PHARMACOGNOSTIC AND TOXICITY STUDIES OF RICINUS COMMUNIS LINN,

BY

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DECLARATION

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

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15-3-96
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CERTIFICATION

This thesis entitled "PHARMACOGNOSTIC AND TOXICITY STUDIES OF RICINUS COMMUNIS LINN" by MARTINS, OLUSEYE LAWRENCE 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

This thesis is dedicated to my mother Mrs. Olufunmilayo Martins who made everything possible.

And to the Almighty God.

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ABSTRACT

Ricinus communis Linn. Family Euphorbiaceae was originally a wild plant, but is now being cultivated in the tropics. The plant is cultivated primarily for the oil obtained from the seed. The seed contains a poisonous protein known as ricin which has been implicated in cases of poisoning in both man and animals. The whole plant has been used over the ages in traditional medicine and is also employed in both pharmaceutical and general industries.

In this work various parts of the plant were screened pharmacognostically and toxicologically. The results of the phytochemical screening indicated the presence of lignin, tannins, oils, starch, cellulose, mucilage, free and combined reducing sugars, alkaloids and flavonoids in all the parts of the plant. Ricin was found to be present only in the seed.

A detailed microscopical study of the anatomical sections and powdered forms of the different parts of the plant were carried out and characteristic features like xylem vessels, fibres, nature of cells, calcium oxalate crystals and cork cells were observed. The moisture content, ash and extractive values were also determined.

The toxicological screening of the aqueous extract (ricin) of the seed gave the median lethal dose (LD_{50}) as 0.0003g per kg. Chronic toxicity studies indicated lung oedema and liver necrosis. The extract also caused haemolysis of red blood cells.

The various results and data obtained from the pharmacognostic screening would be useful in the preparation of pharmacopoeial monographs and help in the realization of the full economic potential of the plant. The results of the toxicity studies would serve as an aid in the treatment and maintenance of cases of poisoning.

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CHAPTER ONE

1.1 INTRODUCTION

Ricinus communis Linn of the family Euphorbiaceae was a native of Asia, but is now being cultivated in tropical countries including Nigeria, where the small varieties grow wild.

The annual production of castor oil seed amounts to 800,000 - 900,000 tonnes and 350,000 tons of oil. Major producers of the plant are China 310,000t of beans, India (185,000t) and Brazil (146,000t). Two thirds of the total world production is exported in form of seed and oil, and of these China exported 32,000t of oil, and 127,000t of seed, Brazil 60,000t of oil and India 31,000t of oil in 1988 alone (Rehm and Espiq, 1991).

The entire seed is very poisonous on account of the presence of a toxalbumm called ricin. Ricin is not present in the grade A oil expressed from the seed used as purgative (castor oil) but small amounts can be found in the grade C and the highest concentration in the cake. The cake has been found to be a contaminant of linseed and maize meal. On account of its poisonous nature the 'cake' is used exclusively as fertilizer. This cake is rich in protein (15-20%) and

therefore it could act as feed material for man and animal (Rehm and Espiq, 1991).

By whatever route it is administered ricin is very toxic. All animals are susceptible to its effect. According to Clark (1947), the horse is definitely the most susceptible animal while sheep, cattle and pigs are least resistant, and ducks and hens more resistant.

True immunity can be produced through the administration of immunizing doses of the phytotoxin, by giving increasing doses (or concentrations) of the phytotoxin. An animal is rendered immuned by injecting gradually, increasing doses of the toxin. The immuned animal can then tolerate amounts of up to 800 times the lethal doses.

Immunity in cattles and calves can be induced by feeding increasing amounts of the bean (Breirem, 1943). Passive immunity can be induced by the injection of the specific antiserum, but this is not likely to be on hand under field conditions (Clarke and Johnson, 1956).

1.2 LITERATURE REVIEW

1.2.1 THE PLANT

MORPHOLOGY

Ricinus communis Linn. Family Euphorbiaceae is originally a tree which can get to a height of 10 metres. The agricultural cultivars are usually grown annually, particularly the dwarf cultivars (60-120m high), which are best suited for dry areas and for mechanical cultivation. Branchy at the base, round alternate leaves, on long leaf stalks, palmately lobed with 7 dentate globular lobes. The shoot formation is sympodial with inflorescences at the end of the shoot, and branching under each inflorescences. With most types, the female flowers are in the upper part of the inflorescence, while the male is in the lower part. Female capsule contains 3 arillated seeds.

The seeds are rounded, oblong and somewhat flattened from 8-12mm long, 6-9mm wide and 4-8 mm thick with an arched dorsal surface and a nearly flat ventral surface. The seed coat which is thin and brittle, is smooth and glassy, varying in colour from greyish brown to grey, and mottled with reddish brown or black spots and stripes.

At one extremity of the seed there is a prominent and usually pale coloured caruncle, from which the raphe runs along the ventral surface as a distinct line to the other extremity, where it terminates in a raised chalaza. The caruncle can be removed easily, disclosing the hilium beneath as a dark spot.

A delicate silvery-white membrane inside the seed coat surrounds a large, yellowish white oily endosperm which encloses the embryo with two large papery cotyledoms.

The seeds have a slight odour and weakly acrid taste. They contain a high proportion of fats and proteins and some ricin. There are many forms of the plant with variations in the shape of the leaves and colour, size and seed markings (African, Pharmacopoiea, 1985).

The epidermis consists of polygonal pitted cells, some with and others without brown contents. The layer of seed coat consist of brown, pitted and sclerenchymatous cells. There are large aleurone grains in the endosperm and abundant fixed oil. The aleurone grains are round or ovoid and measure up to 20μ in diameter.

1.2.2 ECOPHYSIOLOGY

R. communis Linn comes from the tropical regions of the world and it grows well in the wet tropics to the sub-tropical dry regions, wherever its temperature requirements are satisfied. Growing time is 4.5-6 months, optimum temperature 20-25°C and over 38°C the seed setting is poor.

In areas with summer rainfall the dwarf cultivars can be grown with only 500mm rainfall, although 750-1000mm is optimal. Due to its deep reaching root systems, castor is quite drought resistant. Short days delay flowering, however, castor has little sensitivity to daylight. It prefers deep, sandy loam with a pH of 6, but it can be cultivated on a wide variety of soils with a pH range of 5-8. It does not tolerate salt. The nutrient removal for 1 ton of seed (with capsules) is about 30 kg N, 5 Kg P, 12 Kg K, 4 Kg Ca and 3 Kg Mg. Unbalanced nitrogen fertilization encourages the growth of foliage at the expense of flower and seed formation (Rehm and Espiq, 1991).

1.2.3 CULTIVATION AND PROCESSING

In mechanized cultivation, seeds are sown with a spacing of about 90 cm between the rows and 50cm within the rows (seed requirement 12-18 kg/hectare, depending on the seed size). The seed is sown 5cm

deep. Weed control is especially important in the first weeks, as the plant initially develops quite slowly. Mechanized hoeing should be done with care, as the young plants are very sensitive due to their brittle nature.

Good soil preparation and the eradication of persistent weeds before sowing are of substantial help. In the U.S.A, general herbicides are used before germination.

In the main areas of cultivation, which have low atmospheric humidity, diseases do not play a major role. Insect pests are generally held in check by sanitary measures and crop rotation.

Plants begin to bear fruit at about 4-6 months old. Indehiscent cultivars are harvested fully ripe by picking the capsules by hand, possibly using a cup-shaped device, or mechanically; with cultivars that burst open, it is recommended that the infructescences are cut off before fully ripen, and then left to dry. The non-shattering capsules are broken to separate the seeds from the fruit walls and in large-scale cultivation this is done with the help of machines.

The seed is then shelled and the husks removed by winnowing before pressing for oil or extraction (Rehm and Espig, 1991).

1.2.4 USES OF THE PLANT

Ricinus communis has been employed by man over the ages for various medicinal purposes and more recently in industry. It is of great economic and export potential.

Scarpa and Guerci (1980) reported the use of the castor plant in Traditional and modern medicine in over 50 countries for various ailments.

Following is a brief summary of the therapeutic use of R. communis based on pharmacological actions of the different parts of the plant as described by Scarpa and Guerci, (1980).

Respiratory apparatus

Castor bean latex is used when recovering from rhinitis in China, the leaves are used in treatment of angina in Madagascar; splashing leaves with vinegar is used to cure chest inflammation in Italy; Castor oil with infusion of orange leaves is used against bronchitis in Haiti; other countries include India, Pakistan and Ivory Coast (Pierre-Noel, 1959; Scarpa and Guerci, 1980).

Cardiovascular apparatus

In the 10th century, oil was used for treatment of apoplexy in Iran; the oil used for treatment of cerebrial congestion in Haiti and Ceylon, the oil is also used for lubricating inflammed parts in

lymphangitis in Haiti (Pierre-Noel, 1959).

Digestive apparatus

The leaves are used locally for the treatment of dental caries in Madagascar; Roots are used in treating odontalgia in Zululand, and from relief from gastrolgia in Zululand, Columbia and Mexico; and for treatment of intestnal colic in Madagascar and Iran. The plant is also used as an anthelmintic, taeniafuge, treatment of stomach ache, diarrhoea etc (Watt and Breyer, 1962).

Articulations, Bones and Muscles

The plant is used for healing of contusions and related inflammations in Haiti, mending bone deformations in Algeria; Treatment of articular pains in Italy, Indonesia and Libya; It is used as an antirheumatic in Madagascar, Arabia, Somalia, Caylon, India, and Pakistan, The root is used against rheumatism, lumbago and sciata in India; and for massage of afflicted limbs in Haiti (Scarpa and Guerci, 1980).

Urogenital apparatus

The roots are known for diuretic action in Italy; It is also used in treatment of inflammation of genital organs in India and Pakistan, the purified seed is used against vaginal and uterine diseases; the oil is considered to have a spermatopoietic action in

India; and to relieve pains due to renal calculis in Algeria and Sri Lanka and for hydrocele in Sri Lanka (Scarpa and Guerci, 1980).

Infectious diseases

The leaves are used in treatment of small pox by pygmies of Africa, for treatment of tropical framboesia in Eritrea, Libya, Somaliland; The seeds are ingested to contend leprosy in Senegal, Guinea Bissau, Ivory Coast and Upper Volta; and treatment of Bilharziasis in Senegal (Kerharo and Adam, 1974).

Oncology

Scarpa and Guerci (1980) reported the use of the plant in treatment of tumours (and mammary tumours) in ancient Greece. More recently research is being carried out to evaluate its usefulness in anticancer theraphy due to its antineoplastic (cyto-toxic) properties.

Puericulture and pediatrics

The custom of smearing children with oil at birth was reported in Malawi and Central Africa; The oil and leaf are used against bronchial catarrh in Guatemala and Columbia; Treatment of cateralgia in India and flatulance in Pakistan; The oil is used in treatment of diarrhoea in transvaal, Haiti, Brazil and Columbia and as a purgative in Italy; for relieve of mastitis during suckling in India and Pakistan; Treatment of

hupogalactica in Somalia, South Africa, Cape Verde Islands, Madagascar, India, Pakistan and Columbia (Scarpa and Guerci, 1980).

Dermatology

The oil is used for treatment of Tinea and Seborrhea of scalp in Ethiopia and Italy; For scabies and skin diseases in Angola and Guinea Bissau; Against impetigo, eczema and pustules by Basutos in South Africa; Treatment of furuncles in Tanzania; Against warts in South and East Africa; Castor bean is used for its vulnerary action in India - against snake and scorpion bites, the plant is also used for antiseptic and topical action, and in the feild of cosmetology in India and Tanzania (Ganora, 1929; Scarpa and Guerci, 1980).

Veneral diseases

The crushed seeds are used in treatment of blennorhagia in Eritea; Treatment of gonorrhea in Dominican Republic, India and Pakistan; and treatment of Syphilis in Algeria (Scarpa and Guerci, 1980).

Otorhinolaryngology

The crushed seeds or dried leaves are used against otitis in Transvaal, Ceylon, Thailand, Malaya and Haiti, and curing of troubles of speech and hearing in Chinese medicine (Brondegaard, 1976).

Opthamology

Castor oil is used to prepare, as a soothing base for opthalamic compounds in Nigeria; used locally against conjuctivitis in India and Pakistan, and for inflammation of the eye in Indonesia, Italy and Columbia (Brondegaard, 1976).

Obsterics and Gynaecology

The pulverized root is used as an antiseptic in cases of haemorrhage of hymen in South Africa, the oil is used as ammenagogue in Algeria, promotion of menstrual flux in India and Pakistan, treatment of amenorrhea, and against chest and abdominal pains in pregnant women in Ceylon. The seeds are eaten to induce of barreness in Algeria and Mexico (Brondegaard, 1976).

Nervous System

Leaves are used locally against cephalgia in Tonga, South East Africa, Madagascar, Nepal and Phillipines; The oil is used for Massage in Mexico and Paraquay; As a stimulant of motor activity in Russia, Treatment of nervous and mental diseases in Senegal and India and against paralysis in Algeria (Brondegaard, 1976; Scarpa and Guerci, 1980).

Veterinary Science

Castor oil is used for treating skin diseases in animals and eye inflammations in South east Africa; against 'Berne' do gado in Brazil (Scarpa and Guerci,

1980).

The main use of castor seeds is for the production of castor oil. It is used medicinally as a laxative and emollient. The oil contains 91-95% ricinoleic acid, which is used in the production of many important products, such as heptaldehyde used in perfume synthesis; undecenylic acid as starting material for the synthesis of a variety of chemicals; octanol - utilized as a solvent and in perfume synthesis; and sebacic acid - used for synthetic fibres and resins.

Castor oil itself retains its viscosity at high temperatures and is therefore used as a lubricant. Because it does not corrode rubber, it is used as an additive in the production of crumb rubber and in hydraulic systems (for example brake fluids).

In the plastic industry, it is used as a plasticizer. Due to its ability to dissolve certain colours, it finds application in lipsticks, or cosmetic products, printing inks and in textile industry. Polymerized castor oil gives permanent adhesion and plasticity to paint materials.

It is seldom used any more as a purgative. The press cake is poisonous and is only used as a fertilizer. Fermented castor seeds are used as a condiment in Eastern Nigeria, unripe and also roasted

ripe seeds are eaten in Indonesia.

Shells and stems are used as fuels and for mulching. The young leaves can be consumed as a vegetable. When animals are accustomed to the leaves, they can be fed these as fodder, inspite of the ricin content. Castor leaves are the main food of the eri silk worms (Relm and Espig, 1991).

1.2.5 CONSTITUENTS OF THE SEED

Castor oil seed contains 42-56% fixed oils, which is an almost colourless or slightly yellow viscid liquid with a slight characteristic odour.

The oil contains 91-95% ricinoleic acid which is chemically much more reactive than any other fatty acid due to a double bond on the 9th Carbon atom, and a hydroxyl group on the 12th. It can be dehydrated to form a fatty acid with 2 conjugated double bonds which can be easily polymerized ($C_{17}H_{32}OH$ COOH - Ricinoleic acid).

At 275°C in an alkaline medium, the ricinoleic acid is split, forming sebacic acid and octanol; Vacuum distillation results in undecenylic acid and heptaldehyde (Rehm and Espig, 1991). The castor bean seed coat constitute 17-20% of the seed total weight.

Castor oil is composed of glycerides of oleic, linoleic, stearic and dihydrostearic acid though mainly of ricinoleic acid. It also contains globulin,

cholesterol, lipase, vitamin E or tocophenol (Scarpa and Guerci, 1980).

