PHYTOCHEMICAL AND BIOLOGICAL STUDIES OF THE BARK OF SCLEROCARYA BIRREA (A. RICH,) HOCHST.

BY

I

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

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DECLARATION

I hereby certify that the work reported in this thesis was carried out by me under the supervision of Dr. A. Ghani, Dr. E.N. Sokomba and Dr. M. Ilyas of the Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria. The works of other investigators are acknowledged and referred to accordingly. I declare that no part of this thesis has been submitted elsewhere for a degree.

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CERTIFICATION

This thesis entitled PHYTOCHEMICAL AND BIOLOGICAL STUDIES OF THE BARK OF SCLEROCARYA BIRREA (A. RICH.) HOCHST. by EZZELDIN MUKTAR ABDURAHMAN 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 all those who in their lives have endured the bitterness of great pain, and who, from that pain, have been able to go forward, strong in hope, to yet greater achievements.

AND

To my father Alhaji Mukhtar Abdurahman and my mother Hajiya Asama'u M. Abdurahman for their love and sacrifices.

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ABSTRACT

Sclerocarya birrea (A. Rich.) Hochst is a Savannah tree belonging to the family Anacardiaceae.. The different parts of the plant, especially the plant bark, have long been used by traditional healers in the northern parts of Nigeria for treating dysentery, hemorrhoids and skin diseases.

A thorough literature survey indicated that there has been no systematic phytochemical or biological study of the chemical constituents of this plant.

The phytochemical study of the bark involved a preliminary phytochemical screening and a detailed phytochemical study of its various extracts (water, ethanol and petroleum ether) for isolation and characterisation of the major constituents. Some of the constituents were also quantitativel}' estimatedi

The phytochemical examination of the bark revealed the presence of carbohydrates, simple phenolic compounds and sterols. Sucrose, gallic acid and a sterol (characterised as cholesterol) were isolated from the bark.

Various pharmacological properties of the bark's crude water extract were separately studied using isolated rabbit duodenum, guineapig ileum, rat uterus and frog rectus abdominis. Acute toxicity of its

water extract was also investigated, In addition, the water extract of the bark was also tested for its antimicrobial activity.

The crude water extract of the bark contracted the smooth muscles of rabbit duodenum, guineapig ileum and rat uterus,. It also contracted the skeletal muscles of the frog rectus abdominis. The median lethal dose (intraperitoneal) of the water extract of the bark was 1.44g/kg.

The isolated cholesterol sample and the pure cholesterol sample relaxed the smooth muscles of rabbit duodenum only and did not produce any significant effects on any other tissues. However, the similar behaviour of these two cholesterol sampler provided additional support to the phytochemical identity of the isolated sterol as cholesterol.

The microbiological studies indicated that the water extract of the bark has a wide antimicrobial activity against both gram positive and gram negative microorganisms.

All these results have been reported and discussed in this thesis in relation to the traditional uses of the bark. In conclusion it has been derived that these findings offer a

rational basis for the use of the bark by-traditional healers for treating gastrointestinal diseases and microbial infections.

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ABBREVIATIONS and SYMBOLS

°C	-	degree centigrade
g	-	gramme
l	-	litre
mg	-	milligram
ml	-	millilitre
ug	-	microgram

CHAPTER 1

INTRODUCTORY

1. INTRODUCTORY

1.1: INTRODUCTION

Sclerocarya birrea (A. Rich.) Hochst., a Savannah tree, belonging to the family Anacardiaceae, is found in West Africa mainly in the drier Savannah regions extending from Senegal to Ethiopia. In Nigeria it is found in the northern parts of the country, particularly in Sokoto, Zamfara, Kano, Borno, Karasuwa and Bauchi areas (Keay et al, 1964 and Nielsen, 1965). It grows wild in these areas reaching a height of about 40 feet and a girth of 2 to 6 feet, the trunk is covered with a grey fissured bark and its branches bear pale foliage of pinnately compound leaves. Leaflets are elliptic or obovate in shape with short pointed tip. The plant produces greenish white or reddish flowers in December and January when it becomes leafless. Its fruits have a thick skin, yellow colour, acidic taste and pleasant flavour when fully ripe (Hutchinson and Dalziel, 1958).

The various parts of the plant have long been used in traditional medicine for treating a number of different ailments. Hausas call the plant "uwar magani" meaning the mother of medicine, this indicates the usefulness of the different parts of the tree against a broad range of ailments. The leaves of the plant are used for eye diseases, the fruits for boils

and other skin diseases and the bark for the treatment of dysentery and haemorrhoids (Aishatu, 1984). These various medicinal uses of the plant underline its importance as a medicinal plant deserving proper phytochemical and pharmacological evaluation.

1.2: SIGNIFICANCE OF RESEARCH INTO MEDICINAL PLANTS

Random screening of plants depending upon our present knowledge about the traditional uses of plants for treatment of diseases proved to be a correct approach for drug development. The discovery of reserpine from Rauwolfia serpertina root is a typical example. The plant root has been used in India for centuries against snake bites and for the treatment of insane people by traditional healers. It was this application that originally attracted the attention of scientists to the plant and by mid 1950's the plant's main alkaloid reserpine was isolated, which was found to be useful against nervous disorders and schizophrenia (Dasgupta, 1969). More recently still reserpine has been found effective against high blood pressure and other stress diseases (Khayyal, 1978). In the same way traditional healers led the way to the discovery of the pain-killer morphine from Papaver somniferum (Balbaa, 1976), digitoxin, the essential heart stimulator from Digitalis purpurea (Stoll, 1937),

atropine from Atropa belladonna and caffeine from Coffea arabica. From these examples it is obvious that plants produce many organic substances which have potential value in the treatment of diseases. One of the fundamental ways of drug discovery and development is to investigate and study the traditional uses of plants and then screen such plants by phyto-chemical and pharmacological methods. This is how the real value of traditional medicine and medicinal plants in the development of modern medicine can be appreciated and realised. With this point of view the phytochemical and pharmacological evaluation of S. birrea was undertaken in this project.

1.3: HISTORICAL DEVELOPMENT OF HUMAN KNOWLEDGE ABOUT TRADITIONAL MEDICINE AND MEDICINAL PLANTS

Human being, from his earliest existence, is constantly striving for a happy and enjoyable living. Anything good for such a living is directly or indirectly associated with both his body and mind. For a healthy body early man deliberately selected specific plant materials for treating his ailments. The choice was certainly not based upon a knowledge of the plant constituents. It was probably associated initially with seasonal and astronomical changes, which he accepted as factors influencing his life (Seal, 1971). But it is reasonable to suppose that if he found a

certain plant beneficial, he would use it again and thus by means of trial and error select helpful plant material. This assumes that he was able to recognize the same plant every time it was required (Shellard, 1979). These informations and experiences acquired by him could only be communicated by word of mouth, until it was possible for him to communicate by carving pictures on stones or later by writing on paper (Seal, 1971). This is how traditional medicine evolved, which can be described as the total combination of knowledge and practice, whether explicable or not, used in diagnosing, preventing or eliminating a physical, mental or social disease and which may rely exclusively on past experience and observation handed down from generation to generation, verbally or in writing (Sofowora, 1982).

To understand the scope of using plants as a protector of health and even of life itself, one has to follow the progress of medical thought from the primitive stage to the modern age of scientific developments which depended upon man's intelligence and genius in successive periods that followed primitive living. The modern concept of disease evolved through successive stages, namely,
(i) Demonistic theory of the Babylonians and the Chinese (4000 - 1000 B.C.): According to this theory

the disease was caused by a demon. This is still being pursued by most of the aboriginal tribes and primitive races all over the world and subconsciously by many among the so-called advanced races and individuals (Seal, 1971); (ii) Spiritual theory which believed that the spirit of the man after death or the evil spirit, whose very function was to cause harm, was giving the disease. Man therefore tried to protect himself by endeavouring to appease the spirit by various means, for example, magic, folk-lore dance, rites, charm and sometimes torture (Sofowora, 1982); (iii) Deistic theory of the Jewish civilization, when people assumed the existence of God as the creator of the universe and controller of the Destiny and every-thing on earth: According to this theory, it was God himself who administered illness as a punishment for sin. This theory dominated the medical views of the Babylonians, the Persians, the Jews and the Romans for centuries previous to and following the Christian era. The remedies were piety, atonement and prayer;
(iv) Deistic and natural cause theory of the Hindus and Egyptians: The disease, according to them, was associated with both religion and astronomy. Thus they assumed that natural forces are the causes of disease. The Egyptian medicine was more of a craft than a religion as revealed by the Papyri. For instance, Ebers, Hearst and Brugsch Papyri contain some prayers

and incantations. The most important fact is that they also contain prescriptions of Castor oil, Opium, Aloes, Colchicum, Squill and other drugs which are still in use (Arthur and Evelyn, 1965); (v) The theory of rationalism and naturalism of Hippocrates, the Greek physician (460 - 370 B.C.): It was Hippocrates who propounded the natural factors such as sun, air, winds and nature of man as the causes of disease. But these factors were also conceived of as Divine factors. His ideas was that the origin of disease lay in the natural causes and that it was Nature which also cured the patients. The medical application of plants and the physician were her helpmates (Seal, 1971). Hippocrates' Materia Medica consisted essentially of herbal recipes. Some 400 simple remedies have been described by him, some of which included Opium, Mint and Rosemary (Sofowora, 1982).

From what we know from the European history of medicine, the Greeks following Hippocrates excluded the transcendental part of his theory and based their medicine on philosophical reasoning and practised it as a craft of healing art. They made two great discoveries in medical history namely, that the disease is a natural process not basically different from physiological process, and that the human body has an innate healing power that helps to overcome

the effect of disease and to restore the lost balance of health. Thus the seed for a rational system of medicine based on naturalistic theory of disease was sown which gradually grew up into the modern scientific medicine through developments during the past one thousand years (Leake, 1959).

Although as time went on the rational medicine became increasingly efficient, religious medicine continued to spread and reached Rome in 293 B.C. Apollo was supposed to be the God of pestilence (plague) and was to be appeased for relieving a man (Arthur and Evelyn, 1965).

During the second century A.D. the concept of Gallen (131 - 201 A.D.) and his dogmatic system of polypharmacy prevailed. He treated diseases essentially by the use of herbs and those who followed his method eventually developed the sect known as "Eclectics" who employed herbal as well as mineral substances in treating the sick (Sofowora, 1982).

The renaissance which started in the 15th century began with a revolt against dogmatism led by Paracelsus (1493 - 1541 A.D.), one of the outstanding leaders of the renaissance, who not only developed what is known as modern anatomy and surgery but also dealt a death blow to the Galenic system of

polypharmacy. He revised and emphasised the belief of the Alexandrian Surgeon Herophilus that plants were gifts of God. He elaborated this ideology by declaring that God had made plants in shapes which clearly indicated the disease which they could cure. This was the "Doctrine of Signatures", with which he also associated astrological portents (Leake, 1959).

The great helplessness of the sick man, the limitations of medical science and the probability and often nearness of death are some of the reasons for closer relationship of medicine with religion than any other science. This explains why the natural process of disease has been surrounded by a ring of uncertainty or mystery for centuries. Even today there are persons, who are ill with chronic disease and do not get cured or obtain relief with the existing remedies and customary treatments, turn to God or quacks and charms.

However, whatever way the traditional medicine might have been practised throughout the ages or is being practised today, this very primitive method of health management formed the basis of the art of healing, as is practised today.

1.4: DEVELOPMENT OF MEDICINAL PLANT CHEMISTRY

Before 1800 A.D. medicinal plant chemistry science, which is known as phytochemistry, made a slow progress. The early scientific workers in the phytochemical field were almost entirely lacking in the techniques necessary to real progress. Despite this handicap, a number of important medicinal compounds of plant origin were isolated during that period. For example, in 1747 sucrose was isolated from many plants by the German apothecary Marggraf (Balbaa, 1976). The term "tannin" was introduced by Saguin in 1796 to denote substances present in plants, which were able to combine with protein of animal hides, prevent their putrefaction, and convert them into leather. In 1803 narcotine was isolated and in 1805, the German Chemist Serturner detected meconic acid in Opium (Balbaa, 1976). The Portugese chemist Gemiez isolated cinchonine from Cinchona bark in 1811 (Khayyal, 1978). Pelletier and Caventou discovered strychnine in 1818 and brucine in 1919 (Arthur and Evelyin, 1965). Thus a considerable amount of informations as to the existence, mode of occurrence and properties of substances found in some members of the plant kingdom have been accumulated at the end of the first quarter of the nineteenth century.

At the turn of the twentieth century the swift progress of organic chemistry allowed investigation into the secrets of the structures of natural compounds. Some of the synthetics resulted from attempts at duplicating nature by synthesizing isolated plant constituents. This led to the synthesis of many compounds with new physical properties and greater medicinal uses.

Thus it is hoped that the introduction of the newly discovered methods of bioassay and chemical analysis, for example, chromatography, spectrophotometry, infra-red analysis, use of radio-active isotopes, etc., many discoveries will contribute enormously to the field of medicinal plant constituents.

In this context an attempt has been made in this project to widen our knowledge about the plant Sclerocarya birrea (A. Rich.) Hochst, the various parts of which have long been used in traditional medicine in the treatment of dysentery and hemorrhoids. This project looks into the phytochemical constituents of the plant bark and evaluates some of the pharmacological activities of the constituents.

1.5: MEDICINAL USES AND PHYTOCHEMISTRY OF SCLEROCARYA BIRREA (A. RICH.) HOCHST.: A Literature Review

Sclerocarya birrea is a tree member of Anacardiaceae family, locally known in Hausa as "Danya". The various parts of the tree have been widely used by traditional healers in the northern parts of Nigeria for treating a variety of ailments. In Kaduna State, a cold infusion of the tree bark along with local potash is used as a remedy for dysentery and for treating hemorrhoids. The crushed bark is boiled with water over a period of one day and then allowed to cool. The resultant thick mixture is used to sooth the protrusion of the venous haemorrhoidal plexus (Aishatu, 1984). In Bauchi State, the Fulanis use cold water extract of the fine powdered bark for treating vomiting in children, especially when accompanied with severe diarrhoea (Aishatu, 1984). They claim that the symptoms disappear within one day after taking the fine powdered bark mixed with yoghurt about three times a day or more depending on the severity of the diarrhoea and vomiting (Aishatu, 1984). Around Katsina area, Fulanis drink the hot or cold water extracts of the powdered bark for unproductive cough, ulcers, wounds and deep burns (Aishatu, 1984).

In Plateau State, the bark of the tree, which is locally known as ede or yede (Irvine, 1961), is used as an active component or a potion employed in the management of oedema in children (Aishatu, 1984).

The other parts of the tree are also useful. For example, water extract of the leaves is used in treating eye diseases (Aishatu, 1984). A fermented beverage like cider is prepared from the expressed juice of the fruits. The juice is boiled down to a thick black mass and is used by the people of the northern states of Nigeria for sweetening their guinea corn gruel. The kernel (Ku'be in Hausa or Kats in Fulani) is oily and edible. The Fulani use the expressed oil from the seeds to feed their very young children probably to increase appetite (Dalziel, 1937). The ashes are used by the local people along with those of other trees in the process of de-hairing goat skin before tanning (Dalziel, 1937). The fruits are eaten in many parts of Tropical Africa because of their pleasant acid flavour, sometimes sucked or eaten cooked (Irvine, 1961). A clear nearly colourless gum exudate, obtained from injuries on the tree bark, is mixed with soot and is used as ink (Dalziel, 1937).

The multifarious uses of the various parts of the plant indicate that they contain a variety of chemical substances, some of which may have potential

therapeutic value. But the constituents of this plant have not yet been fully studied. It has been reported that the family to which S. birrea belongs contains dyeing and phenolic compounds like tannins some of which cause dermatitis (Trease and Evans, 1978). For example, Rhus typhnia and R. cori contain in their leaves as high as 25% of hydrolysable tannins while Schinopsis lozentzii heartwood contains 24% condensed tannins (Balbaa, 1976). A systematic chemical study of the bark of Sclerocarya caffra, a tree indigenous to South Africa, showed that about 20.5% of tannins is present in its bark when it was collected in October before appearance of leaves (Watt and Breyer, 1962). Glycosides or neutral principles were absent in the bark, while the seeds of Sclerocarya caffra were found to contain a high percentage of non drying edible oil. The fatty acids were identified by gas-liquid chromatography and compared with those of olive oil. Twelve fatty acids were identified (Fernao and Mendes, 1979). A physico-chemical study of the proteins from the fruits of Sclerocarya caffra by fractional precipitation with ammonium sulphate and dilution with water showed the presence of two major components, consisting approximately 90% of the total protein. The defatted meal was analysed for its amino acids composition. The pattern of the

constituents of the essential amino acids in Sclerocarya caffra nuts differed only slightly from that of human milk and from whole hen eggs (Rijke and Joubert, 1967).

