PHARMACOGNOSTIC FEATURES AND ANTIMICROBIAL ACTIVITY OF *MITRACARPUS VILLOSUS* (SW), DC (Family: RUBIACEAE)

BY

G. ONYILOYI ANDREW

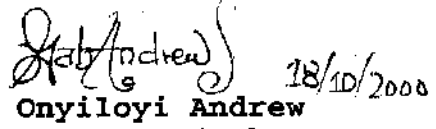
A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL, AHMADU BELLO UNIVERSITY, ZARIA, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN PHARMACOGNOSY

DEPARTMENT OF PHARMACOGNOSY AND DRUG DEVELOPMENT
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APRIL, 1999

DECLARATION

I hereby declare that the work reported in this thesis was carried out and written by me under the joint supervision of Prof. M. Shok and Dr. (Mrs) Illyas in the Department of Pharmacognosy and Drug Development and Dr. (Mrs) Odama in the Department of Microbiology, Ahmadu Bello University, Zaria - Nigeria. It has not been presented in any previous application for higher degree. The work of other investigators have been acknowledged and referred to accordingly.



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CERTIFICATION

This thesis entitled "PHARMACOGNOSTIC FEATURES AND ANTIMICROBIAL ACTIVITY OF MITRACARPUS VILLOSUS (SW) DC. by G. ONYILOYI ANDREW meets the regulations governing the award of the degree of MASTER OF SCIENCE (PHARMACOGNOSY) of Ahmadu Bello University, Zaria - Nigeria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

To the Balm of Gilead who had made my life a challenging paradox of painful anguish and relief, failure and success, this thesis is affectionately dedicated.

It is also dedicated to one man and his wife. The only ones that had cared enough. The only persons that really matter. And to my dear little scatter-brain and my friends the Bakos, who made life worth living. To these dear individuals, my deepest affection.

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ABSTRACT

The medicinal plant, Mitracarpus villosus (SW) DC, formerly (Mitracarpus scaber Zuccarl, family; Rubiaceae is a herb, occurring in both the Savannah and the Forest Zones (Wickens, 1976).

Microscopical examinations of the fresh as well as the powdered root, stem, and leaves of M. villosus were carried out and characteristic features like calcium oxalate crystals and paracytic stomata are noted.

Chemomicroscopy of the root, stem, leaves and inflorescence of M. villosus revealed the presence of lignins, anthraquinones, oil droplets, starch and clusters of calcium oxalate crystals, while the preliminary phytochemical tests showed the presence of alkaloids, carbohydrate (sugars), flavonoids, saponins and tannins. Paper and thin layer chromatographic analyses carried out also confirmed the presence of some of these constituents. Alkaloids were detected and tentatively assigned to the (phenanthrene type) group.

Extracts of the entire plant in different solvents exhibited antimicrobial activity against standard cultures of B^ subtilis (NCTC 8236), E. coli (NCTC 10418), Proteus vulgaris, Pseudomonas aeruginosa (NCTC 67 50), Staphylococcus aureus (NCTC 6571), Streptococcus pyogenes (NCTC 8189) and

Candida albicans (standard).

The activity of the hot water extract of the entire plant was higher than those of the cold water/alcohol extracts on all test organisms.

Extracts of the Unrefined secondary metabolites of the plant showed activity against both Gram negative and positive organisms. The Gram positive organisms were slightly more susceptable.

The rate at which B. subtilis (NCTC 8236), and Streptococcus pyogenes (NCTC 8198), were killed by extracts (alkaloids and steroids) were also tested. The rates of kill were comparable.

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CHAPTER ONE

1.0 INTRODUCTION

The plant Mitracarpus villosus (SW) DC., a herb, is a member of the family Rubiaceae, order; Loganiales. It is widely distributed particularly in the tropics (Lakeb and Wundelin, 1967) where it grows abundantly in farmlands as weeds (Hutchinson, Dalziel and Hepper, 1963). It is found both in areas of high rainfall and in dry locality. The plant is an annual weed. The plant is indigenous in Africa.

1.1.0 THE RUBIACEAE FAMILY

The common name for the Rubiaceae family is the Madder family (Fernald, 1950; Hocking, 1955). It is a family of 500 genera and 6,000 species, and consists of the most important tropical woody plants rich in alkaloids; which are useful in conventional medicine.

Most genera are found in the tropics except the more advanced group (Galieae) which is found in the temperate region. Members of the family may be recognised by their flowers which are crowded into dense cyme (whorled glomerulus). The cyme is sessile in the axils as well as terminals. The flower is perfect (Fernald, 1950).

1.1.1 SOME MEDICINALLY IMPORTANT GENERA OF THE FAMILY

(i) Cephaelis

Cephaelis ipecacuanha (Brot.) A. Rich)

The dried root or rhizome of the plant is known commercially as Rio or Brazilian Ipecac. Cephaelis acuminata, Karsten, is known in commerce as Cartagena or Panama Ipecac. It contains 2% of emetine, the ether soluble alkaloid required in medicine (Trease and Evans, 1982).

(ii) Cinchona

Cinchona barks are rich in quinoline alkaloids.

Drugs obtained from Cinchona barks are very effective against malaria parasite.

Some species important in quinine production are:

(a) Cinchona calisaya

(b) Cinchona ledgeriana

(c) Cinchona officiales

(d) Cinchona succirubra

(iii) Coafea

Caffea arabica L. yields the alkaloid caffeine which is a stimulant. In addition, it contains tannin and chlorogenic acid (Caffeotannic acid) fat, sugar and pentose (Trease and Evans, 1983).

(iv) Nauclea

The roots of Nauclea latifolia Smith, contain bitter principle similar to that of Cinchona. Hence the name "African Quinine". It is used in the treatment of malaria, and is believed to be a possible substitute for Cinchona. The plant possesses an important alkaloid and has been found to exhibit antimicrobial properties (Aminu, 1993).

(v) Uncaria

Uncaria gambier (Hunt. Roxb) Commonly known as Gambier or pale catechu, U. gambier is rich in catechutannic acid. The plant is a native of Malaya (Trease and Evans, 1982).

All these plants are of medicinal importance and most are rich in both alkaloids and tannins. Macek (1972), reported that tannins have antimicrobial activity. Some alkaloids are active against microbes, (Macek, 1972).

1.1.2 DESCRIPTION OF MITRACARPUS VILLOSUS

M. villosus is a herb in both the savannah and forest belts. The leaves are lanceolate and subacute (3.0-6.0cm long and 0.7-1.5cm broad). The flowers are white and densely crowded at the nodes. Calyx are lobed and the bracteoles are filiform in shape. It is an annual herb half woody at the base and erect. The branches alternate and the entire herb is a few centimeters to 60 cm high (Hutchinson, Dalziel and, Hepper,

1963).

Habitat: Abundant on farmland

Locality: It is indigenous to the Sudan.

Distribution: It is well distributed from Mauritania to the Cameroon and eastward to Ethiopia and south through East Africa to Zambia.

Floristic Category: Sudano-Zambezian region.

Synonym: Mitracarpus scaber Zuccar.

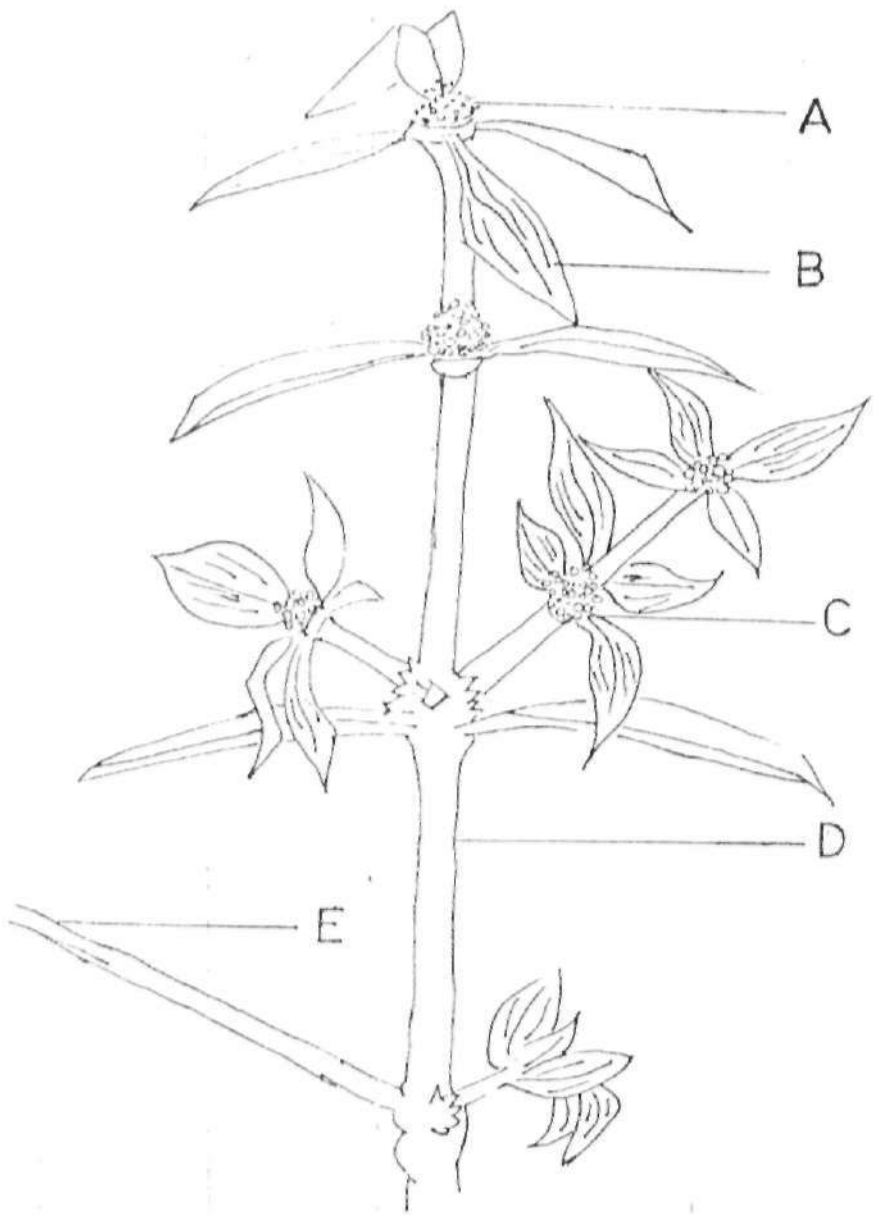
Colloquial names: Button weed (Fernald, 1950) (Fig. 1.10).

Nigerian names: Some Hausa dialects call M. villosus, "Goga masu, harwatsi and yarwatsi" Sokoto Hausa refer to the plant by the name; "Wawa ka je magori" (Dalziel, 1956). Other vernacular names are, Bassa Nge refer to the plant as; "Nda Ediboku" (Abdulsalam Personal Communication, 1996). Yoruba as; "Irawo" or "Irawoile" (Holland, 1922).

1.2.0 MEDICINAL USES OF MITRACARPUS VILLOSUS

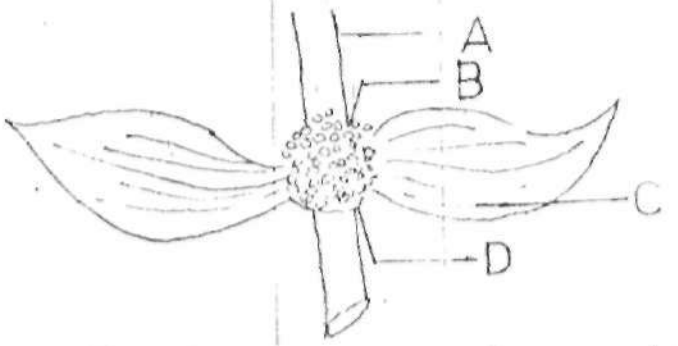
Very little has been reported on the medicinal uses of M. villosus. (Olorintola 1993), reported that most dermatophytes are susceptible to extract from the plant. The locals use the plant medicinally in Central and South Africa (Thonner, 1915). Among the locals in Nigeria M. villosus is used for the treatment of eczema, pruritus, pile, gonorrhea and rheumatism. (Holland 1922), reported the use of the dried leaves as cure for old ulcers and an antidote against arrow poisons.

FIG.10A. FREE HAND DRAWING Of MITRACARPUS VILLOSUS (SW) DC. (BUTTON WEED) "GOGA MASU" (HAUSA VAC;)



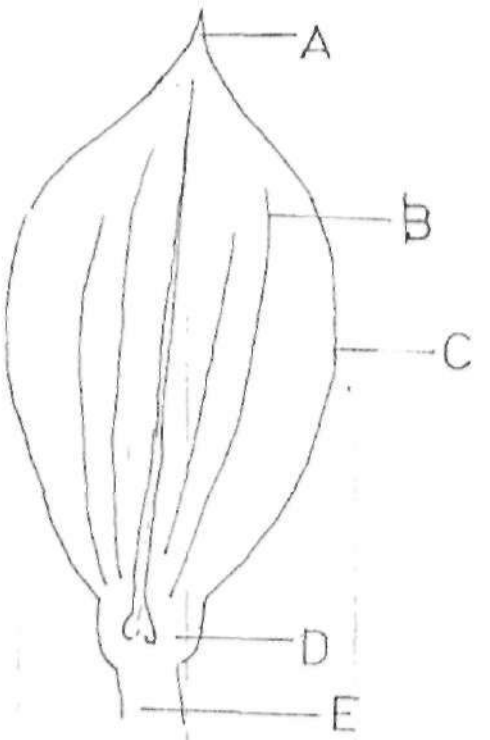
Part of the entire weed M. villosus; (A) Terminal inflorescence (B) Palmate sessile leaf. (C) Node consisting of sessile leaves and an inflorescence attached directly to stem. (D) Main stem. (E) Branch.

FIG.1.10B Inflorescence And Leaf Composition of M. villosus



Drawing of Leaf Composition of M.villosus:(A) Stem (B) Inflorescence directly attached to the main stem at the node. (C) Sessile leaf. (D) Region of the node.

FIG.1.10C Description of the Macromorphology of the whole Leaf:



Leaf (3-6) cm long and (0.7-1.5) cm broad, sessile. Lanceolate with palmate venation. Veins conspicuous on the lower surface. Margin insinuate yet entire. Lateral veins end at margin insinuate. Apex acuminate. Base asymmetrically decurrent.

Drawing of the leaf of M.villosus:(A) Acuminate apex. (B) Palmate Venation. (C) Sinuate entire margin. (D) Asymmetrical decurrent base. (E) Sessile (no petiole).

1.3.0 PHYTOCHEMISTRY OF MITRACARPUS VILLOSUS (SW)DC

1.3.1 ALKALOIDS

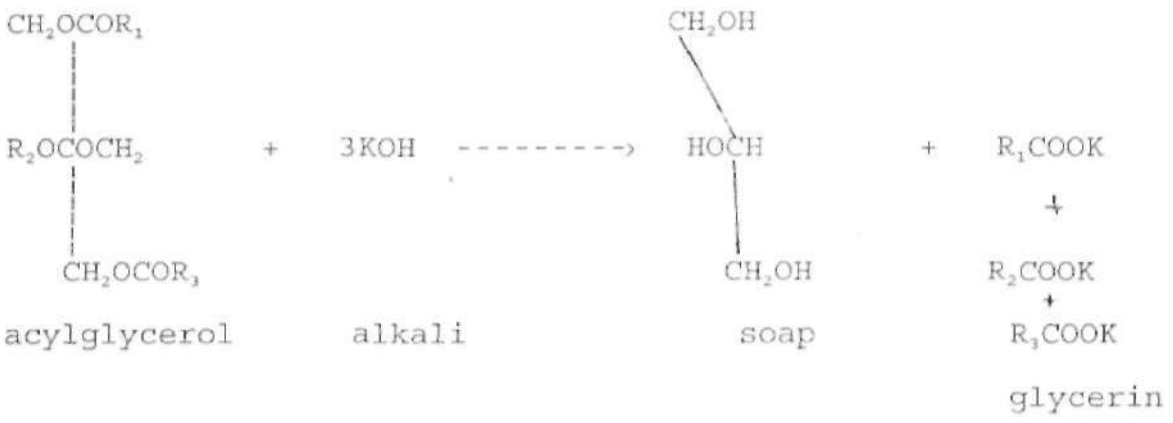
The presence of numerous alkaloids have been reported in the genera of the family, Rubiaceae (one of the outstanding alkaloid-yielding families among the angiosperm) (Hutchinson, 1973).

The literature revealed that the herb has been previously screened for phytochemical constituents (El-Kheir and Salih, 1980; Benjamin et el, 1980; Hussain and Deeni, 1991).

1.3.2 FIXED OILS AND FATS

Fixed oils and fats are naturally occurring mixtures of lipids such as olive oil or oil of theobroma, fixed oils and fats are acylglycerol involving saturated fatty acid solids (fats) and those of unsaturated fatty acids, liquids (oils) fixed oils are obtained from both the vegetative structures and the inflorescence; but chiefly from the inflorescence. All lipids undergo the process of saponification.

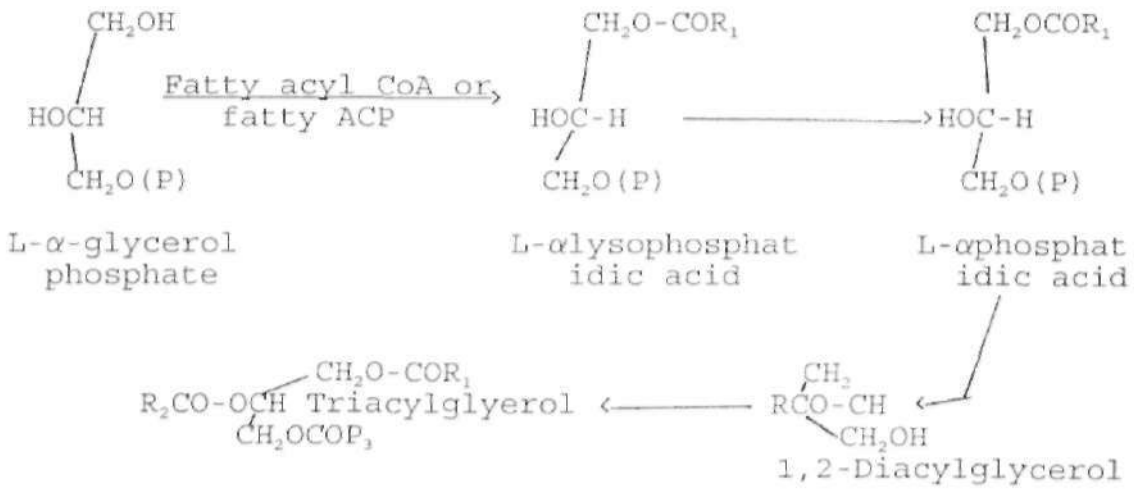
Fig. 1.13 SHOWs THE PROCESS OF SAPONIFICATION



Note: R_1, R_2, and R_3 ≡ Acyl group

BIOGENESIS OF FIXED OILS AND FATS

Acylglycerols are formed from the fatty acyl-CoA or from fatty ACP and (l-α-glycerophosphate) (Trease and Evans 1985)



(Trease and Evans, 1985)

1.3.3 FLAVONOIDS GLYCOSIDES

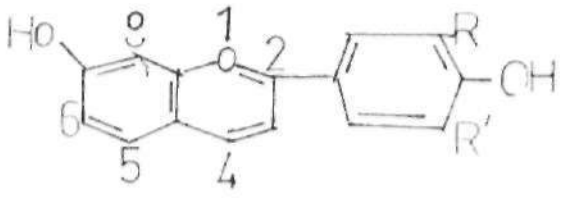
The flavonoids which occur in the free state and as glycosides are the largest group of naturally occurring phenols. Two thousand of these compounds are now known, with nearly 500 occurring in the free state. The flavones (flavone, flavonol, flavanone, chalcones, xanthone) and the anthocyanins (flavanoids) are sap soluble and have Y-pyrone ring (chromone).

Flavonoids dissolve in alkalis, giving yellow solutions which on addition of acid become colourless.

Basic Nucleus of Flavonoids

Flavanoids or anthocyanins have a 2-phenyl benzopyrilium ring or nucleus and differ from each other in the number of hydroxyl or methoxyl group.

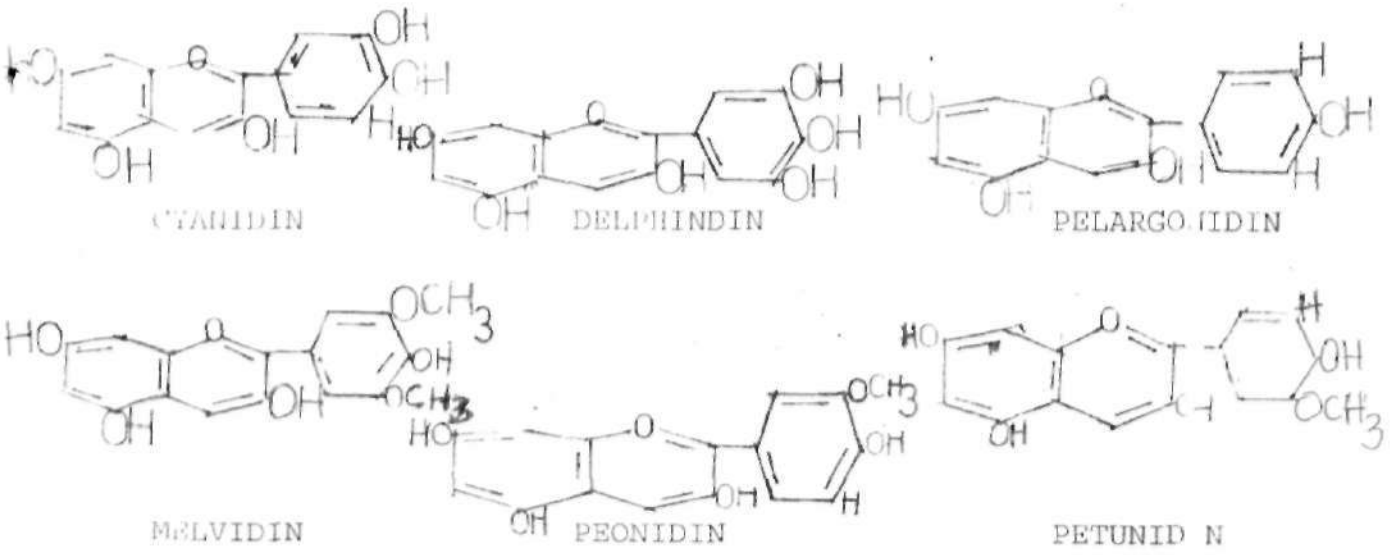
FIGURE 1.15 SHOWS THE BASIC NUCLEUS OF FLAVONOIDS



The sugar components are usually attached in the carbon 3-position rarely in the carbon-5 position. They may be monosaccharides, disaccharides, and trisaccharides. Diglucosides in which separate glucose molecules are attached in both 3-and-5-positions are common.

Anthocyanidins (flavonoids) are structurally related to the flavones. The glucosides (with the carbohydrate moiety) are called anthocyanidins. The anthocyanidins are sap pigments and their actual colour is determined by the pH of the sap. For example cornflower is blue and Rose flower, red. Anthocyanidins consist of the following: cyanidins is found 80% in permanent pigmented leaves, 69% of fruits, and 50% of flowers. Others include Delphinidin, Pelargonidin, Peonidin, Petunidin, and melvidin.

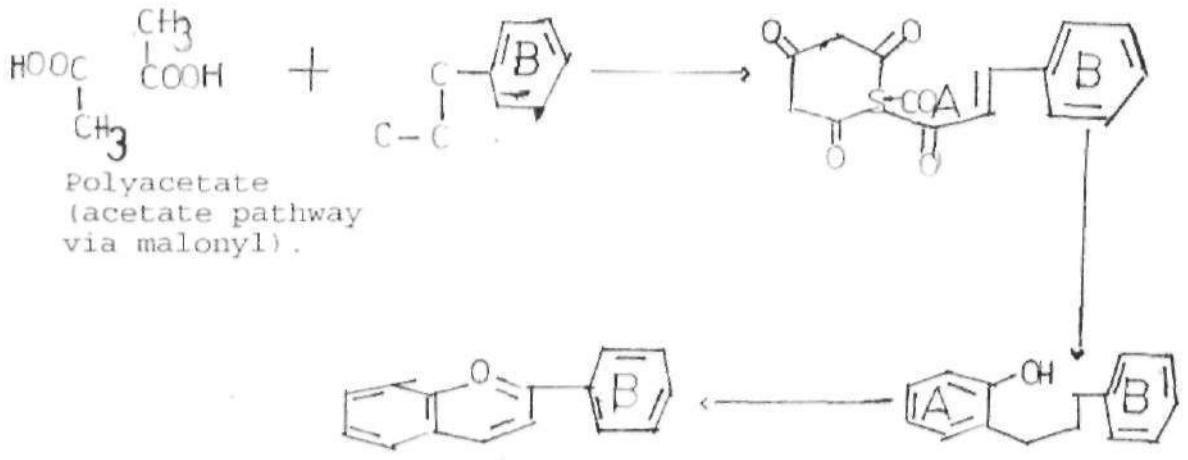
FIGURE 1.16 SHOWS THE STRUCTURE OF ANTHOCYANIDINS



BIOGENESIS OF FLAVONOIDS

Biosynthesis of flavonoids is by the acetate pathway via malonyl substrate.

FIGURE 1.17 SHOWS FLAVONOIDS BIOSYNTHETIC PATHWAY

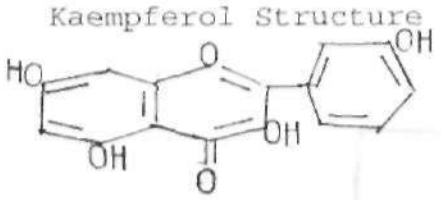


USES

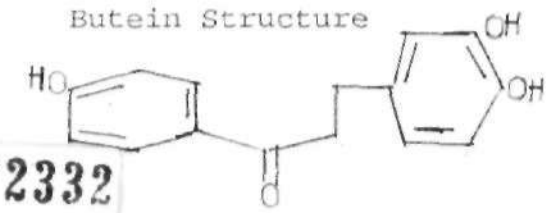
Flavonoids possess the following properties which indicate their uses. They possess anti-inflammatory, anti-allergic anti-thrombotic, vassoprotective, anti-tumor principle, protective from gastric mucosa, diuretic properties, antispasmodic, antibacterial, antifungal properties. Butein is reported to possess anti-infertility activity (Trease and Evans 1989).

Kaempferol finds use in medicine as vitamin - P and has *other generic names, ie swartziol, trifoltin, robigenin etc.* While butein, a chalcone, is also found to be effective in promoting capillary resistance.

Kaempferol Structure



Butein Structure



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1.3.4 **SAPONINS**

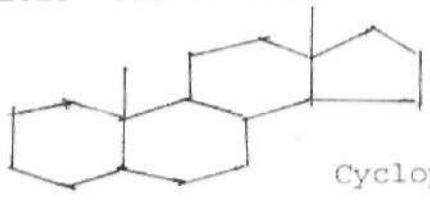
Saponins are a group of amorphous, colloidal glycosides readily soluble in water producing a froth when the aqueous solution is agitated. Saponins are excellent emulsifying agents and possess detergent property. They have acrid taste, the powdered form cause sneezing and chiefly isolated from Quillaja saponaria with the structural formular $C_nH_{n-8}O_{10}$

Many of the saponins are toxic (sapotoxins). Saponins exert powerful hemolytic action on the red blood corpuscles. They have been used as fish poisons. The saponins are widely distributed in the plant kingdom. Commercial saponin product is prepared from the yucca plant (Quillaja) (Tyler et al, 1976).

Properties of Saponins

The isolation of saponin in the pure state is difficult. When pure; saponins are colorless or white, optically active and soluble in alcohol. The glycosides occur in the plant in small amounts (Remington, 1975).

FIGURE 1.18 CYCLOPENTANOPERHYDROPHENANTHRENE NUCLEUS

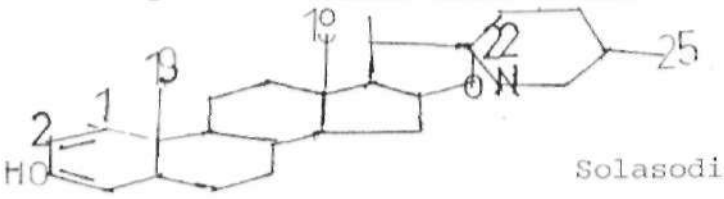


Cyclopentanoperhydo-
phenanthrene Nucleus

Three well differentiated structural types have been identified. Tschsche and Wulff (1964) classify the saponins according to their genin structure in three groups.

1. Steroidal Alkaloid Glycosides: for example solasodin in Solanum sp family: Solanaceae

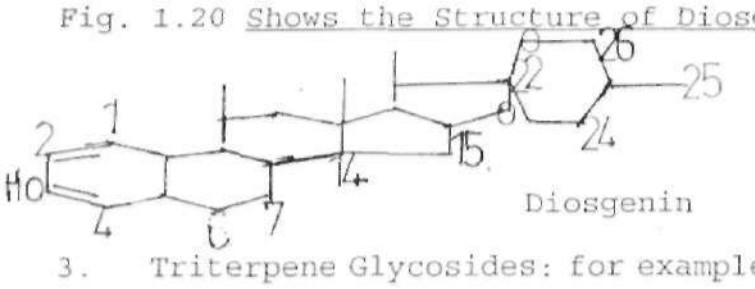
Fig. 1.19 Shows the Structure of Solasodine



Solasodine structure

2. Spirostanol glycosides (Steroidal glycosides) for example Diosgenin in Discorea alata family: Dioscoreacea

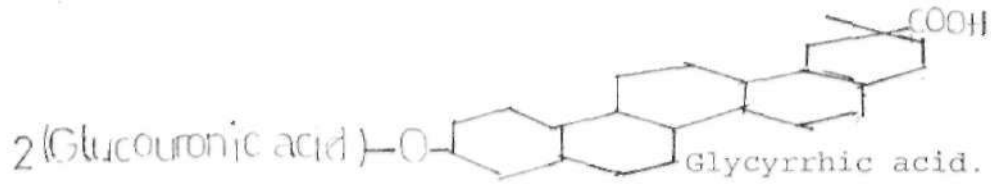
Fig. 1.20 Shows the Structure of Diosgenin



Diosgenin

3. Triterpene Glycosides: for example Glycyrrhizic acid from Glycyrrhiza glabra L. family: Leguminosae.

Fig. 1.21 Show the Structure of Glycyrrhic acid



Triterpenes with carboxylic groups are acid saponins.

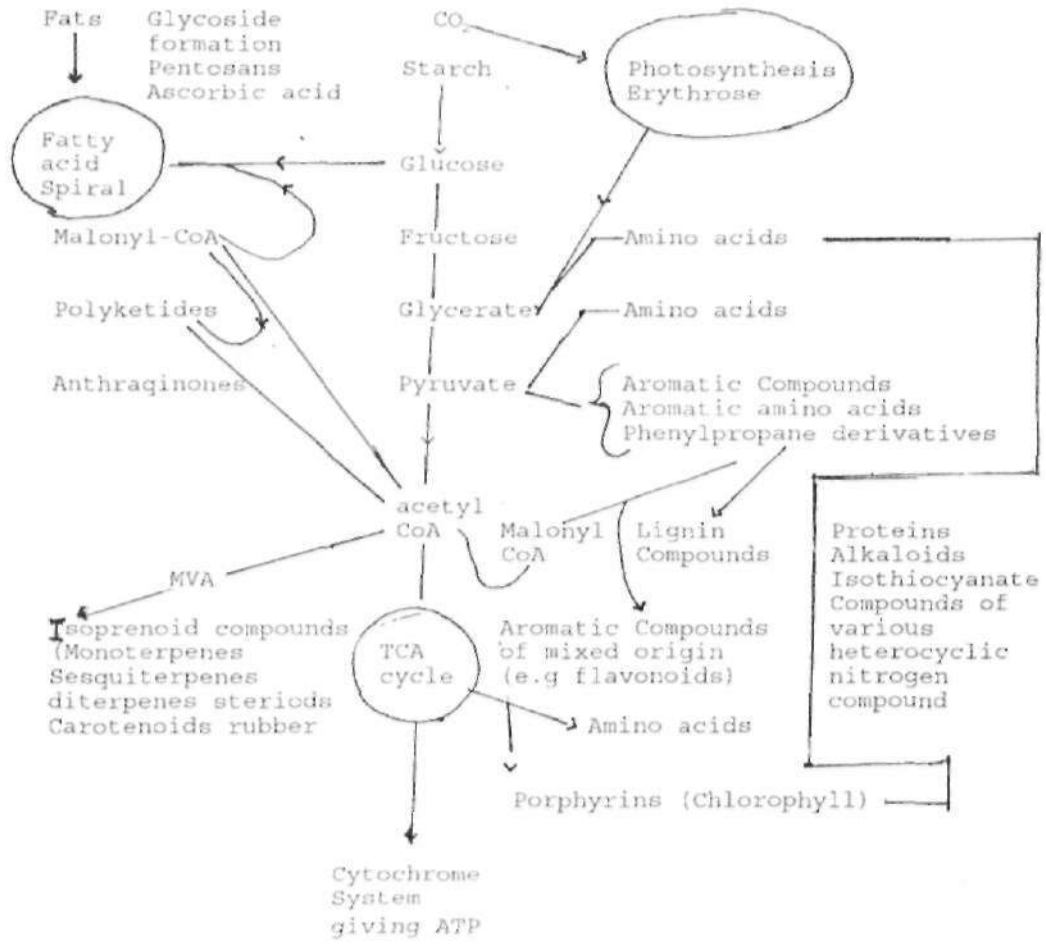
1.3.5 OCCURRENCE OF SPIROSTANOL SAPONINS IN THE PLANT KINGDOM

Saponins of the spirostanol type have been found predominantly in monocotyledon, especially in the genera Smilax and yucca (family; Liliaceace). In the dicotyledons the saponins are found in the genus Strophanthus (family: Apocynaceae). Steroidal alkaloids dominate in the genus Solanum (family; Solanaceae). Triterpene saponins occur mainly in dicots (Hiller et al, 1966). For example in the Phytolaccaceae, Chenopodiaceae, Ranunculaceae, Berberidaceae, Papaveraceae, Rutaceae, Oleaceae, Rubiaceae etc, the triterpene are commonly found (Macek, 1972).