The cake left after expression of the oil contains a crystalline principle ricinine, ricin and a very active lipase.

Ricin, a toxic protein occurs in the form of a white powder that is soluble in water and glycerin, it is a glycoprotein containing in different amounts the amino acids - arginine, histidine, lysine, tryosine, tryptophan, cystine, methionine and glycol. 0.2% of ricin has been isolated from the seed (Watt and Breyer, 1962).

Ricinine has the characteristics of alkaloids but does not form salts with acids. It forms colourless rectangular prisms and laminae, having a slight taste of bitter almonds (Tuson, 1864).

Ricinine (N-methyl-cyano-methoxy-pyridone) is an alkaloid, derived from pyridine and has been isolated in crystalline form. It is odourless and soluble in water, chloroform, alcohol and other organic solvent (Kerharo and Adam, 1974).

The amount of ricinine in the seed increases as the seed germinates, it has also been isolated from the leaves. The pressed seed yields 0.3% of ricinine ($C_8H_8O_2N_2$) m.p 201-5°F subliming at 170-80°; and the whole plant yields 1.1%. Ricinine contains a nitrile

group and a pyridine nucleus.

The seed also contains a powerful allergenic principle which produces on inhalation allergic coryza and bronchial asthma. It is known as Ricinus allergen. Castor bean contains about 1.8% of the allergen. The ricinus allergen passes through membranes whose pores are too small to permit the passage of ricin (Ogundokun, 1991).

It has also been confirmed that in addition to the extremely toxic principle ricin, the seed also contains agglutinies with much lower toxicity (Olsnes, 1978).

The seed also yields 1.16 µg/g of riboflavin, 13.0µg/g of nicotinic acid, tocophenol 1%, Nonsterol 21.6% and 0.0061% of uric acid. A positive test has been obtained for the presence of urease in the seed and for a protein denaturing enzyme (Watt and Breyer, 1962).

Glutamine has been extracted in large quantities from the germinated seed. The product of hydrolysis of the seed was found to contain norleucine.

During germination of the castor oil seed, the formation of volatile, non-volatile and ketonic acids occur, among which formic, acetic, butyric, lactic, succinic, fumaric, malic, citric, pyruvic, α-ketoglutaric and ascorbic acids have been noted

(Kerharo and Adam, 1974).

1.2.6 TOXIC PRINCIPLE - RICIN

The residue left after extraction of the oil by pressing contains the toxin ricin.

Ricin, the active principle of the seed belongs to the group of plant toxins known as 'phytotoxins'. It is a protein of molecular weight 77,000 (Kabet et al, 1947).

Phytotoxins (also known as Toxalbumins) are protein molecules of high toxicity produced by a small number of plants. In vitro, they cause agglutination of erythocytes. It has been suggested that they function in vivo as potent proteolytic enzymes, owing their toxicity to breakdown of crucial natural products. Ricin solutions are coagulated by heat, the coagulation causing complete loss of toxicity but not of antigenicity (Breirem, 1943).

Poisoning has arisen from accidental or deliberate admixture of pellets of castor cake with other feeding stuffs. It can be caused by meal mixed in a plant previously used for castor seed (Fox, 1961).

Ricin is absorbed in the gut. An aqueous extract of castor bean contains ricin. It is highly stable and keeps for months without deterioration (Watt qnd Brayer, 1962).

The minimum lethal dose of ricin by injection is about 0.0001 mg per kg (0.00000001%) of animals weight. Oral administration is several hundred times less toxic - this suggests that ricin is not readily absorbed through the wall of digestive tract, or that it maybe partially destroyed during ingestion. In the case of the ingestion of whole seed, the degree of mastication and digestion determines in part the amount of ricin available for intestinal absorption.

Ricin differs from bacterial toxins and snake venom in the relative ease with which it is absorbed through intestinal wall. Ricin is present in all parts of plant but predominantly in the seed.

Ricin is a mixture of three glycoprotein, one of which is Ricin D which has 493 amino acids and 23 sugar units with a total molecular weight of 55,000 divisible into 2 units.

Ricin D is composed of 2 polypeptide chains linked by a single disulfide bond. One of the chains has an N-terminal isoleucine, a C-terminal serine and one glycopeptide moeity. The other chain has an N-terminal alanine, a C-terminal phenyalanine and 2 glycopeptide moeties. The sugars are attached to either aspartic or asparagine as oligosaccharides (Funatsu et al, 1971).

Funatsu et al (1988) reported that ricin D contains, manose, fucose, xylose and N-acetylglucosamine as sugar compounds; and that the sugar chains are linked to Asn-10 of the A chain and to Asn-95 and Asn-95; Man5-7 Glc NA_{c2} and M_{4x} to Asn 135 of the β chain; and M_5FX and M3X to Asn-10 of the A-chain.

After treatment with β-mercaptoethanol, the ability of ricin to inhibit protein synthesis in a cell-free system is increased 50-100 fold, whereas its toxic effect in living animals and in cells in culture is virtually abolished suggesting that the 2 chains have different functions.

The smaller peptide chain A strongly inhibited protein synthesis in a cell free system from rabbit reticulocytes, whereas the larger peptide B chain lacked this ability.

Human erythocytes pretreated with intact ricin or with B chain were agglutinated by an antiserum directed specifically against ricin, whereas erythocytes pretreated with A chain were not agglutinated under same conditions. Only the B chain was bound to a sephradose column, a binding which could be abolished by the presence of galactose. This shows that the toxic action of ricin is associated with the A chain and that B chain functions as a carrier moeity which binds the toxin to the cell

surface (Olsnes and Phil, 1973).

Cushley (1985) stated that ricin is a heterodimeric glycoprotein derived from the cotyledon of a common bean, R. communis. It has a m.w. of 63,000 and upon reduction with 2-mercaptoethanol is divisible into its 2 structurally distinct sub-units. These are the A-chain (RTA) which acts enzymatically as an irreversible inhibitor of eukaryotic cellular protein synthesis and the B-chain (RTB) which functions principally as a lectin with specificity for galactose containing cell surface macromolecules. Moroney et al (1987) state that ricin has 2 separate binding sites for galactose.

Isolation of ricin from R. communis seed cake is done by aqueous extraction. Purification is carried out by fractionating with $(NH_4)_2SO_4$ and then chromatography on sephadax G-100 (Dirheimer et al, 1965) or Eletrophoresis (Funatsu et al, 1971) to give its fractions. Ramakrishnan et al (1983) prepared ricin A and B by chromatofocusing and by DEAE B10 Gel A chromatography.

A simple and rapid method for the isolation and purification of ricin was designed by He et al (1988). Ricin was isolated from the seeds of castor bean by homogenization and $(NH_4)_2SO_4$ precipitation, then purified by Sephrarose 4B colomn. this method was

found to be simple, efficent and useful for obtaining ricin for the preparation of immunotoxins.

Emmanuel et al (1988), after completely cleaving of ricin inter chain disulfide bridge with 0.05N dithiothreitol in denaturing conditions at 37°C during 1.5H, separated A and B-chains on a lactosaminyl - aminoethyl Biogel D-150 colomn at 4°, in the presence of 0.01M dithiothreitol and 0.5$MgCl_2$ A- and B-chains were characterized by SDS-PAGE and immunol. Cross-contamination was ≈ 0.1%.

Isolated ricin is judged to be homogenous by chromatography or hydroxyapatite, electrophoresis and ultracentrifugation.

Piatak (1987) determined DNA sequences encoding full length percursor proteins of both A and B portions of 2 ricin iso-toxins and ricin agglutinins, as well as the linker regions. The DNA portions or portions of modification thereof were expressed in recombinant hosts to obtain desired proteins which can readily be converted thereto.

The first demonstrable effect of ricin on cells in culture is the inhibition of protein synthesis. The toxin inactivates the ribisomes rather than some of the soluble components. Different cells exhibit different sensitivities to the toxin. Only the intact ricin inhibits protein synthesis in intact cells. The

A chain of ricin can inhibit protein synthesis in cell-free systems but the B-chain does not have this ability. On the other hand, the B-chain binds to cell surfaces, probably to certain glycoproteins containing terminal non-reducing β-galactose residues, and the binding can be easily prevented by adding galactose or lactose to the medium.

The binding is very important for the toxic effect of ricin, since the presence of lactose and galactose in the medium strongly inhibits intoxication. It thus appears that the toxins bind to the cell surface by their B-chain while the A-chain in some way penetrates the cell membrane to get access to its target in the cytoplasm (Olsnes, 1978). In intact cells, the ribosomal 60S unit appears to be the site of action of the ricin. In some experiments, one ricin molecule was found to inactivate a large number of ribosomes indicating that the ricin acts by a catalytic mechanism.

Tsurgi and Ebert (1987) showed that RNA N-glycosidase activity of A-chain of ricin was responsible for the ability of ricin to inactivate eukaryotic ribosomes.

Tsurgi and Ebert (1987) reported that the cytotoxin-ricin retains its specificity when tested on a synthetic oligoribo-nucleotide (35-Mer) that mimics

a region of 28S ribosomal RNA. Ricin depurinated a nucleotide in the synthetic oligomer and in the presence of aniline the phosphoribose backbone was cleaved at a position that conforms to A-4324 in 28S-RNA, the site of the toxin in vivo.

Stirpe et al (1988) confirmed the work of Tsurgi stating that ricin acts to introduce a specific modification into 26-28S rRNA which renders the RNA sensitive to cleavage by aniline.

Van deurs et al (1988) suggested that translocation of toxic ricin A-chain to cytosol occurs in the trans golgi network. Conformation of ricin and its A-chain depends significantly on pH 3-8, for B - 4-5 i.e the conformation changes with pH. The affinity for galactose of ricin and isolated B-chain depends on pH and the maximal binding observed pH was at pH 7 (Bushueva and Tonevitski, 1987).

Methylamine stimulates the action of ricin toxin, this was also found true for some other amines (Mekada et al, 1981).

Proteins extracted from wheat germ 60-S ribosomal sub units and rat liver 60S and 40S ribosomal units with 3M NH_4Cl 75mN $MgCl_2$ were able to prevent the ricin A-chain mediated inactivation of untreated 80S rat liver ribosomes. This data being consistent with the idea that the enzymatic target of ricin A chain is

protein in nature (Chang and Houston, 1981).

Balint (1983) used irradiation with ultraviolet light to decrease significantly acute toxicity of ricin in intraperitoneal administration to mice.

The free chains of ricin A and B were more reactive in the immunoassay than the equivalent quantity of the undivided chains when combined in intact ricin (Ramakrishnan et al, 1982).

Montesano et al (1982) reported that Disuccimydyl suberate cross linked ricin does not inhibit cell-free protein synthesis. Ricin was reacted with dissucinimydyl suberate to yeild a molecule in which the A and B chains were covalently linked through a non-reducible bond. After purification, this cross-linked ricin analog was unable to inhibit protein synthesis in a cell-free translation system from rat liver. In contrast, after modification with the cross-linking agent the isolated ricin A chain maintained its inhibitory activity. These results supported the views that the ricin must be cleaved into its constituent polypeptide chains to elicit its toxicity and suggests that the reduction of the disulfide bond alone is not sufficient for ribosome inactivation in vitro.

Arming α 2-macroglobulin with ricin A chain forms a cyto toxic conjugate that inhibits protein synthesis

and kills human fibroblasts. Ricin was found to be 28,000 times more inhibitory than chain A alone. The presence of α 2-macroglobulins did not increase the cytotoxicity of unconjugated A-chain, and it even protected cells to a slight extent. The inhibitory action of the conjugate was blocked by antibodies specific for α 2-marcroglobulin or ricin and was not prevented by galactose or antibodies specific for ricin B (Martin et al, 1983).

Concanavalin A-induced inhibition of ricin activity parallels with a decrease of the number of toxin-binding uptake-elution circles (Delfini et al, 1982).

Sargiacomo and Hughes, (1982) reported that a cytotoxic photoliable crosslinking derivative of ricin was found to be fully toxic when treated in the dark on intact cells but toxicity decreased with irradiation.

After digestion with papain, pepsin and pancreatic polypeptidase the toxicity is greatly reduced by the transformation of the toxalbumin into fractions of the albuminose type.

Ricin agglutinates not only blood but also cholesterol suspensions and other suspensions of negatively charged particles. Less than 0.1N, obtained by lycophilizing an aqueous extract of

crushed seed, inhibits tumour growth about 60% (Watt and Breyer, 1962).

By whatever route it is administered ricin is very toxic. The single oral lethal doses of the seed in g/kg are said by Miessner (cited by Clark and Clark, 1975) to be as follows:- Horse 0.1, Ox 2.0, Calf 2.5; Sheep 1.25; Goat 5.5; Pig 1.4; Piglet 2.4; Rabbit 1.0; Geese 0.4; and hen 4.0.

However, there appears to be a wide individual variation in susceptibility to the toxin. Miessner found, for example that although, as stated above, the average lethal dose for the rabbit is 1g/kg, some animals were killed by 0.4g/kg while for others 1.25g/kg was necessary to be certain of producing death.

Similarly, Clarke (1947) states that the oral lethal dose of castor seed in the horse may be as little as 0.007g/kg, yet in some animals even 0.3g/kg may not be lethal.

The toxic dose by parenteral administration may be 500 times less than the oral dose. Thus, sub-cutaneous injections of the ricin from 1mg of castor seed is said to be fatal in the rabbit (Clark and Clark, 1975).

There happens to be a lot of disagreement on the possible lethal dose of the toxin (ricin) in man and

other animals. The Zulus regard three seed as a fatal dose (Watt and Breyer, 1962). This workers regard two seeds as a fatal dose in an adult and one to a child. But it is clear, however, that recovery has followed ingestion of larger quantities, the form in which seed is swallowed might also be significant.

1.2.7 SIGNS AND SYMPTOMS OF POISONING

There is a characteristic latent period between ingestion of castor beans (or injection of ricin in experimental poisoning) and the onset of signs.

This lag time decreases with increasing amounts of toxin given and may last for a few hours when high amounts of toxin are injected.

In horses the latent period is usually several hours but may be as long as 2-3 days (McCunn et al, 1945). In birds it is never less than 48 hours.

Steyn (1934) states that if large amounts of castor bean are eaten by horses, general weakness and collapse may be evident almost immediately. Clinical signs are progressive, becoming more serious as the case goes on.

McCunn et al (1945) gave a detailed description of the appearance of horses at all stages of castor bean poisoning. In the early stages animals were dull, later they showed signs of inco-ordination and in

severe cases, there was profuse sweating, tetanic spasms of the muscles and a 'tumultous' heart beat which shook the entire body. All animals showed profusely watery diarrhoea and even in the most severely poisoned animal there was no blood in the faeces, but Anderson (1948) gave an account of poisoning in cattle in which the main sign was bloody diarrhoea.

Geary (1950) noted vomiting and diarrhoea (not bloody) in pigs. The animals were weak and showed some inco-ordination and signs of abdominal pain. The skin of the ears, flanks and hams were cyanotic. Dullness, drooping wings, ruffled feathers and greyish coloured wattles were observed in pullets while adult birds were less severely affected.

In experimental poisoning by parenteral adminstration of ricin, after the initial latent period, the animal rapidly lost weight and developed diarrhoea. Convulsions then suddenly appear, alternating with periods of flaccid paralysis; these persist until the animals die within an hour or so (Clark, 1947).

The most common symptoms are nausea, vomiting, abdominal pain, colics, diarrhoea, hemorrhage from the anus, anuria, cramps, dilation of the pupils, fever, thirst, burning in the throat, headache and shock

symptoms. Death occurs in exhaustion or cramps (Olsnes, 1978).