A thorough literature survey indicated that there has been no systematic phytochemical study of the chemical constituents of Sclerocarya birrea. Githens (1948) and Oliver (1960) reported that the plant contains tannins.

This apparent absence of any significant phytochemical report on this important medicinal plant has prompted us to undertake this phytochemical and pharmacological evaluation of the bark of the plant which has long been used in African, specifically Nigerian, traditional medicine.

1.6: BOTANICAL IDENTITY OF SCLEROCARYA BIRREA (A. RICH.) HOCHST. (Fig. 1)

Sclerocarya birrea is the only species of Sclerocarya genus found in Nigeria. It is a Savannah tree and belongs to the family Anacardiaceae. The tree is locally known in Hausa as "Danya" or "Daniya", in Fulani as "Edi", in Plateau State as "Edere" and the Shuwa Arabs call it "Homeid" (Irvine, 1961).

Sclerocarya birrea is a species with male and female flowers occurring on separate trees (Keay et al, 1964).

The plant is a tree, growing wild to about 40 feet high and 2-6 feet in girth, with short bole, rounded dense crown, stout branchlets with grey corrugated bark and pale foliage. The trunk is scally and reddish grey in colour (Irvine, 1961). Leaves are compound, occur mostly in a bunch at the tip of the branch; leaflets are pinnate opposite or sub-opposite, seven to ten pairs, elliptic or obovate, shortly cunneate at base, very acute at apex, about 3.5 cm long and 2 cm broad, glabrous, sometimes glaucous with very abscure nerves. Flowers appear when the tree is leafless between December and January, dioecious, greenish-white or reddish in colour, male flowers in short spikes, bracts broadly ovate with membranous margin, stamens 12 or more, inserted on an annular disc. Fruits appear between May and June, obovoid, yellow, thick-skinned, resembling a small mango, about 3-3.5 cm long, glaucous with fibrous pulp, taste acidic with pleasant flavour when fully ripe (Hutchinson and Dalziel, 1958). The plant bark is flat, about 1-2 cm thick, occur in large pieces, 10-15 cm long and 8-12 cm broad. The outer surface is corrugated, reddish grey in colour, while the inner surface is reddish white and shows longitudnal striations. Fracture tough and fibrous. Powder dark brown in colour.



FIG. 1: Sclerocarya birrea (A. Rich.) Hochst. (Anacardiaceae).

A, leaflet, showing venation. B, habbit C, flower D. cross-section of ovary E. fruit.

1.7: AIMS AND SCOPE OF THE PROJECT

The objective of this study is to establish some rationale behind the use of this plant for the treatment of various diseases by traditional healers and to evaluate its medicinal value.

Phytochemical studies on different extracts were done to examine the different chemical constituents of the plant bark. Also some pharmacological actions of the bark's water extracts on isolated tissues were studied.

In view of the fact that the plant bark is widely used by traditional healers for treating microbial infections, the bark's water extract was also tested for antimicrobial properties.

CHAPTER 2

RESULTS AND DISCUSSION

2. RESULTS AND DISCUSSION

2.1: PHYTOCHEMICAL STUDIES OF SCLEROCARYA BIRREA BARK

2.1.1: Preliminary Phytochemical Screening

In order to have an idea of the general chemical constituents of the bark, which may be responsible for its various uses, different quantities of the powdered bark were subjected to various physico-chemical tests. These phytochemical screening tests included the following:

(a) Test for sublimable constituents
(Experimental details, page 73)

A microsublimation tests with the bark powder indicated the absence of any sublimable constituent in the bark of this plant. There is, of course, no report of their presence in this or any other species of the genus Sclerocarya.

(b) Test for carbohydrates
(Experimental details, page 73)

The aqueous extract of the bark was treated with Molisch's reagent when it gave a strong positive test for carbohydrates. Carbohydrates like starch, gums and mucilages are normally present in plant barks. So, its presence in the bark of Sclerocarya birrea was not unexpected. Some of these carbohydrates were later

isolated and characterized from a larger extract of the bark powder (see page 23).

(c) Tests for glycosides

Glycosides are a group of complex substances which include all types of organic substances in combination with various sugars. As such, no common tests are available for their detection in plant materials. In order to examine the presence of some common groups of glycosides the following selective tests were performed:

(i) Test for simple phenolic glycosides
(Experimental details, page 74)

A tannin-free extract of the bark gave no colour reaction with ferric chloride (5%) solution. This indicated the absence of any phenolic glycoside in the bark.

(ii) Test for anthraquinone glycosides
(Experimental details, page 75)

Borntrager's tests were performed with extracts of the bark for the presence of both free and combined anthraquinones. But both types of anthraquinones appeared not to be present in the bark.

(iii) Test for flavonoid glycosides
(Experimental details, page 76)

All tests for the different classes of flavonoid glycosides gave negative results, except for anthocyanins which were found to be present in good quantities. Anthocyanins are a group of water soluble plant pigments. They are all based chemically on a single aromatic structure which is that of cyanidin. Different types of anthocyanins are derived from this pigment by addition or substraction of hydroxyl groups or by methylation (Harborne, 1973).

(iv) Tests for cyanophore and cardiac glycosides and saponins
(Experimental details, page 77)

Extracts of the bark powder were separately tested for cyanophore glycosides, cardiac glycosides and saponins respectively by various chemical tests. But none of these compounds were found to be present in the bark of S. birrea. No such compound has so far been reported from any Sclerocarya species. This fact indicates that these plants do not produce or accumulate any glycosidic compounds.

(d) Tests for sterols
(Experimental details, page 79)

Various standard tests for the detection of the presence of sterols were carried out with extracts of the bark. All these tests yielded positive results indicating the presence of these compounds in the bark. Some of them were later isolated from a larger extract of the bark and were characterized and identified by the use of various physico-chemical techniques (see page 31). Some of these compounds were also pharmacologically and microbiologically evaluated to substantiate or discard the rationale of the uses of the bark in traditional medicine (see page 43).

(e) Tests for tannins
(Experimental details, page 80)

A water extract of the bark gave positive result with ferric chloride (5%) solution, indicating the presence of tannins in the bark. Phlobatannins was also indicated to be present by another test.
Tannins are widely distributed in the plant kingdom. Almost every family of plants embodies species which contain tannins. Inspite of their wide distribution, however, only few plants synthesize sufficient amounts of tannin or contain quantities which are large enough to make them worthwhile sources of commercial tannins.

Further isolation and characterization of tannins were carried out from a large extract (see page 25).

(f) Test for alkaloids
(Experimental details, page 80)

A modified form of thin-layer chromatographic (TLC) method described by Farnsworth and Euler (1962) was used to test for the presence of alkaloids in the bark. But alkaloids appeared not to be present in the bark. No alkaloid has so far been reported present in any Sclerocarya species.

2.1.2: Detailed Phytochemical Studies on Larger Extracts of the Bark

As the preliminary phytochemical screening of the extracts of the bark indicated the presence of a number of chemical constituents in it, larger extracts were made to isolate and characterise some of them. In this effort the bark powder was extracted with three different solvents which included water, ethanol (70%) and petroleum ether (60-80°). The choice of the solvents was dictated by the type of compound intended to be isolated, as indicated by the preliminary screening.

(a) Extraction with water
(Experimental details, page 82)

The water extract of the bark (250 g) was shaken with lead acetate solution (5%) to remove tannins and other phenolic compounds and filtered. The filtrate which appeared (on TLC) to contain a mixture of compounds, was divided into two arbitrary portions for further investigation. Each portion was then treated differently. Each of these portions yielded one major compound. The two compounds, designated as compound A and compound B, were later characterised as follows:

(i) Isolation and characterisation of sucrose
(Compound A) (Experimental details page 82)

On concentration to a small volume one of the above portions produced a white crystalline solid after standing for 48 hours at room temperature. The crystals were separated by filtration, purified by recrystallisation from hot water and designated as compound A. This compound was later characterised as Sucrose by its sweet taste, m.p. and mixed m.p. (158-60°) with authentic sample (m.p. 160°), positive Molisch's test, Fehling's test (after hydrolysis) and co-chromatography on TLC and PC with authentic sample of sucrose. The compound gave one spot corresponding to that of

sucrose. Its hydrolytic products were also co-chromatographed on TLC and PC along with authentic samples of sucrose, glucose and fructose. The hydrolysate gave two spots corresponding to those of glucose and fructose.

Isolation of sucrose from a bark is not new in phytochemistry because it is almost universally present throughout the plant kingdom in the juices, fruits, stems, leaves, roots, flowers and seeds of plants.

Sucrose (m.p. 160°) is a non-reducing sugar which was first isolated in 1747 by the German Apothecary, A.S. Marggaraf, from a number of plants including the sugar beet (Balbaa, 1976). On enzymatic or acid hydrolysis sucrose yields equi-molecular quantities of the pyranose forms of D-Glucose and D-Fructose.

The presence of sucrose in the bark of Sclerocarya birrea may have contributed to some extent to its use in traditional medicine, particularly in the treatment of diarrhoea and vomiting, in which case the sucrose in the bark extract, which is given to the patient, helps in replacing the electrolyte lost due to dehydration.

(ii) Isolation and Characterisation of Compound B
(Experimental details, page 83)

The second portion was treated with disodium hydrogen phosphate and filtered. On cooling, the

concentrated filtrate solidified. It was then dissolved in ethanol and on addition of acetone it formed a dirty precipitate which yielded white flakes after several washings with petroleum ether. These tasteless sparingly soluble (in water) flakes gave positive Molisch's and Fehling's tests. All tests carried out on this compound, designated as compound B, indicated it to be a gum. As gums are of common occurrence in plant barks and since they do not normally have any direct therapeutic properties, no attempt was made to determine the chemical nature of the gum, a complex polysaccharide.

(b) Extraction with ethanol (70%)
(Experimental details, page 84)

An ethanolic extract of the powdered bark (500 g) yielded one phenolic compound, designated as compound C, which was isolated and characterised as follows:

Isolation and Characterisation of Gallic Acid
(Experimental details, page 84)

The concentrated ethanolic extract was hydrolysed with 2M hydrochloric acid. Yellowish white crystals were obtained from an ether extract of the hydrolysate. The crystals were later purified by using preparative paper chromatography technique. The crystals (m.p. 250-54°) were later characterised by various

physico-chemical tests and identified as Gallic acid.

Gallic acid (m.p. 253) is a polyhydroxy phenolic acid, readily soluble in water. When heated at its melting point it forms pyrogallol and carbon dioxide. It is a powerful reducing agent and produces a blue colour with ferric chloride solution (Finar, 1977).

Gallic acid is found to be present in many woody plants, bound as hydrolysable tannins. Hydrolysable tannins is a class of tannins characterised by having, as a central core, a polyhydric alcohol, such as glucose, the hydroxy groups of which are esterified either partially or wholly by several molecules of phenolic acids, such as gallic acid. Two main types of hydrolysable tannins have been recognised, derived respectively from gallic acid and ellagic acid. These are known as (1) gallitannins and (2) ellagitannins (Balbaa, 1976). Presence of tannins has been reported in the bark by Githen (1948) and Oliver (1960). But the type of tannins was not identified. This investigation reveals (as indicated above) that only hydrolysable tannins are present in the bark. The amount of hydrolysable tannins was found to be more than 20% (see page 40). This finding, which is apparently the first report about tannins in this plant, compares very well with those of other plants of the family Anacardiaceae. For example,

leaves of Rhus typhnia and R. coriaria contain 25% hydrolyzable tannins and Schinopsis lorentzii heart wood contains 24% condensed tannins (Balbaa, 1976). This occurrence of tannins in Anacardiaceae plants appears to be a common characteristic of this family. This fact may have some chemotaxonomic significance.

The almost universal occurrence of tannins in plants has led some plant physiologists to believe that these compounds serve a fundamental role in the physiology of the plant. The fact that tannins are generally most abundant at sites of intense metabolic activity, such as meristematic regions, unfolding flowers or leaf buds and developing fruits, had led other researchers to conclude that the tannins are waste-products of metabolism (Goodwin, 1965).

Although found in immature fruits, tannins usually disappear during the ripening process. It has been suggested that the fruits employ the energy supplied by the oxidation of these tannins in its metabolic processes and also that the tannins are the source of fruit acids (Harborne, 1964).

Another concept which has been proposed, is that tannins probably serve as a protective to the plant (plant antiseptics) during certain stages of growth (Burton and Nurstein, 1956).

Since all the regions of intense metabolic activity are characterised by a relative high demand for oxygen and by relatively high rates of respiration, and since the tannins, through their phenolic constituents, have a great affinity for oxygen, it seems reasonable to postulate that they may have some common function in respiratory activity, i.e. in the mechanism of hydrogen transfer in plant cells (Dean, 1963). Tannins, because of their protein binding capacity, inhibit many enzyme systems. But it appears unlikely that this property is shown in the living cells, for here it must be assumed that the tannins are present in organelles separated from the cytoplasm. However, their ability to inhibit enzymes may be important in dying or dead cells, and more especially in aiding the resistance of such cells to attack by fungi and other pathogens (Bhatia, 1962).

If it is assumed that tannins are metabolic waste products and that they are of little use to the plant, it is logical to believe that leaves, which normally destined to be separated from the stems, would represent a convenient method of tannin release from the plant. The greatest concentration of tannins appear to occur in dead or dying cells. Whether the increase in tannins in aging tissues has any effect on the normal metabolic function of cells, however, is

not known. It seems more likely that the two are parallel rather than sequential processes (Balbaa, 1976).

Finally, it should be recognised that tannins play an important part in the acceptance of many foods and beverages by consumers. Many unripe fruits are unacceptable, for example, not only because of their lack of sweetness, but also because of their astringency, that is their ability to produce a feeling of dryness in the mouth, due to the presence of tannins of an appropriate amount. As the fruits ripen, such tannins are apparently further polymerized to give substances which are less effective as astringent agents. Astringency is also important in beverages, especially, tea, cocoa and wines. In these cases complete lack of astringency would lead to lack of distinguishing taste and flavour.

The fact that tannins possess ample antiseptic properties and are capable of precipitating proteins rendering them resistant to proteolylic enzymes justifies the use of Sclerocarya birrea bark extracts in traditional medicine for the treatment of various microbial infections of the skin and the gastrointestinal tract (see page 52).

(c) Extraction with Petroleum Ether (60-80°)
(Experimental details, page 86)

A concentrated petroleum ether extract of the powdered bark produced a white precipitate after standing for 48 hours at room temperature. This white precipitate was discarded and the supernatant liquid was further concentrated and subjected to column chromatography. Petroleum ether, chloroform and methanol were employed as successive eluting solvents. Each eluate was examined separately and two compounds designated as compound D and compound E were isolated, purified and then characterised as follows:

(i) Isolation and Characterisation of Cholesterol
(Compound D) (Experimental details, page 87)

The concentrated petroleum ether eluate was subjected to saponification process and then was extracted with ether. The ether extract was evaporated to dryness. The residue was dissolved in hot methanol and allowed to cool down at room temperature when few drops of acetone were added to it. After standing for 12 hours, the mixture yielded a good quantity of white crystals. The crystals were separated from the mixture by filtration and purified by recrystallisation from ether. The crystals were designated as compound D. The compound gave a positive

Liebermann-Burchard and Salkowski tests for sterols. Its i.r. spectrum corresponded with that of cholesterol (Fig. 2 and 3). When the compound was co-chromatographed on TLC along with authentic sample of cholesterol it gave one spot which corresponded to that of cholesterol. It had a mixed m.p. of 148-49° with authentic cholesterol sample (m.p. 149°). Thus compound D was characterised as cholesterol.