1.3.6 BASIC METABOLIC PATHWAYS AND THE ORIGIN OF *SECONDARY METABOLITES*

Some basic metabolic pathway appear to be similar in both plants and animals. It is the secondary plant products (those not involved in the essential metabolism of the cell) that the majority of vegetable drugs owe their therapeutic activity.

However, as shown (Fig. 1.22), the production of these secondary matabolities is dependent on the fundamental metabolic cycles of living tissues.



(Trease and Evans, 1985)

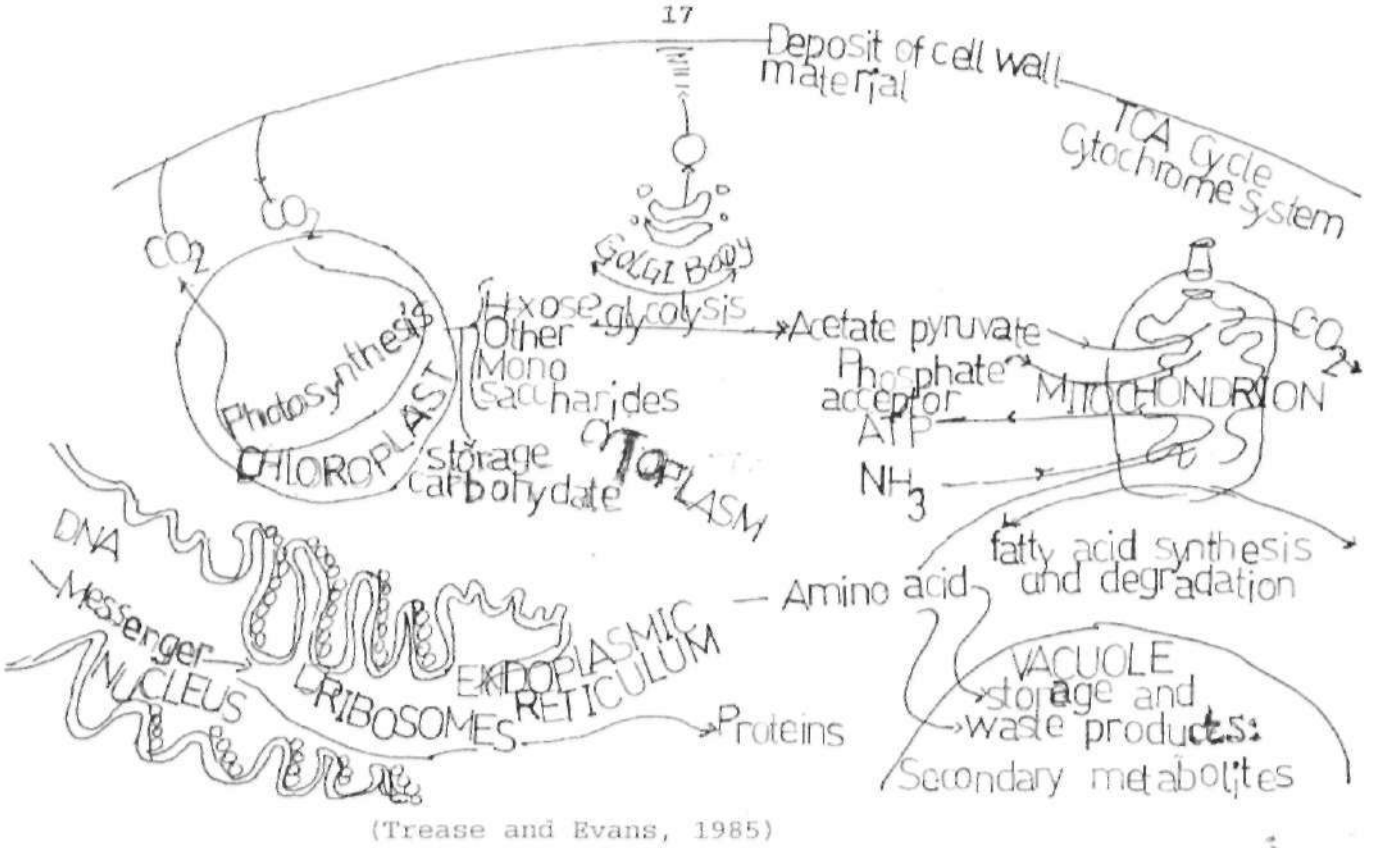
Figure 1.22 ORIGIN OF SOME SECONDARY METABLITIES IN RELATION TO BASIC METABOLIC PATHWAY

The living plant cell possesses a highly organized structure with various organelles having distinct biochemical characteristics. The organelles allow the creation of different chemical enviroments within one cell, and by their structure they increase the area available for surface reaction important in biological systems.

At present little firm information concerning the subcellular distribution of secondary metabolites are known. For instance certain stages in the formation of cardiac glycoside in Convallaria majalis, the biogenesis of tannins and anthocyanins, vacuoles are implicated. Steroidal sapogenins and their glycosides in Discorea tokoro and triterpene in Calendula officinales are reported to be confined to particulate subcellular fractions. The diagram below is a flow chart of some cell metabolites (Figure 1.23).

1.3.7 BIOGENESIS OF STEROIDAL SAPONINS

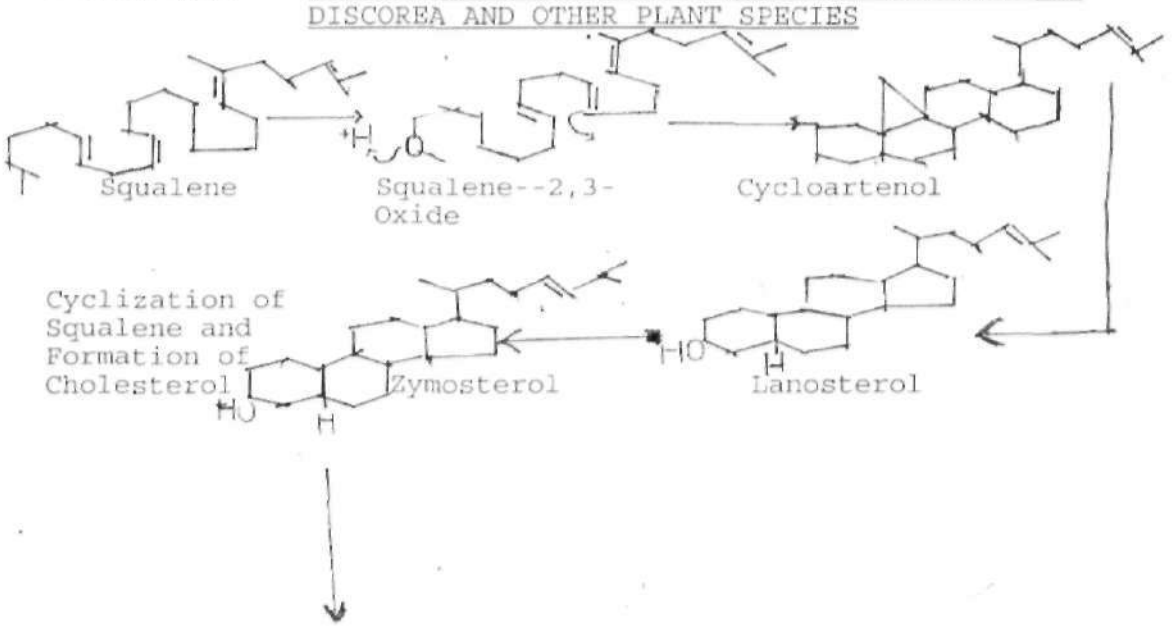
Steroidal saponins arise via the mevalonic acid pathway. The subsequent cyclization of squalene to give cholesterol is shown below (Fig. 1.24). Cholesterol is widely distributed in plants (Trease and Evans, 1985). Cholesterol can be incorporated into a number of C_{27} Sapogenins without side chain cleavage. In Discorea homogenates one such compound has been converted to dioscin.

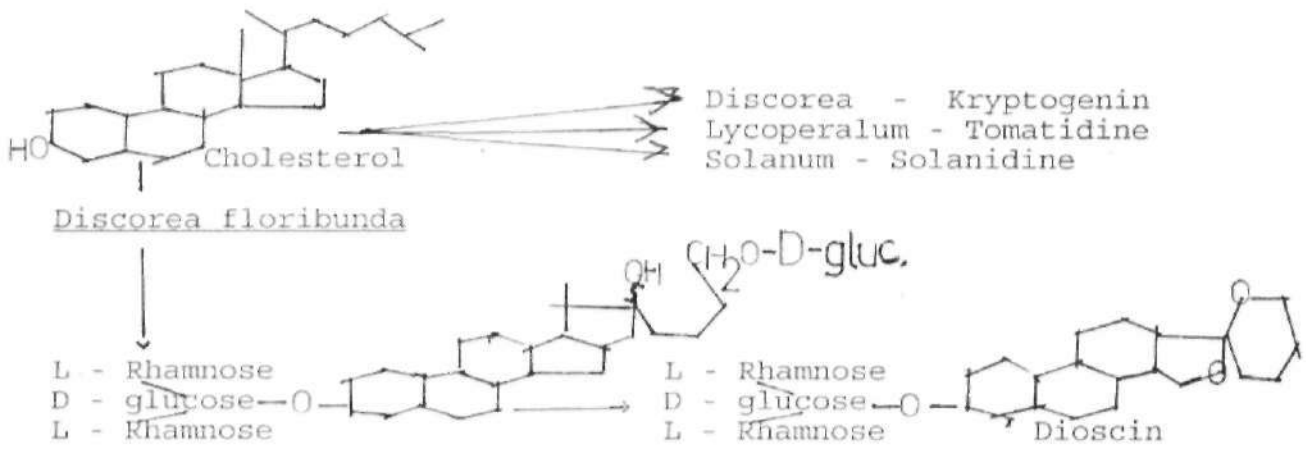


(Trease and Evans, 1985)

FIGURE 1.23 FLOW CHART OF SOME CELL METABOLITES

FIGURE 1.24 FORMATION AND METABOLISM OF SAPONIN IN DISCOREA AND OTHER PLANT SPECIES





1.3.8 **USES**

The use of saponin drugs has recently shifted to different areas. In medieval times these drugs were valued in the treatment of cough, gout and syphilis. Nowadays they find application as expectorants and diuretics (Trease andEvans, 1985).

A variety of specific effects have been observed in several saponins. The anti-exudate activity of the seed saponins from Aesulum hippocastanum, Thea Sinensis and glycyrrhic acid from Glycyrrhiza glabra has been established. Saponin from Panax ginseng and from Aralia manshurica have tonic activity. Saponin of Centella asiatic possess anti-leprous activity. Saponins have antibiotic activities.

The spirostanols are valuable materials for the industrial synthesis of steroid hormones (Macek, 1972).

1.3.9 INDUSTRIAL STERIODS

Steroid hormones (Corticosteroids) are produced in the adrenal cortex; but a wide variety of semisynthetic drugs of this type are in use. They are synthesized using plant steroids as intermediates; diosgenin and hecogenin being the principal sources.

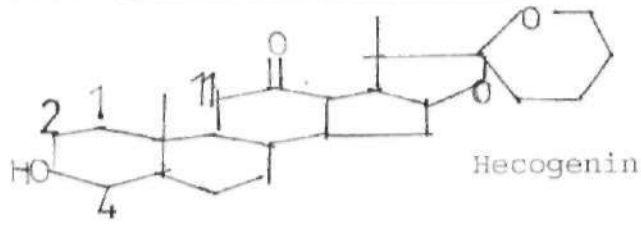
There is a large world demand for these compounds, particularly for the synthesis of oral contraceptive (Trease and Evans, 1985).

The determination of the initial desirability of a given steroidal sapogenin as a cortisone precursor is based on its having hydroxyl group in the 3-and 11-positions, or having the ability to be readily converted to this structure (Tyler et al, 1981).

1.3.10 PRESENT SOURCES OF STEROIDS

Hecogenin from Agave sisalana; the compound has a C-ring substitution and is a practical starting material for the synthesis of the corticosteroids.

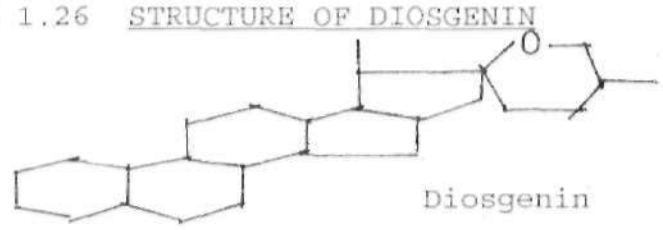
FIGURE 1.25 STRUCTURE OF HECOGENIN



Hecogenin

Diosgenin from Dioscorea alata; the compound is suitable for the manufacture of oral contraceptives, sex hormones, and diuretic steroids. Diosgenin could be used for corticosteroids production by employing suitable microorganisms to introduce O_2 in the 11, α-position of the pregnane nucleus (Trease and Evans, 1985).

FIGURE 1.26 STRUCTURE OF DIOSGENIN



Diosgenin

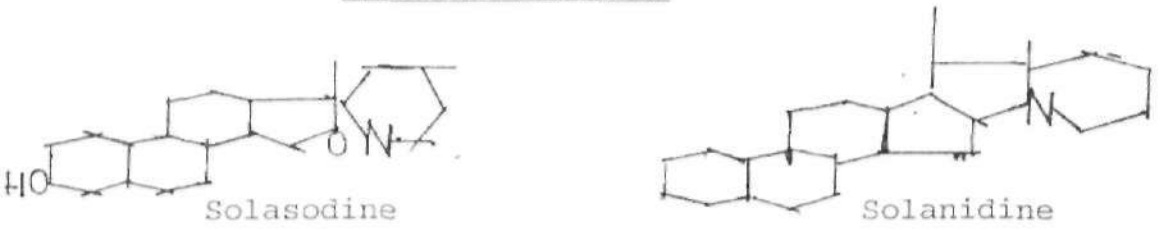
Botogenin and diosgenin of Discorea floribunda (Mexican edible yam), is a rich source of cortisone precursor (Trease and Evans, 1985).

The botogenin steroid nucleus is altered by transferring the O_2 atom from 12 - to the position 11- before it could be utilized as an intermediate in cortisone production (i.e. glucocorticosteroids)

Sarsapogenin from Smilax sp.

Sarmentogenin from Strophanthus sp., are rich in sapogenins. Solasodine and Solanidine are steroidal alkaloids from Solanum sp. (Trease and Evans, 1985).

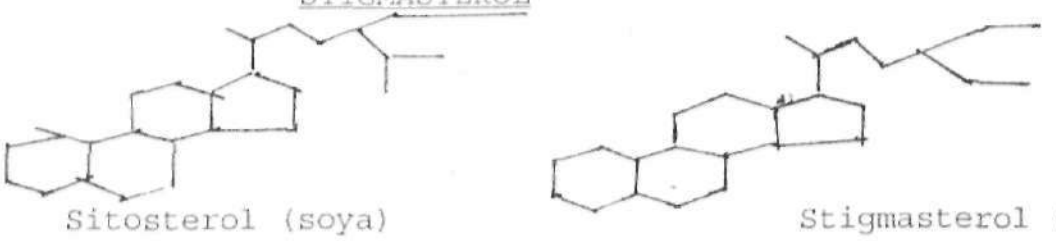
FIGURE 1.27 SHOWS THE BASIC NUCLEI OF TWO STEROIDAL ALKALOID SAPONINS



Solasodine Solanidine

Phytosterols (Stigmasterol, sitosterol) from Glycine max (soya bean) family: Leguminosae, have been used as starting materials for steroid synthesis. Phytosterols, though not sapogenin they are used extensively for steroid production. Stigmasterol can replace diosgenin because its unsaturated side chain is amenable to chemical conversion. Sitosterol with saturated side chain can be modified by microorganism.

FIGURE 1.28 SHOWS THE STRUCTURE OF SITOSTEROL AND STIGMASTEROL



Sitosterol (soya) Stigmasterol (soya)

Similar phytosterols are found in products of cotton seed oil, and sugar cane wax (Trease and Evans, 1989).

USES OF STEROIDS

Some steroids have strong anti-inflammatory action, the clinical indication include use in replacement therapy (Addison's disease), reduction in lymphatic tissues

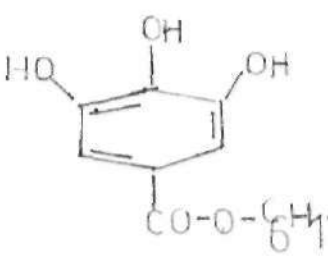
(Leukemias), suppression of lymphopoiesis (lymphomas) and as anti-inflammatory agent against variety of conditions like arthritis, cerebral oedema and raised intracranial pressure (Trease and Evans, 1985).

1.4.0 TANNINS

Tannins are substances present in plant extracts which combine with the protein of animal hides and skin converting them into leather.

Definition: Tannin is a substance detected qualitatively by the tanning test (the gold-beater's skin test) and determined quantitatively by the absorption on standard hide powder. The definition excludes simple phenolic substances present with tannin; such as gallic acid, catechins and chlorogenic acid. The compounds have low molecular weight, sometimes give precipitate with gelatins and may partly be retained by hide powder. True tannins have molecular weights from about 1,000 to 5,000. Many tannins are glycosides (e.g. glycogallins) (Trease and Evans, 1985).

FIGURE 1.29 STRUCTURE OF GLYCOGALLINS



Glucogallin

(Glycoside of gallic acid)

Tannins comprise a large complex substance widely distributed in the plant kingdom. Tannins are usually localized in specific plant parts such as leaves, fruit, barks or stem.

Tannins are non cystallizable, soluble in water and alcohol. In water tannin form colloidal solutions possessing an acid reaction and a sharp "puckering" taste. They precipitate solution of gelatin, proteins and alkaloids. With ferric salts tannins form dark blue or greenish-black soluble compounds. Tannins form deep red color with potassium ferricyanide and ammonia solutions. Tannins are precipitated by salts of copper, lead, tin and by strong solution of potassium dichromate. In alkaline solution many of the tannin derivatives absorb oxygen.

Tannins are complex substances, occurring as mixtures of polyphenols. Complex tannins are considered to have risen from simple polyphenols by polymerization.

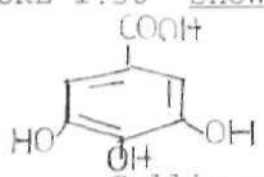
The phenolic groups of tannin are responsible for their astringent and antiseptic action as well as the coloration with iron salts.

The chemistry of tannins is not yet fully understood. However, two main groups are recognised. These are the Hydrolysable tannins and the condensed tannins (Proanthocyanidins).

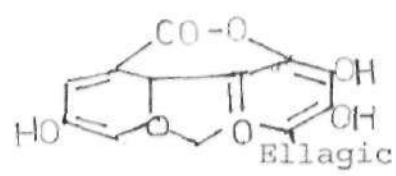
1.4.1 HYDROLYZABLE TANNINS

Hydrolysable tannins may be hydrolysed by acids or enzymes such as tannase. The tannins are formed from several molecules of phenolic acids such as gallic acid and ellagic acid united by ester linkage to a central glucose residue. Elagic acid and gallic acid turn blue with iron salts.

FIGURE 1.30 SHOWS SOME STRUCTURE OF HYDROLYZABLE TANNINS



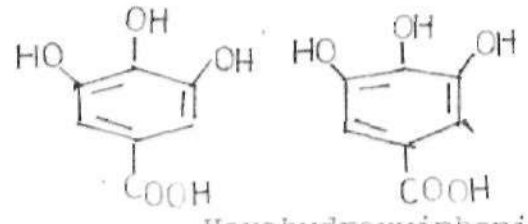
Gallic acid



Ellagic acid

Hydrolysable tannins were formerly known as pyrogallol tannins because on dry distillation gallic acid and similar components are converted into pyrogallol. Two types of hydrolysable tannins are recognised derived from gallic acid and ellagic acid. They are known as gallitannins and ellagitannins. Ellagic acid normally occurs as diester of hexahydroxy-diphenic acid (Fig. 1.31).

FIGURE 1.31 SHOWS THE STRUCTURE OF HEXAHYDROXYDIPHENIC ACID



Hexahydroxyiphenic acid

Drugs containing hydrolyzable tannins are:-

Gallitannins: rhubarb, cloves, redrose petal, hamamelis

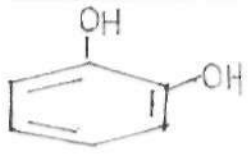
Turkish gall and chinese gall (nut gall).

Ellagitannins: Pomegranate rind/bark; kino and oak.

1.4.2 CONDENSED TANNINS

Condensed tannins include all other tannins. The molecules are more resistant to breakage than hydrolysable tannins. Catechin and flavan -3,4-diol appear to be intermediate in their biogenesis. On treatment with acid or enzymes they are decomposed into red insoluble compounds called phlobaphenes. It gives the tanner's red of leather and the red colour to many drugs; such as the red cinchona bark. The phlobatannins and their decomposition products; on dry distillation yield catechol (Fig. 1.32 below).

FIGURE 1.32 CHEMICAL STRUCTURE OF CATECHOL



Catechol

These tannins are called catechol taninns and turn green with ferric chloride.

DRUGS CONTAINING CONDENSED TANNINS

Hamamelis (leaves and bark) contain hydrolysable and condensed tannins. The followings are rich in condensed tannins.

Bark: Cinnamon, wildcherry, cinchona, hamamelis.

Root and Rhizomes: Krameria (rhatany).

Seed: Cocoa, Kola

Leaves: Hamamelis and green tea

Extract and dried juices: catechu, kino.

1.4.3 PSEUDOTANNINS

Pseudotannins are compounds of lower molecular weight than true tannins and they do not respond to the goldbeater's skin test.

Drugs Containing Pseudotannins

Gallic acid: rhubarb and most material containing gallitannins.

Catechins: Catechu, acacia cutch, cocoa, guarana, and many drugs containing condensed tannins.

Ipecacuanha acid: ipecacuanha, chlorogenic acid, coffee (unroasted) and nux vomica (a small quantity only).

Tannins are found in great quantity in dead or dying cells. They exert an inhibitory effect on enzymes due to protein precipitation, therefore contribute a protective function in barks and heartwoods (Trease and Evans, 1985).

1.4.4 BIOSYNTHESIS OF TANNINS

Tannins being complex in structure, little information is available as regards their biogenesis. Biochemical pathway leading to various individual components of tannins are known. The biosynthetic origin of some of these compounds are shown below (Fig. 1.33).

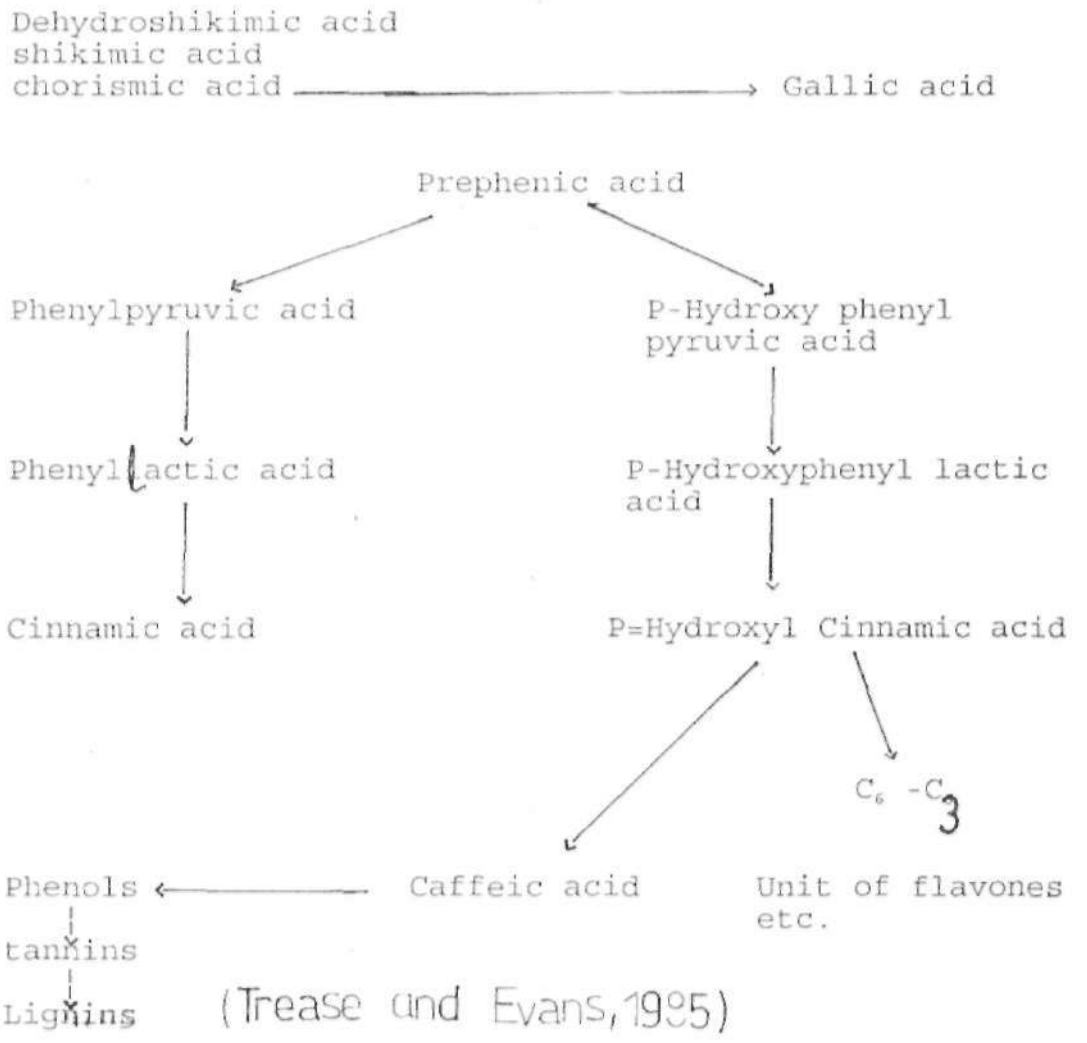
The confirmed pathway is from the shikimic acid homogenate (Trease and Evans, 1985).

1.4.5 USES OF TANNINS

Tannins find wide uses as astringents in gastrointestinal tract and on skin abrasions. They are employed in the treatment of burns, where the protein of exposed tissues are precipitated forming mild antiseptic, protective coat under which the regeneration of new cells may take place. They are employed in tanning industries for their ability to convert hides and skin to leather. Tannins react with iron salts to form deep colour, this property has been employed for ink production. Also as dyes and in cytological laboratory as stains. Tannins have precipitating properties and these have been employed in the laboratory to test for the presence of gelatin, proteins and alkaloids. Tannic acid is employed as antidotes for alkaloids poisoning and arrow wounds. Tannic acids form albumin - tannate drug and are used by ostomates to reduce odour. Gallic acid is used for the formation of Bismuth

subgallate; a developer substance in photography (Abubakar, 1993).

FIGURE 1.33 FORMATION OF TANNINS AND RELATED PHENOLICS



1.5.0 MICROBIOLOGY

Plant products exert bacterostatic/bacteriocidal effects on microorganisms. Susceptibility of organism to plant extract

determines the therapeutic use of the drug (Abubakar 1993). Some test organisms sesceptible to the plant extracts are:

1.5.1 Bacillus subtilis

B. subtilis is a gram positive rod occurring in chains. They form spores and are aerobes. The organism is saprophytic prevalent in soil, water, air and on vegetation. B. subtilis is a common contaminant in the laboratory. The organism rarely produces disease in human. When they become pathogenic they produce disease such as meningitis, endocarditis, endopthalmitis, conjunctivitis or acute gastroenteritis (Jawetz et al, 1978). B. subtilis is susceptible to Ampicillin. Ampicillin is a broad spectrum antibiotics (Carter, 1972)

1.5.2 Escherichia coli (E. coli)

E. coli are short Gram-negative rods that may form a chain. Under unfavourable conditions long filamentous forms occur. Capsules are rare. Most are motile. On differential media, metallic sheen, motile, flat, non viscous colonies are formed. On standard medium E. coli colonies are circular, convex, smooth with distinct edges.

The coliforms along with E. coli constitute the normal aerobic intestinal flora. Within the intestine they do not cause diseases. But become pathogenic when they reach tissue

outside intestinal tract. Also they cause diseases when host defences become inadequate. When they reach the blood stream they cause sepsis. E. coli may cause diarrheal disease. E Coli is susceptible to Gentamycin (Carter, 1972).

1.5.3 Proteus vulgaris

All Proteus organisms are Gram-negative, motile aerobic bacilli. Most are free (living in water, soil and sewage). Proteus vulgaris occurs in normal fecal flora of the intestinal tract. Proteus does not ferment lactose, decomposes urea with the liberation of ammonia and tends to "swarm". Proteus does not grow well in acid pH.

Proteus produces disease in human outside their normal habitat (intestinal tract). They are found in chronic urinary infections, and produce bacteremia and focal lesions in debilitated patients or patients receiving intravenous infusions. Proteus Vulgaris is susceptible to Carbenicillin (Carter, 1972).

1.5.4 Pseudomonas aeruginosa

The group is composed of Gram-negative motile rods which produce water soluble pigments that diffuse through the medium. They occur in soil, water, sewage and the air. Ps. aeruginosa occurs in small amounts in the intestine, found on the human skin and occur in the environment but rarely cause

disease. Ps. aeruginosa may also produce heat labial exotoxins. Ps. aeruginosa becomes pathogenic when introduced into areas devoid of normal defences or when participating in mixed infection. It produces infections of wounds, giving rise to a blue-green pus; meningitis (as a result of lumbar puncture), urinary tract infection (as a result of the use of catheter, instrument, or irritating solutions), and respiratory tract infection (as a result of the use of infected aspirator). In infant with pulmonary cystic fibrosis, mucoid pseudomonas strain occur. The organism is found in otitis external and infection of the eye leading to its destruction. When Ps. aeruginosa invades the blood stream it results in fatal sepsis. Ps. aeruginosa is susceptible to colistin sulphate and development of resistance is rare in all gram negative organism (Carter, 1972).

1.5.5 Staphylococcus aureus

Staphylococcus is a Gram positive spherical cell, arranged in irregular cluster. They grow on a variety of media and produce pigments that vary from white to deep yellow. The pathogenic staphylococci haemolyse blood and coagulate plasma. Some are members of the normal flora of the skin and mucus membrane of human; others cause suppurative, abscess formation, a variety of pyogenic infections and even fatal septicemia. Certain staphylococci produce food poisoning by an

enterotoxin production. Staphylococci develop resistance to many antibiotics by evolving drug resistant mutants. Many strains are penicillin resistant by producing penicillinase, (ß-lactamase), is an enzyme that destroys penicillins by breaking the lactam ring. Its production is controlled by a plasmid that can be transferred by bacteriophages (transduction). The plasmids carry genetic control of resistance to other antibiotics.

Young cocci strains are strongly Gram positive, but on aging cells become Gram-negative. The organisms are non motile and are non-spore formers. Under the influence of certain chemicals they lyse or change into the l-forms.

Staphylococci are involved in minor skin infections (e.g. acne, impetigo etc); could cause exfoliation through the production of exfoliating toxin. They cause osteomyelitis and chronic suppuration (Jawetz et al, 1978). Vancomycin antibiotic to which staphylococcus are never resistant (Carter, 1972).

1.5.6 Streptococcus pyogenes

The streptococci are spherical microorganisms arranged in chains. They are widely distributed in nature. Some members are normal human flora others are associated with important human diseases. The organism produces a variety of extracellular substances and enzymes. Their ability to

haemolyse red blood cell (RBC) to various degree is one important basis for classification. Streptococcus pyogenes is a Gram positive organisms.