Meretic (1980) reports the case of poisoning of 7 adults and 7 children, treated in hospitals and out patient clinics. Symptoms of poisoning appeared a few hours after ingestion as profuse vomitting and watery diarrhoea leading to dehydration. Also arterial blood pressure dropped, leucocytes with neutrophilia and eosinolymphorpenia appeared, occasionally associated with a slight increase in blood glucose and ECG changes with a lowering of final complexes. In one case, ECG changes developed further and persisted for more than 1 month and in another for 8 days. Medical treatment was symptomatic.

Acute poisoning with the seed also results in the production of hyperplastic myelosis from stimulation of the bone marrow, followed by haemolytic anaemia, hyperchromia, neutropenia and eosinophilia (Watt and Breyer, 1962).

1.2.8 POST-MORTEM FINDINGS

The contents of the gut are fluid or semi fluid, there are patchy inflammation of the gastric and intestinal mucous membranes, sometimes accompanied by punctiform haemorrhages. The mesentric lymph

glands are swollen, and there are frequent inflammatory lines along blood vessels and lymphatics of the mesentry. The liver and kidney may be swollen and oedematous or may be unaffected. The lungs may be oedematous and the trachea and bronchi filled with frothy oedematous fluids (Olsnes, 1978).

Geary (1950) states that a fowl that died of castor bean poisoning showed inflammation of the oesophagus, crop and true stomach.

After injection of high doses of toxin, autopsy shows haemorrhages of the intestine, mesentry and omentum, and often a diffuse nephritis; and after injecting smaller doses, the autopsy shows haemorrhages in the ileum and a ret spotted oedema of the Peyer's patches is observed. Multiple necrosis appear in the liver and kidney, and there is a degeneration of the myofibrils in the myocardium. After subcutaneous injection of sub-lethal doses, necrosis and induration occur around the injection site (Kobert, 1906).

More recently it has been shown that in the liver of rats treated with ricin, the sinusoidal cells were more damaged than the hepatocytes (Olsnes, 1978).

Watt and Breyer (1962) reported irritation, congestion and necrosis of the mucosae of the gastro-intestinal tract and in some cases erosion of the

stomach wall.

Henry et al (1987) reported the ultra structure of degenerative changes within the ipsilateral trigerminal ganglion and partes caudalis and interpolaris of the spinal trigerminal nucleus in a cat following the application of ricin to the tooth pulps of unilateral maxillary and mandibular posterior teeth including the cupsids. The survival times ranges from 6-10 days and typical changes identified within the ipsilateral trigeminal ganglion include myelin fragmentation and 'compartmentilization' of the axoplasm of medium-sized myolinated axons, while small myelinated and unmyelinated axons underwent a more variable response ranging from dectron-lucent to electron dense changes. The affected cell body was also characterised by the presence of swollen, electron-lucent mitochondria, a reduction of cytoplasmic ribosomes and a filamentous hyperplasia. Other changes include an eccentric nucleus and satelite cell proliferation. Degenegative changes often occured in isolated elements surrounded by normal profiles, suggesting specificity of ricin within the trigerminal ganglion. Changes within brainstem axons showed both an electron-dense and a lucent fragmenting type of axonal alteration. Terminal changes ranged from electron dense to lucent

and also included filamentous hyperplasia and 'hyperglycogenesis'. The altered axonal knobs contained round synaptic vesicles. The above brainstem alterations were identified specifically in the following areas - ventrolateral, medial and dorsomedial pars interpolaris; the ventrolateral and mid-dorsal to dorsomedial areas of the marginalis and outer substantia gelatinosa layers of pars caudalis, and in ventral pockets corresponding to lamina V of the medullary dorsal horn. Dense alterations within terminals containing flattened synaptic vesicles that are typically presynaptic to primary effects in these areas were rare findings but along with vacuolization of dentritic profiles suggest a trans synaptic effect possibly due to the exocytosis of ricin.

1.2.9 AIMS AND OBJECTIVES OF THIS STUDY

The magnitude of human harzard from plant (toxin) ingestion is suggested by the fact that plants are the second most frequent harzadous substances ingested by children under 5 years of age and the fifth most frequent harzadous material ingested by humans of all ages (Oehme, 1978).

Between 12,000 - 15,000 reported cases of ingestion occur in the U.S.A each year in children

alone (Hardin and Arera, 1978). Poisoning by plant toxins including ricin have not received proper attention although they present an important problem to public health. The most efficient means of prevention is health education. Many of the medications (preparations) prescribed in Traditional medicine using Ricinus communis are not given in specific doses. There is therefore a need to do a toxicological profile on the plant (crude drug) so as to prevent/minimize cases of poisoning.

The specific aims and objectives of this research project are:

- To carry out microscopic, chemomicroscopic and quantitative microscopic studies on the different parts of the plant i.e leaves, stembark, root and seeds, since all these parts are used in Traditional medicine preparations.
- To determine the nature of the chemical constituents present in the different plant parts.
- To determine the various evaluative parameters such as moisture content, total ash value, acid insoluble ash value, alcohol soluble extractive value and water soluble extractive value of the different plant parts.
- To carry out toxicological studies on the aqueous extract of the seed (ricin), this would include the

determination of a median lethal dose LD_{50} and chronic toxicity studies.

- To carry out quantitative estimation of ricin and related substances using haemolysis and spectroscopic measurements.

CHAPTER TWO

2.0 MATERIALS AND METHOD

Materials and methods employed in various qualitative and quantitative determinations have been indicated and described in every case.

2.1 COLLECTION AND IDENTIFICATION OF THE PLANT MATERIALS

The plant parts (roots, leaves, stem bark and seeds) were collected in January 1993 from Kabba in Kogi State. The variety used was the large variety used for making soup in the eastern part of Nigeria.

The plant was identified by comparing with authentic samples preserved in the Herbarium of the Dept. of Biological Sciences, A.B.U, Zaria. All the samples compared favourably. The identity of the plant was further confirmed by comparing it with the description and diagram given in 'Cultivate plants of the tropics' by Rehm and Espig (1991) and 'Flora of West Africa by Daziel (1956). The plant parts conformed strictly to the description given in these books. This was necessary to ascertain that the sample collected was that of Ricinus communis L.

2.2 DRYING AND STORAGE

The plant parts were air dried under shade at room temperature for some days.

After drying the individual parts were ground separately using mortar and pestle. The seed testa were first removed before grinding. The ground stem bark, leaves and roots were sifted using No-44 mesh and the powders obtained after sifting were used for microscopical studies of the various parts.

The oil from the seed was removed by washing in petroleum ether (60-80°) until there was no oil left. The residue was then dried at room temperature for 3 days. These powders were stored separately in air tight glass bottles and kept in a cool dry place and used for microscopical, phytochemical and toxicological studies.

2.3 SOLVENTS AND REAGENTS

All the reagents used were of laboratory or Analytical grades. In all cases water used was distilled water. The reagents used were as follows:-

- acetic anhydride
- Acetone (95%)
- Alcohol (90%, 95%) (Ethanol)
- Ammonia solution

- anihiline pthalate spray reagent
- anisaldehyde - sulphuric acid spray reagent
- Barfoed's reagent
- n-butanol
- chlor-zinc-iodine
- Chloroform
- Chloroform-water (0.25%)
- Copper acetate
- Dragendorffs reagent (Bismuth pottasium iodine)
- Ethyl acetate
- Fehling's solution I (A-copper II oxide) and II (B-solution hydroxide)
- Ferric chloride
- Formaldehyde
- Galactose
- Glacial acetic acid
- D-Glucose
- Hager's reagent (a saturated solution of picric acid)
- Hydrochloric acid (10%)
- N/50 iodine
- Iodine vapour
- Lead acetate
- Mayer's reagent (potassium mercuric iodide solution)
- Maltose
- Magnesium fillings

- Molisch's reagent (10% solution of alphanaphthal in alcohol)
- Phloroglucinol
- Ruthenium red
- Silica gel
- Sucrose
- Sodium acetate
- Xylose

2.4 ORGANOLEPTIC EXAMINATION OF RICINUS COMMUNIS

The following sensory characters of the stem bark, leaves, root and seeds of Ricinus communis were observed. They are colour, odour, taste and texture (Trease and Evans, 1983).

2.5 MICROSCOPICAL EXAMINATION OF RICINUS COMMUNIS

2.5.1 Microscopical Examination

The powdered materials as well as transverse sections of the fresh leaf, stem, root and seed were used for the study and prepared for microscopical examination as follows:- The anatomical sections of the leaf, stem bark, root and seed were taken on a thin slide separately and mounted in a few drops of chloral hydrate solution. These slides were slightly

warmed for 30 seconds (over heating was avoided as this could result in the formation of chloral hydrate crystals giving false observation. The sections were carefully covered with cover slips to avoid the trapping of air bubbles. After the section was cleared properly, the slides were mounted in dilute glycerol and observed under a microscope. These sections mounted in dilute glycerol remained fresh for a long period (Inyenger and Nayak, 1975, Trease and Evans, 1983a).

2.5.2 Chemomicroscopical Examination

In order to differentiate the tissues and also detect some chemical substances such as cellulose, lignin, tannins, fats and oils, starch and calcium oxalate crystals, the various anatomical sections and powders of the different parts of R. communis were stained with the appropriate chemical reagents on microscope slides and observed under the microscope (Inyenger and Nayak, 1975).

(a) Test for Lignin

The powdered stem bark, leaf and root and the section of seed were heated separately in a few drops of phloroglucinol and conc hydrochloric acid for two

minutes. A red colouration produced by any of the tissues such as xylem conducting elements and sclerenchyma etc indicates the presence of lignin. The intensity of red colouration indicates the extent of lignification (Trease and Evans, 1983b).

(b) Test for oils

The powders were separately mounted in Sudan IV reagent to test for the presence of fats and fixed oils. a pink colour in any of the tissues indicates the presence of fats and oils.

(c) Test for tannins

The powders (i.e leaf, stem bark, root and seed) were mounted in Ferric chloride solution. A bluish-black or greenish colour indicates the presence of tannins (Trease and Evans, 1983c).

(d) Test for starch

The powders (i.e leaf, stem bark, root and seed) were mounted in N/50 Iodine. A bluish-black colour indicates the presence of starch (Trease and Evans, 1983e).

(e) Test for Calcium oxalate crystals

The powders as well as the anatomical sections were cleared by warming with chloral hydrate solution.

The calcium oxalate crystals were seen as bright structures. After addition of a few drops of concentrated sulphuric acid, the crystals were dissolved thus confirming the presence of calcium oxalate crystals (Trease and Evans, 1983f).

(f) Test for Cellulose

The powders were mounted separately in chlor-zinc-iodine. A blue black colour indicates the presence of cellulose (Trease and Evans, 1983b).

(g) Test for Mucilage

The powdered samples were separately mounted in Rutherium red. A red or pink colour indicates the presence of mucilage (Trease and Evans, 1983d).

2.6 PHYTOCHEMICAL SCREENING OF THE ROOT, STEM BARK, SEED and LEAF OF RICINUS COMMUNIS

The powdered parts of the plant R. communis were screened to find out the types of carbohydrates and secondary metabolites like glycosides, flavonoids, and alkaloids present in them. Appropriate chemical reagents were used on them and resultant changes recorded.

2.6.1 TEST FOR CARBOHYDRATES

EXTRACTION: 3g of the powdered sample was boiled in 50ml of distilled water on a hot plate for three minutes. The mixture was filtered while hot and the resulting filtrate cooled and used for the following carbohydrate tests.

(a) MOLISCH'S TEST (GENERAL TEST)

5ml of extract was put in a test tube containing 1ml of water and mixed with a few drops of Molisch's reagent (10% solution of alpha napthol in alcohol). 1ml of conc. H_2SO_4 was then allowed to flow down the side of the inclined tube so that the acid forms a layer beneath the aqueous layer without mixing with it. A red or purple ring formed at the interface indicates the presence of carbohydrate. The test tube was then shaken and the mixture allowed to stand for 2 minutes, then diluted with 5ml of water. A dull-violet precipitate indicates presence of carbohydrate (Trease and Evans, 1983g).

(b) BARFOED TEST (GENERAL TEST FOR MONOSACCHARIDES

1ml of extract from extract and 1ml of Barfoed's reagent were put in a test tube and heated in a beaker of boiling water. Red precipitate of cuprous oxide is formed within 2 minutes if a monosaccharide is present (Trease and Evans, 1983g).

(c) FEHLINGS TEST (STANDARD TEST FOR FREE REDUCING SUGARS

To 2ml of the water extract was added 5ml of a mixture of equal volumes of Fehling's solution I (A) and II (B) and boiled for 2 mins in a water bath. Formation of a yellow or red precipitate cuprous oxide indicates the presence of free reducing sugars (Trease and Evans, 1983g).

(d) STANDARD TEST FOR COMBINED REDUCING SUGARS

1ml of extract was hydrolysed with 5ml of dilute HCl. The solution was then neutralized with NaOH solution and Fehlings test (described above) carried out on it. Production of rose colour is indicative for presence of combined reducing sugars (Trease and Evans, 1983g).

(e) SELIVANOFF'S TEST FOR KETOSES

To a small amount of extract, a crystal of resorcinol and an equal volume of HCl containing a little phloroglucinol was added, the solution was then heated. The production of a rose colour is indicative for presence of ketoses (Trease and Evans, 1983g).

(f) STANDARD TEST FOR PENTOSES

To a small amount of extract and equal volume of HCl containing a little amount of phloroglucinol was added, the solution was then heated on a small flame. a red colour indicates presence of pentoses (Trease and Evans, 1983g).

2.6.2 TESTS FOR GLYCOSIDES

(a) TEST FOR SAPONINS

A small quantity of the powdered plant part was added to 95% ethanol and boiled. The mixture was filtered hot and cooled. Then 2-5ml of ethanol extract was dissolved in 10 ml of distilled water in a test tube. This tube was stoppered and shaken vigorously for 30 secs, then the tube was allowed to stand for over 30 minutes. A honey comb froth is indicative of the presence of saponins (Sofowora, 1982).

(b) TEST FOR TERPENES AND STEROLS

EXTRACTION: 5g of the powdered drug was extracted by maceration with 50ml of 95% ethanol. The extract was filtered and the filtrate dissolved in 10ml of anhydrous chloroform and refiltered. This filterate was divided into two equal proportions which were used for the following tests:-

(i) SALKOWSKI'S TEST

The first portion of the chloroform extract was mixed with 2ml conc. H_2SO_4 carefully, so that the acid forms a lower layer. A reddish ring at the interface indicates the presence of steroidal ring (Sofowora, 1982).

(ii) LIEBERMAN-BURCHARD TEST FOR TERPENES

The second portion of the chloroform extract was mixed with 1 ml of acetic anhydride, followed by the addition of 1ml of conc. H_2SO_4 down the wall of test tube to form a layer underneath. The formation of a reddish-violet colour at the interface of the two liquids and a green colour in the chloroform layer indicates the presence of terpenes (Sofowora, 1982).

2.6.3 TESTS FOR FLAVONOIDS

EXTRACTION: 2g of the powdered sample was completely detanned with acetone (Selgelman et al, 1971). The acetone was evaporated to dryness on a water bath. The residue was dissolved in warm water and the mixture was filtered while hot. The filtrate was allowed to cool and then used for the following tests.

(a) LEAD ACETATE TEST

To 5ml of the detanned water extract was added 10% lead acetate solution (2-3 drops). A buff-coloured precipitate indicates the presence of flavonoids.