(ii) Isolation and Characterization of Compound E (Experimental details, page 88)

The chloroform eluate from the column was evaporated to dryness. The residue was dissolved in hot methanol and the solution left to cool at room temperature. Then steam was passed through the mixture until it became saturated with steam when a white fluffy crystalline precipitate was formed.

The precipitate was separated from the mixture by filtration and purified by recrystallisation from hot methanol. The precipitate was designated as compound E. The compound (m.p. 78°) gave positive results with Liebermann-Burchard and Salkowski tests, indicating it to be a steroidal compound. Its i.r. spectrum was almost similar to

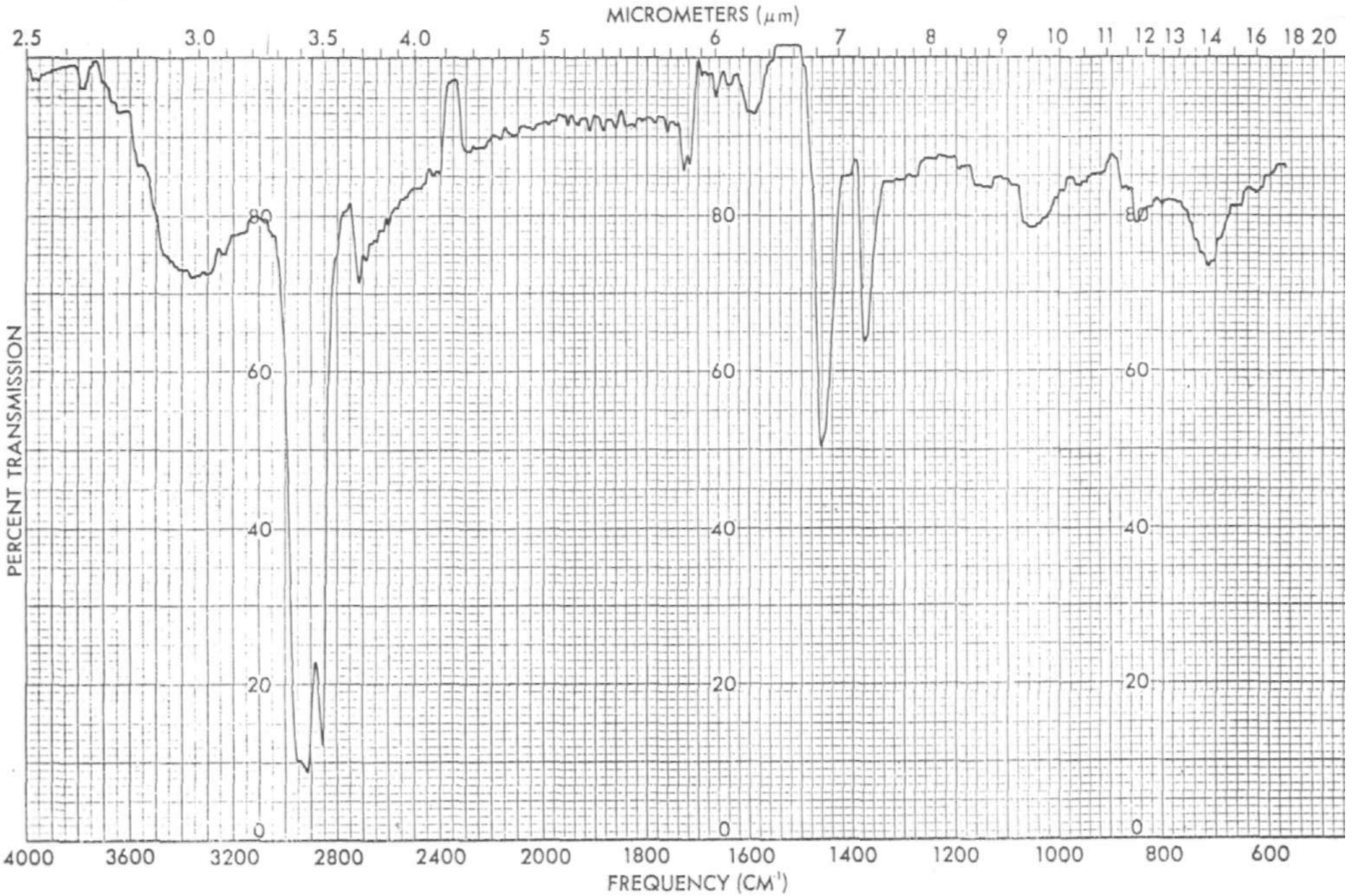


FIG. 2: I.R. SPECTRUM OF COMPOUND D (ISOLATED CHOLESTEROL)

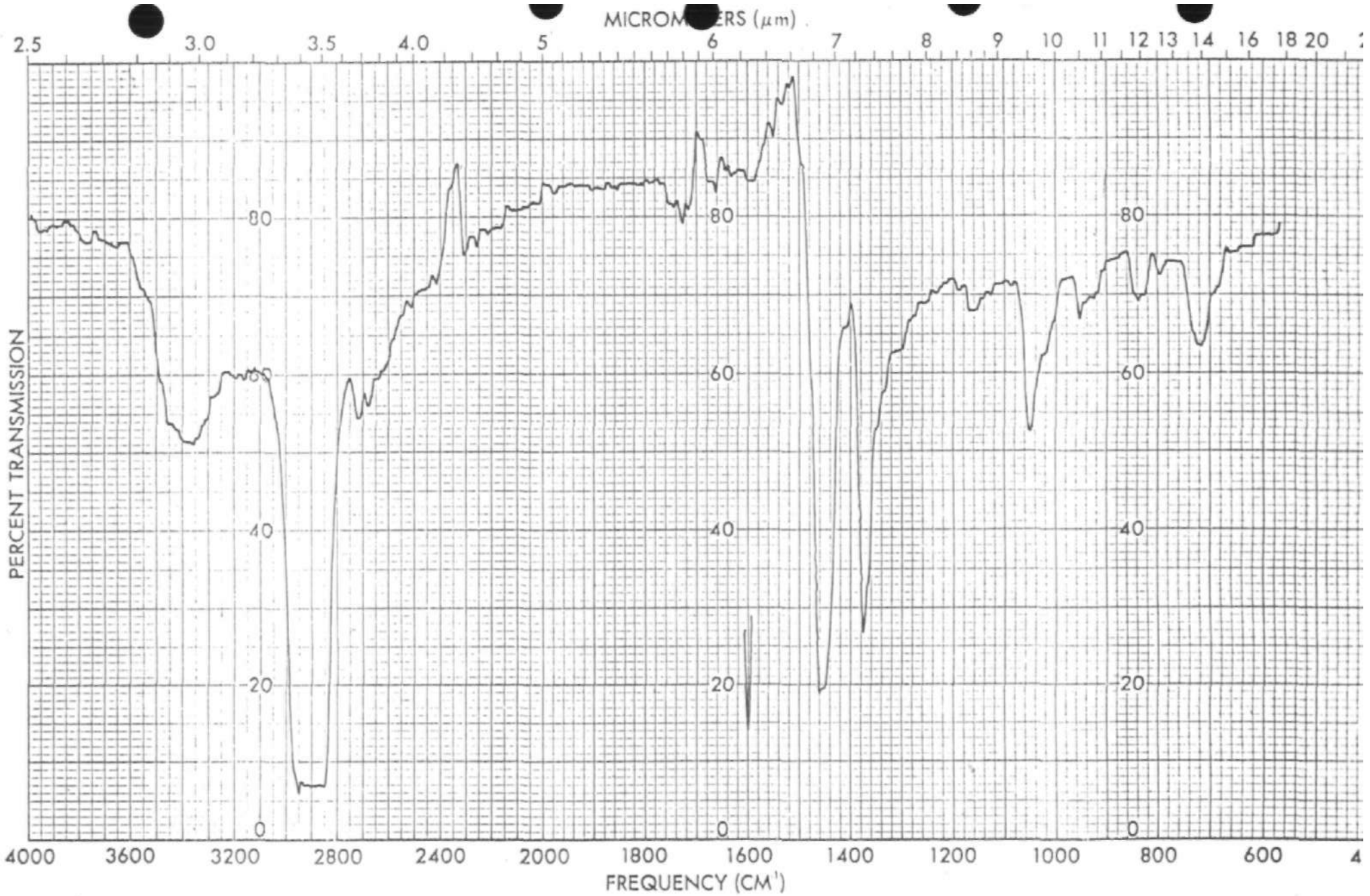


FIG. 3: I.R. SPECTRUM OF PURE CHOLESTEROL

that of cholesterol but had an additional prominent peak at 1720 cm^{-1} (Fig. 4). Since this peak is usually characteristic of a carbonyl (C=O) group, the compound was tentatively called a cholesterol ester. Small quantity of the isolated compound and lack of adequate facilities prevented its further characterisation and identification or any pharmacological study on it.

However, the isolation of cholesterol from Sclerocarya birrea bark is interesting and significant because, cholesterol has aroused scientific curiosity, since its discovery in vertebrate tissues toward the end of the eighteenth century. The brain, spinal cord and gallstones are particularly the rich sources. About 17% of the solid matter of the brain is free cholesterol, while the blood plasma contains mainly esterified cholesterol (200 mg/100 ml) as one of the constituents of plasma lipoproteins (Joseph, 1980).

Although animals absorb ingested cholesterol, biological studies clearly show that the mammalian organism is capable of the total synthesis of cholesterol from nonsteroidal components (Burger, 1970).

Cholesterol, which is perhaps the most important of all sterols, was isolated first from gallstones by Paulleitier de La Salle, in about 1770. In 1815,

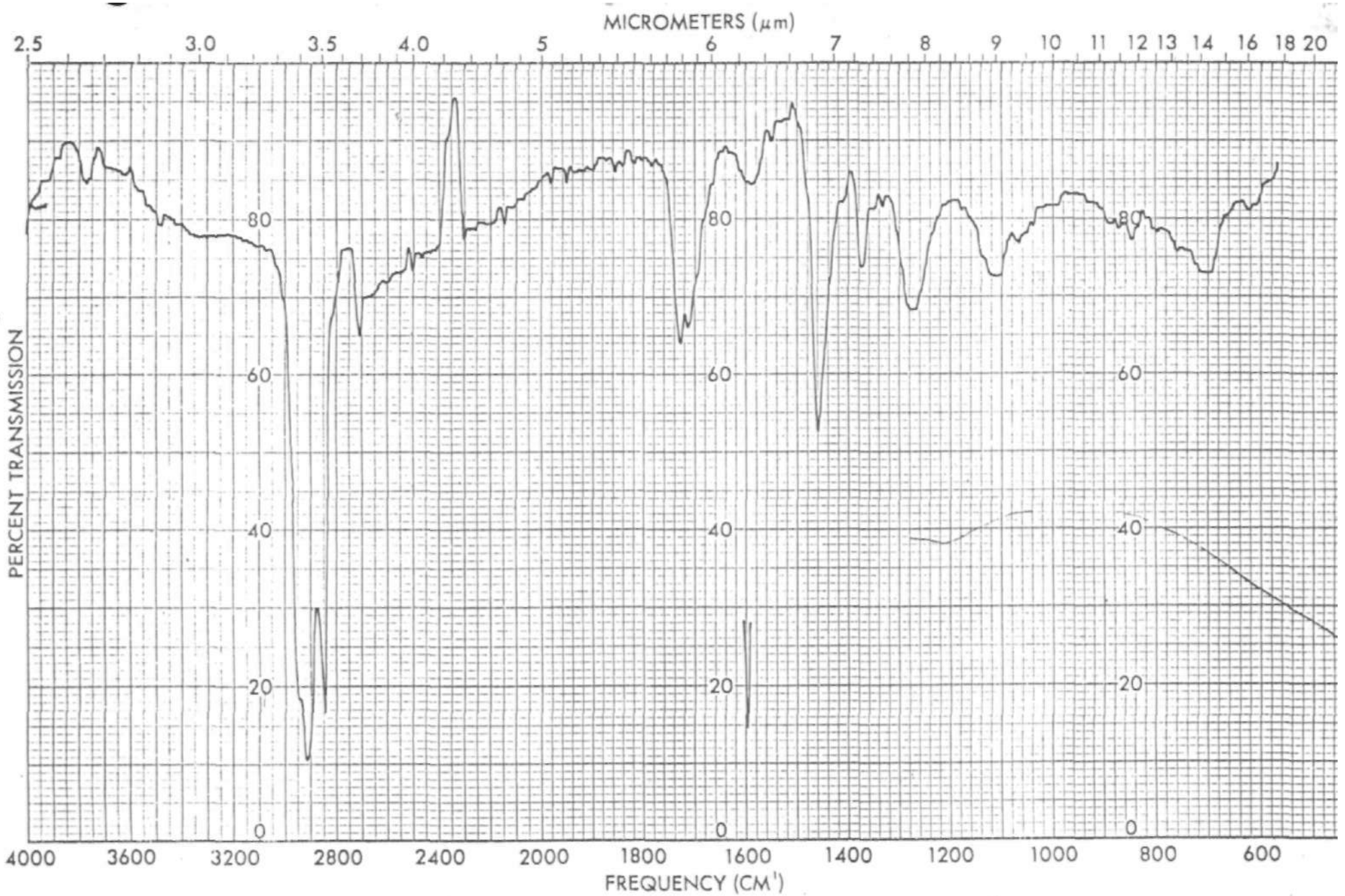


FIG. 4: I.R. SPECTRUM OF COMPOUND E (CHOLESTEROL ESTER)

Chevreul showed that cholesterol was unsaponifiable, and he called it cholesterin (chole, bile; stereos, solid). In 1859, Berthelot established its alcoholic nature, and since then it has been properly designated as cholesterol (Charles and Ole, 1971).

Cholesterol serves mainly a structural role in mammals; it is a major component of cellular and subcellular membranes and is a component of nerve tissues. Cholesterol also is the biosynthetic precursor of all steroid hormones and bile acids (Khayyal, 1978).

The involvement of cholesterol in atherosclerosis has also stimulated a tremendous amount of research in this area. Atherosclerosis, is a disease of the intima (lining) of the arteries. This disease is insidious in that it damages arteries progressively over a long period of time but usually only becomes symptomatic when it is far advanced. Atherosclerosis is caused by the formation of cholesterol containing lesions that proliferate into the arterial lumen thus partially, or completely, blocking blood flow. This in itself may give symptoms, or the lesions thus formed may serve as foci for thrombosis. Ischemic heart disease is usually the result of a thrombotic obstruction that occurs on a coronary artery lesion (Constaninides, 1965).

Cholesterol also serves as a starting material for the synthesis of physiologically active steroids, i.e. those steroids occurring naturally and having a physiological role in some organisms, plus those steroids synthesized for the purpose of imitating or competing with steroids occurring naturally in that organism, for example, estrogens, androgens, progestins and corticosteroids (Klyne, 1965).

Thus it seems reasonable to discuss the biosynthesis, chemistry and pharmacology of cholesterol.