Streptococcus pyogenes cause the following infections Erysipelas, puerperal fever, [illegible]sis, sore throat and impetigo. Strept. pyogenes is found to be susceptible to Penicillin-V (Carter, 1972)

1.5.7 Candida albicans

C albican is an oval, budding yeast producing pseudomycelium both in culture, in tissue and in exudate. C. albicans is a normal flora of the mucus membrane of the respiratory tract, gastrointestinal and the genital tract. It may gain dominance in certain locations and become associated with pathogenic conditions. Sometimes it produces systematic progressive disease in debilitated or immunosuppressed patients. It may produce blood stream invasion, thrombophlebitis, endocarditis or infection of the eyes and organs when introduced intravenously.

In smears of exudates Candida albicans appears as a Gram-positive, oval, budding yeast or elongated budding cells resembling hyphae.

Candida albicans cause the following; oral thrush in children, female genitalia infection, skin disorder, nails, lungs and infection of other organs. There is candida

endocarditis in narcotic addicts and in chronic mucocutaneous candidiasis (Jawetz et al, 1978). The organism is susceptible to Nystatin (Carter, 1972).

1.6.0 PHARMACOGNOSY

It was not until 1815 that the term pharmacognosy was introduced by C. A. Seydler, a medical student in Halle/Saale Germany. The name is formed from two Greek words, "Pharmakon," drug, and, gnosis, knowledge. The most comprehensive idea of the scope of pharmacognosy was presented by Flückiger who stated that it is the simultaneous application of various scientific disciplines with the object of acquiring knowledge of drugs from every point of view (Tyler et al, 1976).

Pharmacognosy may be defined as an applied science which deals with the biological, biochemical and economic features of natural drugs and their constituents (Tyler et al, 1976) used in the treatment of diseases. As such its roots lies deep in the antiquities of world cultures (Shellard, 1958). It is the study of drugs having their origin in the plant and animal kingdom. In a restricted sense, the definition of pharmacognosy implies a particular knowledge of methods of identification and evalution of drugs. In this era of specialization, pharmacognosy deals primarily with information on the sources and constituents of natural drugs (Tyler et al,

1976). Pharmacognosy involve an objective study of crude drug of plant and animal origin so that they could be identified easily and correctly; and given standard in order that adulteration and substitution could be detected (Shellard, 1958).

The factor most responsible for the development of pharmacognosy has been the need to control the quality of drugs used in pharmacy and medicine. The maintenance of certain standards has involved both botanical and chemical examination of drug (Shellard, 1958).

Standards whereby the quality and quantity of crude drugs may be assessed fall into two main groups. Those in the first group are based upon a detailed study of the history and commerce of the drug, upon a knowledge of the biological and geographical source of the drug and upon its morphological, anatomical and histological characters. Such examination will also include a similar study of those materials likely to be accidental adulterants of the drug. A knowledge of the constituents may be useful in many cases. As a result of such study it would be possible to accurately define the drug by giving the botanical name of the plant from which it is obtained and to give an accurate and detailed description of it both in the whole and the powdered condition. It would also be possible to state the likely adulterants and to set numerical limits for those derived from other part of the same

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plantor from closely related plants. In some cases it may be considered desirable to include some definite qualitative chemical test for the exact identity of the drug (Shellard, 1958).

The standards in the second group may be sub divided into the following section:

1) Physical standards: These would be based on physical properties of the drugs.
2) Chemical Properties: These would be chiefly chemical assays, whereby the amount of the main constituents would be determined.
3) Biological Standards: These are based on a biological assay of the drug and are carried out on important drugs when it is not possible for one reason or another to assess their quality either by chemical or physical methods. The quality of drug is controlled by standards given to them in the current issue of the "African Pharmacopoeia, British pharmacopoeia, British pharmacopoeia Codex, European pharmacopoeia and the India pharmacopoeia. Constant reference to the British pharmacopoeia Codex is necessary while carrying out work on the evaluation of drugs (Shellard, 1958). In Nigeria the NAFDAC and NDLEA are charged with the responsibility of insuring compliance of the food and drug act in this Country.

1.6.1 SOME PHARMACOGNOSTIC TECHNIQUES IN CRUDE DRUG STUDY

The analytical problems encountered in pharmacognosy are varied and include the identification and definition of new drug sources, the assessment of the quality of crude drugs for direct use or as a source of valuable constituents, difficulties encountered in extraction or use of a drug (Brain and Turner, 1975).

1.6.2 PREPARATION OF DRUGS INVOLVE THE FOLLOWING TECHNIQUE

Collection; Harvesting; Drying; Garbling; Packaging, Storage and Preservation.

Collection

Collection of drugs from cultivated plants insures a true natural source and a reliable product. This may or may not be the case when drugs are collected from the wild. Since drugs come from all over the world, collection areas are almost universal and collectors may vary from uneducated natives to highly skilled botanists (Tyler, et al, 1976).

Harvesting

The mode of harvesting varies with the drug being produced and with the pharmaceutical requirements of the drug. Some drugs can be collected by hand labour others by

mechanical methods. For leaves and herbs, the cut plant may be hauled directly to the driers. At the appropriate time crop producing fruit and seed are best cut with mower and binder (Tyler et al, 1976).

Drying

Drying the plant material removes sufficient moisture to insure good keeping qualities and prevent molding, the action of enzymes, the action of bacteria and chemical and other possible changes. It fixes the constituents and facilitates grinding and milling. The drug may be "air-dried," sun dried, shade dried or dried with artificial heat (Tyler et al, 1976).

Garbling

Garbling is the final step in preparation of the drug. Garbling consist of the removal of extraneous matter, such as other plant parts, dirt and added adulterants. This is done to some extent during collection but should be carried out after the drug is dried and before the drug is baled or packaged (Tyler et al, 1976).

Packaging

Packaging provide suitable protection as well as economy of space. The packaging of drugs is dependent upon their final disposition. Leaf and herb material is usually baled with power balers into a solid compact mass which is then sewn

into a burlap cover (Tyler et al, 1976).

Storage and preservation

Proper storage and preservation are important factors in maintaining a high grade of quality of the drug. Warehouse should be fire proof, steel, concrete or brick construction; they should be unheated allowing no moisture, rodent proof, insect proof and in some cases even sunlight proof, sometimes storage at low temperation (2° - 8°C) may be required (Tyler et al, 1976).

Animal Drugs

Animal drugs are produced from wild or domesticated animals; if from wild animals they must be hunted (whale) or fished for (cod, halibut), and this is in a sense, their collection parallels the collection of vegetable drugs. Many animal drugs are produced from domesticated animal, and so correspond to the cultivated vegetable drugs (Tyler at al, 1976).

1.6.3 OFFICIAL AND UNOFFICIAL DRUGS

Pharmacopoeias

Drugs of outstanding therapeutic value are standardized in the national pharmacopoeias, of which about forty are issued at intervals by the nations of the world. In these

authoritative compendia drugs are designated as, "official," "unofficial" and "non-official" standards (Tyler et al, 1976).

1.6.4 EVALUATION OF DRUGS

To evaluate a drug means to identify it and to determine its quality and purity. The identity of the drug can be established by actual collection of the drug from a plant or animal which has been positively identified. Researchers must be absolutely certain of the origin of their samples; hence "drug gardens" are frequently established by institution engaged in pharmacognostic research. Another method of identification is by comparing a representative unknown sample with a published description of the drug and authentic drug samples. Quality refers to the intrinsic value of the drug (amount of medicinal principle or active constituents present) (Tyler et al, 1976).

The evaluation of a drug involves a number of techniques which may be classified as follows:

1.6.5. MORPHOGRAPHY

Morphology is the study of the form of an object and morphography is the description of that form. A number of different bases may be used in the classification of drugs. Morphology may be studied as the whole drug (micromorphology or gross morphology), Cell/Cytomorphology), or particulate

level. If a given sample of a known crude drug is to be evaluated as a potential source of therapeutic activity it is necessary to confirm that:

(a) it is, in fact, a sample of the drug stated;

(b) it contains the expected active constituents in reasonable quantity; and

(c) it contain nothing else (Brain and Turner, 1975).

The usual procedure is first to attempt to authenticate the source of material, that is to decide if it is actually the material claimed. The essential of any authentication procedure is a comparison between the test sample and another, standard sample, the origin which is known without doubt. The description can be made most accurate and unambiguous if it is a combination of verbal and pictorial information (Brain and Turner, 1975).

1.6.5.1 SAMPLING

In sampling care must be taken in the selection of samples for examination as they must be truly representative of the material undergoing analysis. For example, the USP lays down very definite sampling procedures, if they are in broken (pieces less than 1cm) or powdered form. Samples are taken by extracting cores from top to bottom of the container in each direction. If the sample size is less than 100kg at least 250g of material is taken. If the sample size is

greater than 100kg repeated samples are taken according to the prescribed procedure, mixed and quartered (Brain and Turner, 1975).

1.6.5.2 ORGANOLEPTIC EVALUATION

This includes the examination of the odour, taste, colour and texture of the drug. Odour and taste are extremely sensitive criteria. Because the description of taste and odour is difficult they can only be described as "characteristic" and reference is made to the analyst's memory. The texture is best examined by taking a small quantity of the material and rubbing it between thumb and forefinger. The texture is described by terms such as "smooth," "rough," and "gritty" (Brain and Turner, 1975).

1.6.5.3 MACROMORPHOGRAPHY

The majority of natural product drugs which occur in more or less entire (intact) form are derived from plants or parts of plants. For example, anthraquinones are found in high concentration only in the bark of the cascara tree so that there is little point in collecting material other than the bark. Macromorphology is of greatest importance to the drug importer and manufacturer, both of whom commonly deal with the drug at this level.

The following groups are commonly used in the description of drugs derived from different parts of the plant:

Herb or aerial parts, = Leaf Wood

flower wood,

Sub terranean organ;- Fruit and seed (Brain and Turner, 1975).

1.6.5.4 Description of Leaf Drugs

(a) Surface Appearance and Texture

The drug may be in whole or broken form and the texture may be described by such terms as "shrivelled," "brittle""leathery" (Coriaceous), "papery," or "fleshy," the surface colour can be important especially with the occurrence of oil glands or hairs. Surface hairs may be present (pubescent) or absent (glabrous). If they are present they are of diagnostic value and may be rough (hispid), long (hirsute) or glandular (Brain and Turner, 1975).

(b) Dimensions

This vary even within a sample but the aspect (length - to - breadth) ratio, mean dimensions, and maximum and minimum values can be useful.

(c) Attachment of leaf

The attachment of the leaf to the stem is known as "sessile" or "petiolate" according to whether the leaf is

attached directly to the stem or via a short stalk (the petiole) (Brain and Turner, 1975).

(d) Lamina Structure

The lamina is the main flat part of the leaf (leafblade) and can show a very wide variation in form (Brain and Turner, 1975).

(e) Shape

Many variations occur (e.g., oval, oblong, round, cordate, linear, lanceolate, ovate and obovate) are common descriptive terms for lamina shape. These terms may be combined to describe intermediate or variable structures e.g., "linear - lanceolate" or "ovate to obovate" where the specimen has been dried the shape can best be determined after soaking (Brain and Turner, 1975).

(f) Composition

Commercially, "leaf" includes both true leaves and the individual leaflets of compound leaves. A true leaf is distinguished from compound leaves by having a bud found in the axil (the angle between the petiole and stem). The simple leaves are classified as "pinnate" or "palmate." The arrangement of leaflets in compound leaves is known as "Paripinnate" or "imparipinnate", dependent upon the presence of a terminal leaflet (Brain

and Turner, 1975).

(g) Incision

This refer to the occurrence of clefts into the edge of the leaf, and increasing depth of the incision is indicated by the addition of the suffixes-fid,-partite, and - sect to the composition term, e.g, palmatifid (Brain and Turner, 1975).

(h) Venation

The arrangement of veins (vascular tissue) on the lamina. Four types are common: parallel, pinnate, palmate and reticulate (net) (Brain and Turner, 1975).

(i) Margin

The margin or edge of the leaf may be entire or may have some indentation. These marginal indentations are much smaller than those considered as incisions. The most common types are entire, serrate, crenate, dentate and sinuate (Brain and Turner, 1975).

(j) Apex

The tip of the lamina. This may be symmetrical or asymmetrical and of a variety of shape (truncate, obtuse, acute and acuminate) (Brain and Turner, 1975).

(k) Base

The lower extremity of the lamina, may be symmetrical or asymmetrical and of a variety of shapes (e.g., symmetrical, asymmetrical, decurrent and cordate).

Example of the description of the macromorphology of a whole leaf (X2) specimen: Mitracarpus villous

Organoleptic examination:

Dark-green to brownish-green, fleshy-papery, characteristic odour and taste.

1.6.5.5 MACROMORPHOLOGY

Leaf (3-6cm. long and 0.7-1.5cm. broad) no petiole, sessile. Lanceolate with palmate venation. Veins conspicuous on the lower surface. Margin entire-sinuate. Lateral veins end at margin in conspicuous sinuate. Apex acuminate, Base asymmetrically decurrent(Fig. 1.10 A,B and C).

(a) Description of herbs

Consist of the entire aerial parts usually a young plant. Herbs are composed of leaves, stem, flowers and young fruits in small quantity (Brain and Turner, 1975).

(b) Description of Underground Organs

This group includes root and also underground structures, such as rhizomes and corms, derived from the stem.

The description of an underground organ should include the form in which the drug occur e.g., as the root or rhizome (or both) in whole, peeled or sliced form. The shape may be described as "straight," "branching," "tortuous," "cylindrical" or "conical". Surface characters, such as scale leaves, root scars, or

lenticels may be present. The fracture (transverse breakage), may be short, fibrous, splintery, or starchy. Observation of transverse surface by means of the naked eye or a hand lens will give information about the tissue arrangement within the structure (Brain and Turner, 1975).

(c) Description of Barks

Bark include all these tissues external to the cambium in the mature, secondarily thickened, stem. The shape of the pieces of bark depends on the type of incision made in removing them from the tree and on the nature and extent of any subsequent shrinkage. The description of the macromorphology of a bark should include the shape, which is most common, the mean dimensions and size range of the pieces, the appearance of both the inner and out surfaces, and the form of the fracture (Brain and Turner, 1975).

(d) Description of Woods

There are only a small number of drugs derived from woods. The material known as wood is generally that tissue which lies inside the cambium and comprises the xylem, with smaller amounts of other cell types. The description is similar to that for underground organs (Brain and Turner, 1975).

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(e) Description of Flowers

Flowers include true flowers, buds and other inflorescences. A few flowers consist of sepals (collectively calyx) petals (collectively corolla; brightly coloured). Within the petals are stamen (male reproductive organs). In the centre of the flower is gynoecium (the female reproductive organ), with a conspicuous style at its apex. The inflorescence is the whole flowering structure of the plant. It varies according to the structure of the individual flower and the arrangement of these relative to the plant axis. For example the commonest inflorescence types are umbel, panicle, Raceme and capitulum. The compositae is the largest family of flowering plants and is characterized by the capitulum inflorescence (Brain and Turner, 1975).

(f) Description of Fruits and Seeds

A fruit consists of the ripened ovary together with the remains of the style. A fruit contain one or more fertile seeds. For the description of the fruit the following points are important: the shape and general appearance of the fruit and its external surface, the number and arrangement of cavities (loculi) in the fruit; and the number of position of the seeds; the point of attachment of the stalk; and the remains of the calyx and other floral parts.

Many drugs derived from fruits include the seeds so that a full description must also include the seed characters. The shape of the seed depend to some extent on the manner in which the ovule are suspended in the ovary. This affect the position of certain surface characters of the seeds. These are the hilum (a scar left by the detachment of the seed stalk) the raphe (the vascular strand which connected the ovary to the ovule), and the micropyle, (a minute pore through which the fertilizing pollen tube entered the ovule). The outer layer of the seed, the testa, is very hard. Internal structure of a seed consists of the embryo (food reserve). The food may be stored in the swollen cotyledon of the embryo (exalbuinous seed) or may occupy a separate endosperm layer surrounding the embryo (albuminous or endos, hermic seed). The material stored in the seeds include fixed oil, starch and other carbohydrates, and proteins. The description of the seed should include the shape, size, and general appearance of the seed, the occurrence of any testa outgrowths, the distribution of the hilum, raphe, and micropyle (if visible), the form of embryo, and the nature of the food reserve (Brain and Turner, 1975).

(g) <u>Description of Acellular Materials</u>

As these materials do not contain cells they may be

described as "acellular products," or more traditionally as, "unorganized drugs." The majority of acellular product are solids. Liquid examples include the fixed and essential oils. In solids the size and shape of the pieces, their colour (single or varied), surface appearance (dull, smooth, cracked), fracture (short, rough) and texture (hard, brittle, crumbling) may be significant careful organoleptic evaluation often gives a hint as to the type of material present (Brain and Turner, 1975). Acellular materials include starch and starch product (e.g absorbable dusting powder), gums (e.g, acacia and tragacanth), resins (e.g. colophony and myrrh), dried extracts and juices (e.g aloes and catechu), minerals and fossil remains (e.g talc and kieselguhr), fixed oils, fats and waxes (e.g, castor oil and oil of theobroma), and essential oils (e.g., oil of cinnamon) (Brain and Turner, 1975).

(i) Gums

Gums are plant exudates and their forms depends on factors such as the type of incision made to release the material, the nature of the exudate, the drying procedure, and any subsequent treatment.

(ii) Resins

Resins are a very heterogeneous group of materials which have certain similar properties. They are brittle amorphous solids which melts to adhesive liquids and burn with a smoky flame (Brain and Turner, 1975).

iii) Dried Extracts and Juices

These yield a number of products of importance. Opium is the dried latex from the capsule of the opium poppy, aloes is the dried juice drained from cut leaves of various species of Aloe; and catechu is a dried aqueous extract of certain tannin-containing plants. These products differ from each other completely in composition (Brain and Turner, 1975).

(h) Description of Micro-Organisms

Certain microbes can synthesize substances, such as antibiotics which have therapeutic activity. They are important as spoilage organisms in food and drugs. They are identified by their general appearance in mass growth (colonial morphology), the detailed structure of the organism and any reproductive bodies and the response of the organism to particular stains. The distinctive characters in a liquid medium include the form and degree of surface growth (ring, pellicle, flocculent);

subsurface growth (turbid and granular) and sediment (flocculent, granular). The description of a culture on a solid medium should take into account the overall shape (punctiform, circular, irregular), texture of the surface (smooth, concentric, contoured, loose); elevation (flat, raised, convex, concave, irregular) and margin (entire, undulate, filamentous, curled), the extent of any subsurface growth, and any features.

If a crude drug has reached the stage where there is visible evidence of microbial growth then it is fair to assume that the material is of unsatisfactory quality and has been inadequately preserved or kept under unsuitable conditions. For accurate identification of the particular organism it is necessary to prepare cultures from the specimen and to grow these under standard conditions (Brain and Turner, 1975). It should be noted that contamination is less easy to detect in the early stages and microscopical examination is necessary.

1.6.6 MICROSCOPICAL TECHNIQUE

Microscopical techniques allow a more detailed examination of a drug. The microscope has two main analytical uses. First, it allows the the fine structure of minute objects to be visualised. Secondly,it can be used in the determination of optical and other properties of a specimen

(Brain and Turner, 1975).

1.6.6.1 Sample Preparation

The precise manner of preparation of a microscopical mount of the sample depends on the form and nature of the sample. The specimen must be placed in the optical path of the microscope in such a form and condition that the details of its structure are seen. The main factors affecting visibility include sample thickness and refractive index, and the presence of pigment and other obscuring material. Sampling thickness can be controlled in many cases by careful "sectioning" of the specimen or by selection of particular portions of a powder for examination. If a fragile specimen is to be sectioned it must be supported. The simplest of this method is to take a piece of elder pith and divide it longitudinally into equal halves. The sample is placed between these and the whole held between the thumb and index finger of the left hand. A razor, held in the right hand, is passed across the preparation from right to left producing a section at each sweep. The thickness of the section depends on the skill and judgement of the worker. A hand microtome enables better control of thickness. This has a tube into which the pit and sample are loaded. At the base of the tube is a screw threaded plunger which can be raised to extrude the sample onto the cutting table. For routine work on vegetable

drug samples a cover slip must be placed on top of the dispersion so that the objective lens cannot come into contact with the liquid. When a cover slip is applied care must be taken to avoid the formation of air bubbles in the preparation (Brain and Turner, 1975).

Where a specimen contain a large amounts of pigments, or other obscuring materials, this can often be removed by solution in a suitable reagent. The reagent most commonly used in this cleaning process is chloral hydrate solution. It is particularly useful in the examination of dried plant material as it not only removes material such as pigment, starch and aleurone, but also expands shrunken cells and tend to restore their natural shape. The activity of the solution is increased by heating, and this also removes trapped air bubbles from the preparation. It does not affect calcium exalate, oils or fats. It is sometimes desirable to study the form of obscuring material such as starch or aleurone grains and glycerol mountant is useful for this purpose. This is 33% solution of glycerol in chloroform water, prolonged contact with starch grains causes swelling. Cresol; does not swell starch grains and is the mountant of choice for quantitative measurements. Cresol is corrosive to the skin. It is also useful in the examination of fibres, minerals and fossil remains. Alcohol (95%) can be used to examine materials which are partially or wholly soluble in aqueous mountants.

It is useful as a preliminary mountant for unknown samples as it is then possible to check for the presence of water - soluble material. The sample is mounted in alcohol, observed microscopically, irrigated with an aqueous mountant and observed again. Alcohol is also useful as a wetting agent for hydrophobic materials which tend to trap air bubbles (Brain and Turner, 1975).

The overall contrast of the image can be altered by changing the refractive index of the mountant and also by general staining. Details of some of the common useful stains are as follows:

i) Methylene Blue

Methylene blue (0.1 percent aqueous) stains all cellular material blue and can be used to display microorganisms. As it is strongly absorbed by mucilages it can be used to detect their presence inside cells.

ii) Ruthenium Red

Ruthenium red is another stain for mucilaginous material. (Brain and Turner, 1975).

iii) Iodine

Iodine (N/50 aqueous) is useful in the examination of starch and aleurone. It may be applied directly or preferably, by irrigation. It colour starch blue-black and the ground substance and globoids of aleurone grains yellow. Various other carbohydrate materials such as

gums and dextrin give a range of colours which are of diagnostic value. The striations on starch grains are seen most readily on partially stained grains (Brain and Turner, 1975).

(b) Iodine

Iodine followed by 80 percent v/v sulphuric acid is a test for cellulose (Brain and Turner, 1975).

iv) Picric Acid

Picric acid (saturated solution in alcohol) stains protein yellow. Its principal application is in the detection of aleurone and proteinaceous fibres. Note that the positive reaction is the retention of yellow colour after washing (Brain and Turner, 1975).

v) Phloroglucinol

Phloroglucinol (1 percent in 90 percent alcohol) is used in conjunction with hydrochloric acid in a test for lignification of cell walls. Initial soaking of the material in phloroglucinol is preferable to a rapid admixture of the two reagents. This process dissolve calcium salts (Brain and Turner, 1975).

vi) Potassium Hydroxide

Potassium hydroxide (5 percent aqueous can be used to detect the presence of anthraquinones in plant materials (Brain and Turner, 1975).

1.6.7 CYTOMORPHOLOGY IN DRUG IDENTIFICATION

The basis of the analytical application of cytomorphology' is that there is always sufficient difference of one type or another to enable the distinction between similar plants at the cellular level. Such difference may be very marked (e.g, the presence or absence of a particular specialised type of cell such as a sclereid) or may be so marginal that they need to be established by optical micromeretics. As with any comparative procedure the more information which is available to build up a profile of the sample the more accurate the sample becomes.

Very detailed information is presented in pharmacopoeial monographic descriptions, textbook of pharmacognosy, and atlases of powdered drugs.

Not all the feature which are to be found in a particular drug are of equal value in its identification. It is those features which are unusual (unique), which are of greatest value. The simplest presentation method first divides the drugs according to the plant part from which they are derived. From a consideration of the general types of cell and cell inclusions it is not too difficult to classify a given powder into a particular morphological group (e.g., copious starch is indicative of an underground storage organ and aleurone grains of a seed). Within each morphological group the characters of the most distinctive types of tissue in different drugs are

then compared in tabular form. Such a tabular system must be scanned by eye and the characters described compared with those of the sample. An improvement on the tabular layout is the presentation of the information on punched cards. Nelson had applied the same basic principles for the identification of powdered drugs. At present this uses 103 selected parameters to distinguish 80 of the most common plant drugs, and the system is still being extended.

When a tentative conclusion has been reached as to the identity of a sample, then whenever possible this must be confirmed by a direct comparison of all the characters of the sample with these of a standard sample of the drug. Thus whilst the initial selection of likely candidates may be on the basis of minimal information the final identification should be based on a comparison of all available parameters (Brain and Turner, 1975).

ANALYSIS OF ACELLULAR PRODUCTS

There are certain acellular products which have regular form and are of value in microscopical analysis. The liquid materials are a special case.

Form and Organoleptic Characteristics

For example talc is exceptionally smooth in texture, dextrin (starch) has slightly sweetish gummy taste. Other techniques include solubility and Dispersion behaviour; the

tile tests (for example. N/50 iodine give characteristic colour with certain carbohydrates and 5% ferric chloride solution gives a dark blue-green coloration with tannins); Microscopical examination (e.g., for starch: the shape, hilum, striations, polarized light, size) are parameters to be described.

Fossil remains and mineral can be identified under the microscope using suitable mountants. Crude plant exudate containing cellular debris can be examined under the microscope. Kaolin and aluminum silicates bentonite can be differentiated by their solubility and observation under the microscope. Native starch and modified starch granules can be detected using microscopy. Gelatiniation of starch in 1% potassium hydroxide solution is important as a diagnostic parameter. Ruthenium red is a microscopical stain for distinguishing between gums and mucilages (Brain and Turner, 1975). Further differentiation can be made by means of chemical test. The test for carbohydrate materials include (cellulose, fehling, lead acetate and subacetate solution, 5% KoH and 10% H_2so_4, Selivanoff and specific polysaccharide products tests). Others include Resins (general and specific), Dried exudate and juice tests and tests for fixed oils, fats, and waxes (general and specific). The essential oils are detected by chemical means (Brain and Turner, 1975).

1.6.8 OPTICAL MICROMERETICS

If a quantitative approach to the occurrence of a particular characteristic can be adapted then, using this numerical data, the collation of the information, and the comparis m of sample, becomes objectives. Such comparis ms can be applied in terms of the positive identification of the botanical or geographical source of a sample or in the evaluation of the quality of a drug.

On the microscopical scale cellular and subcellular features are more constant, although it must be appreciated that they will always display a range of values. In any randomly selected population the normal distribution occurs. Most population have characteristics which are near to the mean for this population.

Quantitative microscopical methods can be divided into those which involve the measurement of the size of individual features (this feature employs the sizing method, the size parameters) and those which involve estimations of the frequency of the occurrence of a particular feature in a given quantity of material (Brain and Turner, 1975).

1.6.9 QUANTITATIVE EXTRACTIVE VALUES

Evaluation of a drug requires that it be identified in both its entire and powdered form by means of its micromorphological and cytomorphological characters. Whilst

these diagnostic features enable the nature and origin of the material to be stated, it could well be that faulty collection or possibly deterioration due to incorrect or extended storage may have altered the constituent content of the drug to a point where it is no longer acceptable. Drugs must therefore be submitted to evaluation procedures which will indicate their acceptability by criteria other than their morphology.

The procedures normally adapted to give the qualitative information required include the determination of one or more liquid or solid residue remaining after a particular manipulation. These include:

Solvent extractive values

(a) Water soluble extractive

(b) Alcohol - soluble extractive

(c) Petroleum - soluble extractive

Ash Residues

(a) Total ash

(b) Water - soluble ash value

(c) Acid - insoluble ash value

Moisture content determination

Essential oil content determination (Brain and Turner, 1975).

1.6.10

Pharmacognostic techniques are many and varied whilst each is significant; consequently, it is all of these techniques taken together that identify and classify that drug in question. Techniques such as Extraction (includes, galenical products; Liquid extracts of dry and soft of infusions and of tinctures, finally these isolated products of alkaloids, of glycosides, of flavonoids; of essential oil etc); Quantitative Estimation of constituents (include; chemical assay methods, physical assay methods, Biological assay method and quantitation of chromatograms) and the separation and identification of constituents are of tremendous importance in drug evaluation.

1.7.0 AIMS AND SCOPE

Mitracarpus villosus has been used for years in the treatment of eczema and skin disorder by Traditional Medical practitioners in Africa; its efficacy has not been established scientifically. The present work is aimed at the following:

1. To Study the general pharmacognostic profile for the plant.
2. To establish some pharmacopoeial standards that may be useful in preparing a monograph of the plant.
3. To screen the plant extracts for antimicrobial activity.
4. To attempt to determine some of the biologically active constituents

1.6.10

Pharmacognostic techniques are many and varied whilst each is significant; consequently, it is all of these techniques taken together that identify and classify that drug in question. Techniques such as Extraction (includes, galenical products; Liquid extracts of dry and soft of infusions and of tinctures, finally these isolated products of alkaloids, of glycosides, of flavonoids; of essential oil etc); Quantitative Estimation of constituents (include; chemical assay methods, physical assay methods, Biological assay method and quantitation of chromatograms) and the separation and identification of constituents are of tremendous importance in drug evaluation.

1.7.0 AIMS AND SCOPE

Mitracarpus villosus has been used for years in the treatment of eczema and skin disorder by Traditional Medical practitioners in Africa; its efficacy has not been established scientifically. The present work is aimed at the following:

1. To Study the general pharmacognostic profile for the plant.
2. To establish some pharmacopoeial standards that may be useful in preparing a monograph of the plant.
3. To screen the plant extracts for antimicrobial activity.
4. To attempt to determine some of the biologically active constituents

CHAPTER TWO

2.0.0 MATERIAL AND METHOD

2.1.0 PLANT COLLECTION, PREPARATION AND IDENTIFICATION

The fresh whole plant of Mitracarpus villosus (SW) DC., was collected during the months of January and February in its natural habitat on farmlands in Area F, Main Campus, Ahmadu Bello University, Zaria - Nigeria. It corresponded with the morphological description of Mitracarpus Scaber Zuccar which was renamed Mitracarpus villosus (SW) DC. as described by Thonner,(1915) and White,(1962).

The identity of the plant was again compared and confirmed by prepared herbarium specimen with those available at the Department of Biological Sciences, Ahmadu Bello University, Zaria - Nigeria. Mr. Musa M.D., of the Herbarium of the department of of Biological Sciences, Ahmadu Bello University, Zaria Nigeria confirmed and authenticated the specimen brought.

2.1.1 Prepation of Herbarium Specimens.

The plant sample was collected in the morning and was arranged to give complete view between blotting papers.

Then it was inserted between more sheets of paper placed on a flat cardboard. Another cardboard was placed on top and

this was weighed down with two bricks. The outer papers were changed every other day until the sample was completely dry. The dried sample was removed and placed on a white cardboard paper cut to herbarium size. The Specimen was tied to the paper by strips of cellotape.

An information card was prepared noting the general locality, botanical and vernacular names, habitat, date, time of collection, collector's name and local uses. The card was affixed onto the herbarium sheet(Walters, 1963). A free hand drawing of the plant was illustrated (pages 5 and 6).

2.2.0 DRYING AND STORAGE

The plant was collected and morphological organs were separated and dried. After drying each part was ground using mortar and pestle to get fine powder, then stored and labelled for pharmacology, pharmacognostic and microbiological studies.

2.3.0 MATERIAL

SOLVENTS, REAGENTS AND EQUIPMENT

All solvents and reagents used in the work were analytical grade and refer to British Pharmacopoeia solvents. All reference to water indicate distilled water.

2.3.1 SOLVENTS AND REAGENTS

The solvents and reagents used are as follows:

SOLVENTS

Acetone

Benzene

Chloroform

Diethyl ether (ether)

Ethyl alcohol (ethanol)

Methyl alcohol (methanol)

n-Butanol

Petroleum ether

Carbon tetrachloride

Distilled water

Cyclohexane

REAGENTS

Acetic acid

Acetic anhydride

Alcoholic potassium hydroxide

Alcoholic ferric chloride

Ammonia Solution

Barfoed's reagent

Bromocresol green solution

Bromine water

Bromine vapour

Iodine crystals

Indicator paper

Lead acetate solution

Lead subacetate solution

Liebermann - Burchardt

Lime water

Magnesium filling

Mayer's reagent

Million's reagent

Chlor-zinc-iodine
Citric acid
Cuprous Oxide
Diethylamine
Dilute glycerol
Dragendorff's reagent
Fehling reagents (A&B)
Ferric ammonium citrate solution
Ferric chloride solution
Glacial acetic acid
Hagner's reagent
Hydrochloric acid
Human blood serum
Ice block
Sterile water
Sulphuric acid
10% Tannic acid
Vanillin reagent
Wagner's reagent
Yellow Sodium Picrate paper
Molisch's reagent
N/50 Iodine solution
Nutrient agar
Phloroglucinol
Potassium chloride solution
Resorcinol
Ruthenium reagent
Saebro dextrose agar
Silica gel-G
Sodium acetate
Sodium chloride
Sodium hydroxide
Sodium hydrogen citrate
Sudan III & IV

2.3.2 EQUIPMENT

Ultraviolet (UV) Spectrophotometer
Hot plate
Mettler digital balance (P1200)
Bunsen burner
Wooden hood
Refrigerator

Pulveriser (Mortar and Pestle)

Hot air drier

Oven

Filter paper

Chronometer

Thimbol

Waterbath

Autoclave

Inoculating loop

Cork borer

Forceps

Automatic test pipette

Sieve mesh

Compound microscope

camera lucida

Knife

Fume chamber

Water circulator

Transformer

Posters (News paper)

Leather straps

Tared Gooch crucible

Incubator

Automatic stirrer

Mechanical shaker

cotton wool plug

chromatographic paper

Rotatory evaporator

Mantle hot plate

Spatular

Gieger counter

Dissection kit

Cellotape

Ashless filter paper

storage rack

porcelain filter

2.3.3 GLASSWARES

Measuring cylinder
Glass funnel
Round bottom flask
Petri-dish
Test tube
Erlenmeyer flask
Burette
Pipette
Beaker
Glass stirrer
Sterile bottle
Hand lens
Iodine tank
Desiccator
Weighing bottle
Separating funnel
Shanndon Tank
Capillary tube
Chromatographic plate
Soxhlet apparatus
Reflux condenser
Sterile swap
Evaporating dish
Dropper
Microscopic slide
Coverslip
Tiles
Ground glass
Watch glass
Developing Chamber
Buchner funnel

2.4.0 MACROSCOPICAL EXAMINATION OF THE ROOT, STEM, AND LEAVES OF M. VILLOSUS

The following macroscopical features and organoleptic properties (colour, odour, taste and texture) were noted for the root, stem and leaves; (Herbert and Ellery, 1948; Trease and Evans, 1983).