(b) SODIUM HYDROXIDE TEST

A small quantity of detanned water extract was put in a test tube and an equal volume of 10% NaOH was added. a yellow solution indicates the presence of flavonoids. On addition of HCl the solution becomes colourless (Trease and Evans, 1985h).

(c) FERRIC CHLORIDE TEST

2ml of the detanned water extract was diluted with distilled water in a ratio of 1:4 and a few drops of 10% Ferric chloride solution was added. A green or blue solution indicates the presence of phenolic nucleus (Brian and Turner, 1975).

(d) SHINODA'S TEST

0.5g of the powder of plant part was boiled in ethanol in a test tube on water bath for 5 minutes. This was filtered and cooled. 4 pieces of magnesium fillings were added followed by a few drops of conc. HCl. A pink or red colour indicates the presence of flavonoids (Geissman, 1962).

2.6.4 TEST FOR RESINS AND BALSAMS

(a) Test for Resins

10ml of petroleum ether extract was made from 0.1g of the powder of plant part bark and filtered into a test tube. An equal volume of copper acetate solution was added and shaken vigorously, then allowed to separate. A green colour indicates the presence of resins (Trease and Evans, 1983i).

(b) Test for Oleo-Gum-Resins

0.1g of the bark was triturated with 0.5g of sand. This was transfered into a test tube containing 3ml of ether and shaken well. The mixture was allowed to evaporate into a thin film. In the fume cupboard, bromine vapour was passed over the residue. A violet colour indicates the presence of oleo-gum- resins (Trease and Evans, 1983i).

(a) Test for balsams

(i) 2-3 drops of alcoholic ferric chloride solution was added to 5ml of 90% ethanol extract of the powdered plant part. A dark green colour indicates the presence of balsams.

(ii) 5ml of ethanol extract was warmed with a few drops of pottasium permanganate in a water bath. A

benzaldehyde or almond odour indicates the presence of balsams (Trease and Evans, 1983i).

2.6.5 TEST FOR ALKALOIDS

10g of powdered plant part was taken in a beaker and strong ammonia solution was added in an amount sufficient enough to moisten it, this mixture was allowed to stand for about 10 minutes. Some quantity of a mixture of chloroform and ethanol (1:1) was added just to soak and suspend the powder, the mixture was then allowed to stand for 20 minutes with occasional stirring. The mixture was then filtered through a plug of cotton wool in a filter funnel.

The marc was washed twice with 2 ml chloroform and the washings were added to the filtrate. The combined filtrate was concentrated to dryness on a water bath. Care was taken to avoid over heating. The chloroform extract was transferred to a separatory funnel and was shaken with 3ml sulphuric acid. The two layers were allowed to separate, the chloroform layer was drained off and kept aside. 3ml of chloroform was further added, shaken and drained off, until the acid layer was colourless, the chloroform extracts were stored together. The acid layer was made alkaline with strong ammonia and extracted with 3ml of chloroform. The extract was retained and evaporated to dryness.

The residue was dissolved in 3ml of ethanol and the following tests were carried out after neutralising with dilute sulphuric acid (Brian and Turner, 1975).

(a) General Tests

To 0.5ml of ethanolic extract residue obtained from above extraction was added 2-3 drops of the following reagents (Trease and Evans, 1983j).

- Wagner's reagent
- Mayer's reagent
- Dragendorff's reagent
- 10% tannic acid solution
- Picric acid

(b) TEST FOR OPIUM ALKALOIDS

To 0.1g of powdered drug was added 15ml of 20% ethanol, this mixture was boiled for 2 minutes, cooled and filtered. The filterate was used for the various tests.

Nitric acid test

A drop of nitric acid was added to the ethanolic extract of plant part.

Marquis reagent test

1ml of reagent was added to the extract

Ferric chloride test

To 3ml of extract was added a few drops of 5% ferric chloride solution.

Test for Morphine

To 3ml of the extract 0.5ml of hydrogen peroxide and 0.5ml of 10% ammonia solution was added and the mixture shaken. One drop of copper sulphate solution was then added, shaken gently and allowed to stand for 2-3 minutes.

(c) TEST FOR TROPANE ALKALOIDS - VITALI-MORIN TEST

The chloroform extracts obtained from the extraction process described was evaporated to dryness, the residue was dissolved in a few drops of glacial acetic acid and then evaporated to dryness. After cooling 2-3 drops of fuming nitric acid was added, this was again evaporated to dryness. After cooling the residue was dissolved in a few drops of water-free acetone and 1-2 drops of pottasium hydroxide solution in methanol quickly added. A purple colour indicates presence of tropane alkaloids.

2.7 QUANTITATIVE EVAULATION OF RICINUS COMMUNIS Linn

The following quantitative determinations were made on the plant R. communis. this include the

determination of physicochemical constants of the plant parts such as moisture content, Total ash, Acid insoluble ash, Alcohol soluble and water soluble extractive values.

Also the quantitative microscopy was carried out. This include the determination of stomatal number, stomatal index, vein inlet number, vein termination number and palisade ratio.

2.7.1 DETERMINATION OF MOISTURE CONTENT

An evaporating dish was heated to constant weight, 3g of powdered drug was weighed into it. The dish with its content were put in an oven at 105°C and dried to constant weight. The final constant weight was noted and the weight of moisture (i.e total loss of weight) was calculated by subtracting the final weight of the dish and powder from the initial weight of the dish and powder.

This procedure was repeated 4 times for each sample of powdered drug (Trease and Evans, 1983k).

2.7.2 DETERMINATION OF TOTAL ASH VALUE

A nickel crucible was heated at 105°C to constant weight, its accurate weight noted, cooled and stored in a dessicator. 2g of powdered drug was weighed and put into crucible. The crucible and its

content was then heated gently until it was moisture free i.e completely charred. The heat was then gradually increased until residue was free of carbon. It was then cooled and weighed, heating and cooling was continued until the weight of residue become constant. This procedure was carried out 4 times for each sample of powdered drug (B.P. 1980).

2.7.3 DETERMINATION OF ACID INSOLUBLE ASH VALUE

The crucible and ash obtained in the above experiment were each individually transfered into a beaker containing 25ml of dilute HCl, then boiled for 5 minutes then filtered through an ashless filter paper. The beaker and crucible were washed with water and washing passed through the filter paper. Washing was repeated 3 times, each time passing the washing through the filter paper in a manner so that the residue collected in the tip of the cone of the filter paper. The funnel and filter paper were then dried in an oven at 105°C. The weight of a clean and dry nickel crucible was determined, and the filter paper with residue, folded into a small cone and transferred into it. The crucible was heated until the filter paper is completely ashed, then heated strongly for a few minutes until the final constant weight. This procedure was carried out for all cases of ash

produced in the experiment for Total ash determination (B.P. 1980).

2.7.4 DETERMINATION OF ALCOHOL SOLUBLE EXTRACTIVE VALUE

5g of powdered drug was put into a 250ml stoppered concical flask, 100ml of 90% ethanol was then added, and stopper replaced firmly. The flask was shaken on a mechanical shaker for 6 hours, the extract was then filtered quickly. 20ml of the filtrate was put in a clean, heated and weighed round-bottomed evaporating dish and then heated to dryness on a water bath. The residue was then dried at 105°C in an oven to constant weight. This procedure was repeated 4 times (Brian and Turner, 1975).

2.7.5 DETERMINATION OF WATER SOLUBLE EXTRACTIVE VALUE

5g of powdered drug was put into a 250ml-stoppered conical flask. 100ml of chloroform-water (0.25% v/v chloroform in pure water) was then added, and stopper replaced firmly. The flask was shaken on a mechanical shaker for 6 hours, the extract was then filtered.

20ml of the filtrate was put in a clean, heated and weighed round-bottomed evaporating dish, and then heated to dryness on a water bath. The residue was

then dried to constant weight at 105°C in an oven. This procedure was repeated 4 times (Brian and Turner, 1975).

2.8 QUANTITATIVE MICROSCOPY

2.8.1 DETERMINATION OF STOMATAL NUMBER

This is defined as the average number of stomata per square mm of epidermis.

The area of the field of microscope was calculated using a stage micrometer. The stomata in 10 microscopic fields were then counted, using a single peice of leaf. The average number of stomata per sq mm was then calculated.

2.8.2 DETERMINATION OF STOMATAL INDEX

This is defined as the percentage which the number of stomata form of the total number of epidermal cells.

The number of epidermal cells and stomata in 10 fields of microscope were counted. Each stoma consisting of 2 guard cells and pore (ostiole) is counted as a single unit (Shellard, 1958). The index is claculated using the formular.

$$I = \frac{S \times 100}{E + S}$$

where S = number of stomata

E = number of epidermal cells.

2.8.3 DETERMINATION OF VEIN ISLET NUMBER

This is defined as the number of vein islet per square mm of leaf. A peice of leaf about 5sq mm was taken from the central part of lamina i.e midway between midrib and margin and heated in a testube containing chloral hydrate to remove chlorophyll (the veins become distinct).

Camera lucida was set up at low power magnification, with the aid of a ruler, a line 1mm was drawn on drawing surface and a square was constructed on this line. The paper was then rearranged so that the whole square was visible in eye peice. The number of vein islet in square mm was counted. This was repeated 3 times for a continous area of 4 sq mm (Shellard, 1958).

2.8.4 DETERMINATION OF VEIN TERMINATION NUMBER

The procedure described above for vein islet determination was used but in this case the number of veins terminating per sq mm were counted. The position of terminal point of which each vein or veinlet within the measured square terminates was marked and counted (Shellard, 1958).

2.8.5 DETERMINATION OF PALISADE RATIO

This is defined as the average number of palisade cells under one epidermal cell.

Small portions of leaf were mounted under camera lucida at high power (i.e x 40), with their lower surfaces uppermost. The peices of leaf had been partially cleared by heating in chlorohydrate.

The outline of 4 contigous cells of epidermis was traced out. The camera lucida was then focused down to palisade layer and the position of all palisade cells under the selected epidermal cells including those which are more than half covered by epidermal cells traced out also. The average number of palisade cells beneath a single epidemal cell was then calculated. This determination was repeated for 4 other groups of epidermal cells (Shellard, 1958).

2.9 CHROMATOGRAPHIC ANALYSIS

2.9.1 CHROMATOGRAPHIC ANALYSIS OF PROTEINS

2g of the powdered plant part was put into 10ml of distilled water. The mixture was shaken on a mechanical shaker then left to stand for 24 hours. After which the mixture was filtered, the resulting filtrate was used for chromatographic analysis.

Reference sample

- abrin

0.1mg of abrin was dissolved in 10ml of distilled water.

The extracts of the different plant parts and the reference solution of abrin were chromatographed on thin layer and paper chromatography using the following systems

Paper Chromatography

Chromatographic paper: Whatman No 1 (20cm x 20cm)

Solvent system : n-butanol:Acetic acid:Water (3:1:4) shaken, then put in a separatory funnel, the lower layer was drained and the upper layer used for the analysis.

Technique : Ascending

Visualisation : (i) uv

: (ii) Iodine vapour

: (iii) Sprayed with ninhydrin. The chromatograms were then heated in the oven at 105°C until spots attained maximum colour intensity (Macek (1972).

Thin layer Chromatography

Adsorbent : Silica Gel (0.25mm). Air dried and activated at 105°C for 30 minutes.

Solvent system : n-butanol:Acetic acid:water (4:1:5) shaken, the put in a separatory funnel, the lower layer drained and the upper layer used for analysis

Technique : Ascending

Visualization : (i) uv

: (ii) iodine vapour

: (iii) Sprayed with ninhydrin. Then chromatograms were heated in oven at 105°C until spots attained maximum colour (Macek, 1972)

2.9.2 CHROMATOGRAPHIC ANALYSIS OF FLAVONOIDS

2g of the powdered samples were completely detanned with acetone. The acetone was evaporated to dryness on a water bath. The residue was then dissolved in warm water and the mixture was filtered while hot. The filtrates were cooled and then used for chromatographic analysis.

Paper Chromatography

System 1

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

Solvent system : n-butanol:Acetic acid:Water

(4:1:5), shaken, then put in a seperatory funnel, the lower layer drained and the upper layer used for analysis.

Technique : Ascending

Visualisation : (i) uv

: (ii) sprayed with 1 % sodium carbonate solution and observed under daylight and uv (Macek, 1972)

System 2

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

Solvent system : Acetic acid:Water (15:85)

Technique : Ascending

Visualisation : (i) uv

: (ii) Sprayed with 1 % sodium carbonate solution and observed under daylight and uv. (Macek, 1972).

2.9.3 CHROMATOGRAPHIC ANALYSIS OF ALKALOIDS

2g of the powdered plant part was put in a testube. Strong ammonia solution was added to it in a quantity sufficient enough to moisten. The mixture was allowed to stand for 10 minutes, after which chloroform was added to it in sufficient quantity to

soak and suspend the powder, the mixture was allowed to stand for 2 hours with occasional shaking. The mixture was then filtered and the filtrate used for chromatographic analysis.

Paper Chromatograhpy

System 1

Chromatographic paper	:	Whatman No 1 (20cm x 20cm)
Solvent system	:	Ethylacetate:25% formic acid (4:3)
Technique	:	Ascending
Visualisation	:	(i) uv
	:	(ii) Sprayed with Dragendorff's spray reagent
	:	(iii Iodine vapour (Macek, 1972)

System 2

Chromatographic paper	:	Whatman No 1 (20cm x 20cm)
Solvent system	:	n-butanol:Acetic acid:Water (4:1:5) shaken, and put in a separatory funnel, lower layer drained and the upper layer used for analysis.
Technique	:	Ascending
Visualisation	:	(i) uv
	:	(ii) Sprayed with Dragendorff's spray reagent
	:	(iii)Iodine vapour (Macek, 1972)

Thin Layer Chromatography

Adsorbent : Silica Gel (0.25mm). Air dried and activated in oven at 105°C for 30mins

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

Technique : Ascending

Visualisation : (i) uv

:(ii) Sprayed with Dragendorff's spray reagent

:(iii) Iodine vapour (Macek, 1972)

2.9.4 QUANTIFICATION OF FLAVANOID

50g of powdered plant part was completely detanned (extracted) with 100 ml of acetone. This was carried out using soxhlet apparatus, the extraction was said to be complete when the acetone used for extraction became colourless i.e the powder had been completely exhausted (Segelmen et al, 1971).

A clean and dry evaporating dish was then weighed, then heated until the weight became constant, this weight was then noted. The extract was then poured into it and evaporated to dryness on a water bath. The residue was dissolved in warm water and filtered while hot. The filtrate collected was then poured into evaporating dish and put on water bath and then evaporated to drynees. The evaporating dish and residue were then weighed and the weight of residue

was calculated. This experiment was carried out on the powders of leaves, stem, root and seed. Each was carried out twice.

2.10 TOXICITY STUDIES

2.10.1 EXTRACTION OF RICIN

The seeds of Ricinus communis were deshelled by application of slight pressure which led to the cracking of the hard seed coat, followed by the removal of the peices.

The seeds were then ground into a paste using a mortar and pestle, then transfered into an electric grinder for further grinding. 1kg of the paste was weighed out and put in a 2 litre conical flask. Petroleum ether 60-80° was added and the conical flask stoppered, shaken for about 5 minutes, the petroleum ether was decanted. Washing with petroleum ether was continued until the marc became free of oil. This was indicated by the formation of distinct granules and when any fresh petroleum ether added did not change colour. The residue was then spread out to dry.

Aqueous extraction of ricin was then carried out using distilled water, this was continued until any further distilled water added did not change colour.