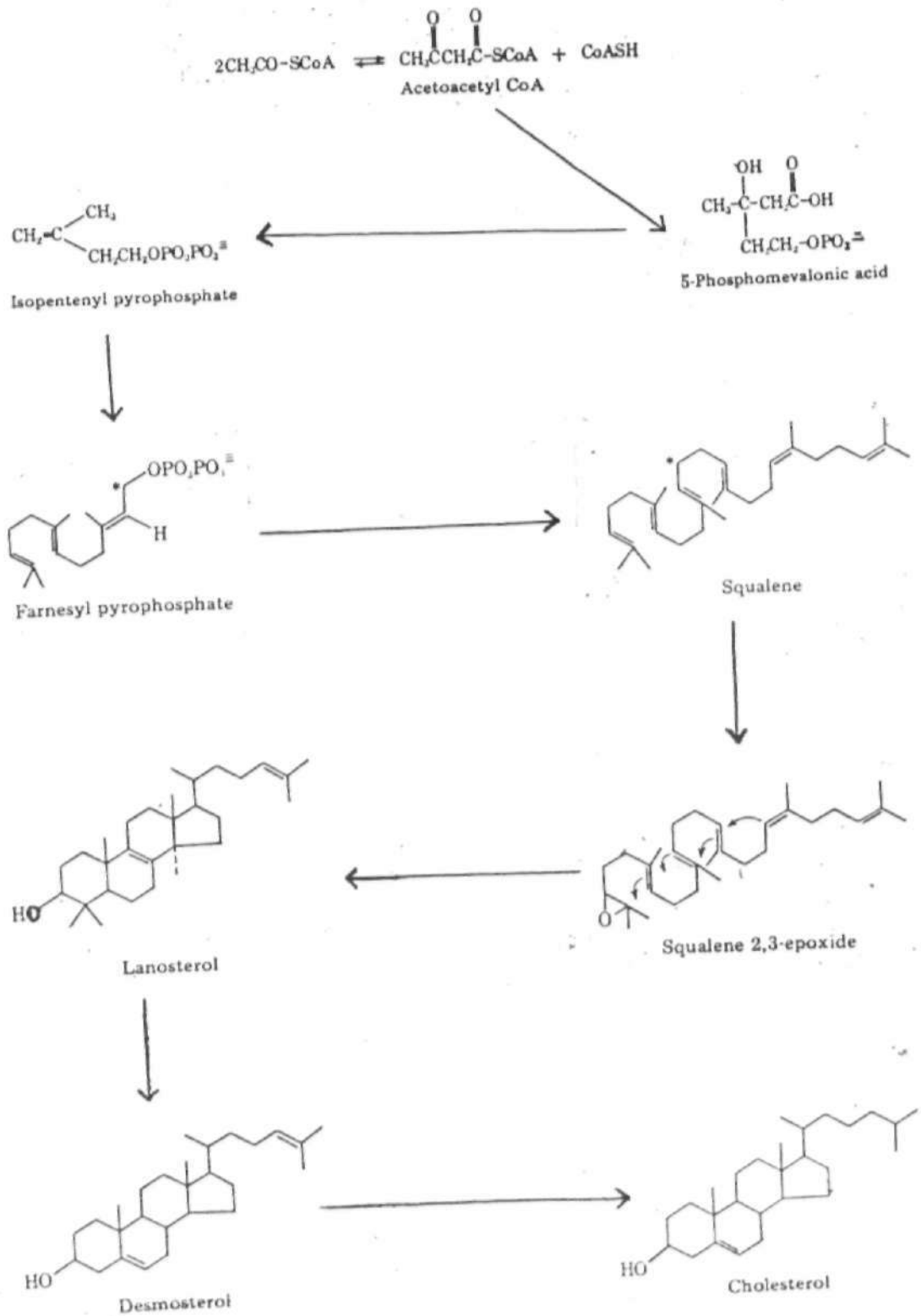
A knowledge of the biosynthesis of cholesterol is necessary for a full understanding of the functioning of the human body. Such a knowledge would be useful, for example, in coping with the condition known as atherosclerosis in which the regulation of cholesterol and other lipids has gotten out of hand and resulted in their deposition on the inner walls of the blood vessels.

In man cholesterol is derived from two sources; (a) exogenous, i.e. from the yolk of egg and animal fats, and (b) endogenous one. Dietary cholesterol is absorbed in an esterified form directly into the lacteals; and it is interesting to note that even if little cholesterol is available from the dietary source, considerable amount can be synthesized endogenously (1-5 gm/day) in such structures as liver, intestines and skin (Dasgupta, 1969).

An inverse relation generally exists between the rate of synthesis and dietary intake of cholesterol, for high cholesterol level inhibits endogenous production (Dasgupta, 1969).

For cholesterol biosynthesis acetyl Co A serves as the building material. Mevalonic acid formation is the first step in this direction which is oxidized to isoprenoid units (C_5H_9), which condense through the formation of farnesyl pyrophosphate to squalene ($C_{30}H_{50}$) which is oxidised to lanosterol ($C_{30}H_{50}O$); and demethylation of the latter produces 14-desmethyl lanosterol. Cholesterol ($C_{27}H_{46}O$) is ultimately obtained from its immediate precursor desmosterol which is derived from desmethyl lanosterol through the intermediaries of symosterol and cholastadienol (Joseph, 1980). It is now clear that dietary restriction of cholesterol has limited value, for endogenous synthesis (about 1.5 to 2 gm per day) rather than dietary intake primarily affects the concentration of cholesterol in the blood (Dasgupta, 1969).

Although cholesterol occurs primarily in the animal kingdom, it is now established that cholesterol also occurs in some plants. It has been identified as the major sterol in many species of Rhodophyta (Gibbons, 1967). It is also found in some members of Chlorophyta and Phaeophyta (Alcaide, 1968). Among higher plants it is found in Digitalis purpurea (Jacobsohn and Frey, 1967), Solanum tuberosum, Dioscorea spiculiflora



Biosynthesis of Cholesterol

(Raymond, 1963), Sinapis alba seeds and Avena sativa stem (Donald, 1981).

Cholesterol (m.p. 149) occurs as white or faintly yellow, almost odorless, leaflets or granules. It usually acquires a yellow to pale - tan colour on prolonged exposure to light or to elevated temperatures. It is insoluble in water and is soluble in alcohol and most organic solvents.

Cholesterol itself does not play a direct vital role in pharmaceuticals. It imparts water-absorbing power to ointments and is employed as an anti-irritant in spirituous "hair tonics" in concentrations of up to 2%. It is also incorporated in many cosmetic ointments (Martindale, 1983). The pharmaceutical value of cholesterol lies on the fact that cholesterol provides a practical starting material for the synthesis of bile acids, D vitamines and sex hormones. Thus with the world-wide sale of hormones estimated at $1500 million per annum (Trease and Evans, 1983), the pharmaceutical importance of cholesterol cannot be over emphasised.

Occurrence of cholesterol in the bark of Sclerocarya birrea has added extra value to the multifarious use of the bark in traditional medicine. Its various pharmacological effects, as established before and also demonstrated by the pharmacological

tests carried out during this investigation (see page 43), justify the use of the bark in traditional medicine.

2.1.3: Quantitative Estimation of Some Constituents of the Bark of Sclerocarya Birrea

Since tannins and cholesterol appeared to be the major active constituents of the bark, attempts have been made to determine their quantities in the bark.

(a) Quantitative estimation of tannins

(Experimental details, page 89)

Tannins were percipitated quantitatively with copper acetate (5%) solution. The precipitate consisting of copper tannate was collected on an ashless filter paper, washed with water till free from copper acetate and ignited. The residue of copper oxide was completely oxidized by the addition of a few drops of nitric acid. The tannins content was calculated using the equivalence that one part of copper oxide is equivalent to 1.305 parts of tannins (Balbaa, 1976). The percentage of tannins in the plant bark was found to be 20.88%.

Tannins containing sugar in the molecule are hydrolysed by tannase, an enzyme which occurs in molds

such as Penicillium and Aspergillus. The mold splits off the sugar and utilize it, leaving the gallic acid and the ellagic acid unchanged (Brown, 1964).

The simplest gallic acid ester of glucose found in plants is B-glucogallin, which has been isolated from Chinese rhubarb (Rheum officinale) and myrobalans (Terminalia chebula) (Olis, 1961). The only other gallotannin which has been carefully investigated is B-hamamelis tannin from the whitch hazel (Hamamelis virginiana) (Balbaa, 1976).

(b) Quantitative estimation of cholesterol
(Experimental details, page 90)

The method used to estimate the quantity of cholesterol in the plant bark involved the rapid precipitation of cholesterol with digitonin and aluminium chloride in the presence of dilute hydrochloric acid. The digitonides formed was then cleaved to their components with hot dimethyl sulfoxide, when the solution was cooled to room temperature. The cholesterol precipitated out and was extracted with hexane. The hexane extract was evaporated to dryness, the residue which was pure cholesterol was weighed and the percentage of cholesterol in the plant bark was calculated as 0.034%.

The ability of cholesterol to form a sparingly soluble complex with an equivalent amount of digitonin was first noted by Windaus in 1910. Some nonsteroid alcohols also form digitonides, but these differ in solubility and stability from those of the sterols. Thus, the precipitation of a sterol with digitonin is an effective method of isolation and purification of the sterols of the 3 -hydroxy series (Windaus, 1910).

2.2: PHARMACOLOGICAL STUDIES OF SCLEROCARYA BIRREA BARK

In order to evaluate and study the effects of the constituents of the bark on animal systems and microorganisms and also to verify the rationality of the use of the bark extracts for various ailments in traditional medicine, crude water extracts of the bark and isolated/pure cholesterol were pharmacologically and microbiologically tested on isolated tissues, living animals and microorganisms. The results are presented below:

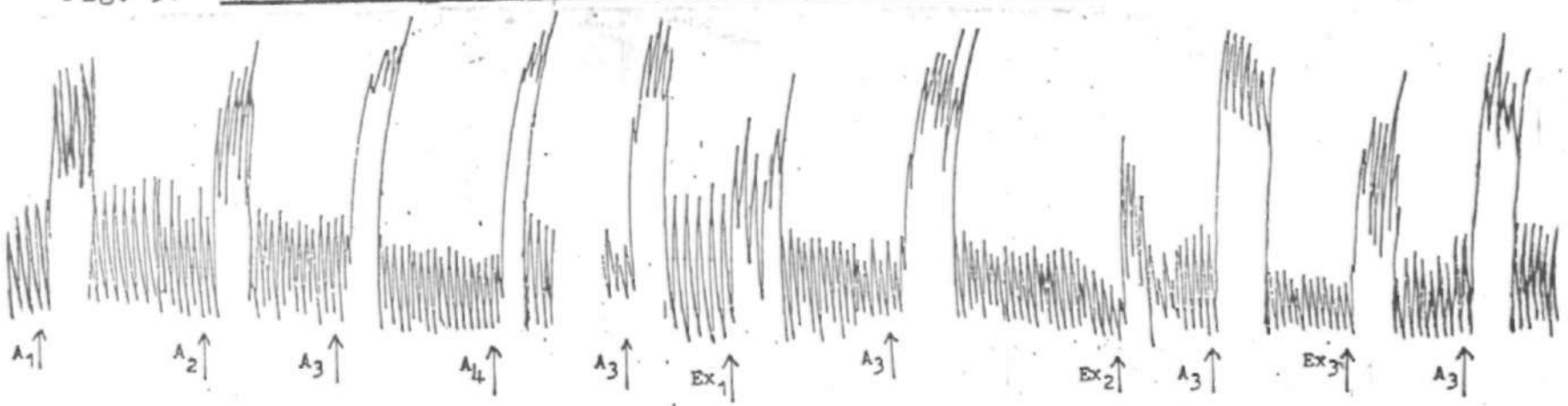
2.2.1: Pharmacological Studies on Isolated Tissue Preparations

(Experimental details, page 95)

(a) Rabbit duodenum

Both the spontaneous contraction and acetylcholine (5 ug/ml) induced contractions were inhibited by atropine (10 ug/ml). The contractions induced by histamine (5 ug/ml) were inhibited by promethazine (2.5 ug/ml). The crude water extract (200 - 300 ug/ml) caused contraction of the rabbit duodenum. Atropine (5 ug/ml) inhibited the contraction caused by the crude water extract (200 ug/ml) (Fig. 5). A pure sample of cholesterol (200 - 500 ug/ml) caused the relaxation of the rabbit duodenum and also inhibited the contraction produced by acetylcholine (5 ug/ml) and histamine (2.5 ug/ml) (Fig. 6). A similar effects

Fig. 5: The effect of the crude water extract of the bark on rabbit duodenum



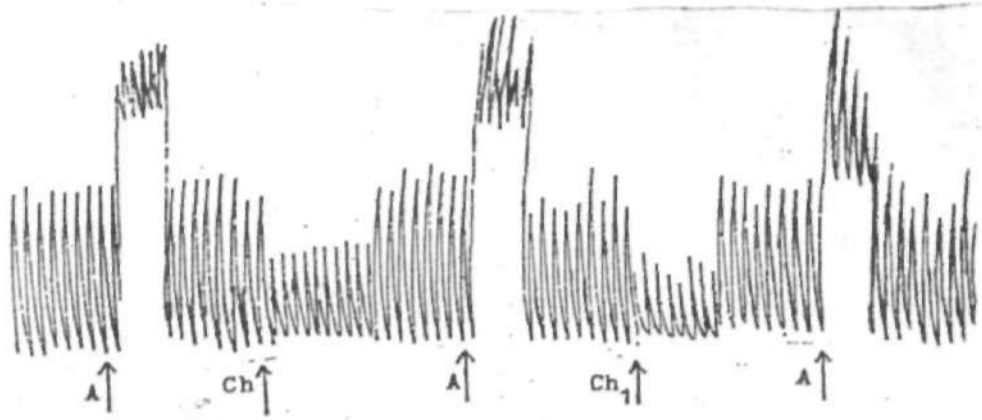
1. Effects of acetylcholine and crude water extract on rabbit duodenum.
2. Effects of atropine on the actions of acetylcholine and crude water extract on rabbit duodenum.

A_1 = (Acetylcholine) = 2 mg/ml, A_2 = 3 mg/ml, A_3 = 5 mg/ml

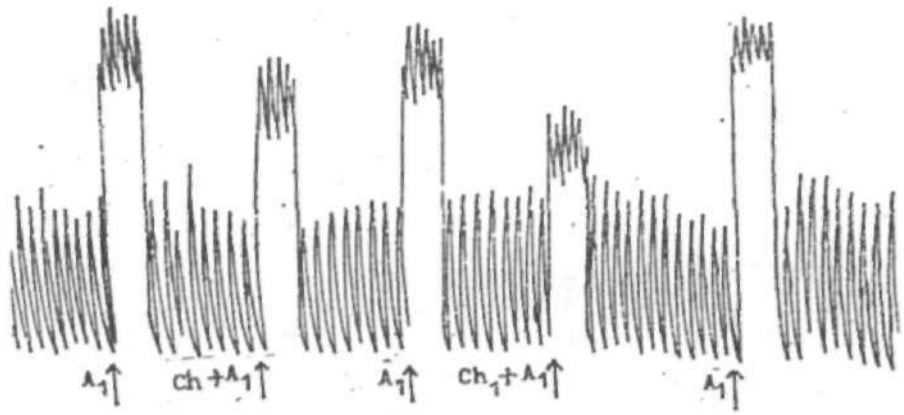
At = (Atropine) = 5 mg/ml, At_1 = 10 mg/ml

Ex_1 = (Crude water extract) = 50 mg/ml, Ex_2 = 100 mg/ml, Ex_3 = 200 mg/ml.

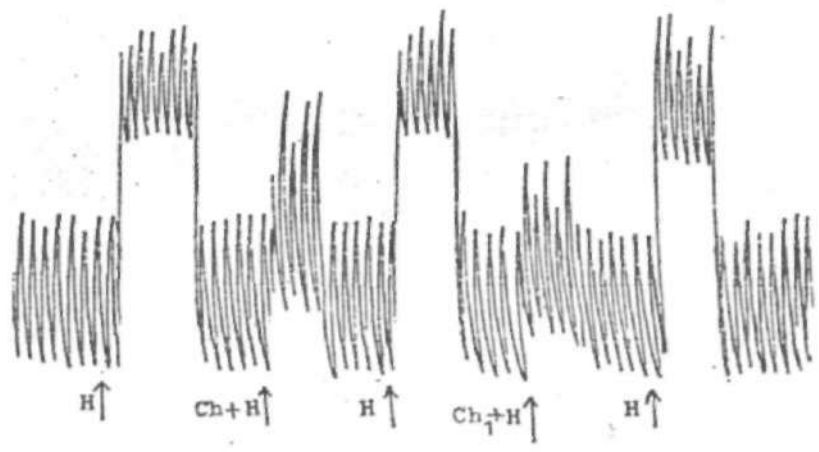
Fig. 6: The effects of cholesterol on rabbit duodenum



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1. Effects of cholesterol on rabbit duodenum
2. Effects of acetylcholine on the action of cholesterol on rabbit duodenum.
3. Effects of histamine on the action of cholesterol on rabbit duodenum.

Ch (Cholesterol) = 200 ug/ml, Ch_1 = 500 ug/ml

H (Histamine) = 2.5 ug/ml

A (Acetylcholine) = 5 ug/ml, A_1 = 10 ug/ml.

were observed when a sample of the isolated cholesterol was similarly tested. This similar behaviour of these two cholesterol samples provided additional support to the phytochemical identity of the isolated sterol as cholesterol.