2.4.1 MICROSCOPICAL EXAMINATION OF ROOT, STEM AND LEAVES OF M. VILLOSUS

2.4.1.1 MICROSCOPICAL EXAMINATION OF ROOT POWDER

The powdered material of the root was pulverized and sieved using number 44 mesh. The powder was then examined for diagnostic characters using the microscope.

2.4.1.2 MICROSCOPICAL EXAMINATION OF THE STEM POWDER

STEM POWDER

The plant stem was ground using the mortar and the pestle, and sieved employing the number 44 mesh. The stem powder and its bark were examined for diagnostic characters, cell inclusions etc.

2.4.1.3 MICROSCOPICAL EXAMINATIONS OF THE FRESH SECTIONS (MOUNTS) AND POWDER OF THE LEAVES OF M. VILLOSUS

The powdered material as well as the transverse and longitudinal sections of the fresh leaves were used for the study and prepared for microscopical examination as follows: a thin anatomical section of the leaf was taken on a clean slide and mounted in a few drops of chloral hydrate solution. The slide was slightly warmed for 30 seconds. Over heating was avoided as this could cause formation of chloral hydrate crystals giving false observation. The section was carefully covered to exclude air bubble creeping in. After the section was sufficiently cleared, the slide was mounted in dilute

glycerol and observed under the compound microscope. The section mounted in glycerol remained fresh for a long time (Iyenger and Nayak, 1975).

2.5.0 CHEMOMICROSCOPICAL EXAMINATION

In order to differentiate the tissue and observe for the presence of a chemical substance such as anthraquinones, calcium oxalate, calcium carbonate, fixed oils, starch, lignin and tannins, the various anatomical sections and powder of the root, stem, leaves and inflorescence were stained with appropriate chemical reagents on microscope slides and observed under the microscope (Iyenger and Nayak 1975).

(i) Anthraquinones Test (Leaf only)

The leaf section was mounted in water. The microscopic field of vision was scanned through for brown patches in the anatomic section (Brain and Turner, 1975).

The transverse section of the leaf was mounted in 5% potassium hydroxide. Red colouration denote the presence of anthraquinones (Gulei and Loan, 1988).

The powdered leaf was treated with 5% aqueous ammonia hydroxide solution. The presence of anthraquinones was indicated by red colouration (Gulei and Loan, 1988).

(ii) Calcium Carbonate Test

The powdered root, stem, leaves and inflorescence on a white tile was treated with dilute sulphuric acid. Presence of calcium carbonate was indicated by effervescence with rapid evolution of gas (Brain and Turner, 1975) and with crumbing of the solid.

(iii) Calcium Oxalate Test

The powdered root, stem, leaves and inflorescence as well as anatomical section was cleared by warming with chloral hydrate solution. The calcium oxalate crystals appear as bright structures. On treatment with concentrated sulphuric acid the crystals were slowly dissolved (Brain and Turner, 1975).

(iv) Cellulose Test

The powdered sample of the root, stem, leaves and inflorescence were mounted in chlor-zinc-iodine. A blue colour would indicate the presence of cellulose (Trease and Evans, 1983).

(v) Fixed Oils Test

Samples of the root, stem, leaves and inflorescence were mounted in Sudan IV separately. The presence of oils in any of the tissues is indicated by pink colour (Trease and Evans,

1983).

(vi) Lignin Test

The powdered root, stem, leaves and inflorescence and their sections were heated for two minutes with a mixture of phloroglucinol and concentrated hydrochloric acid (1:1 V/V). Tissues like xylem, Sclerenchyma etc; because of lignification took up red colour. The intensity of the red colouration is an indication to the extent of lignification (Trease and Evans, 1985).

(vii) Mucilage Test

Various sections and powdered samples of the root, stem, leaves and inflorescence were treated separately with ruthenium-red. Mucilage in tissues stains pink or red (Iyenger and Nayak, 1975).

With haemotoxylin, mucilage take up violet - red colouration (Gulei and Loan; 1988).

(viii) Proteins Test

Red to violet coloured patches were formed on warming the samples (of root, stem, leaves and inflorescence) with million's reagent indicating the presence of proteins (Irvine, 1961).

(ix) Starch Test

The powdered, root, stem, leaves and inflorescence as well as their anatomical sections were mounted on a slide in water, then irrigated with N/50 iodine. The presence of starch is indicated by blue or blue-black colouration (Iyenger and Nayak, 1975).

(x) Suberin Test

Red colour around the periderm of the anatomical section and powdered root, stem, leaves and inflorescence on warming with Sudan IV in alcohol. Suberin in a polysaccharide (Irvine, 1961).

(xi) Tannins Test

The powdered root, stem, leaves and inflorescence, and anatomical sections were mounted in ferric chloride test solution. A blue-black or greenish colour indicate the presence of tannins (Ramstad, 1959).

2.6.0 PHYTOCHEMICAL SCREENING OF THE POWDERED ROOT STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS

Anthraquinone Test:

0.1g. of the powdered leaf was extracted with 10ml. of hot water for 5 minutes. The mixture was filter while hot. The filtrate was extracted with 10ml. Of carbon

tetrachloride. The carbon tetrachloride layer was diluted in 5ml of dilute ammonia solution. A Pink to cheery-red colour in the Ammonia layer indicated the presence of free anthraquinone.

A second 0.1g. of the leaf was mixed with 10ml. of ferric chloride solution and 5ml. of hydrochloric acid. The mixture was hydrolyse by heating on a water-bath for 10 minutes. The hydrolysate was filtered hot and allowed to cool. The filtrate was extracted with 10ml. of carbon tetrachloride. The carbon tetrachloride was taken off and washed in 5ml. of dilute ammonia solution. A more intense pink or red colouration indicated the presence of anthraquinone glycosides. The absence of colouration in the first test and its presence in the second, indicated the presence of only bounded anthraquinones (Brain and Turner, 1975).

2.6.2(a) Test for Alkaloids

10g. of powdered root, stem, leaves and inflorescence were taken in a small beaker and a strong ammonium solution was added in a quantity sufficient to just moisten it, and the mixture allowed to stand for about 10 minutes after thorough mixing was done. Sufficient mixture of chloroform and ethanol (1:1) was added just to soak and suspend the powder. The mixture was allowed to stand for 20 minutes with occasional stirring with a glass rod. The mixture was filtered through a

plug of cotton wool in a filter funnel. The marc was washed twice with 2ml. chloroform and the washing was added to the filtrate. The combined filtrate was concentrated to dryness on the waterbath. Care was taken to avoid overheating. The residue was cooled and dissolved in 5ml. of chloroform. The chloroform extract was transferred to a separatory funnel and was shaken with 3ml. of sulphuric acid. The two layers were allowed to separate, the lower (chloroform) layer was drained off and discarded. Three ml. of chloroform was further added, shaken and drained off and discarded until the acid layer was colourless. The acid layer was made alkaline with strong ammonia and extracted with 3ml. of chloroform. The extract was retained and evaporated to dryness. The residue was dissolved in 3ml. of ethanol and the following tests were carried out after neutralizing with dilute sulphuric acid (Trease and Evans, 1983).

To 0.5ml. of ethanol residue obtained from the above 2-3 drops of the following reagents were added.

(a) Dragendorff's reagent

(b) Mayer's reagent

(c) Hagner's reagent

(d) 10% Tannic acid

(e) Wagner's reagent

Most alkaloids are precipitated in most or all of the above reagents. False positive results could occur when

proteins are present; but heating during the course of extraction eliminates the possibility.

OTHER METHOD OF EXTRACTON OF (ALKALOIDS)

2.6.2(b) DETERMINATION OF TOTAL ALKALOID CONTENT OF MITRACARPUS VILLOSUS (SW) DC.

5g. of powdered leaf/inflorescence were thoroughly mixed with 2.0gm of calcium hydroxide in a beaker. The mixture was moistened with 50ml. of water and allowed to stand for 30 minutes. A large amount of the mixture was transferred into a separating funnel soaked in sufficient ether. The separating funnel and its content were shaken by a mechanical shaker for one hour after which the initial extract was collected in an evaporating round bottom flask. The marc was compressed and percolated with sufficient ether until no more alkaloid was extracted; then the bulk extract was heated until it was freed of the solvent. 10ml. of the chloroform was added to the residue, another 5.0ml. of chloroform was used in transferring the mixture into another clean separating funnel. Two layers are immediately visible, the upper (clear) acid layer and the lower chloroform layer, where the upper acid layer was transferred into yet another separating funnel. The initial mixture in the first separating funnel was washed repeatedly two more times with 5.0ml. of 0.05m sulphuric acid, making sure no acid was left in the first separating funnel. The

lower chloroform layer was then discarded. The bulk acid extract was basified with strong ammonia solution, 10ml. of chloroform was added and shaken.

Meanwhile the mouth of the separating funnel was packed with absorbent cottonwood (plug) soaked in ether. As the content of the separating funnel separates into two layers; the lower chloroform layer was drained off through the plug of cottonwood, while the upper acid layer was collected into a round bottom flask. The same procedure was repeated twice and each time, filtrates were collected into the round bottom flask. The contents of the flask was heated in an oven at low temperature to dryness removing any trace of ammonia. Three drops of acetone was added to dissolve the residue and 10ml. of ether was further added to the solution. The solution was transferred into a 250ml. conical flask and washed with 5.0ml. of ether (to reserve for titration). For titration: a stock of 0.05m sulphuric acid was prepared and transferred into a burette clamped to a stand. Few drops of the indicator (bromocresol solution) was added into the solution in the conical flask. Two immiscible layers were immediately visible which blended together by vigorous shaking.

At the end point a light green colour in the lower aqueous layer was indicative of the conversion from the acid to the alkaloid salt. The mixture was again vigorously shaken, and the volume of the 0.05m sulphuric acid was read off and

noted. The total alkaloids content was obtained by calculation from the given formular. Each 1ml. of 0.05m sulphuric acid was equivalent to 0.028g. of $C_{17}H_{19}NO_3$ (phenanthrene alkaloid). Add 0.052g. to correct for loss of the alkaloid due to its solubility (B.P, 1988).

2.6.3 CARBOHYDRATES

Extraction

3.0g. of powdered root, stem, leaves and inflorescence were boiled in 50ml. of water on a hot plate for 3 minutes. The mixture was filtered while hot and the resulting filtrate cooled, and used for the following tests.

General test (Molisch's test)

To 2ml. of extract in a test tube was added a few drops of Molisch's reagent, then a small quantity of concentrated sulphuric acid, was allowed to run down the side of the test tube at an angle of 45°C to form a lower layer. A purple to violet colour at the interface indicated the presence of carbohydrate. The mixture was then shaken gently and allowed to stand for two minutes and then diluted with 5ml. of water. A dull violet precipitate further indicate the presence of carbohydrate (Trease and Evans, 1982).

Sugar test

Barfoed's test for monosaccharide

To 1ml. of dilute solution of extract in a test tube was added 1ml. of barfoed's reagent and the mixture heated on the water bath for 4 minutes. A red precipitate indicate the presence of monosaccharide (Trease and Evans, 1982).

Standard test for free Reducing Sugars

(i) Fehling's test

To 2ml. of the water extract, 5 ml, of a mixture (1:1) of fehling solution A and fehling solution B was added and the mixture boiled in waterbath for 2 minutes. A brick red colour indicate the presence of free reducing sugar.

(ii) Standard test for combined Reducing Sugars

1 ml. of water extract was hydrolysed by boiling with 5ml. of dilute hydrochloric acid. This was neutralized with dilute sodium hydroxide solution (for the cuprous oxide to be precipitated the fehling's test was repeated). A brick-red colour indicate the presence of combined reducing sugar (Trease and Evans, 1982).

Standard test for Ketoses

(Selivanorff's test)

To a small volume of the water extract, were added to a

few crystals of resorcinol and equal volume of concentrated hydrochloric acid in the water bath. A rose colour indicate the presence of ketoses e.g. fructose (Trease and Evans, 1982).

Pentose test

To a small portion of water extract, was added an equal volume of dilute hydrochloric acid containing little phloroglucinol. The mixture was heated in the waterbath. A red colour indicate the presence of pentose (Trease and Evans, 1982).

2.6.4 GLYCOSIDES TEST

CARDIAC GLYCOSIDES

Legal test

The extract was dissolved in pyridine and a few drops of 2% sodium nitroprusside to gether with a few drops of 20% NaoH were added. A deep red colour which faded to a brownish yellow indicated the presence of cardenolides (Sofowora, 1993)

Kedde test

1ml. of an 8% solution of the extract in methanol was mixed with 1ml of a 2% solution of 3,5-dinitrobenzoic acid in methanol and 1ml. of a 5.7% aqueous sodium hydroxide. An immediate violet colour indicated the presence of

cardenolides in the extract, the colour fading gradually through reddish-brown to browish-yellow with the precipitation of a white crystalline solid. This test indicated the presence of a lactone ring in the cardenolides (Sofowora, 1993).

Liebermann's test

0.5g. of the extract was dissolve in 2ml. of acetic anhydride and cooled well in ice. Sulphuric acid was then carefully added. A colour change from violet to blue to green indicated the presence of a steriodal nucleus(i.e aglycone portion of the cardiac glycoside)(Sofowora, 1993).

Salkwoski test

0.5g. of the extract was dissolved in 2ml. of chloroform. Sulphuric acid was carefully added to form a lower layer. A reddish-brown colour at the interface indicated the presence of a steriodal ring(i.e aglycone portion of the cardiac glycoside, (Sofowora,1993).

Keller-Kiliani test

0.5g. of extract was dissolved in 2ml. of glacial acetic acid containing one drop of ferric chloride solution. This was then underlayed with 1ml. of concentrated sulphuric acid. A brown ring obtained at the interface indicated the presence of a desoxy sugar characteristics of cardenolides. A violet ring

may appear below the brown ring while, inthe acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer (Trease and Evans, 1989).

2. Cyanogenetic Glycosdes

1ml. of the leaf extract in a test-tube containing a strip of moist yellow sodium picrate paper warmed on the water bath for 1 hour. A colour change indicated the presence of Cyanogenetic glycosides (Trease and Evans, 1982).

3(i). Saponins Test

To a small quantity of the powdered root, stem, leaves and inflorescence was added 10ml. of 95% ethanol and boiled on the water bath. The mixture was filtered while hot and allowed to cool. 2.5ml. of the ethanol extract was diluted with 10ml of water in a test tube. The test tube was stoppered with a cork, vigorously shaken for 30 minutes and allowed to stand for 45 minutes. A honey-comb froth indicate the presence of saponins (Sofowora, 1982).

(ii) Haemolytic test for saponins (Steroidal and triterpenoids).

0.2g of the chemical substance from powdered root, stem, leaves and inflorescence were extracted with 10ml. warm water, filtered and the filtrate retained. 2ml. of 1.8% sodium

chloride solution was added to each of the two test tubes. To one of the test-tubes, 2ml of distilled water was added and to the other, 2ml of extract was added. The concentration of sodium chloride in each case is now isotonic with blood serum. Blood was obtained by pricking the thumb at the base of the nail and the blood was drawn into a small pipette. Five drops of the blood was added to each of the test tubes. The tubes were stoppered and inverted gently to mix the contents. Haemolysis in the test tube containing the extract and not in the control indicates the presence of saponins (Brain and Turner, 1975).

Terpenes and Sterols test

Extraction

5g. of the powdered root, stem, leaves and inflorescence was extracted by maceration with 5.0ml of 95% alcohol. The extract was filtered and the filtrate was evaporated to dryness in a beaker. The residue was dissolved in 10ml. of chloroform and re-filtered. The filtrate was divided into two portions which were used for the following tests.

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(i) Salkowski's test (Steroidal Ring)

The first portion of the filtrate above was taken in a test tube, 2ml. of concentrated sulphuric acid was added carefully to form a lower layer. A reddish brown ring at the

interface indicate the presence of a steroidal ring (Sofowora, 1982).

(ii) Liebermann-Burchard test (terpenes)

To the second filtrate from above was added 1.0ml. acetic anhydride. To this mixture was added 1.0ml. of concentrated sulphuric acid down the sides of the test-tube. The formation of reddish violet colour at the interface of the two liquids and a green colour in the chloroform layer indicate the presence of terpenes (Sofowora, 1982).

2.6.5 FLAVONOIDS TEST

Extraction

2g. of the powdered root, stem, leaves and inflorescence was completely detanned with acetone. The acetone was completely evaporated over the water bath. The residue was dissolved in warm water and the mixture was filtered while hot and allowed to cool. The filtrate was used for the following tests (Brain and Turner, 1975).

(i) Lead acetate test

To 5.0ml of the detanned water extract, was added 2-3 drops of the lead acetate solution. A buff coloured precipitate indicate the presence of flavonoids.

(ii) Sodium hydroxide test

5.0ml. of the detanned water extract was taken in a test tube and an equal volume of 10% sodium hydroxide was then added. A yellow solution indicate the presence of flavonoids. On addition of dilute hydrochloric acid the solution became colourless (Trease and Evans, 1983).

(iii) Ferric chloride test

2.0ml. of detanned water extract was diluted within a ratio of (1:4) and a few drops of 10% ferric chloride solution was added. A green or blue colour indicate the presence of phenolic nucleus (Brain and Turner, 1975).

(iv) Shinoda test

0.5g. of the powdered root, stem, leaves and inflorescence was boiled in ethanol in a test tube over the water-bath for 5 minutes, filtered and cooled. Four pieces of magnesium filling was added followed by a few drops of concentrated hydrochloric acid. A pink or red colour indicate the presence of flavonoids (Geissman, 1955).

2.6.6 TANNINS TEST

Extraction

3.0g. of the powdered, root, stem, leaves and inflorescence were boiled in 50ml. of water for 3 minutes on

the hot plate. The mixture was filtered while hot and the resulting filtrate was used to carry out the following tests for tannins.

(i) Ferric chloride test

1ml. extract was diluted with 4ml. of water and a few drops of 10% ferric chloride solution were added. A bluish black colour indicate the presence of hydrolyzable tannins and a greenish colour indicate the presence of condensed tannins (Trease and Evans, 1983).

(ii) Lead subacetate

To 1ml. of the extract was added 2-3 drops of lead subacetate solution. A coloured precipitate indicates the presence of tannins (Trease and Evans, 1983).

(iii) Ferric ammonium citrate test

To 1ml. of the water extract was added 5 drops of 0.25% ferric ammonium citrate solution. To the mixture was added 0.5g. solid sodium acetate to adjust the solution to (PH=8) using the pH indicator paper. The mixture was boiled in the water bath and filtered while hot. A purple, violet, or black precipitate which is insoluble in hot water indicates the presence of tannins (Balbaa, 1976).

(iv) Bromine water test

A few drops of bromine was added to 1ml of the water extract in a test tube. Condensed tannins give a buff coloured precipitate, hydrolysable tannins gives none at all (Balbaa, 1976).

(v) Test for phloroglucinol nucleus

15ml. of the alcoholic extract of the root, stem, leaves and inflorescence were obtained by boiling 0.5g. in 10ml. of ethanol and filtered. 5ml. was taken in an evaporating dish and placed on the waterbath, 2ml. of vanillin reagent was then added. The mixture was evaporated to dryness. A drop of concentrated hydrochloric acid was added. A crimson colour indicate the presence of phloroglucinol which frequently occurs with catechol i.e. condensed tannins (Balbaa, 1976).

(vi) Gelatin test

To 2ml. of water extract was added an equal volume of 0.5% gelatin solution containing 10% sodium chloride. A precipitate indicate the presence of tannins (Trease and Evans, 1983).

2.7.0 CHROMATOGRAPHIC ANALYSES

2.7.1 CHROMATOGRAPHIC ANALYSIS OF CRUDE ALKALOIDS EXTRACTS

Extraction

To 0.5g. of extract of the root, stem, leaves and inflorescence was added 5.0ml. of dilute hydrochloride acid and heated over water bath and then filtered. The filterate is concentrated over waterbath. The concentrated solution was used for Chromatographic analysis.

Paper Chromatography

Condition for Paper Chromatography

Chromatographic paper = Whatman N°1 (20x20 cm)

Solvent System = n-butanol: acetic acid: water (4:1:5)(Lower layer

Technique = Ascending

Visualization (i) UV

(ii) Spraying with strong ammonia vapour.

Thin Layer Chromatography

Condition for Thin Layer Chromatography (TLC)1

Adsorbent = Silica gel - G

Solvent system = chloroform: Methanol (4:1)

Technique = Ascending

Visualization = (i) UV

(ii) Spraying with (a) Dragendorff's reagent

Condition for Thin Layer Chromatography II

Adsorbent = silica gel - G

Solvent system = Chloroform: Methanol: Ammonia (10:20:1)

Technique = Ascending

Visualization = (i) UV

(ii) Spraying with (a) Marquis (Harborne, 1973)

(b) 50% Sulphuric acid (Macek, 1972).

2.7.2 CHROMATOGRAPHIC ANALYSIS OF FIXED OIL

Extraction

30g. of the powdered root, stem, leaves and inflorescence were extracted with 200ml. of water. The solvent was evaporated off leaving a residue. The residue was dissolved in 200ml. of medium range petroleum ether (60°-80°) and evaporated off completely over the water-bath. The oil obtained was diluted in alcohol and chloroform and was used for spotting.

Thin layer chromatography

Condition for Thin layer chromatography

Two Dimensional Thin layer chromatography

Adsorbent = silica gel - G

Solvent system = Chloroform: Methanol; Acetic acid; water

(170:25:25:4)(Lower layer)

Technique = Ascending

Visualization = (i) UV

(ii) Spraying with 50% H_2SO_4 (Harborne, 1973)

Solvent system II (Second Dimension).

Chloroform: Methanol: 7m NH_4OH (65:30:4).

Sprayed with 50% H_2SO_4

2.7.3 CHROMATOGRAPHIC ANALYSIS OF FLAVONOIDS

Extraction

2.0g. of powdered root, stem, leaves and inflorescence was completely detanned with acetone. The acetone was completely evaporated over water bath. The residue was dissolved in a warm water and the mixture was filtered while hot (Brain & Turner 1975).

Paper Chromatography

Condition for two Dimensional Paper Chromatography

Chromatographic paper = Whatman No. 1 (20 x 20 cm)

Solvent system = n-butanol: acetic acid: water (4:1:5). 2nd Dimension Solvent system; 5% acetic acid.

Technique = Ascending

Visualization = (i) UV

(ii) Spraying with 5% alcoholic ferric chloride solution

Paper Chromatography

Condition for paper chromatography

Chromatographic paper = Whatman No. 1 (20 x 20 cm)

Solvent system = n butanol; acetic acid: water (4:1:5)

(Geissman, 1962; Harborne, 1973).

Technique = Ascending

Visualization = (i) UV

(ii) Spray with 5% alcoholic ferric chloride solution (Macek, 1972).

Thin layer chromatography

Condition for Thin layer Chromatography

Adsorbent - Silica gel - G

Solvent System = Benzene: Pyridine: formic acid (36:9:5)

Technique = (i) UV

(ii) Spraying with ammonia vapour (Macek, 1972).

2.7.4 CHROMATOGRAPHIC ANALYSIS OF SAPONIN GLYCOSIDES EXTRACTION AND ISOLATION OF STEROIDAL ALKALOID GLYCOSIDES

The material was extracted twice with 5% acetic acid for 24 hours at 20°C. The filtered acid extract was heated to (60°C-70°C) and adjusted to (pH. 9-10) with concentrated ammonia. The resulting flocculent precipitate is cooled, filtered, washed with water, and dried at (60°- 70°C). This crude precipitate contain substantial amount of inorganic salts, which was then repeatedly refluxed with methanol for

1 hour in order to extract from them the steroidal alkaloid glycosides which are still contaminated with chlorophyll (Macek, 1972).

Paper Chromatography

Condition for Paper Chromatography

Chromatographic paper: Whatman No. 1 (20x20cm):

Solvent system: ethylacetate: pyridine: water (3:1:3)

Technique = ascending

Visualization = (i) UV

(ii) Spray withCarr-price (Antimony trichloride in chloroform) (Macek, 1972).

Thin Layer Chromatography

Condition for Thin layer Chromatography

Adsorbent = silica gel - G

Solvent system = Benzene: Pyridine: formic acid (36:9:5)

Technique = Ascending

Visualization = (i) UV

(ii) Spraying with ammonia vapour (Macek, 1972).

2.7.5 CHROMATOGRAPHIC ANALYSIS OF SAPONIN GLYCOSIDES *EXTRACTION AND ISOLATION OF STEROIDAL SAPONINS FROM* PLANT MATERIAL

Powdered material (10g) was extracted three times with 60ml. of methanol and water (2:1) at room temperature. The

extract was concentrated under vacuum to 50ml. and are then shaken with 50ml., of isobutanol. The isobutanol phase is taken up in 20ml. of methanol. concentrated to 50ml. (may also be taken up repeatedly with the same volume of petroleum - ether to remove non-polar substances). The aqueous layer was evaporated to dryness and extracted repeatedly with 90% alcohol. The ethanol extracts are combined, filtered and concentrated to 20ml. (Macek, 1972).

Paper Chromatography

Condition for Paper Chromatography

Chromatographic paper: Whatman No. 1 (20x20cm):

Solvent system: Benzene: n-butanol: water (10:4:5)

Technique = Ascending

Visualization - (i) UV

(ii) Spraying with Carr-Price

Thin Layer Chromatography

Condition for Thin layer Chromatography

Adsorbent = silica gel - G

Solvent system = Chloroform: Methanol: Water (65:35:10)

Technique = Ascending

Visualization = 1) UV 2)Spraying with Carr-Price

2.7.6 CHROMATOGRAPHIC ANALYSIS OF TRITERPENE GLYCOSIDES EXTRACTION AND ISOLATION OF TRITERPENES

Dried plant material (100g) was first extracted with petroleum ether (root, inflorescence) and with chloroform (stem, leaves) for 16 hours (defat), and then exhaustively extracted with (4:1) mixture of methanol; water (Macek, 1972).

Paper Chromatography

Condition for Paper Chromatography

Chromatographic paper: Whatman No. 1 (20x20cm):

Solvent system: n-butanol: glacial acetic acid:water (4:1:5)

Technique = Ascending

Visualization = (i) UV

(ii) Spraying with Carr-Price

Thin Layer Chromatography

Condition for Thin layer Chromatography

Adsorbent = silica gel - G

Solvent system = Chloroform: Methanol: Water (65:35:10)

Technique = Ascending

Visualization = (1) UV (2) Spraying with Carr-Price (Macek, 1972).

2.7.7 CHROMATOGRAPHIC ANALYSIS OF TANNINS EXTRACTION AND ISOLATION

3g. of powdered root, stem, leaves and inflorescence was boiled in 50ml. of water for 30 minutes over hot plate. The mixture was filtered while hot, the resulting filtrate was concentrated, cooled and stored for using in chromatographic separation and antimicrobial screening. The same procedure was repeated using the mixture 95% alcohol and water in the ratio (1:1 v/v) (Trevor, 1976).

Reference Standard

1% of the following standard samples were prepared in hot water.

i) Tannic acid

ii) Catechol

iii) Phloroglucinol

Paper Chromatography

Condition for Paper Chromatography

Chromatographic paper: Whatman No. 1 (20x20cm):

Solvent system: n-butanol: glacial acetic acid: water (4:1:5) (Lower layer)

Technique = Ascending

Visualization = (1)UV (2)Spraying with 5% alcoholic ferric chloride solution (Balbaa, 1976; Trevor, 1976).

Thin Layer Chromatography

Condition for Thin layer Chromatography

Adsorbent = silica gel - G

Solvent system = n-butanol: acetic acid: Water (4:1:5)(Lower layer)

Technique = Ascending

Visualization = (i) UV

(ii) Light

(iii) Spraying with 5% Alcoholic Ferric chloride Solution (Trevor, 1976).

2.8.0 QUANTITATIVE EVALUATION

QUANTITATIVE EVALUATION OF POWDERED LEAVES AND INFLORESCENCE OF M. VILLOSUS.

2.8.1 DETERMINATION OF MOISTURE CONTENT OF POWDERED LEAVES AND INFLORESCENCE - BY "LOSS ON DRYING METHOD"

A tared evaporating dish was heated at 105°C to a constant weight and its exact weight was noted. 3g. of the powdered leaves/inflorescence was accurately weighed into the dish. The evaporating dish and its contents was placed in an oven at 105°C and dried to constant weight (achieved by checking the weight at 30 minutes interval after an initial drying for 1 hour). The total loss in weight was determined by subtracting the constant weight of the porcelain dish from the combined weight of the porcelain and its content.

The percentage of the moisture content was then

determined with reference to the initial weight of the powdered leaves/inflorescence. Two other determinations were done and the average of these was taken as the moisture content of the drug (B.P., 1980).

2.8.2 DETERMINATION OF SOME PHYSICOCHEMICAL VALUES

(i) Determination of Total Ash Value

A nickel crucible was heated at 105°C to a constant weight in the oven and its accurate weight was determined after cooling in a desiccator. 3g. of the powdered leaves/inflorescence was accurately weighed into the crucible. The crucible and its contents were gently heated over the bunsen flame until it was moisture free and was completely charred. The heat was gradually increased until most of the carbon had vaporized and was heated strongly such that the inorganic ash could be seen (residue free from carbon) almost white. The ash was cooled, weighed and the weight noted. The heating and cooling continued until the weight of the residue was constant. The weight of the ash was obtained by subtracting the weight of the crucible from the final weight (of the crucible and residue or ash) i.e. (b-a) =wt. of residue

where a = wt. of empty nickel crucible.

b = wt of crucible and residue.

The ash value (in percentage) of the powdered leave/inflorescence was calculated with reference to the initial weight of the powdered leaf/inflorescence

$$\text{Ash value} = \frac{(b-a)\ x\ \left(\frac{100}{1}\right)}{3}$$

Three different determinations were carried out and the average of these was taken as the ash value. The ash was retained for the experiment below (BP, 1980).

(ii) Determination of Acid-Insoluble Ash

The nickel crucible and ash obtained from above was transferred into a beaker containing 25ml. dilute hydrochloric acid. The beaker and its content were boiled for 5 minutes and filtered through an ashless filter paper. The beaker was washed into the funnel. The washing was repeated three times, each time passing the washings in a manner as to allow the collection of the residue at the tip of the cone of filter paper. The funnel along with the filter paper and contents were dried in an oven at 105°C.

The weight of the clean heated and cooled nickel crucible was accurately determined. The filter paper with its contents was folded into a small cone and transferred into the nickel crucible (pre-weighed). The crucible was gently heated over a bunsen flame until the filter paper was completely ashed and

then heated strongly for a few minutes. The crucible and contents were cooled and the final weight noted. The weight of the residue (ash) was then calculated by subtracting the weight of the crucible and the residue. Also the acid-insoluble ash-value with reference to the measured weight of the drug was calculated.

i.e. weight of the ash = b-a.

$$\text{Acid - insoluble ash value} = \frac{b - a}{3} (100)$$

where a = weight of nickel crucible

b = weight of crucible and residue

2.8.3 DETERMINATION OF EXTRACTIVE VALUE

(i) Alcohol - soluble value

5g. of the powdered leaf/inflorescence was accurately weighed into a 250ml. stoppered conical flask. 100ml. of 90% ethanol was added and stoppered firmly. The flask was shaken on a mechanical shaker for 6 hours and then allowed to stand for 18 hours. The extract was filtered quickly (by suction filtration). The weight of a clean, heated and cooled flat bottom evaporating dish was accurately determined. 20ml. of the filtrate was taken into the evaporating dish. The filtrate was evaporated to dryness. The residue was dried to constant

weight at 105°C in an oven and the final weight determined.

The weight of the crucible was determined by subtracting the weight of the evaporating dish from the final weight (total weight of the evaporating dish and residue). The alcohol extractive value was then calculated with reference to the initial weight of the powdered leaf/inflorescence and expressed in percentage.

$$\text{Alcohol extractive value} = \frac{c - b}{a} \times 5 \times 100$$

where a = weight of powder.

b = weight of evaporating dish

c = weight of evaporating dish and residue.