The water extract was then dialysed against distilled water for 48 hours. The extract remaining

in dialysis tube was then centrifuged at 8000 pounds for 15 minutes. The supernant collected was referred to as a ricin rich extract.

Quantification of Ricin extract

A clean and dry evaporating dish was heated to constant weight. The weight was noted. 100ml of extract was put in the dish put on a water bath and heated until all the water had evaporated. The dish and residue were then placed in an oven at 105°C, heated until constant weight. The weight of the dish was then subtracted from the final weight of residue to give the weight of solids present in 100ml of extract. This procedure was repeated 3 more times and the average calculated.

2.10.2 ACUTE TOXICITY STUDIES

DETERMINATION OF LD_{50}

Definition of LD_{50}: LD_{50} or median lethal dose is the dose of any substance that will kill 50% of a given population within a specific period of time, usually 24 hours.

Determination of LD_{50} of Ricin

The LD_{50} of ricin in rats was determined using standard procedures (Aliu and Nwude, 1982).

A pilot study was initially carried out to determine the minimum dose that would give 100% death

and the maximum dose that will not produce any death. These two dose levels were further used as focal points for the determination of dose range to be used in the subsequent experiments.

The final acute toxicity test was carried out using 36 healthy male rats (average weight 120g). The animals were about the same weight and from the same litters at the start of the experiment. The animals were divided into 6 groups of 6 rats per group. A 7th group of 5 rats served as control and they were given distilled water while the other five groups were respectively given a pre-determined dose of ricin extract starting from the maximum dose that produced no death to the minimum dose that produced 100% death as was determined from the pilot studies. The route of administration was intraperitoneal with the aid of syringes.

Table 2.1 Doses of ricin used in LD_{50} determination

Group	Dose (ml)
1	0.01
2	0.05
3	0.1
4	0.5
5	1.0
6	5.0
7	Control (given distilled water 1.0ml)

Symptoms of toxicity were observed over the dose range and recorded. The number of animals that died within 24 hours were noted for each group. The LD_{50} was calculated by Arithmetic method of Karber (Aliu and Nwude, 1982). The surviving rats were observed for 14 days for delayed toxicity symptoms.

2.10.3 CHRONIC TOXICITY STUDY (ORAL)

(a) Preparation of Drug-Treated Feed

The chronic toxicity study was designed to last for a period of 12 weeks. Administration of drug was by oral route (through its inclusion in the diet).

Four dose levels were chosen thus. 0.00ml/kg feed (control) 0.5ml/kg feed, 1.0ml/kg feed and 1.5ml/kg feed (0.00g/kg, 0.0006g/kg, 0.0012g/kg and 0.0018g/kg respectively.

The volumes used for various dose groups were properly mixed in water in a flask and then finally mixed with the weighed out feed (in kg). The feed used was Sander's local growers marsh and yam flour which was used as a binder. The well mixed feed were hand moulded and properly sun-dried.

There were 20 animals per group at the start of the chronic toxicity studies. These were adult male albino rats which were kept in plastic cages (53cm x 38cm x 20cm E.K. Brown Ltd London) which were tagged with appropriate labels denoting the dose group.

At the end of the 4th, 8th and 12th weeks, five rats were selected from each experimental group and were sacrificed. The animals were restrained by holding them at the neck. Blood samples were then collected from the neck region directly into sample bottles containing calcium EDTA to prevent clotting. The livers and lungs were also collected after post-mortem and fixed in 10% formalin for histopathological studies.

(b) HISTOPATHOLOGICAL STUDIES

TISSUE PROCESSING AND SECTIONING

Fresh tissue samples of the liver and lungs from both treated and control rats were fixed in 10% formalin.

Tissue slices of 3-4cm thickness were cut from each organ for processing. The tissues were transfered to the automatic tissue processor where they were further fixed in 10% buffered formal-saline for 2 hours, and then dehydrated for ? hours in each of the ascending grades of ethanol 85%, 90% and 100% v/v). there were three mixes of absolute alcohol. The dehydrated tissues were then cleared in Toluene for 2 hours and the tissues impregnated in molten paraffin for 2 hours, after which the tissue slices were embedded in paraffin wax and left to cool. The

blocks were then trimmed and sectioned on the microtoma at 5 microns. The ribbons of sections were then floated in a warm water bath, suitable sections were selected, attached to slides, dried on a hot plate and stained.

STAINING OF SECTIONED TISSUE

Haematoxylin and Eosin stain (H & E stain).

The sections were dewaxed in xylene, and rehydrated in descending grades of alcohol: 100%, 90% and 70%. They were then stained in haematoxylin for about 5min., differentiated in 1% acid alcohol, blued in scott's tap water and stained in eosin for 3min. They were then rinsed and dehydrated in ascending grades of alcohol solution 70%, 90% and 100% and finally cleared in xylene and mounted in pox.

These slides were then examined microscopically for lesions with the assistance of a pathologist at the Ahmadu Bello University Veterinary Teaching Hospital Zaria.

(c) Haematological Studies

The blood samples collected were taken for analysis to determine haematological parameters, these include packed cell volume (PCV), Total protein (TP), Haemoglobin count (Hb), Red blood cell count (RBC) and white blood cell cout (WBC) to find out if the drug had any effect on them.

(i) Determination of Packed Cell Volume

Using a micro-capillary tube, blood sample was sucked up to 75% of its length, then one end was sealed using a flame. The tube was then put on a micro-haematocrit centrifuge for about 3-5 minutes. these separates the blood into 2 layers. They packed cell volume is then read with the aid of Hawksley Micro-haematocrit reader (Unit = percentage (%).

(ii) Determination of Total Protein

The micro-capillary tube from above PCV determination was broken somewhere along the plasma layer. A drop of plasma was put on a refractometer and the Total Protein read off the scale (Unit = g/100ml).

(iii) Determination of Haemoglobin Count

Some blood sample was taken, put on a Coulter diluter which took up 0.02ml and diluted it to 20ml in Isoton 6 drops of Zap-oglobin(R) was added, it lysed the Red blood cell and released the haemoglobin. The haemoglobin was then counted with the aid of a haemoglobinometer (Unit = g/dl).

(iv) Determination of Red Blood Cell Count

A red blood cell pipette was used to take up 0.5ml of blood sample (the sucking was done with the aid of a rubber aspirator), saline was then taken up to fill the pipette to the 101 mark. Rubber aspirator

was taken off and the pipette shaken, after which 3-4 drops of mixture was discarded.

Sample of the mixture was then put on a haemocytometer or counting chamber (this is a special type of microscope slide). Counting was then carried out under the microscope, counting was done in millions (Unit - number of cells per cubic mm).

(v) Determination of White Blood cell count

A white blood cell pipette was used to take up blood to the 0.5ml mark then diluted to the 11 mark with dilute acetic acid. The pipette was then shaken, after which 3-4 drops of mixture was discarded. Sample was then put on the haemocytometer and counting done under microscope. Counting was done in thousands (Unit number of cells per mm^3) (Brown, 1980).

2.10.4 DETERMINATION OF TOTAL PROTEIN CONCENTRATION OF DIFFERENT PARTS OF R. COMMUNIS

Aqueous extracts of the different parts of plants i.e leaves, stem bark, root and seeds were made by putting 5g of powdered plant part in a conical flask and adding 50ml of distilled water. The mixture was put on a mechanical shaker for 6 hours, then filtered and the filtrate collected.

Using a micro capillary tube, some of extract was taken up and put on a clean refractometer and the total protein concentration read off the refractometer. 5 readings were taken for each part and the average calculated.

2.10.5 DETERMINATION OF THE EFFECT OF THE EXTRACTS OF DIFFERENT PARTS OF PLANT ON RATS BLOOD

Blood was collected from a mature albino rat, 0.2ml of this blood was put in a small test tube and an equal volume of extract was added. The blood was then examined under the microscope. This procedure was carried out for the aqueous extracts of leaves, stem bark, root and seed obtained from the above experiment.

2.10.6 DETERMINATION OF RATE AND EXTENT OF HAEMOLYSIS

A mature albino rat was sacrificed and the blood collected. 0.5ml of the blood was diluted to a 100 places by adding 50ml of distilled water. The wavelength of maximum absorption of this blood was then determined with a spectrophotometer using distilled water as standard.

(i) Using the determined wavelength the rate of haemolysis was determined by mixing 1.5ml of blood with 1.5ml of ricin extract and recording the change

in absorbance of the mixture at different time intervals until the absorbance becomes constant and remains so after two consecutive time intervals. This procedure was repeated five times for different blood samples.

(ii) Working with the determined wavelength of maximum absorption of blood, to a fixed amount of blood i.e 1.5ml, increasing amounts of ricin extract was added. The mixtures were shaken and their absorbance recorded after a minute. Quantities of ricin extract added were as follows. 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1.0ml, 1.2ml, 1.4ml, 1.6ml, 1.8ml and 2.0ml. The procedure was carried out for five different samples of blood.

CHAPTER THREE

RESULTS AND DISCUSSIONS

General pharmacognostic investigation which include microscopy organoleptic examination and chemomicroscopy, as well as phytochemical and toxicity studies were carried out on Ricinus communis. The experimental details of the above studies are present in chapter two. The results are given and discussed in this chapter.

3.1 ORGANOLEPTIC EXAMINATION OF RICINUS COMMUNIS

The sensory characters of the various plant parts i.e the stem bark, leaves, root and seeds were observed and recorded. The characters observed were the colour, texture, odour and the taste, the results are shown below in Fig. 3.1.

Table 3.1

RESULT OF ORGANOLEPTIC EXAMINATION OF R. COMMUNIS

Plant Part	Leaf	Stembark	Root	Seed
Colour	green	light brown	light brown (a lighter shade of brown than stem bark)	Yellowish white
Odour	slight aromatic odour	slight aromatic odour	slight aromatic odour	slight aromatic odour

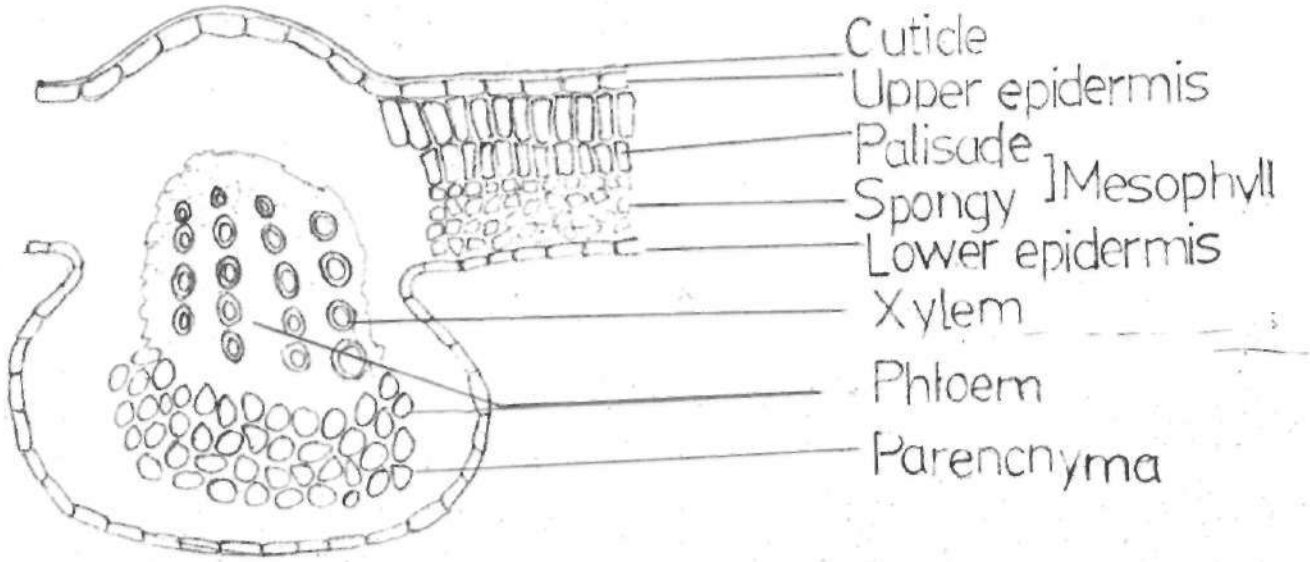
Taste	Tasteless	Slightly bitter	Slightly bitter	Weakly acrid taste
Texture	Smooth	Rough, fibrous	rough fibrous	coarse

3.2 RESULTS OF MICROSCOPIC EXAMINATION OF R. COMMUNIS

(a) Leaves

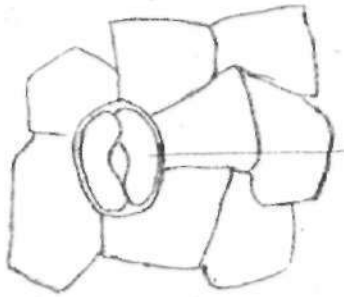
The transverse section of the leaf revealed a dorsi-ventral nature. Features are shown in Fig. 3.1a. The upper epidermis was single layered with cells more or less rectangular with outer walls cuticularized. Trichomes were absent. Mesophyll was divided into two layers - upper palisade and lower spongy mesophyll. The palisade layer was two cells thick and compact. Spongy mesophyll 3-4 layered, loosely arranged with intercullular spaces. The lower epidermis resembled the upper epidermis. The mid rib was occupied prominently by the vascular bundle which is collateral in nature. Xylem positioned towards the ventral surface and phloem towards the dorsal surface. The examination of the powdered leaf revealed the presence of epidermal cells with straight anticlinal walls, anomocytic stomata, more occuring on the lower surface than the upper surface, xylem conducting elements and fibres, calcium oxalate crystals and starch grains (Fig. 3.1b).