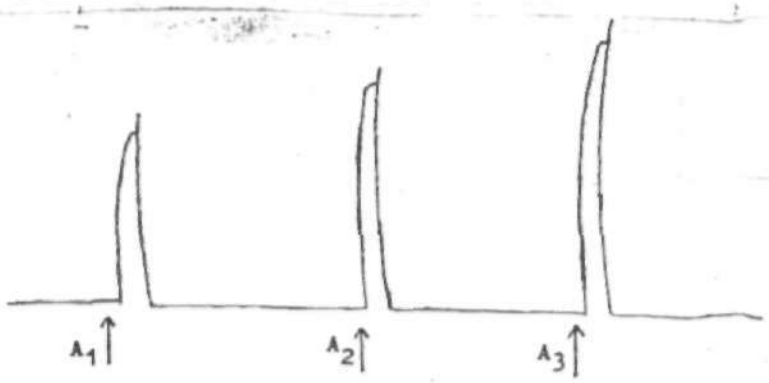
(b) Guineapig ileum

Crude water extract (200 - 600 ug/ml) caused contraction of the guineapig ileum. The contractions produced by acetylcholine (5 ug/ml) were inhibited by atropine (10 ug/ml). Atropine (5 ug/ml) inhibited the contraction caused by the crude water extract (500 ug/ml). The contractions produced by histamine (5 ug/ml) were inhibited by promethazine (2.5 ug/ml). Promethazine (2.5 ug/ml) inhibited the contraction caused by the crude water extract (500 ug/ml). Cholesterol had no effect on the guineapig ileum and also had no effect on the contractions produced by acetylcholine (5 ug/ml) and histamine (2.5 ug/ml) (Fig. 7).

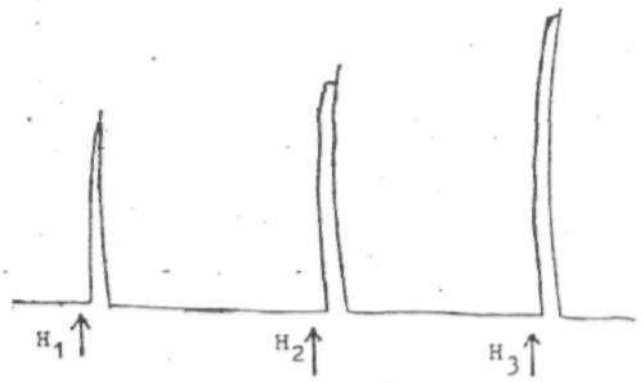
(c) Rat uterus

Acetylcholine (1-5 ug/ml) contracted the rat uterus muscle, similarly the crude water extract (100 - 300 ug/ml) contracted the isolated uterus. Atropine (2.5 ug/ml) inhibited the effect of

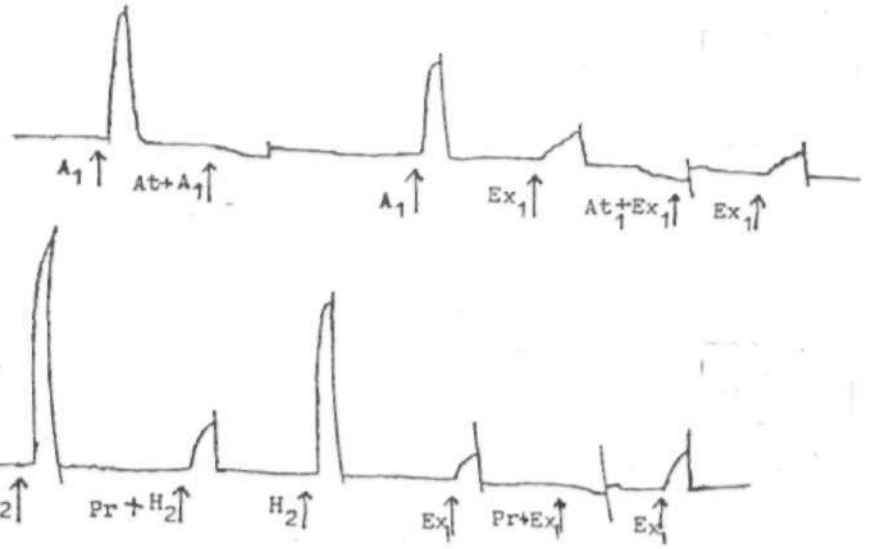
Fig. 7: The effects of the crude water extract of the bark on guineapig ileum



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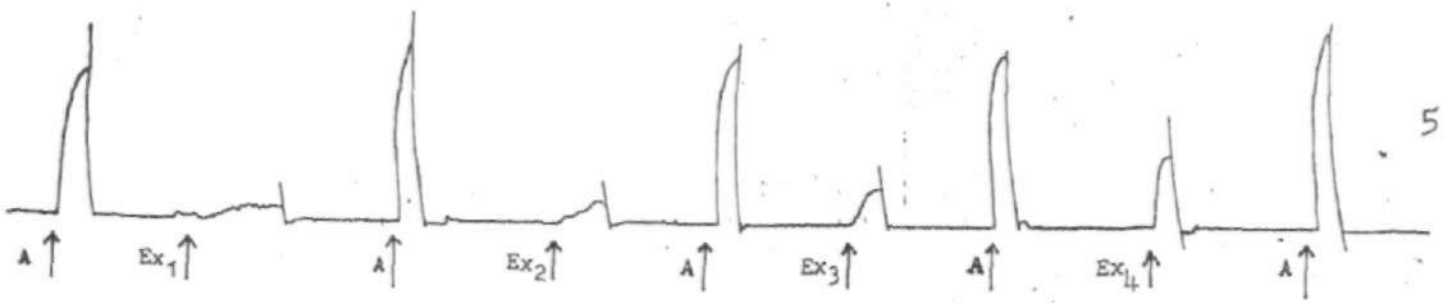


3

4

1. Effects of Acetylcholine on guineapig ileum
2. Effects of Histamine on guineapig ileum
3. Effects of Atropine on the actions of Acetylcholine and extract
4. Effects of promethazine on the actions of histamine and water extract
5. Effects of crude water extract on guineapig ileum.

Fig. 7: The effects of the crude water extract of the bark on guineapig ileum (cont'd)



A_1 (Acetylcholine) = 2 ug/ml, A_2 = 3 ug/ml, A_3 = 5 ug/ml

H_1 (Histamine) = 5 ug/ml, At_1 = 10 ug/ml

Pr (Promethazine = 2.5 ug/ml

Ex_1 (Crude water extract) = 50 ug/ml, Ex_2 = 100 ug/ml
Ex_3 = 200 ug/ml, Ex_4 = 400 ug/ml.

(c)

acetylcholine (5 ug/ml). Atropine (5 ug/ml) also inhibited the contraction produced by the crude water extract (200 ug/ml). Cholesterol (25 - 100 ug/ml) had no effect on the uterus and also had no significant effect on the contractions produced by acetylcholine (2.5 ug/ml) and oxytocin (50 ug/ml) (Fig. 8).

(d) Frog rectus abdominis muscle

Acetylcholine (1-5 ug/ml) caused contraction of the frog rectus abdominis muscle. The crude water extract (100 - 200 ug/ml) caused contraction of the muscle. d-Tubocurarine (2.5 ug/ml) inhibited the contraction caused by acetylcholine (5 ug/ml). d-Tubocurarine (2.5 ug/ml) also inhibited the muscle response to the crude water extract (200 ug/ml). Cholesterol had no effect on the frog rectus abdominis muscle and also had no effect on the contractions produced by acetylcholine (5 ug/ml) and histamine (2.5 ug/ml) (Fig. 9).

2.2.2: Acute Toxicity Studies

(Experimental details, page 97)

In order to study the toxicity of the bark constituents on animals, some toxicity studies were

Fig. 8: The effect of the crude water extract of the bark on rat uterus

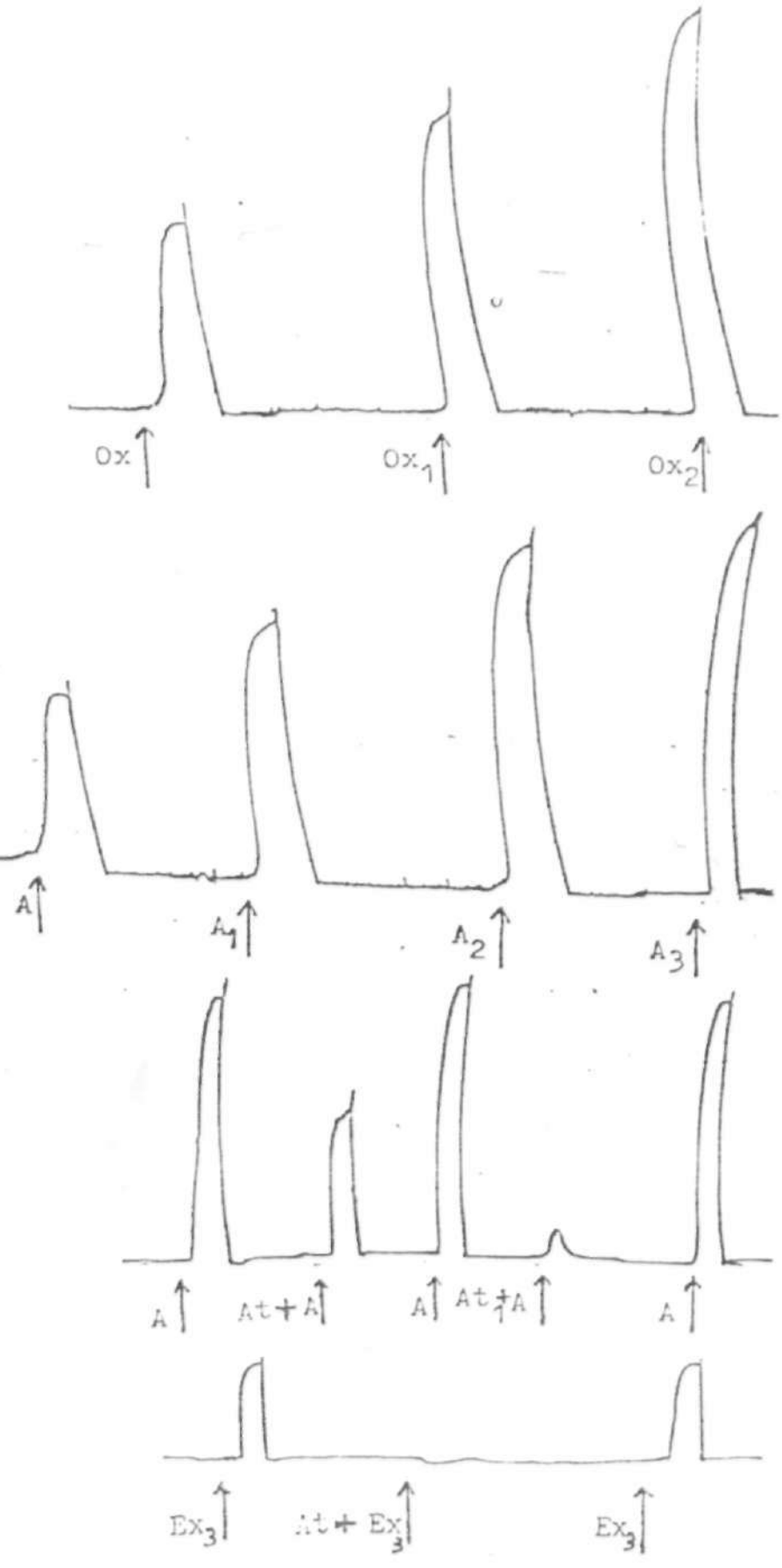
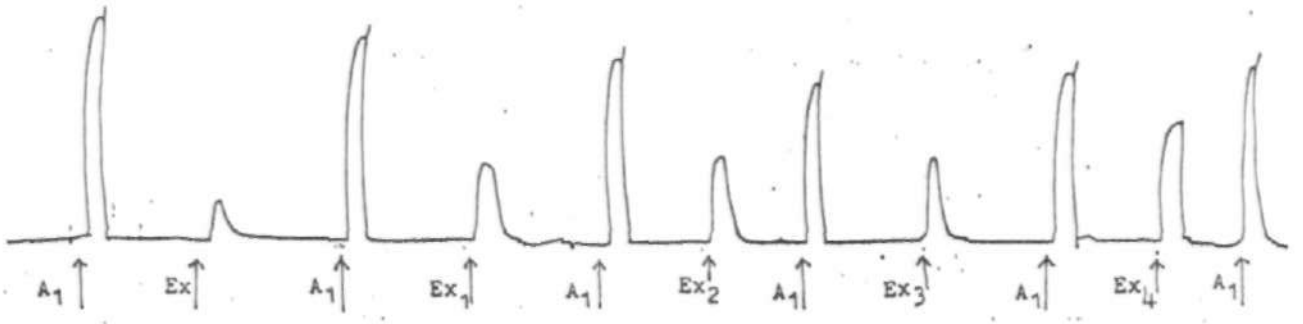


Fig. 8: The effect of the crude water extract of the bark on rat uterus (cont'd)

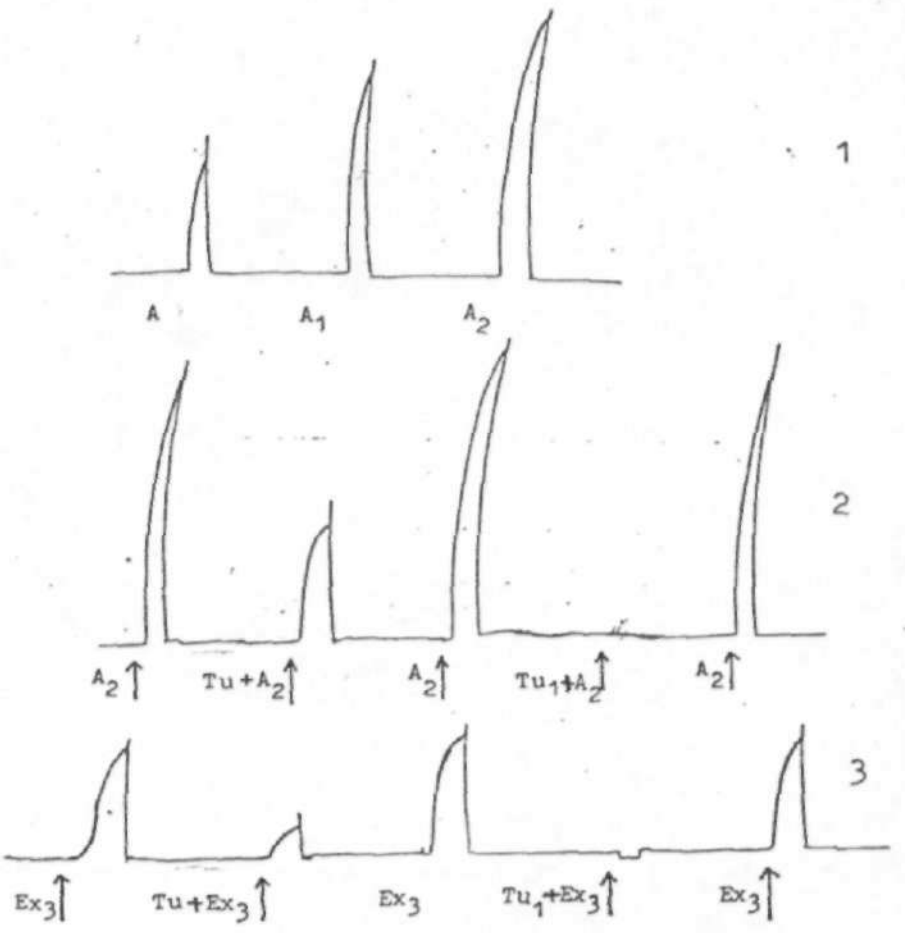


1. Effect of Oxytocin on rat uterus
2. Effects of Acetylcholine on rat uterus
3. Effects of Atropine on the action of Acetylcholine on rat uterus
4. Effects of Atropine on the action of water extract on rat uterus
5. Effects of water extract on rat uterus.

Ox (Oxytocin) = 16 ng/ml, Ox_1 = 32 ng/ml, Ox_2 = 50 ng/ml.
A (Acetylcholine) = 5 ug/ml, A_1 = 3 ug/ml.
At (Atropine) = 2.5 ug/ml, At_1 = 5 ug/ml.
Ex (Water extract) = 50 ug/ml, Ex_1= 100 ug/ml, Ex_2= 150 ug/ml,
Ex_3 = 200 ug/ml, Ex_4 = 300 ug/ml.

