from the spectra.

The melting points of the purified fractions were also determined.

Fraction 2 ⇒ 256 – 258°C (decomp)

Fraction 3 ⇒ 245 – 247°C (decomp)

Fraction 7 ⇒ 251 – 254°C (decomp).