FIGURE 3.1a

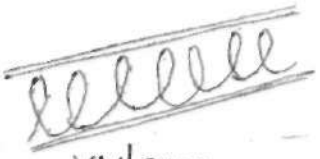


T.S of fresh leaf (through midvein)

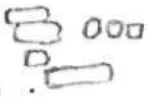
Mag x 400



lower epidermis



xylem conducting vessel



calcium oxalate crystals

KASHIM IBRAHIM LIBRARY



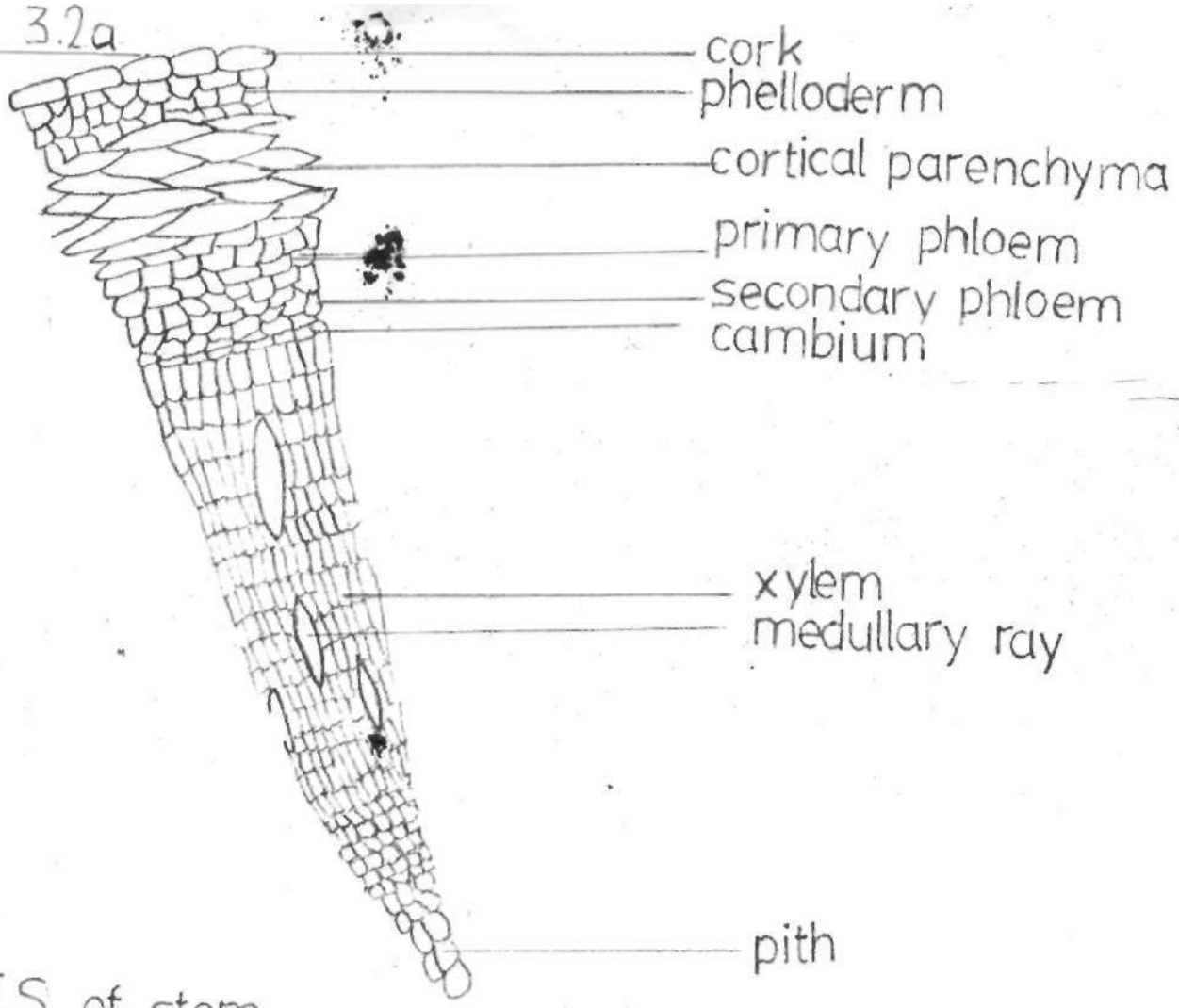
starch grains



fibres

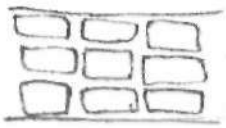
FIG 3.1b FEATURES OBSERVED IN POWDERED LEAF

FIG 3.2a



T.S of stem

Mag x 400



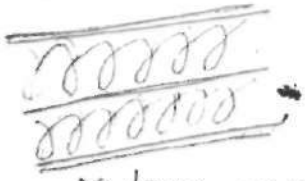
cork cells



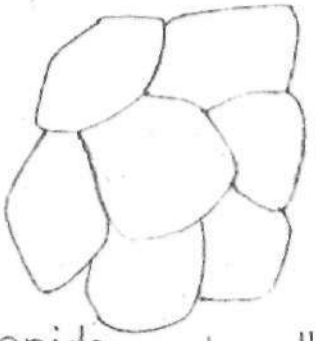
calcium oxalate crystals



fibres



xylem conducting vessels



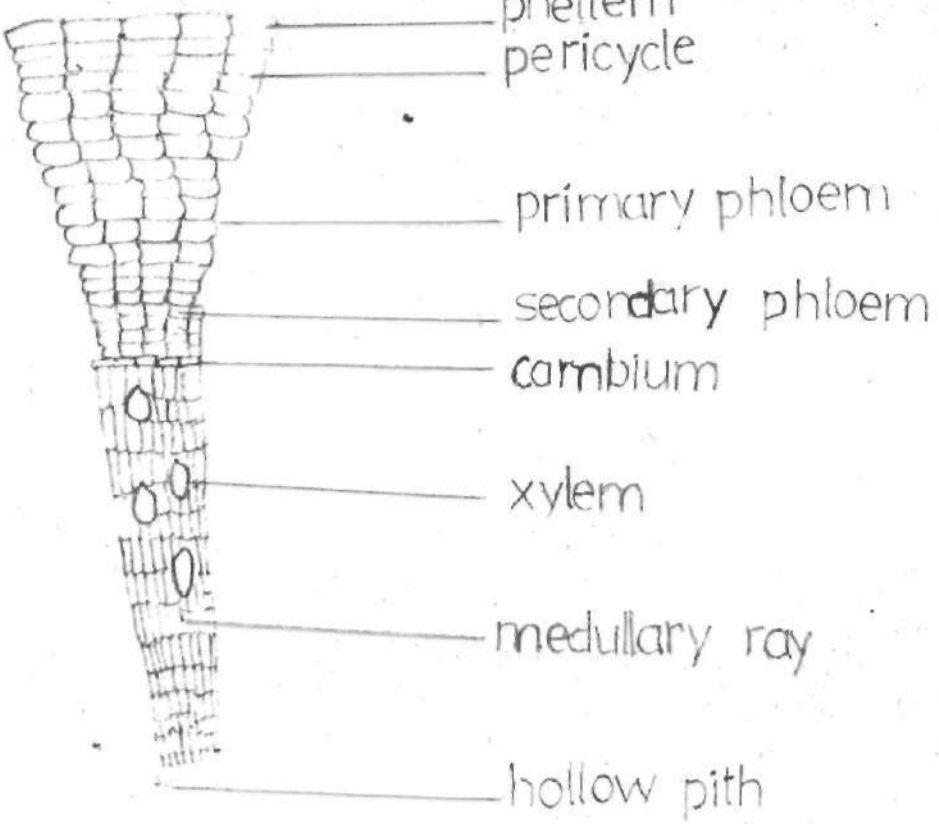
epidermal cells



tracheid

FIG 3.2b FEATURES OF POWDERED STEM BARK

FIGURE 3.3a



TS of root

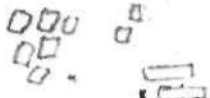
Mag x 400



xylem conducting vessel



fibres



calcium oxalate crystals



starch grains



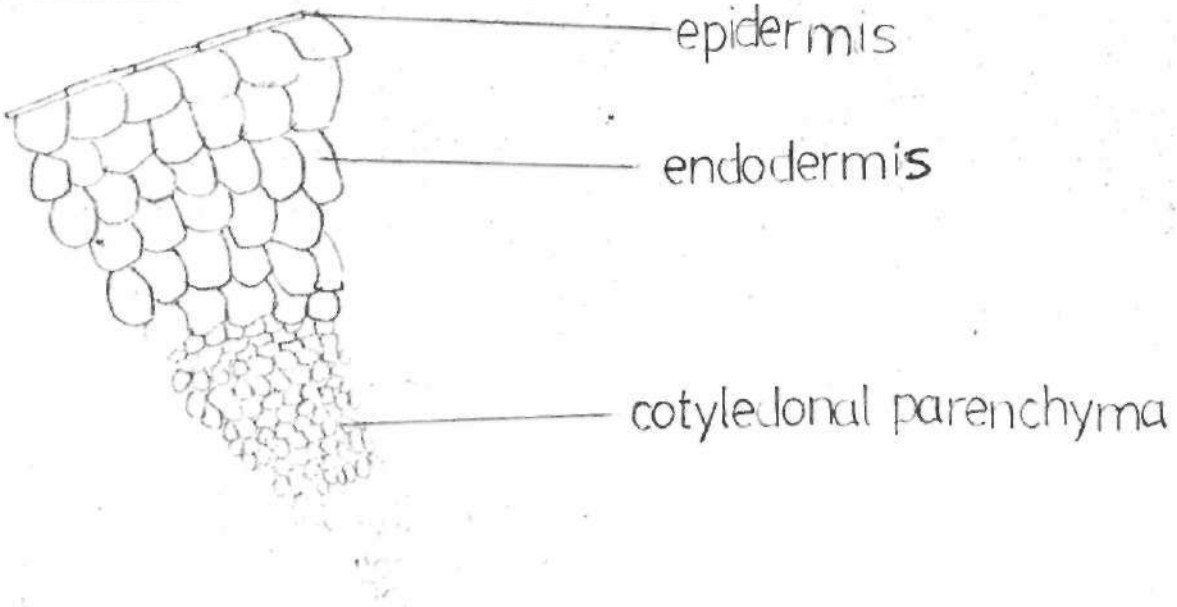
tracheid



cork cells

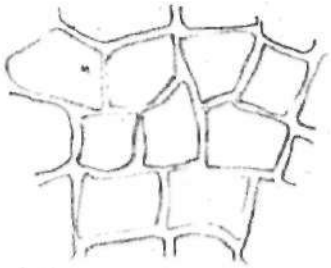
FIG 3.3b FEATURES OF POWDERED ROOT

FIGURE 3.4a

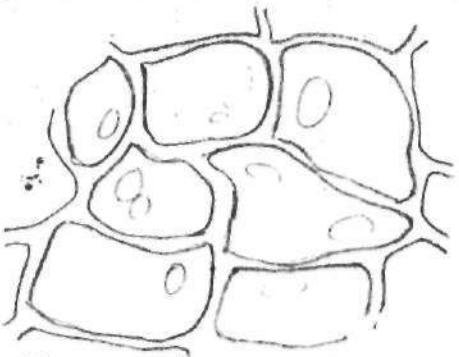


T.S of fresh seed

Mag x 400



epidermal cells



cells with oil ducts



starch grains

FIG 3.4b FEATURES OF POWDERED SEED

(b) Stem

The transverse section of the stem showed a covering layer of cork, followed by a phelloderm 3-4 layers thick with thin walled cells. A wide cortex containing parenchymatous cells, primary and secondary phloem, a one layer cambium and medullary rays. The medullary rays are thin walled radially elongated parenchymatous cells, among them are found the xylem conducting tissues. The central portion of leaves is occupied by the pith which contains large thin walled cells. Fig 3.2a.

Examination of the powdered stem revealed the presence of polygonal cork cells 3-4 cells thick, epidermal cells with thin straight anticlinal walls, lignified fibres (blunt), fibres with tapering apex, xylem conducting vessels of the spiral type, starch grains and calcium oxalate crystals. Fig 3.2b.

(c) Root

The transverse section of the root (Fig. 3.3) showed a covering layer of cork followed by the cortex containing parenchymatous cells which are somewhat rectangular in shape, primary phloem, secondary phloem consisting mostly of phloem parenchyma, medullary rays uniformly arranged, interspersed among which are the xylem conducting elements. The central part of root is occupied by a hollowed pith.

The examination of the powdered root revealed the presence of tracheid vessels with blunt apex, xylem conducting vessels of the spiral type, long stengtening fibres, fibre with tapering apex, calcium oxalate crystals and starch grains. Fig. 3.3b.

(c) Seed

The T.S. of the seed (Fig. 3.4a) showed an epidermal layer one-cell thick, enclosing the endosperm which cells become smaller in size as they become more central. The endosperms encircles the cotyledons. The cells of endosperm and cotyledons are parenchymatous, containing abundant oil globuloles.

The examination of powdered dry seed showed epidermal cells of somewhat rectangular shape and endosperm cells containing oil ducts. Fig. 3.4b.

These results obtained above on the study of the anatomical sections and the powdered drug of the different plant parts can serve as an aid in the identification of the plant during collection of the plant or in the different recipes found in traditional medicine preparations.

3.2.1 RESULTS OF CHEMOMICROSCOPY OF THE PARTS OF R. COMMUNIS

(a) Test for Lignin

The fibres present in the samples of powdered stem bark, leaf and root and also in xylem elements of

these parts gave positive tests (red colour) for lignin. There was no colour change for the seed.

(b) Test for Oils

A red colour was observed in powders of root, seed, leaf and stem bark indicating presence of fixed oils.

(c) Test for Tannins

Bluish black colours were observed in stem bark and root, while in the leaf a greenish-black colour was observed indicating presence of tanins in these parts. No colour change occured for the seed i.e tannins are absent.

(d) Test for Starch

A blue-black colour was observed in the powders of stem bark, seed, leaf and root, indicating the presence of starch.

(e) Test for Calcium oxalate crystals

Calcium oxalate crystals were observed in the powders of stem bark, root and leaves. None were however seen in the seed.

(f) Test for cellulose

Cellulose was found to be present in the powdered stem bark, leaf, root and seed.

(g) Test for Mucilage

A light pink colour was observed in a few cells of the stem bark and root indicating the presence of small amounts of mucilage in these parts. The leaves and seed gave a negative reaction.

Summary of all the above results on chemomicroscopy is found in Table 3.2 below

Table 3.2

CHEMOMICROSCOPICAL EVALUATION OF THE PARTS OF R. COMMUNIS

Constituents	Bark	Leaf	Root	Seed
Lignin	+	+	+	-
Oils	+	+	+	+++
Tannins	+	+	+	-
Starch	+	+	+	+
Calcium oxalate crystals	+	+	+	-
Cellulose	+	+	+	+
Mucilage	+	-	+	-

Key

+ Present

- Absent

3.3 RESULTS OF PHYTOCHEMICAL SCREENING OF R. COMMUNIS

Phytochemical teats were carried out on the various parts of R. communis - stembark, root seed and leaves in order to have an idea of the general constituents present in them.

The results obtained from the various phytochemical tests are described below.

3.3.1 TEST FOR CARBOHYDRATES

(a) Molisch's test - General Test

The aqueous extracts of the bark, root, seed and leaves when treated with Molisch's reagent gave a positive test for carbohydrates (a reddish ring at the interface). After shaking and being allowed to stand for 2 minutes and then a further dilution, a final dull-violet precipitate was observed, which is indicative of the presence of carbohydrates.

(b) Barfoed's test for monosaccharides

A red precipoitate was formed in the case of the leaf extract indicating presence of monosaccharides. No precipitate was formed in the case of stem bark, root and seed extract.

(c) Fehling's test for free reducing sugars

This test was positive for all cases i.e leaf, bark, seed and root i.e red precipitate formed indicating presence of free reducing sugars.

(d) Standard test for combined reducing sugars

The leaf, stem bark, seed and root gave positive reactions to show the presence of combined reducing sugars in their extracts.

(e) Salivanoff's test for ketoses

No colour changes were observed in extracts of leaf, stem, seed and root, indicating absence of ketoses.

(f) Standard test for Pentoses

No colour change was observed in extracts of leaf, stem, seed and root on addition of HCl/Phloroglucinol and heating. This indicates absence of pentoses.

The above results indicate the presence of carbohydrates, monosaccharides, free and combined reducing sugars, in the plant. Carbohydrates are the first products formed in photosynthesis, so they are a convenient starting point for any discussions of vegetable drugs.

3.3.2 TEST FOR GLYCOSIDES

Glycosides are complex compounds present in almost all plants but the type varies from plant to plant. There is no common test for these group of compound, so selective test were carried out to identify the type of glycosides present in R. communis, the results are as follows:-

(a) Test for Saponins

A little honey comb froth was formed in the ethanolic extract of seed which lasted for about 10 seconds, this indicates the presence of saponins in very small amounts. No froth was formed in the extract of leaf, root and stem bark.

(d) Salkowski's test for Sterols

No red ring was formed at the interface of chloroform extracts and concentrated sulphuric acid, giving negative results which indicate absence of sterols in the varios extracts.

(e) Lieberman-Burchard test for terpenes

All the extracts (i.e root, stem bark and leaves) gave negative results for the test for terpenes. No red ring was formed at the interface.