Three different determinations were done and the average of these was taken as the alcohol - soluble extractive value (BP, 1980).

(ii) Water - Soluble Extractive Value

The above procedure was repeated; but using 0.25% chloroform in water instead of 90% ethanol. Chloroform was added as fungistatic. Three different determination were made and the average of these was taken as the water soluble extractive value (BP, 1980).

2.9.0 QUANTITATIVE MICROSCOPY OF THE LEAF OF MITRACARPUS VILLOSUS (SW) DC.

Quantitative microscopy are numerical values like "stomata number," "stomata index", "Palisade ratio," "Vein islet number," and "Vein termination number." These values are based on the microscopical structure of leaves. They are fairly constant for leaf of any particular plant and are used as reliable characters by which botanical origin of a leaf can be established, especially when concerned with closely related species.

(i) Determination of Stomata Number and Stomata Index

To determine the stomata number and hence the stomata index of M. villosus leaf; the leaf was cleared by heating in a test tube with chloral hydrate for about 10 - 15 minutes over water-bath and allowed to cool. After cooling the thin upper layer of cells of the lower epidermis was removed using a razor. It was then transferred to a microscope slide and mounted in a solution of chloral hydrate and a drop of glycerol and covered with coverslip. Using a 4mm objective, a camera lucida, drawing paper and stage micrometer; a line was drawn on the paper equivalent to 1mm, as seen through the microscope. The mounted preparation was placed on the

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microscope stage and marked off, the stomata cells as (O) and the epidermal cells as (X), each enclosed in the square drawn which is equivalent to 1mm. The number of epidermal cells (X) were counted and the stomata index was calculated using the formula:-

$$\text{Stomata Index} = \frac{S}{E - S} \quad 100$$

where S = Stomata

E = epidermal cells

The procedure was repeated for 5 more fields and the average calculated.

(ii) Determination of Palisade Ratio

The fresh leaf of M. villosus was boiled in choral hydrate for 10-20 minutes in a test - tube which was placed over the water bath. A thin transverse section of the leaf was removed with the aid of a razor and the teased section observed under the camera lucida. Four cells of the upper epidermis were traced off and the palisade cells under the epidermal cells were counted; completing the cells that cross the epidermal walls. The procedure was repeated several times taking different fields and the average noted, from whence the palisade ratio may be calculated.

(iii) Determination of Vein-Islet Number

A portion of M. villosus leaf was cleared and the lower epidermis of the uppermost was removed as described above. Using a 4mm objective lens, a camera lucida, drawing paper and stage micrometer a line was drawn; equivalent to 1mm. as seen through the microscope, a square was constructed on the line. The paper was moved so that the square was seen, on looking through the eye piece. The mounted preparation was placed on the microscope stage and traced. The veins enclosed in the square and those necessary to complete the islets that overlap the bottom and the left side of the square. From the drawing the islets were counted, including those that are intersected by the bottom and left side of the square; but excluding those inserted by the top and right side. Five different fields were viewed and the average is the vein-islet number.

(iv) Determination of Vein-Termination Number

The same procedure (iii) above was repeated: but the lower epidermis uppermost was removed from the leaf lamina to include the margin instead of between the midrib and the margin.

2.10.0 PRELIMINARY ANTIMICROBIAL STUDIES OF M. VILLOSUS .HERB

Media:

Media used were nutrient agar, nutrient broth, Tween 40.

The broth dilution method recommended by International Collaborative Studies(ICS) as described by Ecrisson and Sherris (1971) were used. The media were prepared according to the manufacturer's specifications and 5ml. were dispensed into each test tube. The Oxoid media was prepared by the formula set forth therein 10gm of the solid (Oxoid) was measured into 100ml. of water and brought to boil on a bunsen flame. The preparation was stirred from time to time until the medium, on further heating, no longer froth over. The mixture was allowed to cool and just before it solidify was poured into sterile pet ri dishes. All were then sterilized by autoclaving at 121°C for 15minutes (Onaolapo, 1981).

The Tween 40 was prepared and supplied by the manufacturer with specific instructions on the use.

REFERENCE DRUG(ANTIBIOTICS):

Discs of the antibiotics(Polymixin Penecillin, Erythromycin, Oleandomycin, Spiromycin, Novobiocin, Vancomycin, Bactracin, Ampicillin, Tetramycin, Chloraphenicol, Neomycin and Streptomycin) were placed on the inoculated agar surface with sterile forceps and gently pressed down to ensure even contact. The antibiotics discs were evenly spaced and were not closer than 10 - 15mm to the edge of the plate and sufficiently separated from each other to avoid overlapping

zones of inh bition. At each application the pair of forceps were flammed and cooled before use.

The plates were incubated over night and the diameter of the inhibition zones(including diameter of the discs) were measured and recorded to the nearest millimeters. The reading were taken from the underside of the plate without removing the cover(Ondolapo, 1981)

CONTROL

For this screening three controls were set up; two negetive controls to show sterility of the medium and the antibiotic solution respectively; and one positive control to ascertain the growth promotion property of the medium; then incubated at 37°C for 18hour(Ondolapo, 1981).

Test Organism

Bacillus subtilis (NCTC 8236); Escherichia coli (NCTC 10418); Proteus vulgaris, Pseudomonas aeruginosa (NCTC 6750); Staphylococcus aureus (NCTC 6571); Streptococcus pyogenes (NCTC 8198) and Candida albicans (standard). These organisms were obtained as laboratory isolates from Department of Pharmaceutics and Pharmaceutical Microbiology . Ahmadu Bello University, Zaria - Nigeria.

2.10.1 PREPARATION OF EXTRACTS AND TEST ORGANISMS

2.19.1(a) Preparation of Extract

(i) Cold Water/Alcohol Extract

30g. of the powdered whole plant parts was extracted by using 200ml. of water. The extract was then evaporated to dryness over the water-bath until the residue was left. The resulting residue was dissolved in 200ml. of absolute alcohol and concentrated to dryness. The final residue was transferred into sterile bottles and preserved in the refrigerator for use in antimicrobial screening.

(ii) Hot Water Extract

30g. of the powdered whole plant was extracted with 200ml. of water. The extract was heated for 30 minutes over the water-bath, cooled and filtered. The resulting filtrate was evaporated to dryness and the residue packed into sterile bottles and preserved for antimicrobial screening.

(iii) Extraction of the Fixed Oils

30g. of the powdered inflorescence was extracted with 200ml. of water and evaporated to dryness leaving a residue. The resulting residue was extracted with 200ml. of medium range petroleum ether (60°80°) and evaporated leaving the fixed oil. The fixed oil was preserved in the refrigerator for use in antimicrobial screening.

(vi) Extraction of The Unrefined Secondary Metabolites (Constituents) of The herb.

(a) Extraction of the Crude Unrefined alkaloids of the plant:

Extraction procedure [see2.6.2(b)]

(b) Extraction of the Crude Unrefined Flavonoids of the plant:

Extraction procedure [see2.6.5]

(c) Extraction of the Crude Unrefined Saponins(steriodal Alkaloid glycosides of the plant:

Extraction procedure [see2.7.4]

(d) Extraction of the Crude Unrefined Steriodal glycosides of the plant:

Extraction procedure [see2.7.5]

(e) Extraction of the Crude Unrefined Tritepenes glycosides of the plant:

Extraction procedure [see2.7.6]

(f) Extraction of the Crude Unrefined Tanins of the plant:

Extraction procedure [see2.7.7]

2.10.1(b) PREPARATION OF TEST ORGANISM

The culture media were prepared according to the manufacturer's specification and sterilized by autoclaving. 20ml. each of nutrient agar were poured into sterile plates. 24 hour nutrient broth cultures of each of the organism were

prepared from agar slopes and diluted with sterile broth to give a 10,0 x 10^6 viable organisms count per ml; except E. coli and Proteus vulgaris (10,0x10^3 viable organisms per ml). The plate were inoculated and spread; even spreading produce a uniform growth; fresh sterile swab for each organism is recommended for use each time. Streptococcus pyogenes was grown on a special enrichment medium (blood agar).

2.10.2 TESTING FOR ANTIMICROBIAL PROPERTIES

(a) Using the Gutter Method (Gunn and Carter, 1965)

The gutter method is a crude and rough method of estimating the susceptibility of microbes to crude extracts. The gutter is a trench (1x0.5mm), dug into the nutrient agar in the petri dish (plate) with sterile knife. Perpendicular depressions were created by sterile inoculating loop from the base of the plate to a distance short of the small trench and test organisms were seeded into each depression. A measured amount of the plant extract were poured into the trench and allowed to diffuse. Two hours of pre-diffusion was allowed and the plates were inoculated at 37°C for 18 hours.

(b) Using Cup-Plate Method (Gunn and Carter, 1965)

Zone of inhibition method was used for testing the antimicrobial activities of the ordinary crude extract and the extracts of the plant constituents; as this method enables the

use of uniform quantity of extracts.

Four holes (ordinary crude extract) and 5 holes (for the extracts of plant constituents) of 9mm. diameter each were made in two petri plates with sterile cork borer No. 4, and each was filled aseptically with o.1ml. extracts using sterile micropipette. The plates were allowed pre-diffusion time of 2 hours and were then incubated at 37°C for 18 hours. The sterility of the crude aqueous extracts and extract of the plant constituents were determined by inoculating 5ml. of each in a sterile tube, incubated at 37°C for 18 hours.

2.10.3(a) Minimum Inhibitory Concentration (MIC)

The MIC of the plant active constituent on test organism were studied in liquid medium. A set of six test tubes were prepared, the first of which contain 2ml. of double strength nutrient broth while the others contain single strength broth.

2ml. active constituents in solution (10% w/v) was added to the first test tube and thoroughly mixed, after which 2ml. was withdrawn and transferred into the second test-tube. The same procedure was repeated for the other tubes until from the last test tube 2mls. was discarded.

1 drop of standardized organism (obtained by dilution from freshly grown stock culture so that 1ml will be expected to yield between 1 - 100 organism/ml) was added to each tube, thoroughly mixed and inoculated at 37°C for 18 hours. The MIC

for the different extracts of the crude unrefined active constituents, on the test organisms were observed by transferring a loop-full of the over-night contents in the test-tubes into sterile nutrient agar(s) which were then inoculated at 37°C for 24 hours, to check if the organisms have been inhibited. The MIC values were recorded which is the minimum or least concentration of the extract that inhibit the growth of the organism.(Gunn and Carter, 1965).

(b) Minimum Bacteriocidal Concentration (MBC)

The same procedure for MIC was repeated; but the plates were incubated at 37°C for 72 hours. The MBC; defined as the lowest concentration of the agent that showed no colony on subculturing (Waterworth, 1978).

2.10.4 RATE OF KILL

Two test organisms Bacillus subtilis (NCTC 8236), and Streptococcus pyogenes (NCTC 8198), susceptible to the crude unrefined active constituent (10% w/v alkaloids and 10% w/v Steroids) were used.

Nutrient agar was prepared with 3% Tween 40 were poured into culture plates and dried overnight in the incubator. Tween 40 has been found to be capable of inactivating the active constituents of the plant extract. 30ml. of the nutrient broth and a culture previously standardized were put

into sterile conical flasks. 10% (w/v) active constituent were transferred into conical flask in graded volumes of 0.1ml. (first conical flask); 0.25ml. (second conical flask); 0.5ml. (third conical flask); 1.0ml. (fourth conical flask); 1.50ml (fifth conical flask) and 0ml. (sixth conical flask) acting as control. All the flasks were placed in a shaker-bath set at 30°C.

Starting from time zero; o.1ml. was withdrawn from the reacting mixture in the conical flasks and transferred into 9.9ml. sterile nutrient broth which was then thoroughly mixed. 1.0ml. was withdrawn from the latter mixture and seeded into the already prepared nutrient agar containing the tween 40.

The same procedure was repeated every 15 minutes time interval up to the 60th minute for all six conical flasks in the shaker-bath. Plates were allowed to dry for 1 hour and then incubated at 37°C for 18 hours. The developed colonies were then counted.

CHAPTER THREE

3.0 RESULTS AND DISCUSSION

3.0.1 COLLECTION AND IDENTIFICATION OF THE PLANT MATERIAL

The plant sample collected at its natural habitat on farm lands around Area F, Main Campus, Ahmadu Bello University, Zaria Nigeria fitted to the morphological desription of M. Villosus (Rubiaceae)described in the Flora of West Tropical Africa(Hutchinson Dalziel and Hepper, 1963 and useful plants of West Tropical Africa(Dalziel, 1956).

3.0.2 PREPARATION OF HERBARIUM SPECIMEN

The identity of the plant was further confirmed by comparism of the prepared herbarium specimen with those available at the Department of biological Sciences, Ahmadu Bello University, Zaria, as Herbarium number(Specimen) 1074, Taxonomist at the time was S. Ohaeri.

3.0.3 DRYING AND STORAGE

The drying was carried out in the hot air oven and under shade at room temperature to prevent thermolabile constituents from degradation. Ideally fresh plant tissue should be used for analyses and in many instances dried samples were employed where fresh samples are not available or convenient.

3.2.0 MACROSCOPICAL EXAMINATION OF M. VILLOSUS ROOT, STEM, AND LEAVES

Macroscopic Examination of the Root

Origin	:	radicle
Colour	:	reddish-brown
Odour	:	Characteristic
Size	:	medium
Taste	:	bland

Macroscopic Examination of the Stem

Origin	:	Plumule
Colour	:	grey - green
Odour	:	Characteristic
taste	:	bland

Macroscopic Examination of the leaf

Lamina shape	:	ovate
Margin	:	entire
apex	:	obtuse
base	:	asymmetrical
colour	:	yellow
size	:	(4.8 x 1.3)cm
texture	:	smooth

3.2.1 MICROSCOPIC EXAMINATION OF ROOT, STEM AND LEAF

Microscopical Examination of the Root Powder

The macroscopical and microscopical features of the powdered root were recorded as they provide diagnostic characteristics for the identification of the plant.

The microscopy of the root revealed the presence of smooth polygonal parenchymatous cells with straight anticlinal walls. The sclerenchymatous elements have fibres and sclereids. The fibres are long and frequent with thick walls, acute apex, and wide lumen. The sclereids (stone cells) are small to medium range in size. The xylem elements are the tracheids and vessels. Tracheids have septates and are centrally located. The xylem vessels are medium sizes and numerous. They have uniform thickening and blunt at their apices. Various lignification (pitted, annular, reticulate and spiral) forms are found in the conducting elements. Cell inclusions are calcium oxalate, starch grains, oil droplets are present.

Calcium oxalate crystals, oil droplets and starch grains (small and oval in shape) are found scattered all over the powder (Fig. 3.10).

3.2.2 MICROSCOPIC EXAMINATION OF THE STEM POWDER

The powdered stem contains mature cells. Rectangular shaped cells are seen (transverse section) with polygonal straight anticlinal walls in surface view. These are the periderm and are punctuated by vessels. Certain structures arise from the surface of the periderm whose identity are unknown (fig. 3.11).

The sclerenchymatous elements are rich with fibres and sclereids of diverse lignification. Some fibres have tapering

apices while others are blunt. Stone cells (sclereids) are abundant. Xylem trachieds and xylem vessels with (reticulate, spiral and pitted lignification) are numerous. Cell inclusion (calcium oxalate, oil droplet and starch grains) are few.

Microscopic Examination of the leaf Powder

Cell: epidermal

Shape: polygonal and elongated

Anticlinal wall: wavy

Thickening: cellulose

Stomata: Paracytic

Frequency: numerous (lower epidermis)

none - very few (upper epidermis)

Location of stomata = lower epidemis (major)

Cell inclusion = prisms of calcium oxalate crystals; oil globules, starch grains etc.

Xylem vessel

Xylem Element	Vessel	Trachied
Type	Present	Present
Frequency	Frequent	Frequent
Wall	Lignified	Lignified
Thickening	All forms of	Thickening are present
Size	Fragmented	Fragmented

Fibres

Size - fragmented

Frequency - Not frequent

Wall - thickened and lignified

Apex - acute/tapering

Lumen - Wide/narrow

Calcium Oxalate

Form - prism

Location - parenchyma cells

Frequency - Frequent

Arrangement - cluster, prism

3.2.3 MICROSCOPICAL EXAMINATION OF THE LEAF OF M. VILLOSUS

Microscopic examination of the leaf showed the presence of paracytic stomata, numerous prisms and clusters of calcium oxalate crystals of variable sizes with wavy elongated epidermal cells (fig. 3.13 and 3.16). Phloem fibres are numerous. Pitted, reticulate, angular and spiral vessels are abundant; trachea, tracheids and sclereids are present. Oil globules, secretory cells, tannins masses and brown patches of anthraquinone (in fresh section) have been observed (fig. 3.12 and 3.14).

Transverse section (fig. 3.15) through the leaf showed clear differentiation into cuticularised lower and upper epidermis(es). Within the thick layer of parenchyma are prisms of calcium oxalate crystals. Stomata openings are at their

widest indicating at what time of the day the leaves were collected. Palisade cells are observed at the upper epidermis. There are no hydrathods and trichomes are conspicuously absent. Xylem and phloem vessels are collateral and are arranged in rows. Xylem elements are lignified.

Features observed in the leaf are indicative of its morphological group. Special features include presence of oil globules (spongy mesophyll), anthraquinone patches, tannins masses and prisms of calcium oxalate crystals. The cytomorphology are obviously those of dicotyledonous leaf.

3.3.0 CHEMOMICROSCOPICAL EXAMINATION OF ROOT, STEM, LEAVES AND INFLORESCENCE

(i) Anthraquinone test

Brown colour was observed in the anatomical section of the leaves indicating the presence of anthraquinone.
The fresh leaf mount, in 5% potassium hydroxide; showed red coloration, indicative of the presence of anthraquinone .

The 5% aqueous ammonia solution showed intense red coloration, indicative of the presence of anthraquinone in the leaf sample of M. villosus.

(ii) Calcium Carbonate test

The test was conducted; but the result was negative indicating the absence of calcium carbonate.

(iii) Calcium Oxalate test

Cluster of calcium oxalate crystals are observed as shining structures in the free state and in the parenchyma cells of the powder and the anatomic sections of the root, stem, leaves, and inflorescence.

The crystals in the samples were slowly dissolved on addition of 20% (v/v) of sulphuric acid.

(iv) Cellulose test

The result was positive (blue colouration with chlor-zinc-iodine); indicating the presence of cellulose in the root, stem, leaves and inflorescence samples.

(v) Fixed oils and fats test

Distinct red colour was observed in both the anatomical section and the powdered plant parts, (root, stem, leaves and inflorescence). Hence the presence of oil and fats were indicated.

(vi) Lignin test

The root showed little lignification; but the tissues of the stem, leaves and inflorescence gave red colour with phloroglucinol and concentrated hydrochloric acid indicating lignin.

(vii) Mucilage test

Mucilage is absent. Samples do not respond to neither the ruthenium-red nor the haemotoxylin tests.

(viii) Starch test

Blue-black coloration was observed in the sample of the anatomic section of the root, stem, leaves and inflorescence; indicating the presence of starch.

(ix) Proteins test

Million's reagent gave red-colour with the leaf and inflorescence samples. Samples of the root and stem gave negative result indicating that proteins are absent in the root and stem; but present in the leaves and inflorescence of the plant.

(x) Suberin test

Sudan IV (in alcohol) test positive for the stem sample only. Suberin is absent from the root, leaf and inflorescence (anatomical sections). Suberin is present in the stem and the characteristic red colour indicates their presence.

(xi) Tannins tests

An intense blue-black colour turning to green colour was observed in both anatomical and powdered samples of the plant parts (root, stem, leaves and inflorescence).

The result obtained from chemomicroscopical examination are tabulated in table 2.

3.4.0 PHYTOCHEMICAL SCREENING OF THE ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS

In this work the root, stem, leaves and inflorescence of M. villosus have been subjected to various qualitative chemical tests and the results are also discussed below. A summary of the phytochemical constituents detected in the plant parts of M. villosus is presented in Table 3.

3.4.1 Anthraquinone test

The unhydrolysed first mixture of the powdered leaf extract showed no pink or cherry-red coloration, indicating the absence of free anthraquinone. The hydrolysed second mixture of the powdered leaves showed the characteristic intense pink colouration indicating the presence of bounded anthraquinone. The result of the tests indicated the presence of combined (bounded) anthraquinone in the leaf of M. villosus. This confirmed what had been earlier reported in the chemomicroscopic study.

3.4.2 Alkaloids test

Various tests for alkaloids were carried out using the following standard alkaloidal reagents;

Dragendorff's reagent (Potassium bismuth iodide solution)

Orange and orange-yellow precipitate were observed on addition of a few drops of Dragendorff's reagent to extracts of the root, stem, leaves and inflorescence of M. villosus

Mayer reagent (Potassium Mercuric iodide solution)

Buff (white to creamy white) precipitates were observed on addition of Mayer's reagent to the methanolic extracts of the root, stem, leaves and inflorescence.

Hagner's reagent (a saturated solution of picric acid)

Positive test (yellow colour) was observed on addition of a few drops of Hagner's reagent to the ethanolic extracts residue of the root, stem, leaves and inflorescence.

10% Tannic acid

Tannic acid produce buff coloured precipitate when added in drops to the ethanolic extracts residue of the root, stem, leaves and inflorescence.

Wagner's reagent (solution of iodine in Potassium iodide)

Light brown and brownish precipitate were observed with extracts of the root, stem, leaves and inflorescence.
The presence of alkaloids in the entire plant had been reported by several authorities (see introduction).

3.4.3 Carbohydrate test

Most carbohydrates including sugars are wholly or partially soluble in cold or hot water. Therefore, water was used as the solvent for extraction, consequently the water extract of root, stem, leaves and inflorescence were used to carry out the tests for the presence of various classes of

carbohydrates; monosaccharide, reducing sugars, pentoses and ketoses. Sugars are normally contained in various glycosides and tannins and their presence in flavonoids, triterpenes (El-Kheir and Salih, 1980) and saponins has been reported.

Carbohydrate General test

(a) Molisch test

The aqueous extract of the root, stem, leaves and inflorescence treated with Molisch's reagent gave a positive test for carbohydrates. A reddish ring at the common surface of the liquid interface was observed. This is the general test for all classes of carbohydrate which are normally present in all leaves.

(b) Sugar test Barfoed's test for monosaccharids

The red precipitate formed on warming the aqueous extract of the root, stem, leaves and inflorescence with 4.0ml. of Barfoed's reagent. This indicate the presence of monosaccharide. Monosaccharide are found as free sugars in higher plants and are the components of glycosides and polysaccharides (Harborne, 1973). Monosaccharide are absent in the plant inflorescence.

Fehlin's test

(i) Standard test for free Reducing sugars

A brick-red precipitate was observed on boiling 2ml. of each of the extracts with 5ml. each of the mixture of

fehling's A and B, indicating the presence of free reducing sugars in the root, stem but not in the leaves and inflorescence.

(ii) Standard test for Combined Reducing Sugar

A brick-red precipitate was observed on warming the hydrolysed extract with Feling's solution A and B. This indicates the presence of reducing sugars in combined form. Combined sugars are present in all the plant parts.

(b) Standard test for Ketoses

(Selivanorff's Or Resorcinol's) test

All the plant parts (root, stem, leaves and inflorescence) are resorcinol positive. A rose colour was slowly formed on heating 2ml. of each of the extracts over the waterbath with a few crystals of resorcinol and an equal volume of concentrated hydrochloric acid. The test is positive for glucose. Glucose and fructose form osazone. An immediate formation of rose colour indicate fructose while glucose forms the osazone slowly, (on standing for some minutes) (Brain and Turner, 1975). The ketose sugar present in the root, stem, leaves and inflorescence of M. villosus may be glucose as osazone formation was slow.

(c) Standard test for Pentose

The root, stem, leaves and inflorescence are pentose positive, and probably in the free form. The characteristics

rose colour on treating the water extracts of the samples with small volumes of hydrochloric acid and phloroglucinol on heating indicated the presence of pentose e.g. xyloses (Trease and Evans, 1983). The results have indicated the presence of reducing sugars in the free and combined state in the water extracts of the root, stem, leaves and inflorescence. On further investigation the sugar present is the ketoses (e.g. glucose) and the pentoses (e.g. xyloses). The reducing sugars in the combined form could be associated with flavonoids, tannins and saponins in glycosidic linkages (formation). These classes of compounds have been found in the plant (El-Kheir and Salih, 1980).

3.4.4 FLAVONOIDS TEST

Various tests were carried out for the identification of flavonoids in the root, stem, leaves and inflorescence of Mitracarpus villosus (SW) DC.

(i) Lead acetate test

Buff coloured precipitate was observed on addition of three drops of lead acetate solution to each of four test tubes containing detanned water extract of samples of the root, stem, leaves and inflorescence respectively indicating the presence of flavonoids.

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(ii) Ferric chloride test

The presence of OH group in the phenolic nucleus was indicated in the detanned extract of the root, stem, leaves and inflorescence; green colouration on addition of a few drops of 5% ferric chloride solution was observed.

(iii) Sodium hydroxide test

Yellow solution (positive test) was observed on performing the sodium hydroxide test on the detanned water extract of the samples of the root, stem, leaves and inflorescence. The solution became colourless on addition of dilute hydrochloric acid, indicating the presence of flavonoids.

(iv) Shinoda test

The test was applied as a confirmatory test for flanonoids. Red colours were observed in the test tubes containing extracts of root, stem, leaves and inflorescence of M. villosus. This confirmed the presence of flavonoids in the plant.

3.4.5 GLYCOSIDES TEST

1. Cardiac Glycosides

Legal test

A murky precipatate was observed when the extract of the herb was dissolved in pyridine in a few drops of 2% sodium

nitroprusside and 20% NaoH; indicating the absence of cardenolides.

Kedde test

Methanolic extract of the herb mixed with 2% solution of 3,5-dinitrobenzoic acid in methanol and 5.7% sodium hydroxide(aqueous) appeared green. This indicate a conspicuous absence of a lactone ring normally associated with cardenolides.

Liebermann's test

Acetic anhydride react with the extract(herb) and allow to cool in ice. Positive colour reaction was obtained when concentrated sulphuric acid waas carefully added, indicating the presence of steroidal nucleus.

Salkowski test

The extract of the herb when dissolved in chloroform; and sulphuric acid was added carefully, a reddish-brown colour at the interface was observed, indicating the presence of steroidal ring.

Keller-Kiliani

The extract dissolved in glacial acetic acid in one drop of ferric chloride solution and on careful addition of concentrated sulphuric acid formed a green ring at the

interface; indicating the absence of a desoxy sugar characteristic of cardenolides.

The extract contain steroidal ring but the extract is not a cardiac glycoside.

As there are no common tests for this group of compounds, selective tests were carried out to identify the type of glycosides present in the root, stem, leaves and inflorescence of the plant. These tests are as follows:

2. Cyanogenetic Glycosides test

The moist yellow sodium picrate paper did not change when suspended in a test tube containing the root, stem, leaves and inflorescence extracts and warmed over waterbath, for 1 hours; thus indicating the absence of cyanogenetic glycoside in this plant.

3. Saponins test

(i) Frothing test

A honey - comb froth which lasted several days were observed in the aqueous extract of the root, stem, leaves and inflorescence. Persistence of frothing indicated the presence of saponins in all the samples.

(ii) Haemolytic (Steroidal and triterpenoids) test

Haemolysis occurred in the test tube containing extracts of root stem, leaves and inflorescence of M. villosus and none

in the control. This indicated the presence of saponins.

(iii) Sterol and Steroidal Ring test

(a) Salkowski's test (Steroidal ring)

A reddish - brown precipitate at the interface was observed indicating the presence of steroidal ring.

(b) Liebermann - Burchard's test (terpenes)

A reddish violet colour at the interface of the two liquids and a light green colour in the chloroform layer were observed. This indicated the presence of tri terpenes.
The frothing, haemolytic and chemical test have confirmed the presence of steroidal and triterpenoids saponins in the root, stem, leaves and the inflorescence of M. villosus.

TANNINS TEST

The root, stem, leaves and inflorescence of M. villosus responded to the different tannins test as follows:

(i) Ferric chloride test

Tannins in the leaves and inflorescence samples gave blue or green coloured precipitate with 5% ferric chloride solution. Extracts of root and stem, do not show this colour reaction.

The test indicated the presence of condensed, hydrolysable or both in the leaves and inflorescence and their absence in the root and stem.

(ii) Lead subacetate test

The water extract of the root, stem, leaves and inflorescence gave yellow buff precipitate with lead subacetate reagent. This indicated the presence of tannins. Taninns are precipitated by heavy metals.

(iii) Bromine Water

Buff coloured precipitate was observed with water extract of leaves and inflorescence. This indicated the presence of condensed tannins. Hydrolysable tannins do not give this colour reaction (Balbaa, et al, 1976).

(iv) Ferric ammonium citrate test

Violet coloured precipitate is formed when aqueous extract containing tannins are treated with a few drops of ferric ammonium citrate solution (Trease and Evans, 1983). The water extract of the samples of the root, stem, leaves and inflorescence gave violet precipitates (positive result) with ferric ammonium citrate solution indicating the presence of tannins.

(v) Test for Phloroglucinol Nucleus

An alcoholic extract containing phloroglucinol nucleus when treated with a drop of concentrated hydrochloric acid develop crimson colour. Phloroglucinol occur frequently with condensed tannins (Balbaa, et al 1976). The alcoholic extracts

of the leave and inflorescence produced crimson coloured solution indicating the presence of the phloroglucinol nucleus. The root and stem samples did not, indicating, in them, the absence of phloroglucinol nucleus.

(vi) Gelatin test

One of the best known test for tannins is their precipitation with gelatin. Only true tannins precipitate gelatin (Trevor, 1976).

0.5% solution of water extract of the root, stem, leaves, inflorescence added to an equal volume of 0.5% gelatin produce a dirty white precipitate (leaves and inflorescence samples only).

The precipitating quality of tannins is used in the laboratory to detect gelatin, alkaloids and proteins. Therapeutically, tannins have antiseptic properties (Tyler et al, 1981). Also tannins are used as antecdote for alkaloid poisoning.

3.5.0 CHROMATOGRAPHIC STUDIES OF EXTRACTS OF MITRACARPUS VILLOSUS

3.5.1 CHROMATOGRAPHIC STUDIES OF CRUDE ALKALOIDS IN M. VILLOSUS

Paper chromatographic studies of the aqueous extracts of the root, stem, leaves and inflorescence, using solvent system; n-butanol; acetic acid: Water (4:1:5)(Lower layer) were carried out; the spray reagent was strong ammonia vapour.

The aqueous extract of the plant parts revealed one spot each with (hRf = 95). Under day light, colour was brown-yellowish. The spots with (hRf=95) compared well with an alkaloid in terms of mobility and colour reaction.
After developing with ammonia it was again viewed under UV, and it did not florescence at 366nm. Paper chromatography revealed only one spot which corresponded to an unknown alkaloid in terms of spot mobility and colour reaction (Table 4a).

Thin layer chromatography of the aqueous extracts of the entire plant and parts of the plant were studied. Using solvent system; chloroform; methanol (4:1); the spray reagent; Dragendorff's; two spots were obtained in each extract. The yellow spots (hRf=77) which compared well with the same alkaloid (on paper chromatography) and the green spots (hRf=39) compared favourably well with an unknown alkaloid.

Replacement of the solvent system with chloroform: methanol: ammonia (10:20:1); spray reagent; Marquis's the aqueous extract of root, stem, leaves and inflorescence revealed a single pink spot each (hRf=76) which corresponded with the same alkaloid (unknown fraction above) in terms of spot mobility and colour reactions.

Thin layer chromatography result herein revealed the presence of two alkaloids; both fraction are unknown.

3.5.2 CHROMATOGRAPHIC STUDIES OF THE FIXED OILS OF THE ROOT, STEM, LEAVES AND INFLORESCENCE OF M.VILLOSUS

A two dimensional thin layer chromatography of the aqueous extracts of the plant root, stem, leaves and inflorescence was carried out using silica gel-G(adsorbent). The solvent system; chloroform: methanol: acetic acid: Water (170:25:25:4); spray reagent; 50% H_2SO_4 and the second dimension solvent system was chloroform: methanol: 7M ammonia hydroxide solution (65:30:4). Two spots were obtained (hRf-91) brown and (hRf=98) yellow-orange; these spots compared with oleic acid (hRf=95). The brown spots (hRf=91) are fatty acids whose identity is yet unknown.

The result of the thin layer chromatography by two dimension revealed the presence of oleic acid on one hand and unidentified fatty fraction on the other hand (Table 5). The finding that all the plant parts (root, stem, leaves and inflorescence) contains fixed oils agree with preliminary phytochemical screening.