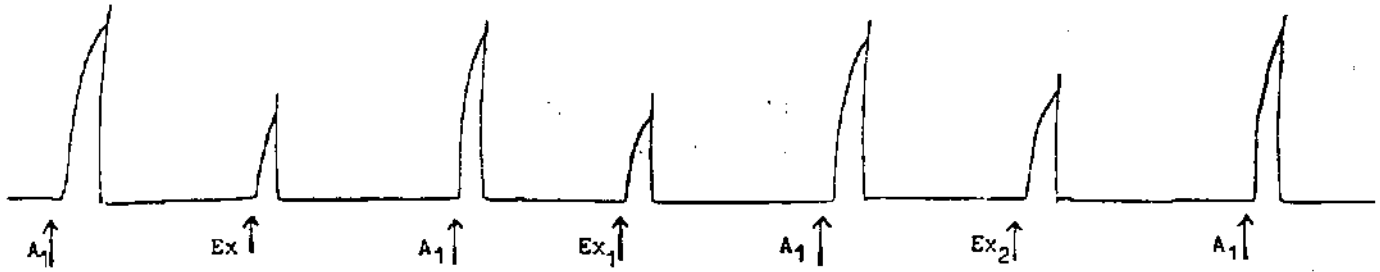
5

Fig. 9: The effects of the crude water extract of the bark on frog rectus abdominis muscle



1. Effects of Acetylcholine on the muscle
2. Effects of tubocurarine on the action of Acetylcholine
3. Effects of tubocurarine on the action of water extract
4. Effects of water extract on the muscle.

Fig. 9: The effects of the crude water extract of the bark on frog rectus abdominis muscle (cont'd)



4

A (Acetylcholine) = 1 ug/ml, A_1 = 3 ug/ml, A_2 = 5 ug/ml

Tu (Tubocurarine) = 1 ug/ml, Tu_1 = 2.5 ug/ml

Ex (Water extract) = 50 ug/ml, Ex_1 = 100 ug/ml

Ex_2 = 150 ug/ml, Ex_3 = 200 ug/ml.

also carried out using standard methods. The results of the toxicity studies are summarized in tables 1 and 2.

(a) Intraperitoneal route

Doses below 1 g/kg had no observable effects on mice. When the animals were given doses of 1 g/kg and above, they had clinical signs of depression, followed by death. The median lethal dose was 1.44 g/kg (Fig. 10). The control animals had no clinical signs.

(b) Oral route

No clinical signs and no death occurred among the animals given the extract by oral route. 16 g/kg was the maximum amount that could be physically administered to the animals. The control animals also had no clinical signs.

Fig. 10: CHART OF MEDIAN LETHAL DOSE.

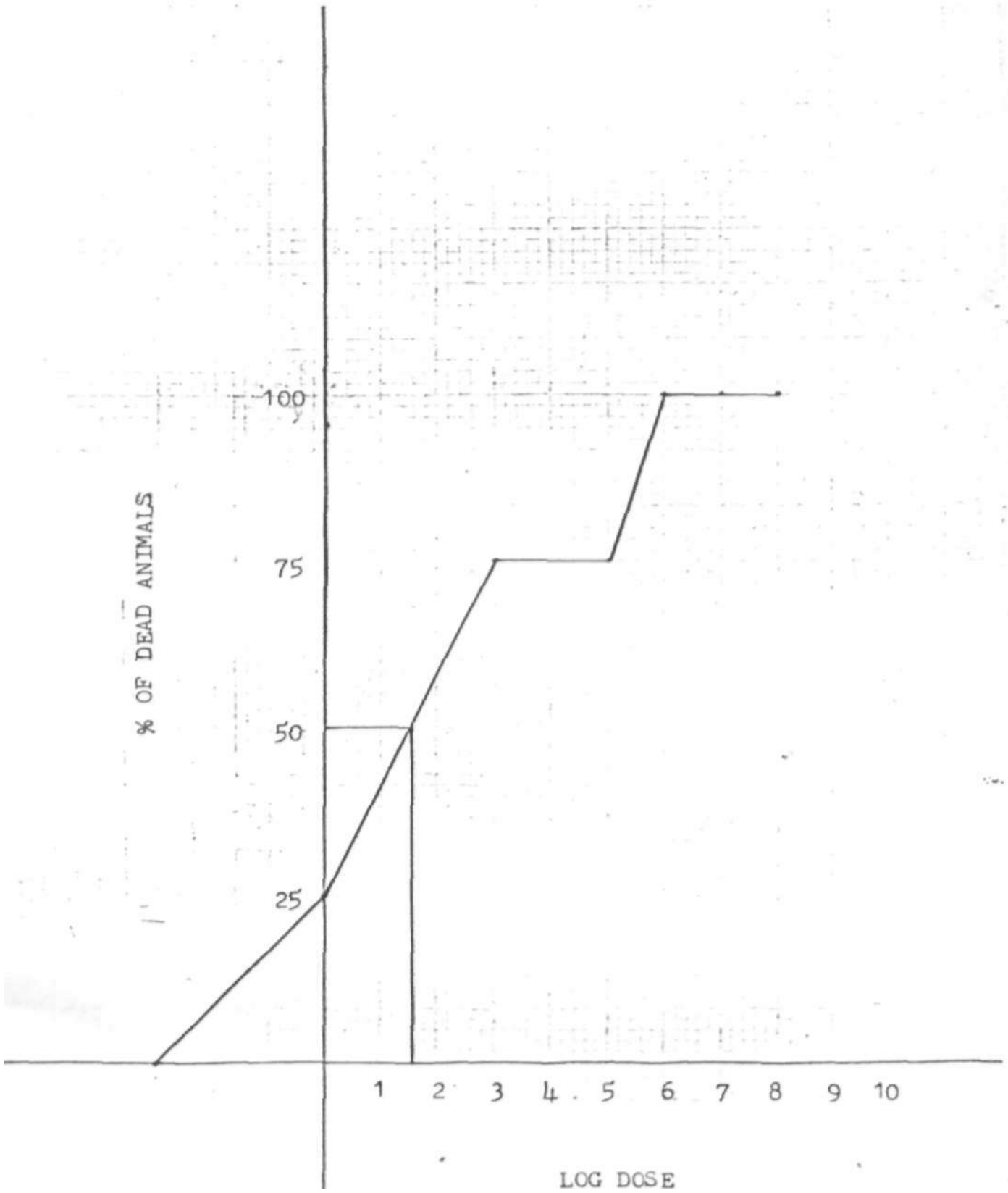


Table 1: Acute toxicity of the crude water extract of S. birrea bark on mice

(Intraperitoneal Route)

Group	Dose (g/kg)	Route of administration	No. of animals treated	No. of animals died	No. of animals survived	Percentage of death	LD_{50} (g/kg)
1	0.5	Intraperito-neal	4	0	4	0%	
2	1	"	4	1	3	25%	
3	2	"	4	3	1	75%	1.44
4	3	"	4	3	1	75%	
5	4	"	4	4	0	100%	
6	5	"	4	4	0	100%	
Control	0.4 ml (Solvent)	"	4	0	4	0	

Table 2: Acute toxicity of the crude water extract of S. birrea bark on mice

(Oral Route)

Group	Dose (g/kg)	Route	No. of animals treated	No. of animals dead	No. of survivors	% dead	LD_{50} (g/kg)
1	1	Oral	4	0	4	0	
2	2	"	4	0	4	0	
3	4	"	4	0	4	0	
4	8	"	4	0	4	0	
5	16	"	4	0	4	0	
Control	0.4 ml Solvent	"	4	0	4	0	
							Not determinable

2.3: MICROBIOLOGICAL STUDIES OF SCLEROCARYA BIRREA BARK

In order to study the antimicrobial activities of the bark, water extracts of the bark were tested microbiologically using standard microbiological techniques. The results are given below:

(a) Determination of sensitivity of the crude water extract (Experimental details, page 98)

The crude water extract had a significant effect on Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Proteus vulgaris and Salmonella typhi, their zones of inhibition were 18, 13, 18, 18 and 15 respectively. But the extract had no effect on Escherichia coli and Serratia marcesens (Table 3).

(b) Determination of minimum inhibitory concentration

The crude water extract showed highest activity against B. subtilis followed by S. aureus and P. vulgaris, their M.I.C. were 1.25 mg/ml and 2.5 mg/ml respectively (Table 4).

From the foregoing results of the pharmacological and microbiological tests carried out on the crude water extract and pure cholesterol the following observations become apparent and the traditional uses of the bark may be connected to these various effects.

The effects of the crude water extract on the rabbit duodenum, guineapig ileum and rat uterus suggest that it has a contractile effect on smooth muscles. The crude water extract also contracted the skeletal muscle of the frog rectus abdominis. These contraction effects may be due to the presence of a

Table 3: Comparative antimicrobial effects of the crude water extract of the bark on various microorganisms

(Agar Diffusion Method)

Organism	Diameter of zone of inhibition (mm)					Mean Diameter of inhibition (mm)
Staphylococcus aureus (2)	18	17	19	18	18	18
Pseudomonas aeruginosa (2)	12	14	13	13	13	13
Bacillus subtilis	17	19	18	18	18	18
Proteus vulgaris	19	18	18	18	17	18
Salmonella typhi	14	14	15	16	16	15
Escherichia coli (2)	10	10	10	11	10	10
Serratia marcesens	10	10	10	10	10	10

Note: Cup diameter = 10 mm.

Table 4: Minimum Inhibitory Concentration for Crude Water Extract of the Bark

Organism	Crude Water Extract mg/ml									
	20	10	5	2.5	1.25	0.625	0.313	0.156	0.076	0.039
Staphylococcus aureus (2)	-	-	-	-	+	+	+	+	+	+
Pseudomonas aeruginosa (2)	-	-	-	+	+	+	+	+	+	+
Bacillus subtilis	-	-	-	-	-	+	+	+	+	+
Proteus vulgaris	-	-	-	-	+	+	+	+	+	+
Salmonella typhi	-	-	-	+	+	+	+	+	+	+
Escherichia coli (2)	+	+	+	+	+	+	+	+	+	+
Serratia marcesens	+	+	+	+	+	+	+	+	+	+

(-) denotes no bacterial growth

high quantity of tannins (20.88%) in the plant bark. The presence of a high percentage of tannins in the bark may explain the wide use of the plant bark for the treatment of burns, haemorrhoids and diarrhoea.

Tannins have a strong anti-inflammatory effect (Arnost, 1960). Application of tannins on the skin for treatment of burns and small wounds causes the closure of dilated blood vessels, shrinkage of oedematous tissues, and it forms a protective coagulated protein film over surfaces where there is excessive exudation of fluid as in small burns, a weeping eczema and small bleeding points, thus protect it from irritating substances (Hawary, 1978). Application of a dilute solution of tannins on a living mucous membrane precipitates a fine pellicle of mucus and protein, which protects the cells beneath and lessens their sensitiveness to external stimuli. But a stronger solution may cause some precipitation in the cells themselves and thus injure them and cause irritation. Thus they are applied externally in cases of excessive secretion, as in local sweating or weeping ulcers, and occasionally to harden the skin (Grollman, 1965).

The anti-inflammatory effect of tannins has been demonstrated by Arnost (1960), who observed that administration of tannins resulted in a marked

suppression of swellings in rats, induced by hyaluronidase, egg white and phenol.

However, tannins are no longer used for treatment of burns and wounds because the cracking of the crust allows bacterial infection to develop beneath it, hepatotoxicity (central lobular necrosis) has also been observed in some cases, due to absorption of tannins from large areas of application.

The results of the acute toxicity study show that the toxicity of S. birrea bark water extract is very low as the extract is not toxic to mice by the oral route,

It was not possible to determine the LD_{50} by this route as the highest dose (16 g/kg) that could physically be given to the animals had no effect on them.

The median lethal dose (1.44 g/kg) obtained by intraperitoneal route is also indicative of very low toxicity of the bark extract. This explains why the bark extracts are used so commonly in traditional medicine without any apparent adverse effects.

As apparent from the microbiological experiments carried out with the extracts, both gram negative (Pseudomonas aeruginosa, Salmonella and Proteus vulgaris) and gram positive (Staphylococcus aureus

and Bacillus subtilis) organisms were sensitive to the crude water extract. This shows the extract has a broad spectrum of activity against both types of microorganisms. Out of these two groups the extracts were more effective against the gram positive bacteria (1.25 mg/ml and 2.5 mg/ml for B. subtilis and S. aureus respectively), which are the caustive agents in many skin and eye diseases.

The crude water extract (5 mg/ml) inhibited the growth of Salmonella, which is the causative agent of Salmonella gastroenteritis, the symptoms and signs of which consist of fever, nausea and vomitting, cramping abdominal pain and diarrhoea which may be bloody (Marcus, 1977).

The crude water extract (2.5 mg/kg, 5 mg/ml)also inhibited the growth of Proteus vulgaris and Ps. aeruginosa which are very common in urinary tract infections (Arthur and Evelyn, 1965).

All the above pharmacological and antimicrobial effects of the water extracts of the bark are in agreement with their uses in traditional medicine. Therefore, it can be safely commented that the traditional uses of the water extracts of the bark of Sclerocarya birrea (A. Rich.) Hochst. is not without clinical justification or scientific basis. Thus, by quoting Dr. Halfdan Mahler, Director-General

of the World Health Organisation, it can be concluded that "Modern medicine still has much to learn from the collector of herbs. Many of the plants familiar to the witch-doctor really do have the healing powers that tradition attaches to them. The age-old art of the herbalist must be tapped". (Norman Myers, 1982).

CHAPTER 3

EXPERIMENTAL

EXPERIMENTAL

3.1: PHYTOCHEMICAL STUDIES OF SCLEROCARYA BIRREA BARK

PLANT MATERIAL

The bark of Sclerocarya birrea (A. Rich.) Hochst. was obtained along with the leaves from Hajiya Aishatu Bubayero Musa, a traditional healer in Kaduna, in October 1983. The plant was identified as Sclerocarya birrea (A. Rich.) Hochst. in the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria. A voucher specimen has been deposited to this Herbarium under Voucher No. 1957.

The bark was obtained in a large quantity and was left to dry at room temperature for two days after which it was dried in an oven at 40 to 50°C for twenty four hours. The dried bark was ground into a coarse powder by an electric grinder. The powdered bark was kept in an air tight glass container and stored in a dry place.

SOLVENTS

All solvents used in this work were of analytical grade and refer to B.P. solvents. All reference to water indicates distilled water.

REAGENTS

(1) α-Naphthol-sulphuric acid

The reagent was prepared by mixing 10.5 ml of 15% ethanolic α-naphthol, 6.5 ml of concentrated sulphuric acid, 40.5 ml ethanol and 4 ml water (Jacin, 1965).

(2) Anisaldehyde-sulphuric acid

1 ml concentrated sulphuric acid was added to a solution of 0.5 ml anisaldehyde in 50 ml acetic acid (Stahl, 1961).

(3) Ferric chloride solution (5%)

This refers to a 5% solution of ferric chloride in 0.5 N hydrochloric acid (Fink, 1949).

(4) Potassium ferricyanide - ferric chloride

Solution a : 1% aqueous potassium ferricyanide solution.

Solution b : 2% aqueous ferric chloride solution.

Spray reagent : Equal amounts of a and b are mixed just before use. (Barton and Evans, 1952).

(5) Antimony (III) chloride

In preparing this reagent 25 gm antimony (III) chloride was dissolved in 75 gm of chloroform (Takeda, 1963).

(6) Acetic anhydride - sulphuric acid

5 ml of acetic anhydride were carefully mixed under cooling with 5 ml of concentrated sulphuric acid; this mixture was added cautiously to 50 ml of absolute ethanol with cooling (Tschesche, 1961).

(7) Vanillin - sulphuric acid

1 gm of vanillin was dissolved in 100 ml of concentrated sulphuric acid (Stahl, 1969).

(8) Dragendorff's reagent according to Munier and Macheboeuf

Solution a : 0.85 gm of basic bismuth nitrate was dissolved in a mixture of 10 ml of acetic acid and 40 ml of water;

Solution b : A solution was made of 8 gm of potassium iodide in 20 ml of water.