3.3.3 TEST FOR FLAVONOIDS

The results for the various test for flavonoids are described below.

(a) Lead acetate test

Buff-coloured precipitates were observed in the detanned water extract of leaf, stem bark, seed and root indicating presence of flavonoids.

(b) Sodium Hydroxide test

Yellow solutions were observed in the extracts of leaf, stem bark, seed and root indicating presence of flavonoids.

(c) Ferric chloride test

Blue solutions were observed in the extracts of leaf, stem bark, seed and root indicating presence of flavonoids.

(d) Shinoda's test

Pink colour observed in ethanolic extract of leaf, stem bark, seed and root indicating presence of flavonoids.

3.3.4 TEST FOR RESINS AND BALSAMS

Negative results were obtained with tests for the resins and balsams. This indicates the absence of this group of compounds in the plant R. communis

3.3.5 TEST FOR ALKALOIDS

Various general test were carried out on the extracts of the leaves, stem bark and root of *R. communis* to find out if they contain alkaloids. The results were as follows.

(a) Wagner's test

Brownish precipitates were observed in the extracts of the seed, leaves, stem bark and roots of the plant indicating presence of alkaloids.

(b) Mayer's test

Whitish precipitates were observed in extracts of the different plant parts tested. This indicates presence of alkaloids.

(c) Picric acid test

Yellowish precipitates observed in the extracts of the different parts of plant tested which is indicative of the presence of alkaloids.

(d) 10% Tannic acid test

Buff coloured precipitates were observed in the extracts of all the different parts of the plant tested, indicating presence of alkaloids.

(e) Dragendorff's test

Orange precipitates were observed on addition of the reagent to the extracts of all the plant parts. This indicates presence of alkaloids.

The results of the test described above are conclusive in indicating the presence of alkaloids in the plant R. communis. Further tests were carried out to determine the type of alkaloids.

(f) Test for Opium Alkaloids

All the tests carried out to determine the presence of opium alkaloids such as morphine, codeine, phebaine, papaverine and meconic acid proved negative. This is indicative of possible absence of opium alkaloids in the different parts of plant.

(g) Test for Tropane Alkaloids

The test carried out to determine the possible presence of tropane alkaloids in the parts of plant were negative, indicating absence of this class of compounds.

An alkaloid Ricinine has been reported to be present in the seed of R. communis (African Pharmacopoiea, 1985). Ricinine has been described to belong to the pyridine class of alkaloids. It has been isolated from the leaf and the whole plant yields

1.1% (Kerharo et al, 1974).

This alkaloid is possibly present in the other parts of plant from which samples were made, which may indicate why they gave positive test for the presence of alkaloids but were negative for tropane and opium alkaloids, since ricinine is a pyridine alkaloid. The summary for the phytochemical screening is given in Table 3.3.

Table 3.3

PHYTOCHEMICAL CONSTITUENTS OF DIFFERENT PARTS OF R. COMMUNIS

CONSTITUENTS GROUP	STEM BARK	LEAF	ROOT	SEED
Carbohydrates	+	+	+	+
Monosaccharides	-	+	-	-
Free reducing sugars	+	+	+	+
Combined reducing sugars	+	+	+	+
Ketoses	-	-	-	-
Pentoses	-	-	-	-
Glycosides				
Saponins	-	-	-	+
Sterols	-	-	-	-
Terpenes	-	-	-	-
Flavonoids	+	+	+	+
Resins and Balsams	-	-	-	-
Alkaloids	+	+	+	+

3.4 RESULTS OF QUANTITATIVE EVALUATIONS OF R. COMMUNIS

Five physical constants of the different parts i.e stem bark, leaves, seed and roots of R. communis were dtermined. These were moisture constant, ash value, acid insoluble ash value, water and alcohol soluble extractive values. Standard procedures were used in the determination of this constants. The calculations and results are given below.

3.4.1 MOISTURE CONTENT

The moisture content was calculated for each part in the following manner.

Weight of sample = 3.0g

Constant weight of empty crucible = (a)

Weight of crucible + sample = (b)

Constant weight of crucible + drug after drying = (c)

weight of moisture = (b) - (c) = (d)

$$\% \text{ Moisture} = \frac{d}{\text{weight of drug}} \times \frac{100}{1} \%$$

This calculation was carried out for 5 samples of drug and the mean calculated. This is taken as the moisture content of the plant part.

The moisture content of the leaves was found to be 5.82%, stem 5.4%, Root 5.88% and seed 6.2% (Table 3.4).

3.4.2 TOTAL ASH VALUE

The ash values were calculated as follows:-

Weight of sample = 2.0g (a)

Constant weight of crucible = (b)

Weight of crucible + sample = (c)

Constant weight of crucible + redisual ash = (d)

∴ Weight of ash = (d) - (b) = (e)

$$\therefore \%\ \text{ash (value)} = \frac{(e)}{(a)} \times \frac{100}{1}\ \%$$

This calculation was carried out for the 5 samples of the different plant parts and the mean was then calculated. This mean is taken as the ash value for that plant part.

The Ash value of the leaf was 11.1%, stem 12.8%, root 12.2% and seed 9.7%.

3.4.3 ACID-INSOLUBLE ASH VALUE

The acid insoluble ash value was calculated as follows:-

Initial weight of drug = 2g (a)

Constant weight of nickel crucible = (b)

Weight of crucible + residual ash = (c)

∴ Weight of acid insoluble ash = (c) - (b) = (d)

$$\% \text{ acid insoluble ash} = \frac{(d)}{(a)} \times \frac{100}{1} \%$$

This calculation was carried out for each of the 5 samples of the different plant parts, the mean was then calculated. This mean is taken as the ash value for each particular part. The acid-insolble Ash value of the leaf was 4.7%, stem 5.8%, root 5.0% and seed 4.1%.

3.4.4 ALCOHOL SOLUBLE EXTRACTIVE VALUE

The alcohol soluble extractive value was calculated as follows:-

Weight of sample = 5.0g (a)

Constant weight of empty crucible = (b)

Constant weight of crucible + residue = (c)

∴ Weight of residue from 20ml = (c) - (b) = (d)

Weight of residue in 100ml = (d) x 5 = (e)

$$\therefore \% \text{ alcohol soluble extractive} = \frac{(e)}{(a)} \times \frac{100}{1} \%$$

This was calculated for each of the 5 samples for each plant part. The mean of this values was taken as the alcohol soluble extractive value of the given part.

The alcohol soluble extractive value for the leaf was 16.96%, stem 15.96%, root 16.76% and seed 18.6%

3.4.5 WATER SOLUBLE EXTRACTIVE VALUE

The water soluble extractive value was calculated as follows:-

Weight of sample = 5.0g (a)

Constant weight of crucible = (b)

Weight of crucible + residue = (c)

∴ Weight of residue from 20ml = (c) - (b) = (d)

∴ Weight of residue from 100ml = (d) x 5 = (e)

$$\% \text{ Water soluble extractive} = \frac{(e)}{(a)} \times \frac{100}{1}\%$$

This was calculated for each of the 5 samples for each plant part. The mean of this values was taken as the water soluble extractive value for the given part.

The water soluble extractive value for the leaf was 19.4%, stem 17.8%, root 19.24% and seed 24%.

The results (values) obtained from the determination of the 5 physical constants for the different plant parts are presented in Table 3.4

Table 3.4

FIVE PHYSICAL CONSTANTS OF DIFFERENT PARTS OF R. COMMUNIS

PHYSICAL CONSTANT	LEAF	STEM	ROOT	SEED
Moisture content	5.82%	5.4%	5.83%	6.2%
Total ash value	11.1%	12.8%	12.2%	9.7%
Acid insoluble ash value	4.7%	5.8%	5.0%	4.1%
Alcohol soluble extractive value	16.96%	15.96%	16.76%	18.6%
Water soluble extractive value	19.4%	17.8%	19.24%	24.0%

*Each value represents a mean of five separate determinations. The physical constants are important parameters used in pharmacognostical investigations, they are used in the evaluation of drugs, most especially the purity and quality of drugs. The physical constants are generally constant for a particular drug thus they are used as means of evaluation.

Majority of drugs will contain 10 to 20 percent of moisture, this shows that the samples of R. communis used in this research have been properly dried since the moisture content of its parts falls below 10%. This means that enzyme action on its constituents will not have occured, i.e the constituents will be stable. The samples used had a moisture content below 10%

indicating their acceptability. The total ash value and acid insoluble ash values were quite low. These standards are used in investigations of purity of drug. The results of this evaluations of R. communis indicate the acceptability of these samples.

The percentage of soluble extractives of drugs depend on the solvent used and the chemical constituents of the drug. In all the different parts of the plant the water soluble extractive value were found to be higher than the corresponding values for alcohol extractives indicating the use of water as a vehicle in traditional medicine as valid.

The variations between the values of the physical constants for the different plant parts do not appear to be very much indicating some similarity in many cases as shown in Fig. 3.4.

3.5 RESULTS FOR QUANTITATIVE MICROSCOPY OF RICINUS COMMUNIS LEAF

The procedures used in the quantitative microscopy of the leaf of a plant are conducted in order to be able to comfirm the identity of the drugs. These are numerical standards based upon various anatomical features of the leaf in question. These munerical standards include - stomatal number, stomatal index, vein islet number, vein termination

number and palisade ratio. The determination of these values for any leaf will involve the use of a camera lucida. The values calculated for the leaf of R. communis are as follows.

Stomatal number-

Upper surface - 17 - 19 - 22 stomata per sq mm

Lower surface - 25 - 29.7 - 35 stomata per sq mm

Stomatal index -

Upper surface - 9.6 - 11.1 - 12.5%

Lower surface - 13.0 - 15.2 - 16.6%

Palisade Ratio - 3.5 - 4.4 - 5.3 per epidermal cell

Vein Islet Number - 12 - 13.25 - 15 per sq mm

Vein Termination Number - 8 - 10.25 - 12 per sq mm

Key - Average are underlined.

The numerical standards derived above can be used when attempting to standardize traditional medicine preparations, they are used to identify the plant in order to make sure that the plant being used is actually R. communis not closely resembling species. For instance the values i.e stomatal number and index give the frequency of the occurence of stomata on leaves, this numerical values will help in differentiating it from other plants. It is possible

that some treatment of diseases attributed to R. communis in traditional medicine in past ages which cannot now be substantiated by scientific study might have been due to mis-identification.

3.6 RESULTS OF CHROMATOGRAPHIC ANALYSIS

Chromatographic techniques are useful methods in the separation, isolation and identification of compounds. They provide information such as Rf values which can be used in identifying various components of a mixture of compounds.

Chromatographic analysis were carried out on the proteins, flavonoids and alkaloids of R. communis in order to try and determine the number and if possible the types of this class of compounds present in the plant.

3.6.1 PROTEINS

In the first solvent system (n-butanol 3: Acetic acid 1: Water 4) the extract of seed separated into five spots, all of which gave a purple colour with ninhydrin reagent. Two of the spots also gave yellow spots with iodine vapour, while one of these two gave a light blue inflorescence under uv light.

The root extract separated into three spots, two of which gave purple spots with ninhydrin, one flouresce yellow under uv and two gave yellow spots with iodine vapour.

The stem extract separated into four spots, three gave purple colour spots with ninhydrin, one floresce yellow in uv and another one gave a yellow spot with iodine.

The last extract separated into five spots, only one gave a purple coloured spot with ninhydrin, four gave yellow spots with iodine vapour and three floresce light blue.

In the second solvent system (n-butanol 4: acetic acid 1: water 5) carried out on thin layer plates the seed extract separated into five spots, three of which gave red to reddish brown spots with ninhydrin and one gave a yellow spot with iodine. None of the components of extracts floresence in this solvent system.

The root extract separated into four spots, only one gave red sopt with ninhydrin and three gave yellow spots in iodine. The stem extract gave only one spot which was yellow in iodine. In both solvent systems, the reference compound abrin appeared, giving a yellow florescence in both cases, also yellow spots in iodine vapour, but a purple spot in ninhydrin in the first

system, while in the second system it gave a reddish brown spot with ninhydrin.

In both cases there was a component of the seed which shared similarities with the reference abrin. It had the same colour reaction with ninhyrin and very close Rf value. This compound is probably ricin which shares similarities with abrin i.e they share a lot of similarities in their chemical structure, both are made of two polypetide chains joined by a sulphur-sulphur bond. The amino acid sequence of their A-chains are closely related.

No similar corresponding spot occured in the extracts of leaf, stem and root. Ricin is probably absent in this parts.

3.6.2 FLAVONOIDS

In the first solvent system (n-butanol 4: acetic acid 1: water 5) carried out on paper, the seed extract gave one spot which floresce yellow under uv after spraying with 1% sodium carbonate solution. The root extract gave two spots, one floresce yellow under uv without spraying with reagent, while the other floresce yellow after spraying with 1% sodium carbonate solution.

The extract of stem gave two spots which floresce under uv without spraying. The leaf extract separated

into four spots, one floresce blue under uv without spraying the other three floresce after spraying, two floresce yellow and the other one violet.

In the second solvent system (acetic acid 15%: water 85%) carried out on paper, the seed extract gave two spots, one floresce yellow without spraying with reagent while the other floresce yellow after spraying with 1% sodium carbonate.

The root extract separated into two spots, one floresce yellow in uv light without spray and the other floresce yellow after spraying. The stem extract gave a spot which floresce yellow under uv. The leaf extract separated into five spots, two floresce under uv without spray as blue and yellow, while the other three floresce after spraying, one was yellow green while the other two floresce yellow.

With the aid of information given in Macek (1972) i.e Rf values and colour reactions it was possible to try and identify some of this separated flavanoids which were chromatographed under the same conditions. These suspected flavanoids are as follows:-

- Vitexin, Rf value 0.82 - occuring in the seed
- Linarin (a acacetin derivative) Rf value 0.61 - occuring in the leaf

- Luteolin Rf value 0.66 - occuring in the leaf
- Quercetin, Rf value 0.41 - occuring in the stem
- Quercetin derivative + 3 - rhamnoglucoside, Rf value 0.36, found in the root.

The flavonoids listed above had corresponding spots on the chromatogram with the same Rf value and colour reactions, which were run under the same conditions given in the literature. It was not possible to further confirm them because the required reference solutions were not available to be run on the same chromatogram.

3.6.3 ALKALOIDS

In the first solvent system (Ethyl acetate 4: 25% formic acid 3) carried out on paper, one spot was found to occur at the same position (Rf 0.89) in all of the extract. Each of the extracts also gave one spot each all of which floresce under uv light. The other spot did not floresce under uv, but all the spots gave yellow colour reaction with iodine vapour. The spots that floresce in the case of the seed and root also gave a brick red colour with dragendorff's reagent.

In the second solvent system (n-butanol 4: acetic acid 1: Water 5) carried out on chromatographic paper, each extract of the different plant parts gave one

spot each. This spots gave yellow colour reaction with iodine and floresce under uv.

In the third solvent sytem (chloroform 10: Methanol 20: Ammonia 1) performed on thin layer plates, each of the extracts gave a spot which had the same Rf values and the same yellow colour reaction in iodine as shown in Table 3.7. Two of these spots floresce yellow under uv. These spots are similar to those found in the chromatogram run in the first solvent system, and there is a strong possibility that this compound is ricinine which is alkaloid reported to be common to all parts of the plant. No reference solution was available to be used in confirming the presence of the ricinine.