3.5.3 CHROMATOGRAPHIC STUDIES OF FLAVONOIDS EXTRACTS IN THE ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS

Two dimensional paper chromatography and paper chromatographic studies of the aqueous extract of the root, stem, leaves and inflorescence reveal two spots brown and yellow which fluoresced. The solvent system: (n-butanol: acetic acid: water) (4:1:5) and the second dimension solvent

system is 5% acetic acid, spray reagent; 5% alcoholic ferric chloride solution. The two chromatograms each with (hRf=84) brown and (hRf=82) yellow were noted. The spot with (hRf=84), green fluorescence compared with 3,4- dihydroxflavonol in terms of spot mobility and colour reaction. The second spot (hRf=82), brown fluorescence compared with Kaempferol (Table 6a). From this result paper chromatography reveal the presence of 3,4-Dihydroxflavonol and kaempferol (Macek, 1972; Harborne, 1973) in the plant M. villosus.

Thin layer chromatography of the aqueous extract of the root, stem, leaves and inflorescence of the plant were carried out (Solvent system; Benzene; Pyridine; formic acid, (4:1:1); spray reagent; alcoholic ferric chloride solution). Thin layer chromatography of the aqueous extract of the parts revealed two spots with (hRf=39) and (hRf=97). The (hRf=39) compared with kaempferol (hRf=41) (Macek, 1972) and (hRf=97) could probably, well correspond with 3,4-Dihydroxflavonol (Table 6b).

Chromatographic analyses of the plant revealed the presence of 3,4-dihydroxflavonol and kaempferol which are flavonoids. Preliminary phytochemical screening has earlier revealed the presence of flavonoids.

3.5.4 CHROMATOGRAPHIC STUDIES OF SAPONIN GLYCOSIDES OF EXTRACTS OF THE ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS

3.5.4 STEROIDAL ALKALOIDS GLYCOSIDES CHROMATOGRAPHY

Paper and thin layer chromatographic studies of root, stem, leaves and inflorescence of M. villosus was carried out (Solvent system; Ethylacetate: Pyridine: Water, (3:1:3); spray reagent: Carr-Price.

Paper chromatography of the aqueous extract of the same plant parts revealed two spots each with (hRf=88) and (hRf=16), Table 7a. The spot with (hRf=88) compared with steroidal alkaloid glycoside while (hRf=16) corresponded with no given standard works of reference.

Thin layer chromatography of the same aqueous extract revealed two spots each (hRf=20) and (hRf=16) Table 7b. One of the spots (hRf=20) compare with steroidal alkaloid glycoside while the other (hRf=16) is yet to be identified.

The chromatographic analyses suspected the presence of solasonin in terms of spot mobility and colour reaction. From paper and thin layer chromatography of the samples two spots which were consistent in the root, stem, leaves and inflorescence (hRf=16 and hRf=16) do not correspond with any standard works of reference.

3.5.5 SPIROSTANOL (STEROIDAL) GLYCOSIDES CHROMATOGRAPHY

Paper chromatography of the aqueous ethanolic extract of the root, stem, leaves and inflorescence revealed the presence of buff spot that fluorescence in (solvent system: n-butanol; Benzene: Water), (10:4:5); spray reagent; Carr-Price: with spots (hRf=39) which compared with dioscin in terms of spot mobility and colour reaction (Table 8a). The same aqueous ethanolic extract under the same chromatographic conditions revealed another spot (hRf=95); this does not compare with any standard works of reference; therefore, the constituent is unknown.

Thin layer chromatography of the aqueous ethanolic extract of the root, stem, leaves and inflorescence in (solvent system. Chloroform: Methanol: Water), (65:35:10); spray reagent: Carr Price, revealed two spots each with (hRf=60) and (hRf=99). The spot (hRf=60) compared with dioscin (hRf=61) whilst (hRf=99) is unknown.

Hence paper chromatography and thin layer chromatography reveals the presence of dioscin and an unknown fraction which were consistent (hRf=99) and (hRf=95) in the root, stem, leaves and inflorescence and did not compare with any of the standard works of reference (Macek, 1972, Harborne, 1973).

3.5.6 TRITERPENES GLYCOSIDES CHROMATOGRAPHY

Paper chromatography of the aqueous methanolic extracts of the root, stem, leaves and inflorescence were carried out

using the solvent system: n-butanol; glacial acetic acid; water (4:1:5); spray reagent. Carr-Price; two spots each (hRf=63) and (hRf=57) were revealed. The (hRf=63) compared with araloside - B(hRf=60) (Macek, 1972) while the spot with (hRf=57) is an unknown fraction of triterpene glycoside (Table 9a).

The thin layer chromatography of the aqueous methanolic extract of the same plant parts were studied: Solvent system; chloroform: methanol: water (65:35:10); spray reagent; Carr-Price. Thin layer chromatography revealed two spots (hRf=96) and (hRf=97) each. However, both are consistent in all the samples; but did not correspond with any standard work of reference. Perhaps, (hRf=97) may well compare with aralosides-B on TLC. (Table 9b).

The result of chromatographic studies (analyses) of the saponin glycosides have revealed the presence of solasonin, (steroidal alkaloid glycoside) dioscin and araloside-B in each of the samples. This is consistent with earlier findings by phytochemical studies of the presence of steroidal alkaloids, spirostanol and triterpenes glycosides which have been mentioned above with specific names.

3.5.7 CHROMATOGRAPHY STUDIES OF TANNINS IN THE ROOT, STEM, LEAVE AND INFLORESCENCE

Paper and thin layer Chromatography studies of the root, stem, leaves and inflorescence were performed. Using solvent system; n-butanol; glacial acetic acid: Water (4:1:5); spray

reagent; 5% alcoholic ferric chloride solution.

Paper chromatography of the aqueous extracts of the root, stem, leaves and inflorescence revealed three spots each with (hRf=75), (hRf=80), (hRf=98) and (hRf=74). The spots of the root, stem, leaves and inflorescence with (hRf=74), (hRf=80) and (hRf=98) (Table 10). These spots compared with phloroglucinol (hRf=72); catechol (hRf=80) and tannic acid (hRf=95) in terms of spot colour and mobility (Table 10a).

Thin layer chromatography of the aqueous alcoholic extracts of the root, stem, leaves and inflorescence revealed three spots each with (hRf=74), (hRf=80), (hRf=75) and (hRf=95) respectively (Table 10). (hRf=74), (hRf=80) and (hRf=95) correspond with catechol, phloroglucinol and tannic acid respectively in terms of spot mobility and colour reaction. While (hRf=75) did not compare with any standard work of reference.

Chromatographic analyses of the root, stem, leaves and inflorescence have confirmed the presence of tannins (catechol, phloroglucinol, and tannic acid) as some of the tannins present in the plant. Phytochemical tests had earlier revealed the presence of some of these compounds of tannins in the leaves and inflorescence. The gelatin test had been negative for the root and stem, yet positive for the other chemical tests, indicating the presence of pseudotannins in the plant root and stem.

3.6.0 QUANTITATIVE EVALUATION OF POWDERED LEAVES AND INFLORESCENCE

The evaluation of a drug requires that it be identified in both its entire and powdered forms by means of its macro morphological and cytomorphological characters. Whilst these diagnostic features enable the nature and origin of the material to be stated, it could well be that faulty collection or possibly incorrect or extended storage have altered the content of the constituent of the drug to a point where it is no longer acceptable.

Drugs must be subjected to evaluation procedures which will indicate that their acceptability by criteria other than their morphology (Brain and Turner, 1975).

The following evaluation parameters were determine on the leaves and inflorescence of M. villosus

All the readings represent an average of three determinations.

3.6.1 DETERMINATION OF TOTAL ALKALOID CONTENTS

The total amount of alkaloid found in a plant up to a given level would determine the commercial importance of that plant.

The total alkaloid content of the leaf and inflorescence in M. villosus was 12.45% w/v and 1.753% w/v respectively (Appendix A).

3.6.2 DETERMINATION OF MOISTURE CONTENT

The moisture content of the air dried leaves and inflorescence were determined to be 16% and 13.3% respectively (Table 11) and this appears to be within acceptable limits when compared with monograph entries of (BP, 1968).

3.6.3 DETERMINATION OF SOME PHYSICOCHEMICAL VALUES

(i) Determination of total Ash Value

The result shows that the leaves and inflorescence have total ash values of 12.7% and 14.3% respectively (Table 11) and Appendix A.

(ii) Acid-Insoluble Ash

The result obtained for the acid-insoluble ash values for M. villosus leaves and inflorescence are 4.7% and 3.3% respectively (Table 11). Comparing the total ash from the same parts of the plant (12.7% and 14.3%) respectively against (4.7% and 3.3%) acid -in soluble; the difference appear normal and compare well with results in any standard works of reference.

3.6.4 DETERMINATION OF EXTRACTIVE VALUES

(i) Water Soluble Extractives

The leaves and inflorescence of M. villosus water soluble extractive values are 13.6% and 12.8% respectively (Table 11), indicating that the water soluble extractive value of the

leaves is higher than the inflorescence. Furthermore, the results have shown that the (cold water/alcohol) and hot water extracts gave higher percentage of water soluble substances in both the leaves and the inflorescence.

(ii) Alcohol Soluble Extractive

The alcohol soluble extractive are higher in the leaves than the inflorescence. The alcohol and water soluble extractive signify the presence of fairly large amounts of constituents that are leached into water and alcohol respectively. Therefore, the use of water as a medium and vehicle in the traditional preparation for the treatment of ailments and skin diseases in Northern Nigeria is valid. Leven et al, (1979), have reported that certain plant constituents like flavonoids, steroids and tannins have antimicrobial activity against some organisms which are the common causative agents of wound infections, gastrointestinal disorders and skin diseases (Cruickshank, et al, 1973). These active constituents are present in therapeutic quantities in all the parts of M. villosus plant.

However, the alcohol soluble extractive for the leaves and inflorescence are 7.6% and 4.0% respectively (Table 11) Alcohol soluble materials within the plant parts are small compared to the water soluble constituents.

Quantitative evaluation of M. villosus are computed in appendix A.

3.7.0 QUANTITATIVE MICROSCOPY OF THE LEAF OF M. VILLOSUS

It is very often impossible to distinguish between the leaves of closely related plants when in small pieces by means of an examination of their morphological features. This may happen to pairs of leaves which are closely related.

Quantitative microscopy aids to identify and thus serves as a means of distinguishing between such leaves by the establishment of certain numerical standards based upon various anatomical features of the leaves. The numerical standards include (a) stomata number (b) stomata index (c) palisade ratio (d) Vein islet number (e) Vein termination number.

The leaves of the plant was examined and numerical criteria for the authentication of the plant were determined and recorded (Table 12).

The numerical standards are useful in determining purity and botanical source of the powdered drugs. The palisade ratio, Vein islet number and Vein termination number are fairly uniform and more reliable for identification and detection of adulteration than the stomata index and the stomata number.

For efficiency and identification, determination of purity, sources, and detection of adulteration; all the anatomical diagnostic characters, with the various physicochemical constants and finally the numerical values should be applied together especially in terms of powdered drugs (Dasniyam, 1991).

3.8.0 PRELIMINARY ANTIMICROBIAL STUDIES OF M. VILLOSUS ENTIRE PLANT.

3.8.1 Gutter Method

An organism is considered susceptible to the effect of an extract, if a zone of inhibition is observed. Only the (cold water/alcohol) and the hot water extracts of the entire plant are active. They showed absence of microbial growth. But the other extracts (alcohol, Petroleum ether and fixed oils) showed dense microbial growths (isolates are not sensitive).

The streaked depression of B. subtilis and Strept. pyogenes showed inhibition zones when (cold water/alcohol) and hot water extracts were housed in their gutters. Though, the extracts inhibited proliferation the difference was not distinguishable remarkable, probably, because of the presence of swarming and motile organisms (Proteus and E. coli), seeded in the same petri-dishes.

Both Gram positive and Gram negative organisms are sensitive (their zones of inhibition quite, negligible); but the most susceptible are B. subtilis and Strept. pyogenes

The disadvantage of this method is that swarming organisms impair with the result; also that the result cannot be measured quantitatively.

A clear and universal susceptibility testing is the CUP PLATE METHOD.

3.8.2 SUSCEPTIBILITY TEST (CRUDE UNREFINED EXTRACT) DETERMINATION OF ZONE OF INHIBITION

Cup Plate Method

Results were recorded in millimeter as diameter of zone of inhibition.

There was growth in the fixed oils, an extract from the plant inflorescence, indicating that the fixed oils showed no antimicrobial activity.

The hot water extract of the entire plant showed greater activity compared with the (cold water/alcohol) extract. This may indicate that the hot water extracted more of the antibacterial principles compared to the (cold water/alcohol) solvent.

The fact that B. subtilis is the most susceptible among the two (Table 13), and because it is a Gram positive organism, might be due to the fact that the plant metabolites act directly on the cell wall envelop by lysing the components of the cell wall or attacking the 30s or 50s ribosomes. Streptococcus pyogenes is yet another Gram positive organism susceptible to the extracts; the drug mechanism of action compared with the above.

Susceptibility of the organism (B. subtilis and Strept. pyogenes) to the two extracts is consistent with the finding obtained by the Gutter method.

3.8.3 SUSCEPTIBILITY TEST OF CRUDE UNREFINED EXTRACTS OF ACTIVE PRINCIPLES OF M. VILLOSUS. DETERMINATION OF ZONE OF INHIBITION

Cup Plate Method

The compounds present in the plant were extracted using different methods (Balbaa, 1975; Brain and Turner, 1975; Harborne, 1973; Macek, 1972; Trease and Evans, 1983; Trevor, 1975; and Tyler et al, 1981) and their activities tested separately on the test organisms. The result showed that tannins have greater effect, followed by steroids, triterpenes, flavonoids, alkaloids and steroidal alkaloid glycosides respectively; but the fixed oils did not show any activity (Table 14). This result also showed that all the compounds have greater effect on B. subtilis and least effect on Pseudomonas aeruginosa and Staphylococcus aureus which is in accordance with previous test carried out with the crude extract.

M. villosus which has been used in traditional medicine for the treatment of most forms of skin diseases (especially eczema), gonorrhea, pruritus, pyle and rheumatism have proved to have inhibitory activity on test organism in the laboratory which were selected based on the fact that they are the most common causative agents of skin disorder (Cruickshank et al, 1973), also boils on the skin are commonly associated with Staphylococcus aureus infection (Obaseiki-Ebor et al, 1990). The crude extract of the plant constituents showed activity

against Gram negative organisms (Pseudomonas aeruginosa and Proteus vulgaris) as well as Gram positives (Table 14). Furthermore, the large size of zones of inhibition shown by the extracts on test organisms indicated its potency in the treatment of the above mentioned infections.

3.8.4 CRUDE UNREFINED EXTRACT OF CONSTITUENTS MINIMUM INHIBITORY CONCENTRATION (MIC)/MINIMUM BACTERIOCIDAL CONCENTRATION (MBC).

The MIC is a helpful parameter used to assess bacteriostatic activity of a particular antimicrobial agent, while MBC is used to detect the bacteriocidal activity under similar condition.

Antimicrobial agents are required to act in a wide variety of environments, each of which may modify the intensity of response of a species to a particular agent. It is therefore important that the determination of antibacterial activity of an antibacterial agent should be conducted under use condition. Extrapolation of data from different organisms or from different condition without first checking the validity of this lead to considerable errors. This as far back as 1947, Salle and Gatlin recommended that test should be performed to determine the lowest concentration of an antiseptic necessary to kill the most resistant organism found on the skin and the extent of bacteriostasis and the influence of organic matter on the activity.

The MIC which is directly related to the activity of the extract supported the result obtained from the agar diffusion (cup plate) method of the extracts on the test organisms. The organisms were found to be inhibited at concentrations, as low as 31.3μg/ml); but most of the organisms were inhibited at concentrations 125μg/ml and 250μg/ml) (Table 15). But for Pseudomonas aeruginosa, Staphylococcus aureus and Proteus vulgaris, the MIC was greater than the highest concentration used.

The MBC was found to be equal to the MIC in two cases, of the unrefine crude extracts of alkaloids and the triterpenes on Streptococcus pyogenes and Candida albicans respectively. The other exceptions are of the unrefine crude extracts of steroids and tannins 62.5 (250); - (-); -(-); 150 (1000); 500 (-); and 62.5 (250); 125(100); >1000(-); 125 (250); 250 (1000); 650 (250) and 250 (1000) respectively (Table 17). This is in line with what was found by Ashley (1983), who studied the antibacterial properties of two mouth washes oraldene (hexitidine) and corsodly (chlorhexidine) and found that the MIC for these agent against the test organisms were either same or slightly lower than the minimum bacteriocidal concentration (MBC).

The MIC and MBC which have been employed to evaluate the efficacy of the agents, such as antiseptic, disinfectants and indeed chemotherapeutical agents (Waterworth, 1978; Croshaw, 1983) under standard conditions also support the result of the

susceptibility test.

3.8.5 RATE OF KILL

The bacteriocidal activity of an antibacterial agent may be assessed by performing viable cell counts at varying time intervals on the surviving members of a bacterial population in contact with a solution of the agent. Also the degree of inhibition of the bacterial growth in the presence of antibacterial agent had been found to depend on the rate of growth of the growing culture (Anderson and Richard, 1981; Acheampong, 1985).

In this study the rate of kill of B. subtilis and Strept. puogenes by the unrefine crude extracts of alkaloids and steroids 1000μg/ml of the entire plant in different concentrations were observed. It was observed that the number of viable organisms decreases with concentration and time (Tables 18a, b, c, d) which is in line with logarithmic order of death (Esselen and Pflug, 1956) that is, when bacterial population is subjected to adverse influence of an antibacterial agent, the number of cells decreases, gradually in such a manner that the logarithm number of surviving cells at any time when plotted against time falls on a descending straight line.

The results have shown that steroids have more effect on the organism, showed more reduction in viable counts at different times and concentrations than the alkaloids from the

same plant. These results are in line with that of the susceptibility test. Both organisms are Gram positive and the activity of the crude extract (of the secondary metabolites) on them are comparable. The result was consistent with the result of the control where viable count increase with time (Fig. 3.17 and 3.18, page 215).

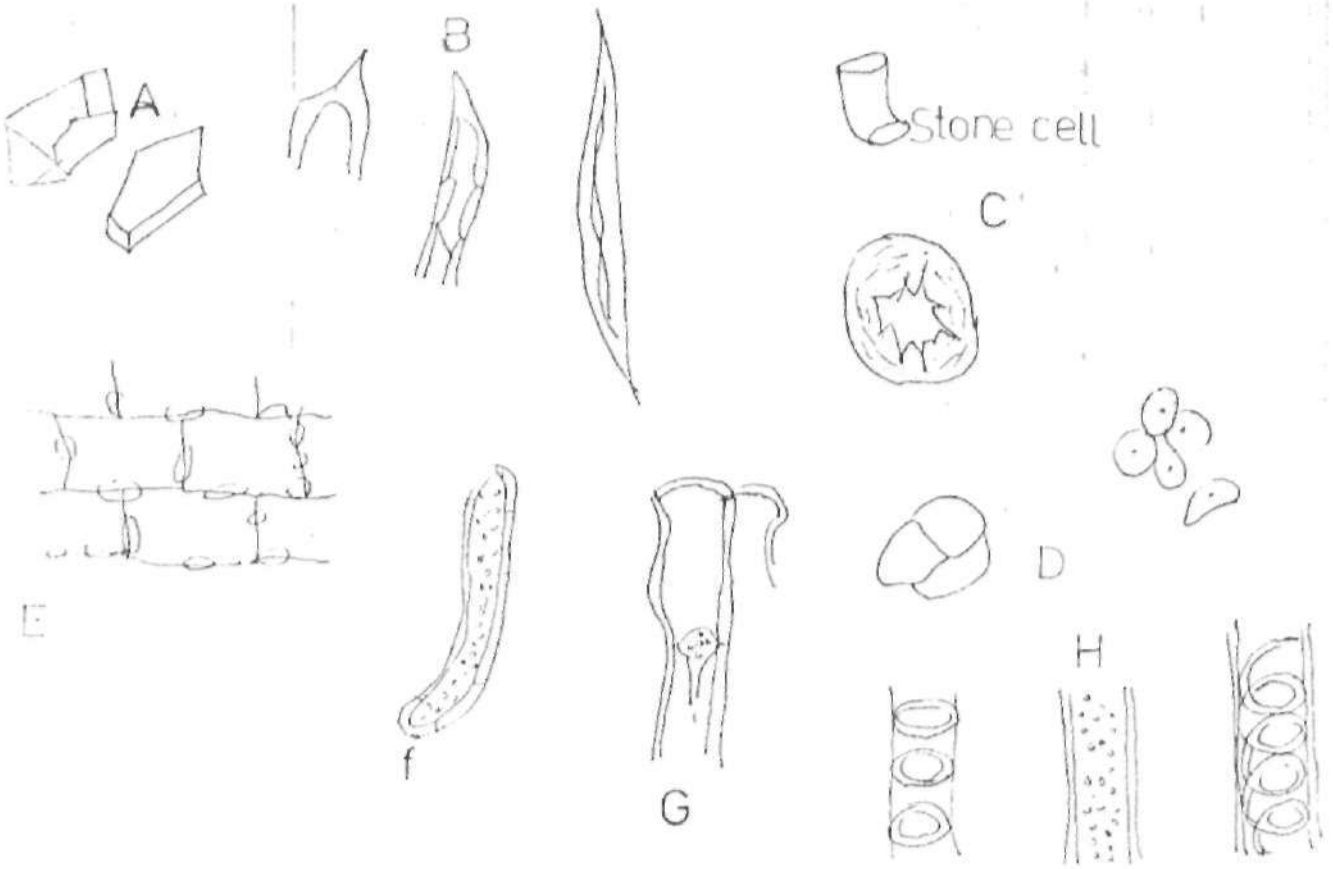
The determination of the rate of kill had made it possible to compare the resistance of one particular organism at different concentrations of the agent. Also Garret and Brown (1964) had suggested that there was no single concentration of agent at which all the cells in a suspension would be killed instantaneously. The process of killing occurs chiefly as a function of time within a range of concentration. This probably explains the reduction in the number of viable cells at a higher concentration and a longer time period. This activity is due to the presence of certain constituents like, alkaloids, flavonoids, tannins and saponins which have been associated with antimicrobial properties (Leven et al, 1979; Scherbanvaskii, 1971) and had also been confirmed by the results obtained on testing these compounds separately on test organisms (Table 18).

The crude unrefined extracts of the plant metabolites showed more effect compared to the crude extracts; that it had wider zones of inhibition irrespective of the concentration. Also the effect of all the secondary metabolites are more on B. subtilis and least on Pseudomonas aeruginosa and

Staphylococcus aureus (Table 13 and 14). Unlike in the crude extracts, the plant metabolites have comparable effect on all the test organisms. This might be as a result of similarity in the mechanics of inhibition inherent in the extracts.

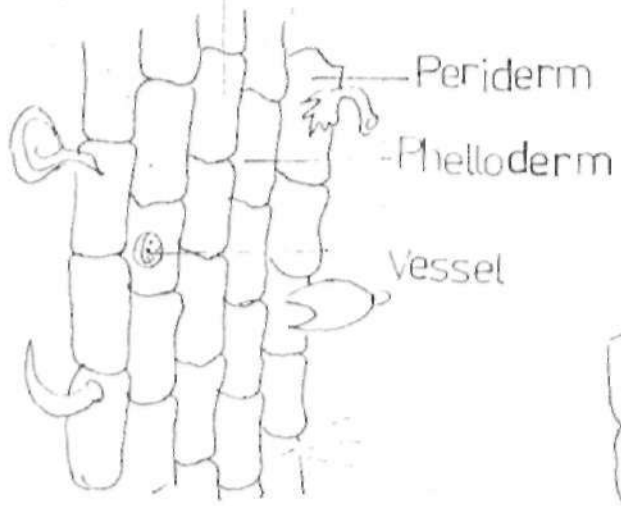
In conclusion, it has been shown in this work that M. villosus has useful antimicrobial properties and the medicinal property tends to support the traditional usage of the plant.

FIG.3.10 Microscopy of the Root Powder of M.villosus

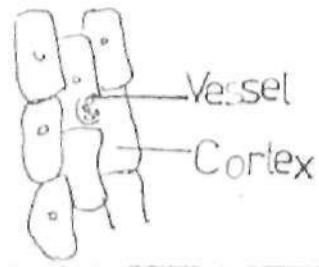


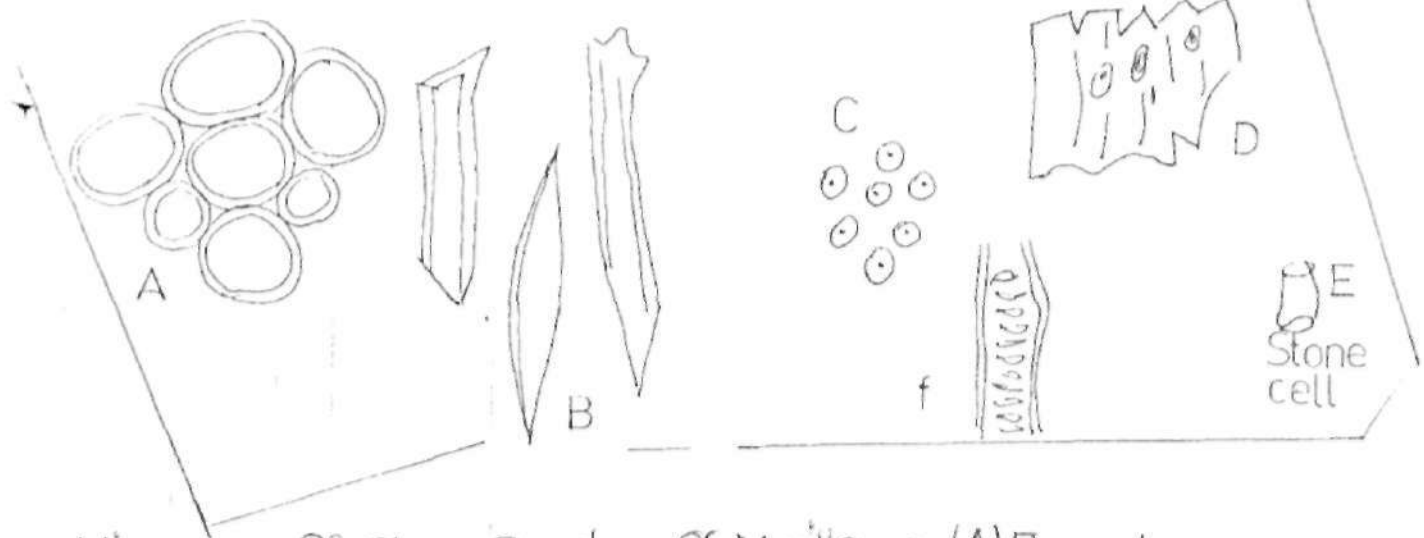
M.villosus Powder(Root):(A) Prism of calcium oxalate crystals.(B) fibres with wide lumen, thin walled fiber and fibre with pointed ends. (C) Stone and sclereids. (D) Starch grain (E) Ground parenchyma tissue (f) Tracheid vessel, (G) Sieve vessel with companion cell.(H) Annular, pitted and spiral lignification of vessels.

FIG-3-11. MICROSCOPY Of THE STEM Of M.VILLOSUS



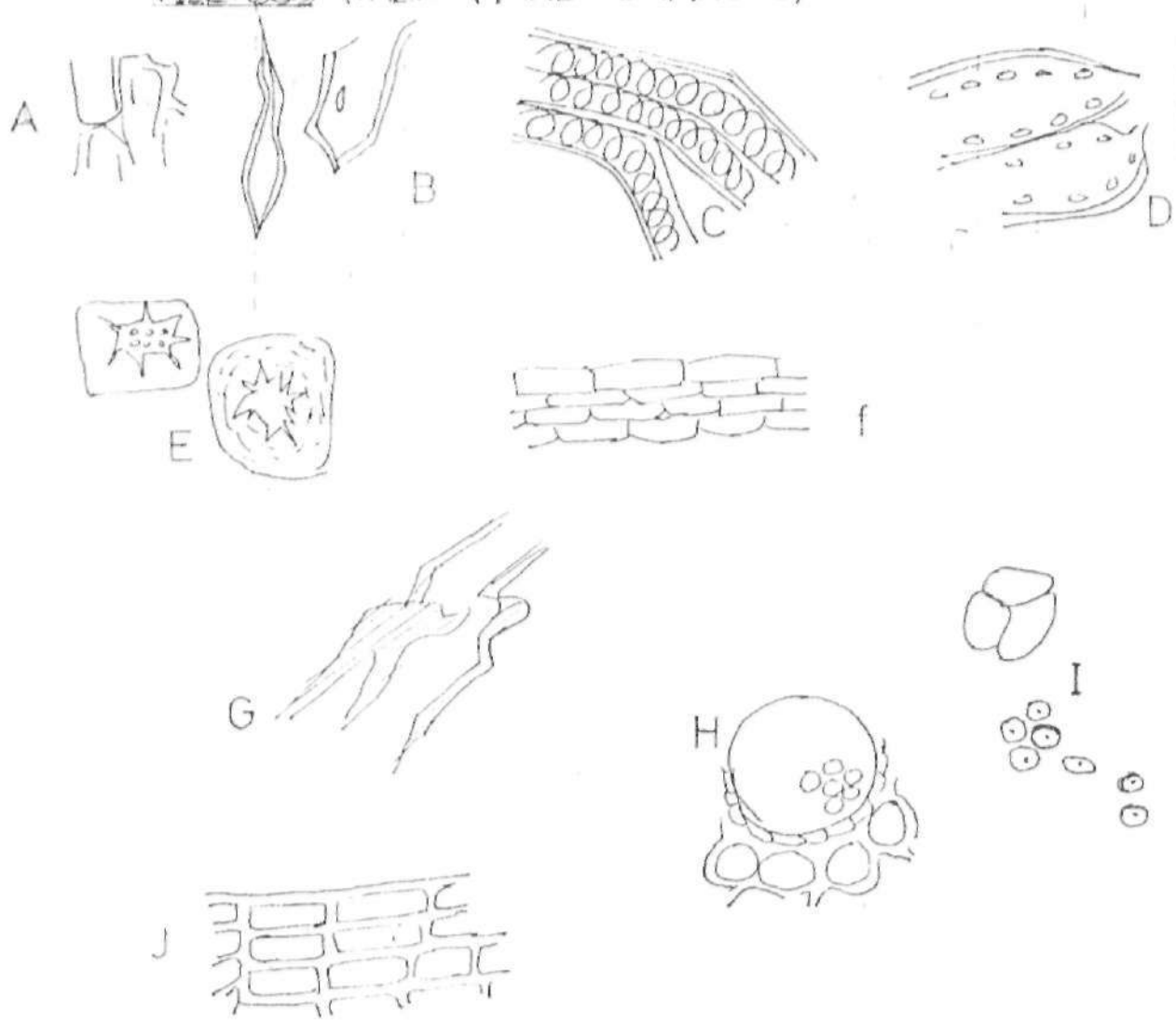
Longitudinal Section Through fresh sample Of The Stem.





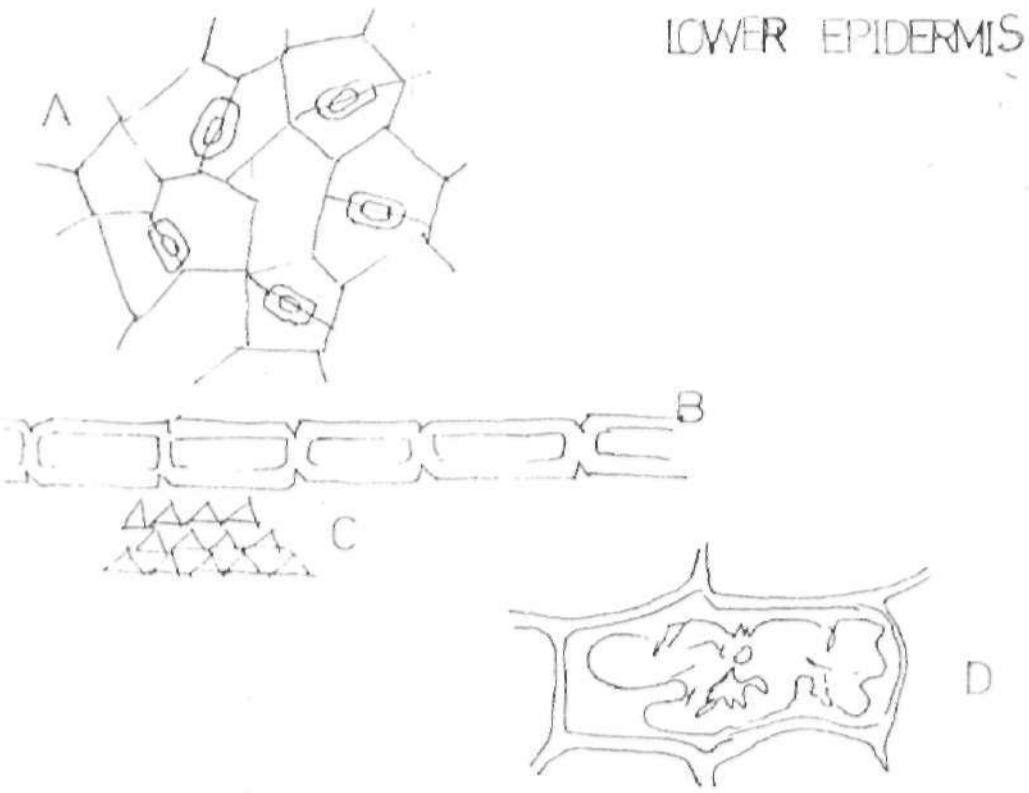
Microscopy Of Stem Powder Of M. villosus: (A) Parenchyma tissue. (B) fibres with: blunt end, pointed ends, and wide lumen (C) Simple starch grains. (D) fragment of pitted vessel. (F) Scalariform lignification.