Spray reagent : Equal volumes of a and b were mixed, 1 ml of the mixture was added to 2 ml of acetic acid and 10 ml of water before use (Stahl, 1969).

CHROMATOGRAPHIC SYSTEMS

A. Thin-layer chromatography (TLC)

System 1_T

Stationary phase : Silica gel G type 60 plates (0.25 mm thick).

Solvent system : Methanol : benzene : glacial acetic acid (60 : 20 : 20)

Visualization : Sprayed with α-naphthol-sulphuric acid reagent. The plates were heated for 3-6 minutes at 100°C after spraying.

System 2_T

Stationary phase : Silica gel G type 60 plates (0.25 mm thick).

Solvent system : Benzene : ethyl acetate (3 : 1).

Visualization : Sprayed with antimony (III) chloride reagent. The plates were heated at 120°C for 10 minutes after spraying.

System 3_T

Stationary phase : Silica gel G type 60 plates (0.25 mm thick).

Solvent system : Chloroform : methanol (6 : 4)

Visualization : Sprayed with acetic anhydride - sulphuric acid reagent. The plates were heated for 10 minutes at 120°C after spraying.

System 4_T

Stationary phase : Silica gel G type 60 plates (0.25 mm thick).

Solvent system : n-Hexane : ethyl acetate (3 : 1).

Visualization : Sprayed with vanillin-sulphuric acid reagent. The plates were heated for 10 minutes at 100°C after spraying.

B. Paper chromatography (PC)

System 1_P

Technique	:	Ascending
Stationary phase	:	Whatman paper No. 1 (57 x 23 cm).
Solvent system	:	n-Butanol-acetic acid-water (4 : 2 : 1)
Visualization	:	Sprayed with anisaldhyde reagent. The chromatogram was heated at 105°C for 5 minutes after spraying.

System 2_P

Technique	:	Descending
Stationary phase	:	Whatman paper No. 1 (57 x 23 cm)
Solvent system	:	n-Butanol : acetic acid : water (4 : 1 : 5)
Visualization	:	The air dried papers were examined under U.V. and then sprayed with ferric chloride reagent.

System 3_P

Technique : Descending

Stationary phase : Whatman paper No. 1 (57 x 23 cm).

Solvent system : Formic acid : conc. hydrochloric acid : water (5 : 2 : 3)

Visualization : Examined under U.V. light and then sprayed with potassium ferricyanide - ferric chloride reagent.

System 4_P

Technique : Descending

Stationary phase : Whatman paper No. 1 (57 x 23 cm).

Solvent system : Acetic acid (10%) in chloroform.

Visualization : Sprayed with potassium ferricyanide - ferric chloride reagent.

System 5_P (Preparative)

Technique : Descending

Stationary phase : Whatman paper No. 3 (57 x 23 cm).

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

Visualization : The air dried papers were examined under U.V.

C. Column chromatography

System 1_C

Column size	:	25 x 1.5 cm
Adsorbent	:	Silica gel G type 120
Eluting solvents	:	Petroleum ether - chloroform - methanol.
Technique of packing	:	Wet slurry technique

INFRA-RED SPECTROSCOPY

The i.r. spectra were recorded on a Perkin-Elmer spectrophotometer using paraffin and KBr discs.

MELTING POINT

The m.p. of all the compounds were recorded using a Gallenkamp capillary melting point instrument and are uncorrected.

The phytochemical evaluation of the bark was carried out in the following three ways:

1. Preliminary phytochemical screening of the bark by using different methods to have an idea about the chemical constituents present in it.

2. Detailed phytochemical studies by extracting larger quantities of the powdered bark with various solvents (water, 70% ethanol and

petroleum ether, b.p. 60-80°) to isolate and characterise some of the constituents present, as indicated by the preliminary tests.

3. Quantitative determination of some of the active chemical constituents present in the bark.

3.1.1: Preliminary Phytochemical Screening of the Bark

In order to have an idea about the chemical constituents present in the bark, various quantities (1 - 10 gm) of the dried powdered bark were subjected to the following phytochemical tests:

A. Microsublimation

About one gram of the dried powdered bark was subjected to careful microsublimation. The powder was taken in a porcelain crucible and covered with a watch glass, the crucible was heated gently using bunsen burner for ten minutes. Then the watch glass was examined under the microscope for the presence of any crystalline sublimate. The same observation was also made after adding sulphuric acid (50%) to the watch glass.

B. Test for carbohydrates (Molisch's test)

About ten grams of the dried powdered bark was mixed with about 60 ml of ethyl alcohol (25%)

containing tartaric acid (2%). The whole mixture was refluxed on a water bath for three hours and then filtered. The marc was washed several times with hot ethyl alcohol (25%). The filtrate and the washings were combined and evaporated to dryness under reduced pressure at 40 to 50°C. The residue was dissolved in 5 ml of hot water and transferred to a test tube. 0.5 ml of freshly prepared alcoholic solution of α-naphthol (in 90% ethanol) was added to it. Concentrated sulphuric acid was then poured carefully by the side of the test tube to form a separate lower layer. Formation of a violet ring at the interface of the two layers indicated the presence of carhohydrates (Hilal, 1976).

C. Tests for glycosides

Because of complexity and different chemical nature of the naturally occurring glycosides, various chemical tests were used to examine their presence in the bark.

(a) Test for simple phenolic glycosides

About 10 gm of the powdered bark was extracted by macerating it in hot water for two hours. The liquid was filtered and concentrated to a small volume over a water bath. It was treated with 2% lead

acetate solution. The mixture was filtered and excess lead was removed by treating the mixture with hydrogen sulphide gas. The precipitated lead sulphide was removed by filtration and the filtrate was hydrolysed by boiling with hydrochloric acid and tested with ferric chloride solution (5%) (Balbaa, 1976). Formation of blue, green or red colour indicated the presence of phenolic glycosides.

(b) Test for anthraquinone glycosides (Borntrager's test)

(i) About 5 gm of the bark powder was shaken with 10 ml of benzene and filtered. To the filtrate 5 ml of 10% ammonia solution was added. The mixture was shaken and the two layers were allowed to separate. Production of a purple colour in the aqueous layer indicated the presence of free anthraquinones.

(ii) About 5 gm of the powdered bark was boiled with 10 ml of aqueous sulphuric acid and filtered while hot. The filtrate was shaken with 5 ml of benzene and the two layers were allowed to separate. The benzene (upper) layer was separated and shaken with 10% ammonia solution (Trease and Evans, 1978). Production of a purple colour in the aqueous layer indicated the presence of combined anthraquinones.

(c) Test for flavonoid glycosides

About 10 gm of the powdered bark was macerated in 150 ml of hydrochloride acid (1%) and filtered. The filtrate was subjected to the following tests for flavonoids:

(i) 10 ml of the filtrate was rendered alkaline with sodium hydroxide solution (20% w/v) and the colour of the mixture was observed. Production of a yellow colour indicated the presence of flavonoids.

(ii) 4 ml of the filtrate was shaken with 5 ml of amyl alcohol and the colour produced was observed. Production of a yellow colour indicated the presence of free flavonoid aglycones.

(iii) 10 ml of the filtrate was shaken with 7 ml of amyl alcohol and the mixture transferred to a separating funnel. The amyl alcohol layer was discarded and the aqueous layer boiled with 10 ml of hydrochloride acid for two minutes. The acidic solution was cooled and divided into two portions. The first portion was shaken with amyl alcohol and the colour produced was observed. Production of a yellow colour indicated the presence of combined flavonoids. To the second portion magnesium turnings were added and the colour of the solution observed (Hilal, 1976). Production of a red colour indicated the presence of flavonone and flavonol glycosides.

(d) Test for cyanophore glycosides

About 5 gm of the powdered bark was soaked in 25 ml of alcoholic potash (5%) for about two minutes and filtered. 1 ml of ferrous sulphate (2.5%) and 1 ml of ferric chloride (1%) solutions were added to 5 ml of the filtrate and the mixture kept at 60 to 70°C for about ten minutes. 5 ml of hydrochloric acid (20%) was then added to the mixture. The colour of the mixture was observed (Balbaa, 1976). Production of Prussian blue indicated the presence of hydrocyanic acid.

(e) Tests for cardiac glycosides

About 10 gm of the dried powdered bark was boiled with 100 ml of water for ten minutes and then filtered. The filtrate was shaken with 50 ml of chloroform for a few minutes. 25 ml of ether and 25 ml of ethyl alcohol (95%) were then added to it and the whole mixture was shaken. The mixture was left aside to form two layers. The chloroform (lower) layer was separated and evaporated to dryness. The residue was dissolved in 10 ml of ethyl alcohol (95%) and divided into two portions and the following tests were performed:

(i) Legal test for unsaturated lactones

The first 5 ml portion of the alcoholic solution was mixed with 1 ml of sodium nitroprusside (1%) solution and 1 ml of alcoholic sodium hydroxide (5%). The colour of the mixture was observed (Hilal, 1976). The production of a deep red colour indicated the presence of unsaturated lactones.

(ii) Keller-Kiliani test for 2-desoxy sugars

The second 5 ml portion of the alcoholic solution was evaporated to dryness. The residue was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solution (5%). Then, with a pipette, 1 ml of concentrated sulphuric acid was placed at the bottom of the test tube containing the mixture. The colour at the junction of the two liquids was observed (Trease and Evans, 1978). The production of a brown ring at the junction of the two liquids indicated the presence of a desoxy sugar characteristic of cardenolides.

(f) Test for saponins

About two gm of the dried powdered bark was boiled with 10 ml of water and then filtered. The filtrate was shaken vigorously for two minutes and set aside to observe any persistant froth formation (Hilal, 1976), which indicated the presence of saponins.

D. Test for sterols

About 5 gm of the powdered bark was extracted by maceration with 50 ml of ethyl alcohol (95%), filtered and the filtrate evaporated to dryness. The residue was dissolved in 10 ml of anhydrous chloroform and then filtered. The filtrate was divided into two equal portions and the following tests were carried out:

(a) Liebermann-Burchard's test

The first portion of the chloroformic solution was mixed with 1 ml of acetic anhydride, followed by the addition of 1 ml of concentrated sulphuric acid down the walls of the test tube to form a layer underneath. The formation of a reddish violet colour at the junction of the two liquids, and a green colour in the chloroformic solution indicated the presence of sterols (Hilal, 1976).

(b) Salkowski test

The second portion of the chloroformic solution was mixed with 2 ml of concentrated sulphuric acid carefully so that sulphuric acid formed a lower layer. A reddish-brown colour at the interface indicated the presence of a steroidal ring (Balbaa, 1976).

E. Test for tannins

About 5 gm of the powdered bark was stirred with 10 ml of distilled water and then filtered. To the filtrate 1 ml of ferric chloride (5%) solution was added. A blue-black, green or blue-green precipitate indicated the presence of tannins (Trease and Evans, 1978).

F. Test for phlobatannins

About 5 gm of the powdered bark was extracted by maceration with water and then filtered. The filtrate was boiled with hydrochloric acid (1%). The formation of a red precipitate indicated the presence of phlobatannins (Trease and Evans, 1978).

G. Test for alkaloids

A modified form of the thin-layer chromatography (TLC) method described by Farnsworth and Euler (1962) was used. 2 gm of the powdered bark was thoroughly moistened with calcium hydroxide (40%) solution and then dried on a steam bath. The mixture was then placed in a 50 ml Erlenmeyer flask fitted with condenser. 15 ml of chloroform was added and the mixture refluxed on a steam bath for 30 minutes. The refluxed mixture was cooled to room temperature and filtered. The marc was returned to the flask and another portion of 15 ml of chloroform added to it. The 30 minute reflux period was repeated.

After cooling and filtering, the extracts were combined and evaporated on a steam bath to about 2 ml. The concentrated extract was then introduced into a micro- separatory funnel and 1 ml of hydrochloric acid (1%) added. The mixture was shaken and allowed to separate into two layers. The chloroform layer was removed and sufficient ammonium hydroxide (28%) solution was added to the aqueous acid layer in the micro-separatory funnel to render it distinctly alkaline. The alkaline mixture was then extracted with two successive 5 ml volumes of chloroform. The chloroform extracts were combined and evaporated to dryness. The residue was dissolved in 1 ml of chloroform and chromatographed on silica gel plates using benzene; benzene-methanol (95:5), xylene-toluene (50:50), and n-butanol-acetic acid - water (4:1:1) as solvent systems. The developed plates were sprayed with Munier and Macheboeuf modification of Dragendorff's reagent, the plates were air dried and examined for the presence of orange red coloured spots which indicated the presence of alkaloids (Farnsworth and Euler, 1962)

3.1.2: Detailed Phytochemical Studies on Larger Extracts of the Bark

(a) Extraction with water

The powdered bark (250 g) was shaken with water (1 l) in a closed conical flask for six hours using a mechanical shaker and then filtered. Lead acetate solution (5%) (25 ml) was added to the filtrate dropwise to remove tannins and other phenolic compounds, until no further precipitate was formed. The mixture was then shaken, allowed to stand and filtered. The filtrate was divided into two portions. Each portion was examined and two compounds were isolated. The compounds were designated as compound A and compound B.

(i) Isolation and characterisation of compound A

One portion of the filtrate was concentrated at 60°C over water bath to a small volume and left aside at room temperature. After 48 hours a white crystalline solid material settled down. The crystals were separated by filtration and purified by recrystallization from hot water. The crystals were designated as compound A.

The compound (m.p. 158-160°) was soluble in water but insoluble in ether and chloroform and had a sweet taste. It gave positive Molisch's test and negative Fehling's test before hydrolysis, but on hydrolysis

with 2M hydrochloric acid it gave positive Fehling's test. This indicated that the compound was a non-reducing sugar.

The compound was co-chromatographed on TLC (system 1_T) and on PC (system 1_P) along with authentic samples of sucrose, glucose and fructose. The compound gave one spot corresponding to that of sucrose.

The hydrolytic products were also co-chromatographed using system 1_T and system 1_P along with authentic samples of sucrose, glucose and fruotose.

(ii) Isolation and characterisation of compound B

The other portion of the filtrate was treated with disodium hydrogen phosphate solution (5%) in order to eliminate some of the unwanted constituents. The mixture was left aside for 12 hours and then filtered. The filtrate was concentrated to a small volume at 60°C over water bath. The concentrated solution solidified after standing for 30 minutes at room temperature. The solid compound was dissolved in ethanol (95%) and when acetone was added to the mixture dropwise a dirty white precipitate formed. The precipitate was separated by filtration and left to dry at room temperature. The precipitate was dissolved in ethanol (95%) and the solution was shaken with petroleum ether (b.p. 60-80°) in a separating

funnel. On standing for sometime an oily compound settled at the bottom of the funnel. This oily substance was separated from the clear solution which was then concentrated at 60°C over water bath to a small volume and left aside at room temperature when a good amount of a crystalline substance (white flakes) was formed.

The crystals were separated by careful filtration and dried at 60°C. The crystals were designated as compound B. The compound was sparingly soluble in water but fairly soluble in alcohol (95%), tasteless and gave positive Molisch's and Fehling's tests indicating it to be a complex polysaccharide which behaved like a gum.

(b) Extraction with ethanol (70%)

The powdered bark (500 g) was extracted with hot ethanol (70%) (2.5 l) using Soxhlet apparatus for 12 hours. The alcoholic extract was concentrated under vacuum to a small volume. One compound was isolated and later characterised from this extract. The compound was designated as compound C.

Isolation and characterisation of compound C

The concentrated alcoholic extract (150 ml) was hydrolysed by refluxing with 2M hydrochloric acid for 30 minutes over water bath. The resultant mixture

was cooled and filtered. The filtrate was transferred to a separating funnel and was extracted two times with ether (2 x 100 ml). The ether extracts were combined and concentrated over a water bath to a small volume and left aside. Yellowish white crystals were formed on standing for 12 hours. The crystals were dissolved in a small volume of ether (3 ml) and the solution was applied as a band on Whatman paper No. 3 (System 5). The developed chromatogram was observed under u.v. light (366 n m) and a bright blue band was observed. The band zone was marked and the compound was obtained by cutting off the marked zone and extracting it with ether (2 x 25 ml). The ether extract was left aside at room temperature for 6 hours when yellowish white crystals were obtained. The crystals were designated as compound C. The compound was soluble in water and ethanol and gave a blue colour with ferric chloride solution (5%) and a blue flouresence under u.v. light indicating it to be a phenolic compound. So, it was then co-chromatographed on PC (systems $^{2}_{P}$, $^{3}_{P}$ and $^{4}_{P}$) along with authentic samples of tannic acid, pyrogallol and gallic acid. It gave one spot corresponding to that produced by gallic acid. It had a m.p. of 250-53° which was undepressed when mixed with an authentic sample of gallic acid.