FIG.3.12 MICROSCOPY OF THE LEAF POWDER OF M. VILLOSUS (FREE HAND DRAWING).



Leaf Powder of M.villosus:(A)fragment of the phloem. (B) fibres. (C) Conducting xylem element with spiral thickening. (D) Pitted thickening. (E) Sclereids. (f) Broken fragment of parenchyma tissue. (G) Broken fragment of tracheids. (H) Oil gland. (I) Starches (compound and simple). (J) Cork cell.

FIG.3.13 LONGITUDINAL SECTION OF THE FRESH LEAF OF M. VILLOSUS.



Anatomical Section Through The Fresh Leaf of M.villosus: (A)Paracytic stomata (B)Elongated epidermal cells. (C)Cluster of calcium oxalate crystals. (D)Oil gland.

FIG.3.14 MICROSCOPY OF FRESH MOUNT OF THE LEAF SHOWING THE UPPER AND LOWER EPIDERMIS(ES) AND OTHER MICROSCOPICAL STRUCTURES WITHIN M.VILLOSUS LEAF

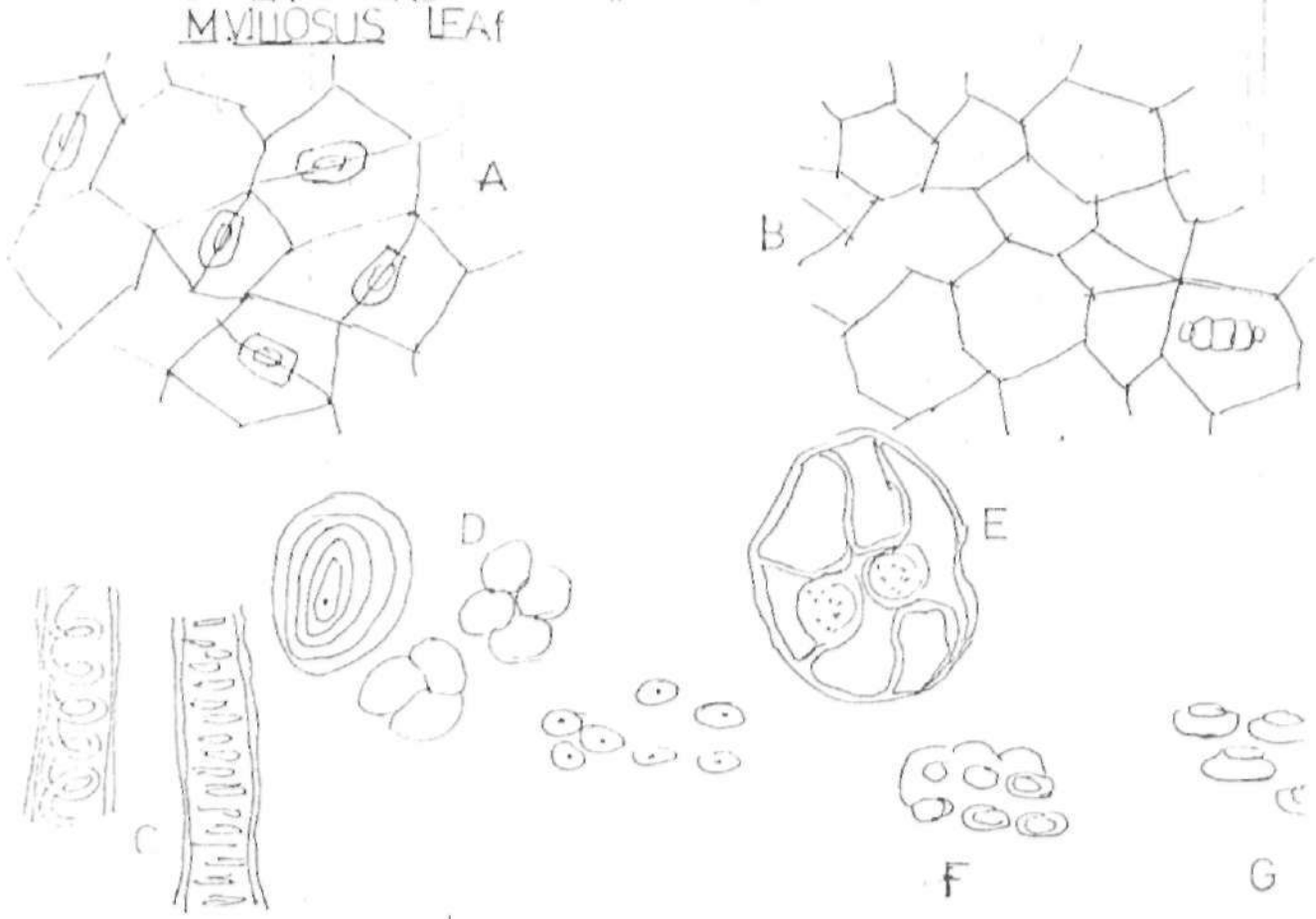
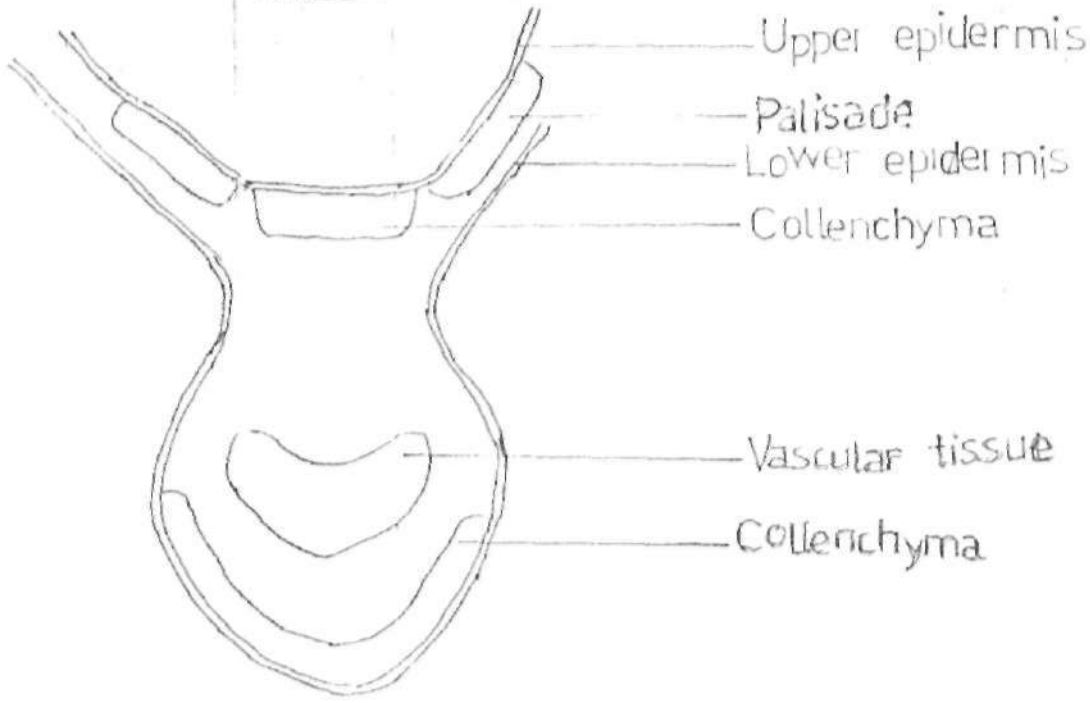
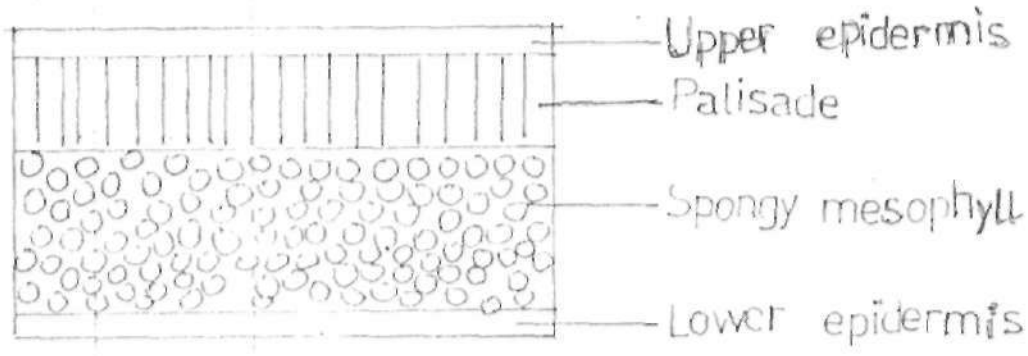


Diagram showing some diagnostic character of the leaf of M. villosus. (A) Lower epidermis with numerous stomata (B) Upper epidermis. (C) Phloem in spiral/reticulate thickenings. (D) Starch grains. (E) Oil gland. (F) Anthraquinone units. (G) Tannins Patches.

FIG.3.15 TRANSVERSE SECTION (T.S.) THROUGH THE MID-RIB AND (T.S.) OF LEAF LAMINAR OF M. VILLOSUS (FRESH SAMPLE).



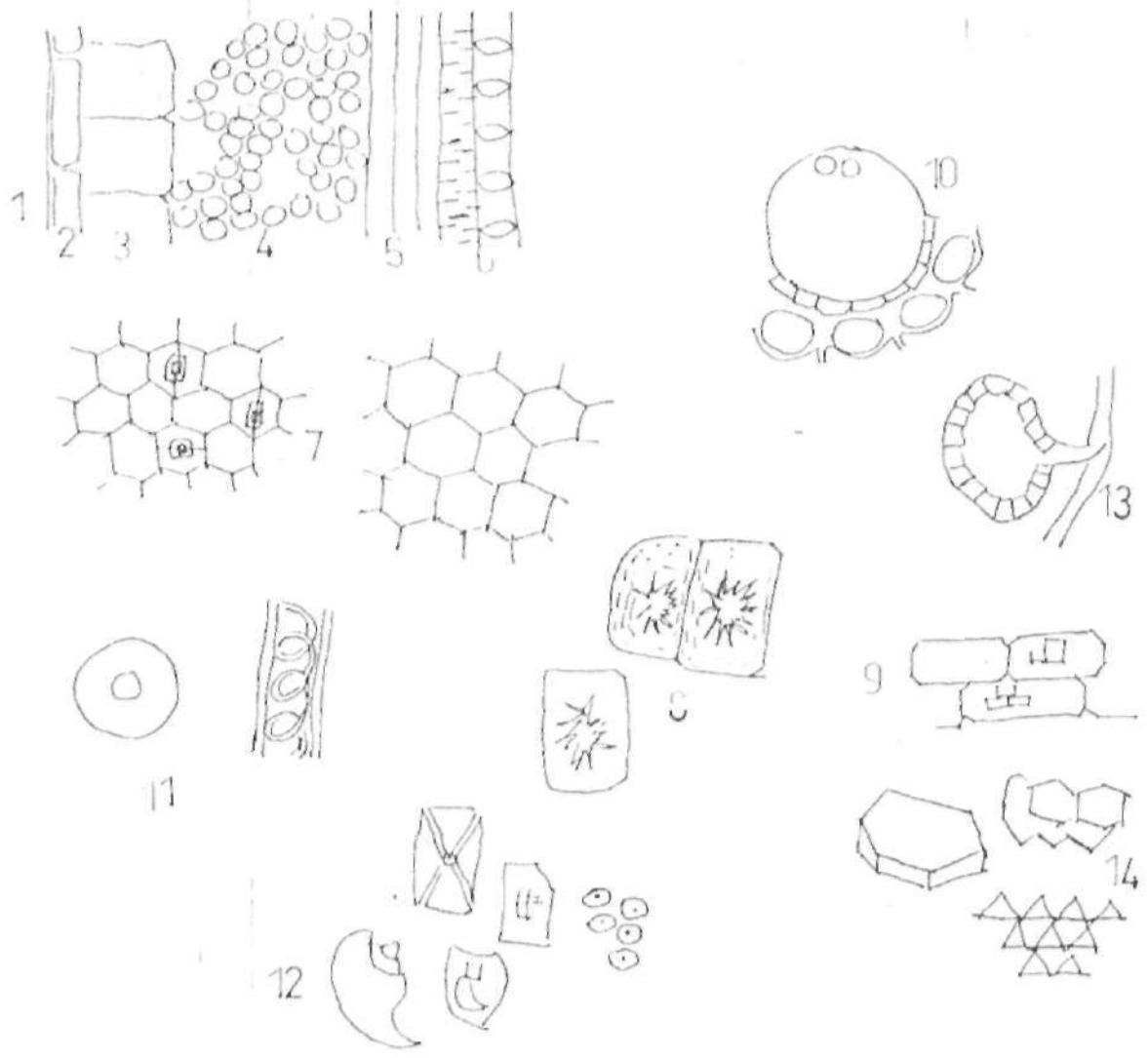
Tissue distribution diagram of mid-rib of leaf of Mitracarpus villosus (Sw) Dc.



Dorsoventral Structure

Arrangement of leaf tissue in transverse section

FIG.3.16 MICROSCC. OF THE LEAF POWDER SHOWING LEAF LAYERS AND CELL INCLUSIONS



Leaf powder of M. villosus: (1) Cuticle, (2) Epidermis, (3) Palisade, (4) Spongy mesopyll, (5) Phloem, (6) Xylem, (7) fragment of lower epidermis with stomata, (8) sclereids, (9) Prisms of calcium oxalate crystals, (10) Ol gland, (11) thick walled vessel/spiral lignification, (12) starch, (13) secretory unit, (14) Isolate prism of calcium oxalate crystals.

TABLE 1: MICROSCOPICAL FEATURE OF MITRACARPUS VILLOSUS LEAF

CHARACTER		DESCRIPTION
Calcium Oxalate Crystal		Present
Stomata	Upper surface	None-to-very few paracytic
	Lower surface	paracytic
Trichomes		none
Epidermis	Upper surface	wavy to straight
	Lower surface	straight to mark wavy
Mesophyll		One layer of palisade
Special character		Oil globules, starch grains, secretory cells, with columnar epithelia cells

TABLE 2: SUMMARY OF CHEMOMICROSCOPICAL EXAMINATION OF THE ROOT, STEM, LEAVES AND INFLORESCENCE OF M. villosus

CONSTITUENT GROUP	ROOT	STEM	LEAVES	INFLORESCENCE
Anthraquinone	0	0	1	0
Calcium Carbonate	0	0	0	0
Calcium Oxalate	1	1	1	1
Cellulose	1	1	1	1
Fixed oils and fats	1	1	1	1
Lignin	1	1	1	1
Mucilage	0	0	0	0
Proteins	0	0	1	1
Starch	1	1	1	1
Suberin	0	1	0	0
Tannins	0	0	1	1

Key

1 = Present (+ve)

0 = Absent (-ve)

Table 3 PHYTOCHEMICAL CONSTITUENTS OF THE ROOT, STEM LEAVES AND INFLORESCENCE OF M. VILLOSUS

CONSTITUENT GROUP	ROOT	STEM	LEAVES	INFLORESCENCE
1. Alkaloids	1	1	1	1
2. Carbohydrates (Barfoed's)	1	1	1	0
Combined Reducing Sugars	1	1	1	1
(Fehling's) Reducing sugars	1	1	0	0
(Molisch's) Reducing sugars	1	1	1	1
Pentose	1	1	1	1
Selivenorff's) Reducing sugars	1	1	1	1
3. Flavonoids				
(Ferric chloride)	1	1	1	1
(Lead acetate)	1	1	1	1
(Shinoda)	1	1	1	1
(Sodium hydroxide)	1	1	1	1
4. Glycosides				
Cyanogenetic glycoside	0	0	0	0
Saponin glycoside	1	1	1	1
Liebermann-Burchard	1	1	1	1
Salkowski's (steroid) test	1	1	1	1
5. TANNINS				
Bromine water				
Ferric chloride	0	0	1	1
Ferric ammonium citrate	0	0	1	1
Gelatin	1	1	1	1
Lead sub acetate	0	0	1	1
Phloroglucinol	1	1	1	1
	0	0	1	1

Key
1 = positive
0 = negative

TABLE 4A hRf VALUES AND COLOUR CHARACTERISTICS OF THE ALKALOID OF THE ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS IN PAPER CHROMATOGRAPHY

Aqueous extract	Solvent system	hRf	spray reagent	colour reation	colour daylight	response UV (366nm)
Root	n-butanol acetic acid water (4:1:5)	95	Strong ammonia Vapour	yellow	brown	orange
Stem		95		yellow	brown	orange
Leaves		95		yellow	brown	orange
Inflore-scence		95		yellow	brown	orange

Paper Chromatography of Drug Containing Alkaloid

Samples: Extracts of the root, stem, leaves and inflorescence of M. Villosus as described in section (2.7.1) extraction.

Method: One way Ascending.
Paper: Whatman No.1 (20x20cm).
Solvent System (Mobile phase): n-Butanol: acetic-acid: Water(4:1:5)
Time: 24hours or several cm. distance.
Location Sequence:
(1) Dry Chromatogram.
(2) Examine in daylight.
(3) Examine in ultra-violet light.
(4) Spray: Using strong ammonia vapour.

TABLE 4B hRf VALUES AND COLOUR CHARACTERISTIC OF ALKALOIDS OF ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS IN THIN LAYER CHROMATOGRAPHY (TLC)

Aqueous Extract	Solvent System	hRf	Spray Reagent	Colour reaction	colour day light	response UV (366nm)
Spot 1	Chloro-form Methanol (4:1)	-	Dragen-dorff's reagent			
Root		77		brown	not visi-ble	Yellow
Stem		77		brown	,,	Yellow
Leaves		77		brown	,,	Yellow
Inflor e-scence		77		brown	,,	Yellow
Spot 2						
Root		39		black	not visi-ble	green
Stem		39		black	,,	green
Leaves		39		black	,,	green
Inflor e-scence		39		black	,,	green
Root	Chloro-form	76	Marquis reagent	greenish	,,	y/green
Stem	methanol	76		greenish	,,	y/green
Leave	Ammonia	76		greenish	,,	y/green
Inflor e-scence	10:20:1)	76		greenish	,,	y/green

TLC Separation of Alkaloids

Samples: Extracts of the root, stem, leaves and inflorescence of M. Villosus as described in section (2.7.1) and (2.8.5).

Method: One way Ascending TLC.
Adsorbent: Silica gel-G.
Solvent System (Mobile phase):Chloroform: Methanol(4:1)
Time: 24hours.
Location Sequence:
(1) Dry Chromatogram.
(2) Examine in daylight and ultra-violet light
(3) Spray with Dragendorff.

TABLE 5 hRf VALUES AND COLOUR CHARACTERISTIC OF THE FIXED OILS OF THE ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS BY TWO DIMENSIONAL THIN LAYER CHROMATOGRAPHY

Aqueous Extract	Solvent System	hRf	Spray reagent	Colour reaction	Colour day light	Response UV (366nm)
Spot 1						
Root	Chloroform Methanol acetic acid water (170:25:25:4)	91	50% H_2SO_4	Yellow	not visible	White florescence
Stem		91		Yellow	,,	,,
Leaves		91		Yellow	,,	,,
Inflor e-scence		91		Yellow	,,	,,
Spot 2	Second Dimension Chloroform methanol; 7M NH_4OH (65:30:4)					
Root		98		Brown	not Visible	-
Stem		98		Brown	,,	-
Leaves		98		Brown	,,	-
Inflor e-scence		98		Brown	,,	-

Two Dimension TLC of Fixed Oil M. Villosus

Samples: Extracts of the root, stem, leaves and inflorescence of M. Villosus as described in section (2.10.1(iii)).

Method: Two dimension (Two way Ascending) TLC.
Adsorbent: Silica gel-G.
Solvent System(I): Chloroform: Methanol:Acetic acid: Water(170:25:25:4)
Solvent System(II): Chloroform: Methanol:7M Ammonia Solution(65:30:4)
Time: left overnight; second run more than 50 minutes.
Location Se
quence:

(1) Dry Chromatogram.
(2) Examine in daylight and ultra-violet light
(3) Spray with 50% H_2SO_4.

TABLE 6a hRf VALUES AND COLOUR CHARACTERISTIC OF THE FLAVONOIDS OF THE ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS IN TWO DIMENSIONAL PAPER CHROMATOGRAPHY (PC)

Aqueous Extract	Solvent System	hRf	Spray reagent	Colour reaction	Colour day light	Response UV (366nm)
Spot 1						
Root	n-butanol	82	5% alcoholic	Yellow	not visible	Brown
Stem	acetic	82	ferric	Yellow	,,	Brown
Leaves	acid	82	Chloride	Yellow	,,	Brown
Inflor e-scence	water (4:1:5)	82	Solution	Yellow	,,	Brown
Spot 2						
Root	Second dimen-	84		Brown	not Visible	green
Stem	sion 5%	84		Brown	,,	green
Leaves	acetic acid	84		Brown	,,	green
Inflor e-scence		84		Brown	,,	green

Paper Chromatography of Drug Containing Flavonoids

Samples: Extracts of the root, stem, leaves and inflorescence of M. Villosus prepared as described in section (2.7.3) extraction.

Method: Two dimensional Two way Ascending) paper Chromatography.
Paper: Whatman No.1 (20x20cm).
Solvent System (I): n-Butanol: acetic-acid: Water(4:1:5)
Solvent System (II): 5% acetic acid.
Time: left overnight; second run 3hours plus.
Location Sequence:
(1) Dry Chromatogram.
(2) Examine in daylight.
(3) Examine in ultra-violet light.
(4) Spray with 5% alcoholic ferric Chloride solution

TABLE 6b hRf VALUES AND COLOUR CHARACTERISTIC OF THE FLAVONOIDS OF THE ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS IN THIN LAYER CHROMATOGRAPHY (TLC)

Aqueous Extract	Solvent System	hRf	Spray reagent	Colour reaction	Colour day light	Response UV (366nm)
Spot 1						
Root	Benzene Pyridine formic acid (36:9:5)	39	Ammonia Vapour	Yellow	not visible	-
Stem		39		Yellow	,,	-
Leaves		39		Yellow	,,	-
Inflor e-scence		39		Yellow	,,	-
Spot 2						
Root		97		Brown	Yellow	-
Stem		97		Brown	Yellow	-
Leaves		97		Brown	Yellow	-
Inflor e-scence		97		Brown	Yellow	-

TLC Separation of Flavonoids

Samples: Extracts of the root, stem, leaves and inflorescence of M. Villosus as described in section (2.7.3) extraction.

Method: One way Ascending TLC.
Adsorbent: Silica gel-G.
Solvent System: Benzene: Pyridine: Formic acid (36:9:5)
Time: One hour plus and 10cm. distance.
Location Sequence:
(1) Dry Chromatogram.
(2) Examine in daylight and ultra-violet light
(3) Spray using Ammonia Vapour

TABLE 7a hRf VALUES AND COLOUR CHARACTERISTIC OF THE STEROIDAL ALKALOID GLYCOSIDES OF THE ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS IN PAPER CHROMATOGRAPHY (PC)

Aqueous etha-nolic Extract	Solvent System	hRf	Spray reagent	Colour reaction	Colour day light	Response UV (366nm)
Spot 1						
Root	Ethyl acetate Pyridine water (3:1:3)	16	Carr-Price reagent	Buff	Dark	Brown
Stem		16		Buff	Dark	Brown
Leaves		16		Buff	Dark	Brown
Inflor e-scence		16		Buff	Dark	Brown
Spot 2						
Root		88		Buff	Dark	Brown
Stem		88		Buff	Dark	Brown
Leaves		88		Buff	Dark	Brown
Inflor e-scence		88		Buff	Dark	Brown

Paper Chromatography of Drug Containing Steroidal alkaloid glycoside

Samples: Extracts of the root, stem, leaves and inflorescence of M. Villosus prepared as described in section (2.7.4) extraction.

Method: One way Ascending.
Paper: Whatman No.1 (20x20cm).
Solvent System Ethylacetate: Pyridine:Water(3:1:3)
Time: overnight.
Location Sequence:
(1) Dry Chromatogram.
(2) Examine in daylight.
(3) Examine in ultra-violet light.
(4) Spray with Carr-Price

TABLE 7b hRf VALUES AND COLOUR CHARACTERISTIC OF THE STEROIDAL ALKALOID GLYCOSIDES OF M. VILLOSUS ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS IN THIN LAYER CHROMATOGRAPHY (TLC)

Aqueous etha-nolic Extract	Solvent System	hRf	Spray reagent	Colour reaction	Colour day light	Response UV (366nm)
Spot 1						
Root	Ethyl acetate Pyridine water (3:1:3)	16	Carr-Price reagent	Buff	Dark	-
Stem		16		Buff	Dark	-
Leaves		16		Buff	Dark	-
Inflor e-scence		16		Buff	Dark	-
Spot 2						
Root		20		Buff	Dark	-
Stem		20		Buff	Dark	-
Leaves		20		Buff	Dark	-
Inflor e-scence		20		Buff	Dark	-

TLC Separation of Steriodal Alkaloid Glycoside

Samples: Extracts of the root, stem, leaves and inflorescence of M. Villosus as described in section (2.7.4) extraction.

Method: One way Ascending TLC.
Adsorbent: Silica gel-G.
Solvent System: Ethyl acetate: Pyridine: water (3:1:3)
Time: One hour plus and 10cm. distance.
Location Sequence:
(1) Dry Chromatogram.
(2) Examine in daylight and ultra-violet light
(3) Spray with Carr-Price reagent.

TABLE 8a hRf VALUES AND COLOUR CHARACTERISTIC OF THE STEROIDAL GLYCOSIDES OF ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS IN PAPER CHROMATOGRAPHY.

Aqueous etha-nolic Extract	Solvent System	hRf	Spray reagent	Colour reaction	Colour day light	Response UV (366nm)
Spot 1						
Root	n-butanol Benzene water (10:4:5)	39	Carr-Price reagent	Greyish	not visible	Blue Flore-scence
Stem		39		Greyish	,,	,,
Leaves		39		Greyish	,,	,,
Inflor e-scence		39		Greyish	,,	,,
Spot 2						
Root		95		Brown	not visible	-
Stem		95		Brown	,,	-
Leaves		95		Brown	,,	-
Inflor e-scence		95		Brown	,,	-

Paper Chromatography of Drug Containing Steroidal glycosides

Samples: Extracts of the root, stem, leaves and inflorescence of M. Villosus prepared as described in section (2.7.5) extraction.

Method: One way Ascending.
Paper: Whatman No.1 (20x20cm).
Solvent System n-butanol:Benzene:Water(10:4:5)
Time: Left overnight.
Location Sequence:
(1) Dry Chromatogram.
(2) Examine in daylight.
(3) Examine in ultra-violet light.
(4) Spray with Carr-Price reagent.

TABLE 8b hRf VALUES AND COLOUR CHARACTERISTIC OF THE SPIROSTANOL GLYCOSIDES IN THE ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS IN THIN LAYER CHROMATOGRAPHY (TLC).

Aqueous etha-nolic Extract	Solvent System	hRf	Spray reagent	Colour reaction	Colour day light	Response UV (366nm)
Spot 1						
Root	Chloro-form Methanol Water (65:35:10)	60	Carr-Price reagent	Light Blue		
Stem		60		,,		-
Leaves		60		,,		-
Inflor e-scence		60		,,		-
Spot 2						
Root		99		Buff	dark	-
Stem		99		Buff	dark	-
Leaves		99		Buff	dark	-
Inflor e-scence		99		Buff	dark	-

TLC Separation of Spirostanol(Steriodal) Glycoside;

Samples: Extracts of the root, stem, leaves and inflorescence of M. Villosus as described in section (2.7.5) extraction.

Method: One way Ascending TLC.
Adsorbent: Silica gel-G.
Solvent System: chloroform: Methanol: water (65:35:10)
Time: One hour plus.
Location Sequence:
(1) Dry Chromatogram.
(2) Examine in daylight and ultra-violet light
(3) Spray with Carr-Price reagent.

TABLE 9a hRf VALUES AND COLOUR CHARACTERISTIC OF THE TRITERPENES GLYCOSIDES IN THE ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS IN PAPER CHROMATOGRAPHY (PC).

Aqueous etha-nolic Extract	Solvent System	hRf	Spray reagent	Colour reaction	Colour day light	Response UV (366nm)
Spot 1						
Root	n-butanol Glacial acetic: acid Water (4:1:5)	57	Carr-Price reagent	Buff	not visible	White Flore-scence
Stem		57		Buff	,,	,,
Leaves		57		Buff	,,	,,
Inflor e-scence		57		Buff	,,	,,
Spot 2						
Root		63		Buff	not visible	Light blue
Stem		63		Buff	,,	,,
Leaves		63		Buff	,,	,,
Inflor e-scence		63		Buff	,,	,,

Paper Chromatography of Drug Containing Triterpenes Glycosides:

Samples: Extracts of the root, stem, leaves and inflorescence of M. Villosus prepared as described in section (2.7.6) extraction.

Method: One way Ascending.
Paper: Whatman No.1 (20x20cm).
Solvent System n-Butanol:Glacial acetic-acid:Water(4:1:5).
Time: Left overnight.
Location Sequence:
(1) Dry Chromatogram.
(2) Examine in daylight.
(3) Examine in ultra-violet light.
(4) Spray with Carr-Price reagent.

TABLE 9b hRf VALUES AND COLOUR CHARACTERISTIC OF THE TRITERPENES GLYCOSIDES IN THE ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS IN THIN LAYER CHROMATOGRAPHY.

Aqueous Methanolic Extract	Solvent System	hRf	Spray reagent	Colour reaction	Colour day light	Response UV (366nm)
Spot 1						
Root	Chloroform Methanol: water (65:35:10)	96	Carr-Price reagent	Buff	not visible	Yellow
Stem		96		Buff	,,	,,
Leaves		96		Buff	,,	,,
Inflorescence		96		Buff	,,	,,
Spot 2						
Root		97		Brown	not visible	-
Stem		97		Brown	,,	-
Leaves		97		Brown	,,	-
Inflorescence		97		Brown	,,	-

TLC Separation of Triterpenes Glycoside;

Samples: Extracts of the root, stem, leaves and inflorescence of M. Villosus as described in section (2.7.6) extraction.

Method: One way Ascending TLC.
Adsorbent: Silica gel-G.
Solvent System: chloroform: Methanol: water (65:35:10)
Time: 2hour plus.
Location Sequence:
(1) Dry Chromatogram.
(2) Examine in daylight and ultra-violet light
(3) Spray with Carr-Price reagent.

TABLE 10a hRf VALUES AND COLOUR CHARACTERISTICS OF TANNINS IN THE ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS IN PAPER CHROMATOGRAPHY (PC).

Aqueous alcoholic Extract Reference	Solvent System	hRf	Spray reagent	Colour reaction	Colour day light	Response UV (366nm)
Spot 1						
Root	n-butanol Acetic acid water (4:1:5)	74	5% Ferric chloride solution	Yellow Brown	not visible	-
Stem		75		Orange Brown	,,	-
Leaves		80		blue black	,,	-
Inflorescence		98		Orange	,,	-
Reference						
Standard						
Catechol		80		Bluish Black		
Pholoro-glucinol		72		Brown		
Tannic acid		95		Orange		floresce
Spot 2						
Root		74		Yellowish	not visible	-
Stem		75		No colour	,,	-
Leaves		80		Black	,,	bright
Inflorescence		98		Yellow Orange	,,	floresce
Spot 3						
Root		74		Brown	not visible	-
Stem		75		Orange Yellow	,,	-
Leaves		80		Brown	,,	-
Inflorescence		98		Orange	,,	floresce

Paper Chromatography of Drug Containing Tannins

Samples: Extracts of the root, stem, leaves and inflorescence of M. Villosus prepared as described in section (2.7.7) extraction.

Standard: 1% Tannic acid; catechol and phloroglucinol.
Paper: Whatman No.1 (20x20cm).
Solvent System n-Butanol:Acetic-acid:Water(4:1:5).

Time: Left overnight.
Location Sequence:

(1) Dry Chromatogram.
(2) Examine in daylight.
(3) Examine in ultra-violet light.
(4) Spray with Carr-Price reagent.

TABLE 10b hRf VALUES AND COLOUR CHARACTERISTICS OF TANNINS IN THE ROOT, STEM, LEAVES AND INFLORESCENCE OF M. VILLOSUS IN THIN LAYER CHROMATOGRAPHY (TLC).