(c) Extraction with petroleum ether (b.p. 60-80°)

About 1125 g of the bark powder was extracted with petroleum ether (60-80°) (5 l) using Soxhlet extractor for 48 hours. The extract was concentrated under vacuum to a small volume and left aside in a glass evaporating dish at room temperature. After 48 hours a white precipitate was observed at the bottom of the dish covered with a greenish oily layer. The oily layer was separated from the white precipitate with a pipette, then the precipitate was washed three times with 100 ml each of hexane (b.p. 60-80°). The washings were combined and concentrated under vacuum then added to the oily fraction. This bulked oily fraction was then subjected to adsorption column chromatograph (system 1_C). The column was successively eluted with petroleum ether b.p. 60-80° (2.5 l), chloroform (500 ml) and methanol (250 ml) collecting fractions of 100 mls in each case. The eluates were examined separately and two compounds were isolated from the petroleum ether and chloroform eluates which were designated as compound D and E. The methanol eluate appeared to contain a mixture of small amounts of the above two compounds mixed with other substances. It was not worked out.

(i) Isolation and characterisation of compound D

A brown yellowish oil was obtained after evaporating the petroleum ether eluate to a small volume under vacuum. This oil was subjected to saponification by refluxing it with 50 ml ethanol (95%) and 7.5 g of potassium hydroxide dissolved in a small volume of water. This mixture was placed in a round-bottomed flask fitted with a condenser and refluxed for two hours on a water bath. After saponification 50 ml of water were added to it and the mixture was extracted five times with 100 ml each of ether. The extraction of the unsaponifiable materials from the soap was accomplished by stirring the mixture for one hour with a stirrer and allowing the ether layer to separate. The ether extracts were combined, concentrated to about 200 ml and washed free of alkali with water. It was then concentrated to a small volume under vacuum at 40°C. The product was dissolved in petroleum ether (b.p. 40-60°), shaken with methanol (10%) and the petroleum ether fraction was collected and evaporated under vacuum to dryness. The residue (a gummy solid) was dissolved in methanol over water bath and left to cool at room temperature when 50 ml of acetone was added to it. The mixture was then left aside at room temperature for 12 hours when white crystals were formed. The crystals were separated by filtration and purified by recrystallization from ether. The crystals were designed as compound D.

The compound was then subjected to Liebermann-Burchard and Salkowski tests for sterol. Its m.p. (149-52°C), solubility characters and i.r. spectrum were then determined.

It was soluble in petroleum ether, benzene and chloroform but insoluble in water and gave a positive Liebermann-Burchard and Salkowski tests for sterols. Its i.r. spectrum corresponded with that of cholesterol. The compound was then co-chromatographed on TLC (systems 2_T, 3_T and 4_T) along with authentic sample of cholesterol. It gave one spot which corresponded with that of cholesterol. It had a mixed m.p. of 148-49° with authentic cholesterol sample (m.p. 149°).

(ii) Isolation and characterisation of compound E

A brown gummy material was obtained after the chloroform eluate was evaporated under vacuum. It was dissolved in a small volume of methanol over water bath and then left to cool at room temperature. Steam was allowed to pass through the mixture until it was saturated with moisture when a white fluffy crystalline precipitate was observed. The mixture was left to stand for 12 hours and then the white crystalline precipitate was separated by removing the supernatant liquid carefully with a pipette. The crystals were left to dry at room temperature. It was then purified by

recrystallisation from hot methanol. It was designated as compound E.

The compound, m.p. 78°, was soluble in ether and chloroform but insoluble in water. It gave positive results with Liebermann-Burchard and Salkowski tests for steroids. The i.r. spectrum of the compound indicated it to be a cholesterol ester as its i.r. spectrum corresponded with that of cholesterol except that it had an additional peak at 1720 cm^{-1}, probably due to a carbonyl (C=O) group of an esterifying acid. Thus compound E was tentatively identified as an ester of cholesterol.

3.1.3: Quantitative Estimation of Some Constituents of the Bark

A. Quantitative estimation of tannins

2 gm of the powdered plant bark was extracted by boiling for one hour with 100 ml of water and then filtered. The filtrate was boiled and 30 ml copper acetate solution (5%) was added to it with stirring. The precipitate formed was then collected on an ashless filter paper. The precipitate was washed several times with water until it became free from copper acetate. The precipitate with the filter paper was ignited in a tared metal crucible. The residue of copper oxide was completely oxidised by the addition of a few drops of

concentrated nitric acid. The residue was ignited several times till a constant weight was obtained. The weight of the residue was then calculated out by subtracting the weight of the empty crucible from the total weight of the crucible and residue. The percentage of tannins in the bark was calculated out as follows: 1 gm of copper oxide ≡ 1.305 gm tannin. (Balbaa, 1976).

B. Quantitative estimation of cholesterol

150 gm of the powdered plant bark was extracted with petroleum ether (b.p. 60-80°) using Soxhlet apparatus for 48 hours. Then the petroleum ether extract was concentrated under vacuum to a small volume and 100 ml of a mixture of acetone and ethanol (1:1) was added to it. 10 drops of hydrochloric acid (3.5%) and 10 ml of digitonin solution (0.5% in aqueous ethanol) were added to the mixture. Then 10 ml of aluminium chloride (10%) was added and the mixture heated at 45°C for 15 minutes. After cooling to room temperature the mixture was centrifuged for 30 minutes. The supernatant was decanted and discarded. The sterol digitonide precipitate was dissolved in 20 ml of methanol by gentle warming and 10 drops of hydrochloric acid (3.5%) and 20 ml of aluminium chloride were added to it, and the mixture was heated at 45°C for 20 minutes and cooled to room temperature. It was

then centrifuged for 30 minutes. The supernatant was removed and the precipitate, which was then free from digitonin, was mixed with 20 ml of dimethyl sulphoxide and the mixture warmed on a water bath for 15 minutes. The mixture was allowed to cool at room temperature, whereupon cholesterol precipitated. The mixture was transferred to a separatory funnel and extracted with 70 ml of n-hexane. The dimethyl sulphoxide layer was extracted further with two 30 ml portions of n-hexane. The combined hexane layers were allowed to stand for 20 minutes and filtered through anhydrous sodium sulphate to free it from water and evaporated under vacuum to dryness leaving the cholesterol as residue. The weight of cholesterol was taken and its percentage was calculated out (Raphael, 1969).

3.2 PHARMACOLOGICAL STUDIES OF SCLEROCARYA BIRREA BARK

3.2.1: Pharmacological Studies on Isolated Tissue Preparations

Test animals

Adult female albino rats (Wistar Strain) were obtained from the Animal House of the Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria.

Rabbits and guinea pigs were purchased locally from the market and kept in the animal house prior to use.

Frogs (Bufo regularis) were obtained from water ponds within the Ahmadu Bello University Campus.

Preparation of drug samples

A. Crude water extract of the bark of S. birrea

10 g of the powdered bark of S. birrea were extracted with 50 ml of de-ionised water using Soxhlet apparatus over a period of 24 hours.

The water extract was poured on a flat porcelain dish and left in open air at room temperature (32-35^{o}C) for 48 hours. At the end of this period of exposure a thin brown solid mass of the plant extract was obtained.

The test solutions of the crude water extract were prepared by taking a known weight of the dry extract in a known volume of the physiological solutions (see appendix) to make 4-10 mg/ml concentration.

B. Cholesterol and compound D

Cholesterol is insoluble in water, so it was necessary to make a suspension of cholesterol in water using gum acacia as a suspending agent.

A known weight of cholesterol was taken in a small porcelain mortar and ground into a very fine powder. Then a known volume of 3% gum acacia solution was mixed thoroughly with the powder to make a suspension of 1 mg/ml.

The same procedure was used to make a suspension of compound D.

C. Standard drugs

Standard drugs used included the following:

1. Acetylcholine chloride (Sigma Chemical Co.)
2. Histamine dihydrochloride (Sigma Chemical Co.)
3. D-tubocurarine chloride (Sigma Chemical Co.)
4. Atropine sulphate (Sigma Chemical Co.)
5. Promethazine HCl (Sigma Chemical Co)
6. Propranolol (Sigma Chemical Co.)

7. Stilboesterol
8. Oxytocin (Richter International Generics Ltd., London).

Acetylcholine, histamine, D-tubocurarine, atropine and propranolol solutions were prepared by dissolving known amounts of each drug in a definite volume of de-ionised water to make a solution of 1 mg/ml strength.

Appropriate dilutions were made with the physiological solutions when required. Promethazine and oxytocin were obtained in ampoules, and appropriate dilutions were prepared using de-ionised water.

Equipments

Recording microdynamometer, transducer (Ugo Basile Italy, model No. 7050), and thermostatically controlled tissue bath (25 ml).

Recording techniques

In all the experiments on isolated tissue preparations, tissues were removed from freshly killed animals and quickly placed in a petri-dish containing physiological solutions which varied with the different tissues.

The tissues were then set up in the organ bath at 37°C and aerated with air. They were allowed to stabilize for about thirty minutes before studying the effects of drugs on them.

Responses were recorded by a Ugo Basile recording micro-dynamometer with appropriate transducer. The speed of the recorder was set at 6 mm/min.

Physiological solutions (see appendix)

Dejalon solution was used for the rat uterus (British Pharmacopoeia). Tyrodes solution was used for the rabbit duodenum and guinea pig ileum. The frog Ringer's solution was used for the frog rectus abdominis muscle (Burn, 1952).

Isolated tissue preparations

Effects of the extracts and the standard drugs were studied on the following isolated tissue preparations:

A. The rabbit duodenum and the guineapig ileum

Rabbits and guineapigs were starved twenty four hours before the experiments. The animals were killed by a blow on the head prior to incision of the throat. The abdomen was then cut, and pieces of the duodenum

and ileum were excised and placed in petri dishes containing Tyrodes solution. The tissues were set up with temperature maintained at 37°C and aerated with air.

B. The rat uterus preparation

The rat uterus was prepared following the procedure contained in the British Pharmacopoeia (1968).

Adult female rats (between 120 - 150 gm in weight) were injected with stilbestrol (0.1 mg/kg s.c.) 24 hours before the experiments, in order to bring the rats to oestrous.

The animals were killed and the abdomen cut open to reveal the uterine horns. The two horns were removed and each used for a different set up. The uterine horns were aerated with oxygen and maintained at a temperature of 37°C.

C. The frog rectus abdominis muscle (Burn, 1952)

The frog was stunned and decapitated, and the spinal cord was destroyed with a probe. The skin of the abdomen was cut to expose the whole of the abdomen. The two rectus abdominis muscles were seen running along each side of the midline from the base of the sternum to the fork.

Each muscle was used for a set-up and was constantly aerated with air. The temperature of the tissue bath was maintained at room temperature at about 28°C. The muscle was stretched for about 30 minutes with a weight before the experiment was commenced.

3.2.2: Acute Toxicity Studies

Test animals

Swiss white mice of either sex weighing 15-20 gm were used for the acute toxicity studies. They were kept in groups of four. The mice were maintained on commercial pellets throughout the period of the experiment.

Administration of the extract

Acute toxicity was determined by intraperitoneal as well as by oral routes. The dried crude water extract was dissolved in physiological saline solution.

A. Intraperitoneal route

Seven groups of four mice each were used for this experiment. Groups 1, 2, 3, 4, 5 and 6 were given the extract intraperitoneally (i.p.). The doses given were 0.5, 1.00, 2.00, 3.00, 4.00 and 5.00 g/kg respectively.

Group 7 served as the control and each mouse of this group was given 0.4 ml physiological saline intraperitoneally. (This corresponded to the highest volume of extract used in the test animals).

B. Oral route

Six groups of four mice each were used for this experiment. Groups 1, 2, 3, 4 and 5 were given 1, 2, 4, 8 and 16 g/kg of the extract respectively. Group 6 was used as control and each mouse was given 0.4 ml of physiological saline.

Determination of median lethal dose

The median lethal dose (LD_{50}) was determined by plotting the percentage mortality against log dose on a graph paper.

3.3: MICROBIOLOGICAL STUDIES OF SCLEROCARYA BIRREA BARK

(a) Determination of sensitivity of crude water extract

Materials and microorganisms used

Syringes, Membrane filters (Sterile), Samples bottles (Sterile), Nutrient agar (Plates), Nutrient broth (Tubes), Swabs (Sterile), Pipettes 1ml, 5 ml (Sterile), Cork borer (No. 5), Disinfectant (Cetrimide in Ethanol).

Cultures of Staphylococcus aureus (2 spp.), Pseudomonas aeruginosa (2 spp.), Bacillus subtilis, Proteus vulgaris, Salmonella typhi, Escherichia coli (2 spp.), Serratia marcesens. All obtained from Ahmadu Bello University Teaching Hospital, Zaria.

Methods

10 grams of the dry povdered bark was extracted with 50 ml of distilled water using Soxhlet apparatus. The crude water extract was filtered using sterile membrane filters and stored in sterilised sample bottles. The organisms were incubated in nutrient broth at 37°C for 18 hours. After incubation, the density of the cultures was adjusted by diluting with nutrient broth and comparing with that of the opacity tube. The plates of nutient agar were inoculated by streaking with sterile cotton swabs. Streaking was done successively in three different directions to obtain even inoculum. The plates were allowed to dry for about five minutes at room temperature. Four holes, about 5 mm in diameter, were then cut with a sterile cork borer number five.

0.1 ml of the extract was placed in each of the holes and left at room temperature for two hours, after which incubation was done at 37°C for 24 hours. The size of the inhibition zones were then measured. The diameter of the zones (including diameter of the holes) were measured and recorded to the nearest millimetres reading to the point of complete inhibition as judged by the unaided eye with the aid of a plastic ruler.

(b) Determination of the minimum inhibitory concentration (MIC)

Materials

1. 10 racks of 10 tubes each containing 2.5 ml of Mueller - Hinton Broth.
2. Sterile pipettes
3. Membrane filters
4. 8-hour cultures of Staphylococcus aureus (2 spp.), Pseudomonas aeruginosa (2 spp.), Bacillus subtilis, Proteus vulgaris, Salmonella typhi, Escherichia coli (2 spp.), Serratia marcesens.
5. Stock solution of extract (40 mg/ml).

Method

The culture (in nutrient broth) was suitably diluted with sterile normal saline such that 1 ml of the broth contained approximately 10^6 organisms. The dilution was done by comparing the density with a suspension of barium sulphate prepared by adding 0.5 ml of 1% barium chloride solution to 99.5 ml of 0.36 N-sulphuric acid.

Serial double dilution of the extract was made by adding 2.5 ml of the stock solution to 2.5 mls of double strength Mueller-Hinton Broth and the dilution carried on by transferring 2.5 ml of the extract broth to 2.5 ml of the Mueller-Hinton broth. One drop (0.02 ml) of the standardised culture was added to each tube and the set incubated overnight for at least 18 hours.

The minimum inhibitory concentration is the least concentration of extract showing no growth of the bacteria.

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APPENDIX

1. Physiological Solutions

The concentrations of solutes in Frog-Ringer solution (g/l of solution) NaCl = 6.5, KCl = 0.14, NaH_2PO_4 = 0.01, D-Glucose = 2.0, $NaHCO_3$ = 0.4 and $CaCl_2$ = 0.12.

Concentrations of solutes in Dejalon solution (g/l of solution) NaCl = 9.0, KCl = 0.42, D-Glucose = 0.5, $NaHCO_3$ = 0.5, $CaCl_2$ = 0.27.

Concentrations of solutes in Tyrode solution (g/l of solution) NaCl = 9.0, KCl = 0.20, NaH_2PO_4 = 0.05, D-Glucose = 1.0, $NaHCO_3$ = 1.0, $CaCl_2$ = 0.20, $MgCl_2$ $6H_2O$ = 0.01.