Aqueous alcoholic Extract Reference	Solvent System	hRf	Spray reagent	Colour reaction	Colour day light	Response UV (366nm)
Spot 1						
Root	n-butanol Acetic acid water (4:1:5)	74	Ferric chloride solution	Light-Brown	not visible	-
Stem		80		-	,,	-
Leaves		75		blue black	,,	-
Inflorescence		95		Light Orange	,,	-
Spot 2						
Root		74		Yellow brown	not visible	-
Stem		80		Blackish	,,	-
Leaves		75		-	,,	-
Inflorescence		95		Orange	,,	floresce
Spot 3						
Root		74		dark brown	not visible	-
Stem		80		bluish	,,	-
Leaves		75		-	,,	-
Inflorescence		95		Orange Yellow	,,	Bright Floresce
Reference						
Standard						
Catechol		81		Black	-	-
Phloro-glucinol		74		Brown	-	-
Tannic acid		96		Orange	-	Bright-floresce

TLC Separation of Tannins

Samples: Extracts of the root, stem, leaves and inflorescence of M. Villosus as described in section (2.7.7) extraction.

Standard: 1% Tannic acid; Catechol; and phloroglucinol.
Adsorbent: Silica gel-G.

Solvent System: n-Butanol: Acetic acid:Water (4:1:5)
Location Sequence:

(1) Dry Chromatogram.
(2) Examine in daylight and ultra-violet light
(3) Spray with 5% ferric Chloride Solution.

TABLE 11 QUANTITATIVE EVALUATION OF POWDERED LEAF AND INFLORESCENCE OF M. VILLOSUS

PARAMETER	LEAF (%)	INFLORESCENCE
Moisture content	16.0%	13.3%
Some Physicochemical Values		
(a) Total Ash Value	12.7%	14.3%
(b) Acid-Insoluble Ash	4.7%	3.3%
(c) Alcohol-Soluble Extractive	7.6%	4.0%
(d) Water soluble extractive	13.6%	12.8%
Total Alkaloid content	12.45% w/v	1.753% w/v

TABLE 12 THE SUMMARY OF QUANTITATIVE MICROSCOPY OF *MITRACARPUS VILLOSUS*

PARAMETER		VALUE
Stomata number		70
Stomata Index	Upper epidermis	18 - 32 - 60
	Lower epidermis	28 - 48 - 80
Palisade Ratio		14 - 18 - 26
Vein-islet number		12 - 34 - 54
Vein-termination number		8 - 22 - 36

TABLE 13 ANTIMICROBIAL ACTIVITY OF THE HOT WATER AND WATER/ALCOHOL EXTRACTS OF M. VILLOSUS ENTIRE PLANT

ORGANISM	ZONE OF INHIBITION (MM)				
	COLD WATER/ALCOHOL EXTRACT (μg/ml)		HOT WATER EXTRACT (μg/ml)		FIXED OILS
	500	1000	500	1000	O
Bacillus subtilis (NCTC 8236)	10.5	14.5	12.0	15.5	O
Streptococcus pyogenes (NCTC 8198).	10.3	13.5	12.5	13.5	O

UNREFINED CRUDE EXTRACT OF METABOLITES

TABLE 14 SUSCEPTIBILITY OF STANDARD CULTURE OF CONSTITUENTS OF M. VILLOSUS - DETERMINATION OF ZONE OF INHIBITION (IN M.M.) CUP PLATE METHOD

ORGANISM	ZONE OF INHIBITION (IN MM)							
	Alka-loids 500 1000 (µg/ml)	Fixed-Oils	Flavo-noids 500 1000 (µg/ml)	Saponils 500 1000 (µg/ml)	Steroids 500 1000 (µg/ml)	Triter-penes 500 1000 (µg/ml)	Tannins 500 1000 (µg/ml)	REFERENCE DRUG AMIKACIN 10(µg/ml)
B. subtilis (NCTC 8236)Gram Positive	15 18	0	17 20	14 -	0 25	13 19	20 28	0
E. coli(NCTC 10418) Gram -ve	0 16	0	10 17	0 -	0 0	10 15	15 17	16
Proteus vulgaris (Gram -ve)	13 15	0	0 0	0 -	0 0	17 14	0 0	0
Ps. aeruginosa (NCTC 6750)(Gram -ve)	0 0	0	0 0	0 -	15 23	0 0	0 0	21
Staph. aureus (NCTC 6571)(Gram -ve)	0 0	0	0 0	0 -	0 0	0 0	15 18	22
Strept. pyogenes (NCTC 8198)G'	0 14	0	0 14	0 -	0 0	17 20	20 26	25
Candida albicans (Std)	0 8	0	10 17	14 -	0 12	0 0	0 0	0

Note: The plant constituents (secondary metabolites) in the table are; in actual fact, unrefined crude extracts of Alkaloids; Flavonoids; Saponins; Steriods; Tritepenes and Tannins obtained from extraction procedure from the herb.

ANTIMICROBIAL ACTIVITIES OF THE UNREFINED CRUDE EXTRACTS OF METABOLITES OF M. VILLOSUS

TABLE 15 SUMMARY OF MIC (µg/ml) OF ALL THE EXTRACTS OF PLANT METABOLITES OF M. VILLOSUS AGAINST TEST ORGANISM.

Organism	Alkaloid µg/ml	Flavonoids µg/ml	Steroids µg/ml	Triter-penes µg/ml	Tannins µg/ml
B. subtilis (NCTC 8236)	125	31.25	>1000	125	62.5
E. coli (NCTC 10418)	500	>1000	62.5	>1000	125
Proteus vulgaris	500	>1000	>1000	500	>1000
Ps aeruginosa (NCTC 6750)	>1000	>1000	125	>1000	125
Staph. aureus (NCTC 6571)	>1000	>1000	>1000	250	250
Strep. pyogenes (NCTC 8198)	250	500	>1000	250	62.5
Candida albicans	1000	250	500	500	250

NOTE: The plant metabolites(plant constituents) in the table are in actual fact, unrefined crude extracts of Alkaloids; Flavonoids; Saponins, Steroids; Triterpenes and Tannins.

TABLE 16 SUMMARY OF MBC (μg/ml) OF ALL THE EXTRACTS OF CONSTITUENTS OF M. VILLOSUS AGAINST TEST ORGANISM.

Organism	Alkaloid (μg/ml)	Flavonoids (μg/ml)	Steroids (μg/ml)	Triter-penes (μg/ml)	Tannins (μg/ml)
B. subtilis (NCTC 8236)	500	500	250	500	250
E. coli (NCTC 10418)	1000	>1000	>1000	>1000	1000
Proteus vulgaris	1000	>1000	>1000	500	>1000
Ps aeruginosa (NCTC 6750)	>1000	>1000	1000	>1000	250
Staph. aureus (NCTC 6571)	>1000	>1000	>1000	1000	1000
Strep. pyogenes (NCTC 8198)	250	>1000	>1000	500	250
Candida albicans	1000	1000	>1000	500	1000

Key

> = MIC greater than the highest concentration

NOTE: The plant constituents (secondary metabolites) in the table are in actual fact, unrefined crude extracts of Alkaloids; Flavonoids; Saponins, Steroids; Triterpenes and Tannins.

TABLE 17 THE MIC AND MBC OF THE CRUDE EXTRACTS OF CONSTITUENTS OF M. VILLOSUS AGAINST TEST ORGANISM.

Organism	Alkaloid MIC MBC (µg/ml)	Flavonoids MIC MBC (µg/ml)	Steroids MIC MBC (µg/ml)	Triter-penes MIC MBC (µg/ml)	Tannins MIC MBC (µg/ml)
B. subtilis (NCTC 8236)	125 500	62.5 500	62.5 250	125 500	62.5 250
E. coli (NCTC 10418)	500 1000	>1000 -	- -	>1000 -	125 1000
Proteus vulgaris	50 1000	>1000 -	- -	500 -	>1000 -
Ps aeruginosa (NCTC 6750)	>1000 -	>1000 -	125 1000	>1000 -	125 250
Staph. aureus (NCTC 6571)	>1000 -	>1000 -	- -	250 1000	250 1000
Strep. pyogenes (NCTC 8198)	250 250	500 -	- -	125 500	62.5 250
Candida albicans	1000 -	250 -	500 -	500 500	250 1000

Key

> = MIC greater than the highest concentration

NOTE: The plant constituents (secondary metabolites) in the table are in actual fact, unrefined crude extracts of Alkaloids; Flavonoids; Saponins, Steroids; Triterpenes and Tannins.

RATE OF KILL

TABLE 18a REDUCTION IN VIABILITY OF BACILLUS SUBTILIS (NCTC 8236) DURING A 60 MINUTES CONTACT WITH VARYING VOLUMES OF THE PLANT ALKALOIDS (10% W/V) RAW DATA

TIME (IN MINUTES)	VOLUME (ML)					
	0.10	0.25	0.50	1.00	1.50	0.00
0	140 2.1461	130 2.1139	100 2.0000	75 1.8751	60 1.7782	140 2.1461
15	110 2.0414	98 1.9912	85 1.9294	63 1.7993	45 1.6532	167 2.2227
30	80 1.9031	70 1.8451	49 1.6902	30 1.4771	25 1.3979	220 2.3424
45	68 1.8324	50 1.6990	41 1.6128	23 1.3617	20 1.3010	240 2.3802
60	40 1.6021	35 1.5441	27 1.4314	17 1.2304	15 1.1761	295 2.4698

Key

Superscript = natural number

Subscript = Logarithm number

NOTE: The table is the raw data obtained(ie. decrease in the number of organism, B. subtilis by graded change in drug (unrefined crude alkaloids) volume with time in minutes.

The graph obtained is the rate of kill (Fig. 3.17)

The graph is the logarithm number(subscript) per unit time.(pp 215)

TABLE 18b REDUCTION IN VIABILITY OF STREPTOCOCCUS PYOGENES (NCTC 8198) DURING A 60 MINUTES CONTACT WITH VARYING VOLUMES OF THE PLANT STEROIDS (10% W/V) RAW DATA

TIME (IN MINUTES)	VOLUME (ML)					
	0.10	0.25	0.50	1.00	1.50	0.00
0	127 2.1038	119 2.0755	80 1.9030	60 1.7782	40 1.6021	170 2.2304
15	100 2.0000	88 1.9445	70 1.8491	47 1.6721	33 1.5185	210 2.3222
30	89 1.9494	70 1.8451	55 1.7404	40 1.6021	28 1.4472	225 2.3529
45	69 1.8388	50 1.6990	40 1.6021	30 1.4771	21 1.3221	240 2.3802
60	40 1.6021	32 1.5051	25 1.3979	18 1.2553	16 1.2041	290 2.4624

Key

Superscript = natural number

Subscript = Logarithm number

NOTE: The table is the raw data obtained(ie. decrease in the number of organism, Strept. pyogenes) by graded change in drug (unrefined crude steroids) volume against time in minutes.

The graph obtained is the rate of kill (Fig. 3.18)

The graph is the logarithm number(subscript) per unit time.(pp 215)

TABLE 18c COLONY FORMING UNITS (CFU) OF VIABLE BACTERIA COUNT
CFU = 10^{x} ORGANISM/ML

Bacillus subtilis (NCTC 8236) in 10%(w/v) Alkaloids

TIME (IN MINUTES)	VOLUME (ML)					
	0.10	0.25	0.50	1.00	1.50	0.00
0	140 $1.4x10^{5}$	130 $1.3x10^{4}$	100 $1.0x10^{4}$	75 $7.5x10^{4}$	60 $6.0x10^{4}$	140 $1.4x10^{5}$
15	110 $1.1x10^{5}$	98 $9.8x10^{4}$	85 $8.5x10^{4}$	63 $6.3x10^{4}$	45 $4.5x10^{4}$	167 $1.67x10^{5}$
30	80 $8.0x10^{4}$	70 $7.0x10^{4}$	49 $4.9x10^{4}$	30 $3.0x10^{4}$	25 $2.5x10^{4}$	220 $2.2x10^{5}$
45	68 $6.8x10^{4}$	50 $5.0x10^{4}$	41 $4.1x10^{4}$	23 $2.3x10^{4}$	20 $2.0x10^{4}$	240 $2.4x10^{5}$
60	40 $4.0x10^{4}$	35 $3.5x10^{4}$	27 $2.7x10^{4}$	17 $1.7x10^{4}$	15 $1.5x10^{4}$	295 $2.95x10^{5}$

Key

Superscript = natural number

Subscript = CFU/ml (10^{x} Organism/ml)

NOTE: TABLE 18d is a conversion table from natural number to colony forming unit (CFU) applying the formulae

$$CFU = 10^{x}\text{organism/ml}$$

This has nothing to do with the ordinates of the "Rate of Kill" graph.

TABLE 18d COLONY FORMING UNITS (CFU) OF VIABLE BACTERIA COUNT
CFU = 10^{X} ORGANISM/ML

Streptococcus pyogenes (NCTC 8198) in 10% w/v) Steroids.

TIME (IN MINUTES)	VOLUME (ML)					
	0.10	0.25	0.50	1.00	1.50	0.00
0	127 $1.27x10^{5}$	119 $1.19x10^{5}$	80 $8.0x10^{4}$	60 $6.0x10^{4}$	40 $4.0x10^{4}$	170 $1.7x10^{5}$
15	100 $1.0x10^{5}$	88 $8.8x10^{4}$	70 $7.0x10^{4}$	47 $4.7x10^{4}$	33 $3.3x10^{4}$	210 $2.1x10^{5}$
30	89 $8.9x10^{4}$	70 $7.0x10^{4}$	55 $5.5x10^{4}$	40 $4.0x10^{4}$	28 $2.8x10^{4}$	225 $2.25x10^{5}$
45	69 $6.9x10^{4}$	50 $5.0x10^{4}$	40 $4.0x10^{4}$	30 $3.0x10^{4}$	21 $2.1x10^{4}$	240 $2.4x10^{5}$
60	40 $4.0x10^{4}$	32 $3.2x10^{4}$	25 $2.5x10^{4}$	18 $1.8x10^{4}$	16 $1.6x10^{4}$	290 $2.9x10^{5}$

Key

Superscript = natural number

Subscript = CFU/ml (10^{x} Organism/ml)

Note: TABLE 18C is a conversion table from natural number to colony forming Unit(CFU) applying the formulae:

$$CFU = 10^{x}\text{organism/ml}$$

This has nothing to do with the ordinates of the "Rate of Kill" graph.

CHAPTER FOUR

4.0 SUMMARY, CONCLUSION AND RECOMMENDATION

4.1 SUMMARY AND CONCLUSION

Pharmacognostic studies of root, stem, leaves and the inflorescence of M. villosus have been carried out. These include microscopic and chemomicroscopical studies; preliminary phytochemical studies as well as the determination of some physicochemical constant for purity evaluation.

Paper and thin layer chromatography were also carried out to further continue the phytochemical tests.

The (cold water/alcohol) and the hot water extracts of the entire herb as well as the unrefined crude extracts of the alkaloids, fixed oils, flavonoids, saponins and tannins obtained from the plant parts as principal constituents exhibited some antimicrobial activity against standard laboratory isolates of B. subtilis, E. coli, Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes and Candida albicans. The effect was most on B. subtilis and least on Pseudomonas aeruginosa and Staphylococcus aureus. However, the plant metabolites showed activity against Gram negative and Gram positive organisms.

The MIC/MBC (minimum Inhibitory concentration and Minimum Bacteriocidal Concentration) further confirmed their activity. The Rate of Kill was also determined to evaluate the activity of B. subtilis (NCTC 8236) and Streptococcus pyogenes (NCTC 8198) only.

Microscopy, Chemomicroscopy, phytochemistry, chromatography, pharmacopoeial evaluation, Quantitative microscopy and the antibacterial susceptibility profile; The results obtained here justify the traditional usage of the plant as an antimicrobial agent against skin disease in general.

Phamacopoeial Standards of The Powdered Leaf And Inflorescence of Mitracarpus Villosus herb		
Parameter	Leaf	Inflorescence
Moisture content	16.0%w/w	13.3% w/w
Some Physicochemical Values		
(a) Total Ash Value	12.7% w/w	14.3% w/w
(b) Acid-insoluble Ash value	4.7% w/w	3.3% w/w
(c) Alcohol Soluble extractive	7.6% w/w	4.0% w/w
(d) Water soluble extractive	13.6% w/w	12.8% w/w
Total alkaloid Content	12.455 W/W	1.753% w/w

4.2 RECOMMENDATIONS

Several plant constituents have been identified. Further work should include isolation and characterization of the constituents and structural elucidations.

Search should be made to find out if the stability of the extracts could be maintained after a long time of storage.

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APPENDIX A

EVALUATION OF CRUDE VEGETABLE DRUG (PHARMACOPOEIAL VALUE) OF POWDERED LEAF AND INFLORESCENCE.

DETERMINATION OF MOISTURE CONTENT (LEAF POWDER)

I constant weight of crucible = 29.10g.

II weight of crucible and contents = 32.10g.

III weight of crucible and contents after heating = 31.62g

IV Moisture content (Step II - Step III) = 0.48g

V Initial weight of leaf powder = 3.00g

Example of Calculation of Moisture contents

$$\text{(Leaf)} = \frac{\text{Step II - Step III}}{\text{Step V}} \times 100$$

$$= \frac{0.48}{3} \times 100$$

$$= \frac{48}{3}$$

$$= 16.0\% \text{ w/w}$$

Moisture Content (powdered Inflorescence)

I constant weight of crucible = 27.85g.

II weight of crucible and contents before heating = 30.85g

III weight of crucible and contents after heating = 30.45g

IV Moisture content (Step II - Step III) = 0.40g

V Initial weight of powder Inflorescence = 3.00g

% Moisture contents
(Inflorescence)

$$= \frac{\text{Step II - Step III}}{\text{Step V}} \times 100$$

$$= \frac{0.40}{3} \times 100$$

$$= \frac{40}{3}$$

$$= 13.3\% \text{ w/w}$$

DETERMINATION OF SOME PHYSICOCHEMICAL CONSTANTS
TOTAL ASH VALUE (LEAF)

I constant weight of tared crucible = 29.10g.

II weight of crucible and contents
before heating = 32.10g.

III weight of crucible and contents
after ashing = 29.42g

IV Total ash of leaf powder (Step III &IV) = 0.38g

V Initial weight of leaf powder = 3.00g

Example of Calculation of total ash
of the leaf (Leaf)

$$= \frac{\text{Step III - Step I}}{\text{Step V}} \times 100$$

$$= \frac{0.38}{3} \times 100$$

$$= \frac{38}{3}$$

$$= 12.67\% \text{ w/w}$$

Total Ash (Inflorescence)

I constant weight of tared crucible = 27.85g.

II weight of crucible and contents before heating = 32.10g.

III weight of crucible and contents after ashing = 28.28g

IV Total weight of ash (Step III - I) = 0.43g

V Initial weight of the powdered Inflorescence = 3.00g

% Total Ash for the Inflorescence $= \frac{\text{Step III - Step I}}{\text{Step V}} \times 100$

$= \frac{0.43}{3} \times 100$

$= \frac{43}{3}$

$= 14.33\%$ w/w

DETERMINATION OF ACID-INSOLUBLE ASH

LEAF INFLORESCENCE

The ash from the total ash experiment was transferred into the beaker with dil.HCL

	Leaf Powder	Powdered Inflorescence
I	Constant weight of tared crucible = 29.10g	= 27.85g
II	Weight of Ash and acid content = 29.24g	= 27.95g
III	Weight of residue (acid in soluble ash) = 0.14g	= 0.10g
IV	Initial weight of	

powdered sample	= 3.00g	= 3.00g

Example of calculation of Acid In-soluble Ash value (%) (Leaf/Inflorescence)

$= \frac{0.14}{3} \times \frac{100}{1}$	$= \frac{0.10}{3} \times \frac{100}{1}$
$= \frac{14}{3}$	$= \frac{10}{3}$
= 4.67% w/v	= 3.33%

DETERMINATION OF ALCOHOL SOLUBLE EXTRACTIVE

Leaf Extract		Inflorescence Extract
I Constant weight of clean washed crucible	29.10g	= 27.85g.
II Weight of residue after 18hrs shake	29.19g	= 27.90g.
III Weight of 20ml. alcohol soluble extractive)	0.09	= 0.05g.
IV Initial weight of powder	2.5g	= 2.5g.

Example of calculation of the percentage alcohol soluble extractive	% alcohol soluble extractive(inflorescence)
$\frac{\text{Step II - Step I}}{\text{Step IV}} \times 100 = \frac{(0.09)}{2.5} 100$	$= \frac{0.05}{2.5} \times 100$
$= \frac{9}{2.5}$	$= \frac{5}{2.5}$
= 3.6%w/v	= 2.0% w/v

WATER SOLUBLE EXTRACTIVE VALUES

Leaf Extract		Inflorescence Extract
I Initial weight of clean crucible	29.15g	= 27.82g.
II Final weight of content after 6 hrs. shake; 16hrs	29.32g	= 27.98g.
Standing		
III Weight of residue (Water soluble extractive)	0.17g	= 0.16g.
IV Initial weight of powder	2.5g	= 2.5g.
Example of calculation of percentage water soluble extractive value		% alcohol soluble extractive(inflorescence)
$\frac{\text{Step II - Step I}}{\text{Step IV}} \times 100 = \frac{0.17}{2.5} 100$		$= \frac{0.16}{2.5} \times 100$
	$= \frac{17}{2.5}$	$= \frac{16}{2.5}$
	= 6.8%w/v	= 6.4% w/v

DETERMINATION OF TOTAL ALKALOIDS

Leaf		Inflorescence Extract	
I Initial burette reading	= 0.00ml	=	0.00ml.
II Final burette reading	= 20.00ml	=	1.25ml
III Total Alkaloids (Step II - I)	= 20.00ml	=	1.25ml.
IV Initial weight of powder	= 5.0g	=	5.0g.

Example of calculation of total alkaloids leaf/inflorescence

Each ml. of 0.05m sulphuric acid is equivalent to 0.028 53g. of $C_{17}H_{19}NO_3$ (morphine). Add 0.052g. to correct for loss of morphine due to its solubility (BP, 1988).

Leaf	Inflorescence
20ml. of 0.05M sulphuric acid	1.25ml. of 0.05M sulphuric acid
= 20x0.02853g. of morphine	= 1.25 x 0.02853g. of morphine
= 0.285 3x2	= 0.035 662g gram
= 0.5706mg.	
To this add 0.052g to correct	for loss of morphine due to
:. Total weight of morphine	solubility :. Total weight of
in leaf = 0.5706	morphine in the inflorescence
+ 0.0520	= 0.035 66 25
0.6226	+0.052
	0.087 66 25g.
% Total morphine-like alkaloid	% Total alkaloids as morphine
in the leaf = $\frac{0.6226}{5} \times \frac{100}{1}$	in the inflorescence = $\frac{0.0876625}{5} \times 100$
= $\frac{62.26}{5}$	= $\frac{8.76625}{5}$
= 12.4502%	= 1.735 25% not significant

Morphine in opium exist from 8-20% or even more (BPC, 1934). However, morphine - like alkaloid in M. villosus leaf (not the inflorescence) is within the standard range of production. The yield from the plant is significant for large scale production.

APPENDIX B

CONCENTRATION OF CRUDE DRUG FOR INOCULATION

Cold Water/alcohol and Hot water Extracts

[1% w/v]

$\frac{1}{100} \times \frac{100}{1} = 1\%$ w/v

$\frac{1}{100} = \frac{0.1}{10} = 0.01$gm/ml

Since 4ml of drug is required: :. Concentration

$= \frac{0.01}{1} \times 4$ g/ml

= 0.04g/4ml

[2% w/v]

$\frac{2}{100} \times \frac{100}{1} = 2\%$ w/v

$\frac{2}{100} = \frac{1}{50} = \frac{0.1}{5} = \frac{0.02}{1}$

But 2.5ml. of drug is required

:. Conc $\underline{n} = \frac{0.02}{1.0}$ 2.5

$= \frac{0.05}{2.5}$

= 0.05g/2.5ml

[5% w/v]

$\frac{5}{100} \times 100 = 5\%$

$\frac{5}{100} = \frac{0.05}{1}$

2ml of drug is required

$\frac{0.05}{1}$ 2 $\frac{0.1}{2}$

= 0.1g/2ml

[10%]

$\frac{10}{100} \times 100 = 10\%$

$\frac{10}{100} = \frac{0.1(g)}{1(ml)}$

1ml of drug was required

:. Dose $= \frac{0.1}{1} \times 1$

= 0.1g/ml.

[15%]

$\frac{15}{100} \times 100 = 15\%$

$$\frac{15}{100} = \frac{0.15\ (g)}{1\ (ml)}$$

Since 2ml. of drug was required

$$\text{Dose} = \frac{0.15}{1}\ 2$$

$$= \frac{0.3\ (g)}{2\ ml}$$

$$= 0.3g/2ml.$$

APPENDIX C

ACTIVE CONSTITUENT CONCENTRATION

For example 10% w/v alkaloids is obtained by weighing out 0.1g. of the residue into 1ml of pure water.

Proof 0.1g/1.0ml = $\frac{1}{10} \times \frac{100}{1}$ w/v

= 10% w/v

With mass kept constant the volume required for 5% (w/v) can be obtained by applying the formular

$$M_1V_1 = M_2V_2$$

Where M_1 = mass at 10% w/v

V_1 = Volume at 10% w/v

M_2 = mass at 5% w/v

V_2 = ?

$\therefore \quad M_1V_1 = M_2 V_2$

$0.1 \times 1 = 0.05 \times V_2$

$V_2 = \frac{0.1}{0.05} 1$ ie $\frac{0.1}{0.05} \times 1ml$

= $\frac{10}{5}$ ml

= 2ml.

APPENDIX D

RATE OF KILL

Calculation of Colony forming Unit (CFU)

for example susceptibility of Bacillus subtilis against alkaloids.

Average plate count = (140 + 170) x ½

= 155 colony count (CFU)

Given: original bacteria load at 3 step dilution (1:1,000) or $10^{-3} = 10^{6}$ organism/m

For a 6 step dilution (1:1,000,000) or $10^{-6} = 10^{3}$ organism/ml

For viable count the formular below is applicable. Viable count = Average number of colonies x reciprocal dilution (Collins, 1976).

DATA

Average number of colonies ≡ 155 CFU

Dilution = 10^{-3}

Reciprocal dilution = 10^{3}

Viable count (CFU/ml) = 155×10^{3}

= 1.55×10^{5} CFU/ml.

APPENDIX E

Pharmacology - Dose Calculation

Calculate the dose and volume of drug to be administered to the mice; if the following data are available.

Weight of mouse = 16.7 grams

Dose = 100mg/kg

Concentration = 10mg/ml

Dose 100mg/kg

1kg = 100mg

1000g = 100mg

∴ 1gm = 0.1mg

Weight of mouse = 16.7grams

Amount of mg. to be administered to 16.7g. mouse

$$= \frac{16.7}{1000} \times \frac{100}{1} = (0.1\text{mg} \times 16.7)$$

$$= 1.67\text{mg}$$

But the given concentration is 10mg/ml. The volume (ml) of the drug administered to the mouse will be as follows:-

10mg = 1ml

$$1.67\text{mg} = \frac{1.67}{10} \times 1\text{ml}$$

$$= \frac{1.67}{10}\ \text{ml}$$

$$= 0.167\text{ml}$$

Given

Dose = 10mg/kg

Concentration = 10mg/ml

Weight of mouse = 20g.

Dose = 10mg/kg
1000g = 10mg

Weigh of mouse = 20g.

Dose require by mouse = $\frac{20}{1000} \times \frac{10}{1}$

= 0.2mg

Concentration (1mg/ml) given

1mg = 1ml

for 0.2mg = $\frac{0.2}{1} \times 1$

= 0.2ml.

APPENDIX F

STOICHOICHEMISTRY

Preparation of Stock Solution

Prepare 10N Hydrochloride Acid

If 10ml. is required

Given

Volume required = 10ml molar mass = 36.46

Specific gravity = 1.18

Percentage Purity (P%) = 36%

Molarity = 10N

Applying the formula

$$\text{Weight (wt) in gm} = \frac{\text{molality} \times \text{molar Mass} \times \text{volume required}}{1000}$$

$$\text{Volume (vv) in ml.} = \frac{\text{wt. x 100}}{\text{Specific gravity x \%P (sp.gr)}}$$

Substituting

$$wt = \frac{10 \times 36.46 \times 10}{1000} = 3.646 \text{ gram}$$

$$vv = \frac{3.646 \times 100}{1.18 \times 36}$$

$$= \frac{364.6}{42.48}$$

$$= 8.583 \text{ml.}$$

Withdraw 8.583ml. of Hcl and made it up to 10ml. with distilled water (Illya Personal Communication, 1997).

Sodii hydroxidum

0.1m NaoH

Data

APPENDIX G

PREPARATION OF SOME IMPORTANT REAGENTS

Dragendorff's Reagent:

Solution A: Bismuth subnitrate (850mg) is mixed with water (40ml) and to this mixture 10ml. of acetic acid is added.

Solution B: Potassium iodide (8g) is dissolved in 20ml. of water the two solutions are mixed and may be stored in a brown bottle for several months.

Dragendorff's Spray Reagent: The spray reagent is prepared by diluting the stock solution (10ml) with acetic acid (20ml) and water (100ml). Alkaloids and the nitrogenous bases are revealed as red spots against a light yellow background (Macek 1972).

70% Alcohol: Prepared by taking 70ml; of 95% alcohol and dilute to 95ml. (A.O.A.C, 1955).

Hydrochloric acid, dilute (10%): Prepared by mixing 236ml. acid with sufficient water to make 1,000 ml. [(USP XVIII), 1970]

Liebermann's Reagent (Sulphuric acid - nitrous acid)

Dissolve 10g. of Potassium nitrite in sufficient sulphuric acid to produce 100ml (Clarke E.G.C, 1971).

Marqui's Reagent (Formaldehyde-sulphuric acid)

Mix 2 drops of formaldehyde solution with 1ml. of sulphuric acid (Clarke, 1971).

Picric acid (Trinitrophenol solution = Hagner's reagent): A saturated solution of Picric acid in water.

Potassium iodate solution(m/5): An aqueous solution of Potassium iodate containing 1000ml; 42.81gm. of potassium iodate (Clarke, 1971).

Sulphuric acid Solution (N/1):

An aqueous dilution of sulphuric acid containing 1000ml; 49.04gm of H_2SO_4 (A.O.A.C; 1955).

Sulphuric acid, Dilute (2N):

Mix 104g. of sulphuric acid with 896g. of water, and cool. The acid should be added gradually to the water (Clarke, 1971).

Sulphuric acid, spray (0.2M); Add gradually, 1.98 gram of sulphuric acid to 80ml. of water, cool and adjust to 100ml. with water (Clarke, 1971).

Mayer's Reagent: Potassium Mercuri iodide solution.

FIG. 3.17 EFFECT OF VARIOUS VOLUMES OF 10%(W/V) ALKALOIDS OF M. VILLOSUS ON VIABILITY OF B.SUBTILIS (NCTC 8236)

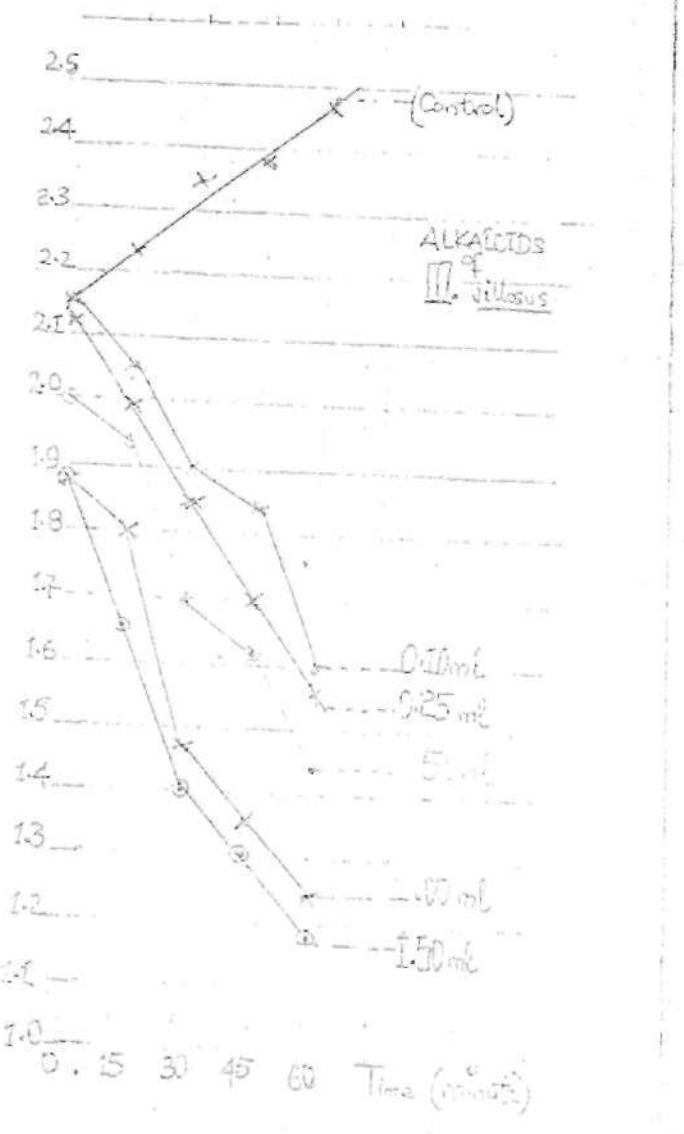


FIG. 3.18 EFFECT OF VARIOUS VOLUMES OF 10%(W/V) STEROIDS OF M. VILLOSUS ON VIABILITY OF S. PYOGENES (NCTC 8198)