TABLE 3.5

CHROMATOGRAPHIC SEPARATION OF PROTEINS IN DIFFERENT PARTS OF RICINUS COMMUNIS

PAPER CHROMATOGRAPHY

Plant part/ Reference	Solvent	Spots	Rf	Colour Reactions			
				Daylight	uv	Iodine vapour	Ninhydrin spray
Abrin	n-butanol:	1	0.738	-	Yellow	Yellow	Purple
	Acetic acid						
Seed	Water						
	(3:1:4)	1	0.38	-	-	-	Purple
		2	0.46	-	-	-	Purple
		3	0.48	-	Light blue	Yellow	Purple
		4	0.58	-	-	Yellow	Purple
		5	0.744	-	-	-	Purple
Root	"	1	0.38	-	-	-	Purple
		2	0.48	-	Yellow	Yellow	Purple
		3	0.63	-	-	Yellow	-
Stem	"	1	0.41	-	-	-	Purple
		2	0.54	-	-	-	Purple
		3	0.60	-	Yellow	-	Purple
		4	0.57	-	-	Yellow	Purple

Leaf	"	1	0.40	-	-	-	Purple
		2	0.55	-	Light blue	Yellow	-
		3	0.48	-	-	Yellow	-
		4	0.62	-	Light blue	Yellow	-
		5	0.80	-	Light blue	Yellow	-

THIN LAYER CHROMATOGRAPHY

Abrin	n-butanol: Acetic acid Water (4:1:5)	1	0.64	-	Yellow	Yellow	Reddish-brown
Seed		1	0.23	-	-	-	Red
		2	0.42	-	-	-	Reddish-brown
		3	0.51	-	-	Yellow	-
		4	0.56	-	-	Yellow	-
		5	0.66	-	-	-	Red
Root	"	1	0.20	-	-	Yellow	-
		2	0.31	-	-	-	Reddish-brown
		3	0.51	-	-	Yellow	-
		4	0.71	-	-	Yellow	-
Stem	"	1	0.2	-	-	Yellow	-
Leaf	"	1	0.2	-	-	Yellow	-
		2	0.29	-	-	-	Reddish brown
		3	0.47	-	-	-	Reddish-brown
		4	0.69	-	-	Yellow	-

Table 3.6

CHROMATOGRAPHIC SEPARATION OF FLAVONOIDS IN DIFFERENT PARTS OF R. COMMUNIS

PAPER CHROMATOGRAPHY

Plant part	Solvent System	Spots	Rf	Colour Reaction			
				Without reagent		1% sodium carbonate	
				Daylight	uv	Daylight	uv
Seed	n-butanol: acetic acid Water 4:1:5)	1	0.33	-	-	-	Yellow
Root	"	1	0.36	-	-	Yellow	Yellow
		2	0.57	-	Yellow	-	-
Stem	"	1	0.50	-	Yellow	-	-
Leaf	"	1	0.52	-	Blue	-	-
		2	0.61	-	-	-	Violet
		3	0.31	-	-	-	Yellow
		4	0.71	-	-	Yellow	Yellow
	Acetic acid 15%:						
Seed	Water 85%	1	0.82	-	-	-	Yellow
		2	0.88	-	Yellow	-	-
Root	"	1	0.40	-	Yellow	-	-
		2	0.88	-	-	Yellow	Yellow
Stem	"	1	0.41	-	Yellow	-	-
Leaf	"	1	0.32	-	-	Yellow	Yellow
		2	0.66	-	-	Yellow-green	Yellow-green
		3	0.74	-	Blue	-	-
		4	0.91	-	Yellow	-	-
		5	0.56	-	-	Yellow	Yellow

Table 3.7

CHROMATOGRAPHIC SEPARATION OF ALKALOIDS IN DIFFERENT PARTS OF R. COMMUNIS

PAPER CHROMATOGRAPHY

Plant part	Solvent system	Spot	Rf	Colour Reactions			
				Daylight	uv	Iodine	Dragendoff's reagent
Seed	Ethyl acetate						
	25% formic	1	0.53	-	Yellow	Yellow	orange
	acid (4:3)	2	0.89	-	-	Yellow	-
Root	"	1	0.51	-	Yellow	Yellow	orange
		2	0.89	-	-	Yellow	-
Stem	"	1	0.50	-	Orange	Yellow	-
		2	0.89	-	-	Yellow	-
Leaf	"	1	0.57	-	Red	Yellow	-
		2	0.89	-	-	Yellow	-
	n-butanol:						
	Acetic acid:						
Seed	Water (4:1:5)	1	0.69	-	Yellow	Yellow	orange
Root	"	1	0.86	-	Yellow	Yellow	orange
Stem	"	1	0.94	-	Orange	Yellow	-
Leaf	"	1	0.93	-	Red	Yellow	-
	THIN LAYER CHROMATOGRAPHY						
	Chloroform:						
	Methanol:						
Seed	Ammonia	1	0.88	-	Yellow	Yellow	-
	(10:20:1)						
Root	"	1	0.88	-	Yellow	Yellow	-
Stem	"	1	0.88	-	-	Yellow	-
Leaf	"	1	0.88	-	-	Yellow	-

- No colour reactions

3.6.4 QUANTIFICATION OF FLAVONOIDS

The flavonoid content of the different plant parts were determined and the results (figures obtained) were compared with those obtained from chromatographic analysis to find out if the variations in the amount of flavonoids present in the different parts were related to the number of flavonoids observed in the chromatograms i.e no of spots. The results were as shown in Table 3.8 below.

Table 3.8

FLAVONOID CONTENT OF DIFFERENT PARTS OF R. CONNUNIS

Plant Part	I	II	Average
Leaves	- 3.5%,	3.9%	3.7%
Stem	- 1.04%,	0.98%	1.01%
Root	- 0.86%,	0.82%	0.84%
Seed	- 0.42%,	0.36%	0.39%

The leaves had the highest amount of flavonoids and it also had the highest number of spots, 5 in each case, suggesting that the large number of different flavonoids contributed to the high amount of flavonoids present in the leaf. Flavonoids are coloured pigments and in plants the highest concentration of colouring matter are found in the leaves which are the site where sunlight is trapped to be used in photosynthesis.

Such a corelation could not be said to be the case for the stem, root and seed. The stem had only one spot in both chromatograms but contains a larger amount of flavonoids than the root and seed which both showed two spots each.

There has been no previous attempt to screen the various parts of the plant Ricinus communis for their phytochemical constituents except for the seed due to fact that only the seed has shown economic value i.e for its oil (castor oil) content. Other plant parts have been reported to be used in traditional medicine preparations but no experiment was carried out to find out which particular constituent was responsible for the various actions reported or even if the reports were true. In this work the different plant parts were investigated pharmacognostically i.e microscopically, chemomicroscopically and phytochemically so as to provide a reference for future studies.

The results obtained in the case of the seed compared favourably with that in literature. Oil was also found in the other plant parts but not as in large quantities like the seed. Lignin, tannins, starch, calcium oxalate crystals and cellulose were found in these other parts. Mucilage was present in trace amounts in the stembark and root. Resins,

balsams, terpenes, sterols, saponin, pentoses and ketoses were absent in the various plant parts. Free and combined reducing sugars were present in all the parts while only the leaves contained monosaccharides, this probably due to the fact that photosynthesis occurs in the leaves, and monosaccharides are the first products and have probably been converted to more complex compounds when they get to the other parts. Flavonoids and alkaloids were present in all the plant parts including the seed.

Ricin, the toxic protein which the plant is famous for, was found to be absent in the other plant parts except for the seed, this is as reported in literature. This was shown chromatographically when no spot compared favourably to the spot of the reference compound abrin in the case of the leaf, stem bark and root, as was the case in the case of the seed, when the proteins of the various plant parts were chromatographed.

3.7 RESULTS OF TOXICITY STUDIES

3.7.1 RESULTS OF THE EXPERIMENTAL DETERMINATION OF LD_{50} OF RICIN EXTRACT IN RATS (INTRAPERITONEALLY) ACUTE TOXICITY STUDIES

The sign and symptoms observed were not dose related, the severity of symptoms did not increase with increasing dose of the extract and there were individual variations in symptoms between the animals.

In the early stages the animals became dull and sluggish, with signs of general weakness. As time passed, the animals began to show signs of incoordination, palor and prostration. They showed watery eyes and passed watery diarrhoea. In some cases there was respiratory distress with spasmic breathing and hypersalivation. This symptoms of diarrhoea, dullness and respiratory distress agree with the findings of Anderson (1948) and Geary (1950).

The animals prostrated and were found dead in these position. Most of the symptoms observed were in agreement with that already reported in the literature.

Table 3.9

The toxic effect of ricin extract on the mortality of rats

Group	Number of rats	Dose ml/kg	Number of rats dead	Percentage (%) death	Survival Time
1	5	0.01	0	0	Over 24 hrs
2	5	0.05	1	20	under 24hrs
3	5	0.1	3	60	"
4	5	0.5	4	80	"
5	5	1.0	5	100	"
6	5	5.0	5	100	"
Control	5	1ml of distilled water	0	0	-

Calculation of LD_{50} value was done using the Arithmetic method of Karber (Aliu and Nwude, 1982). In this method the difference between two consecutive doses and the mean of the number of animals that died at two dose levels (mean dead) were calculated (Table 3.9). The sum of the products of the mean dead and the dose difference was divided by the number of animals in each group (Table 3. 10). The resulting quotient was subtracted from the dose that killed all the animals in order to obtain the LD_{50} value.

Table 3.10

Calculation of LD_{50} of Ricin Extract

Group	Dose ml/kg	Dose difference	Number dead	Mean dead	Dose difference x mean dead
1	0.01		0		
2	0.05	0.04	1	0.5	0.02
3	0.1	0.05	3	2.0	0.10
4	0.5	0.4	4	3.5	1.40
5	1.0	0.5	5	4.5	2.25
					Total = 3.77

∴ From the arithmetic method of Karber (Aliu and Nwude, 1982)

LD_{50} = 1.0 − 3.77/5 = 0.25ml/1kg

From the experiment of quantification of crude ricin extract 100ml of extract was found to contain in 4 samples - 0.12g, 0.11g, 0.12g, 0.13g of ricin (total solids)

$$\text{Average ricin content} = \frac{0.12 + 0.11 + 0.12 + 0.13}{4}$$

$$= 0.12\text{g}$$

$$\therefore \text{0.25ml contains } \frac{0.25}{100} \times 0.12$$

$$= 0.0003\text{g}$$

∴ LD_{50} of ricin in rats = 0.0003g of ricin per kg

3.7.2 RESULTS OF CHRONIC TOXICITY STUDIES

During the course of the chronic toxicity studies, animals were sacrificed at four weeks interval. The lungs, liver and blood were collected and taken for histopathological studies. The results of these findings are presented according to the time intervals, based on the different dose levels that is Group I, 0.5ml/kg feed; Group II, 1.0ml/kg feed and Group III, 1.5 ml/kg feed.

(a) RESULTS OF HISTOPATHOLOGICAL STUDIES OF LUNGS

- At four weeks -

Group I (0.5ml/kg of feed) animals showed congested lungs.

Group II (1.0ml/kg of feed) animals showed congested lungs.

Group III (1.5ml/kg of feed) animals showed congested lungs.

Pulmonary capillaries were distended and there was escape of red blood cells into the lumen of bronchioles.

Control animals were normal.

- At eight weeks -

Group I animals showed congested alveolar capillaries.

Group II animals showed congested alveolar capillaries.

Group III animals showed congested alveolar capillaries.

The alveolar were filled with fluid which was completely cell free.

Control Lungs were normal.

- At twelve weeks -

Group I animals showed oedema of the lungs.

Group II animals showed oedema of the lungs.

Bronchioles of the lungs were filled with some inflammatory exudate of polymorphs with red blood cells and fibrin.

Group III animals showed oedema of the lungs with damage to the interstitial walls of the alveolar.

The lungs of control were normal.

Oedema is the excessive accumulation of fluid in the body tissues, popularly known as dropsy. In pulmonary oedema this occurs in the air spaces of the lungs, it may be caused by drugs. (Concise Medical Dictionary, 1985). In this case the drug involved is ricin. The severity of the oedema increased as the dose of the extract increased and became more pronounced with increasing time of exposure. Death would have occurred due to respiratory failure.

These findings of lung oedema conformed with that of Olsnes (1978) who reported oedematous lungs and the filling of the trachea and bronchi with frothy oedematous fluids.

(b) RESULTS OF HISTOPATHOLOGICAL FINDINGS OF LIVER

- At four weeks -

No pathology was reported for livers of Group I , II, III and control animals. The livers were all normal.

- At eight weeks -

In Group I (0.5ml/kg of feed) animals, the hepatocytes were undergoing degeneration.

In Group II (1.0ml/kg of feed) animals, the hepatocytes were undergoing degeneration.

In Group III (1.5ml/kg of feed) animals, the hepatocytes were undergoing degeneration and patchy grey areas were observed in the focal points of the liver.

- At twelve weeks -

Group I animals showed pyknosis (gradual fading away of nucleolus).

Group II animals showed pyknosis.

Group III animals showed pyknosis and necrosis was starting to show in focal areas of the liver without any loss of architecture of cells.

Necrosis is defined as the death of some or all of the cells in an organ or tissue caused by disease, physical or chemical injury, or interference with the blood supply (Concise Medical Dictionary, 1985).

Multiple necrosis has been reported in the liver and kidney due to ricin poisoning (Kobert, 1906). More recently, it has been shown that in the liver of rats treated with ricin, the sinusoidal cells and hepatocytes were damaged (Olsnes, 1978).

(c) RESULTS OF EVALUATION OF BLOOD

There was no apparent change in the composition of blood throughout the period of twelve weeks. The red blood cell count, haemoglobin, packed cell volume and total protein showed no marked fluctuation for any of the dose ranges.

In conclusion, the chronic toxicity findings conform with those already reported in literature, that is lung oedema and liver necrosis.

Haemolytic anaemia has been reported in the blood for acute

toxicity cases (Watt and Breyer, 1962). Absence of any effect of the blood under chronic studies might give an indication why immunity can be induced in animals by feeding them on increasing amounts of the bean.

3.7.3 RESULTS FOR PROTEIN CONCENTRATION DETERMINATION

Ricin is a poisonous protein reported to be found in the seed of R. communis. An attempt was made to relate the total protein concentration of the different plant parts to the presence of ricin in them. Using the refractometer, the total protein concentration of four samples from each part of plant were determined, the results were as follows.

Table 3.11

TOTAL PROTEIN CONCENTRATION OF THE DIFFERENT PLANT PARTS

Plant part	Conc (g/100ml) n				Mean	Standard error
	I	II	III	IV		
Seed	1.011	1.000	1.002	0.999	1.003	± 0.001
Leaf	0.953	0.947	0.947	0.945	0.948	± 0.001
Stem	0.902	0.896	0.905	0.901	0.901	± 0.001
Root	0.899	0.900	0.906	0.903	0.902	± 0.001

There was very little variation between the protein concentrations found in the different parts. The seed had the highest value, this could be attributed to the presence of ricin in it, however, this cannot be said to be the case because from the results of chromatographic analysis the number of proteins detected appear to be the same, especially in the case of seed and leaf. Ricin occurs in very small amounts - (0.2%) in the seed (Watt and Breyer, 1962) and would not cause a substantial increase in the protein concentration of seed. Table 3.11.

3.7.4 RESULTS FOR EFFECT OF EXTRACTS ON BLOOD

The extract of the seed was the only one which caused haemolysis i.e lysis (destruction) of red blood cells (plate 1 & 2), the other parts did not. The haemolytic property of the seed is due to the presence of ricin, a toxic protein present in the aqueous extract of the seed. Ricin appears to be absent in

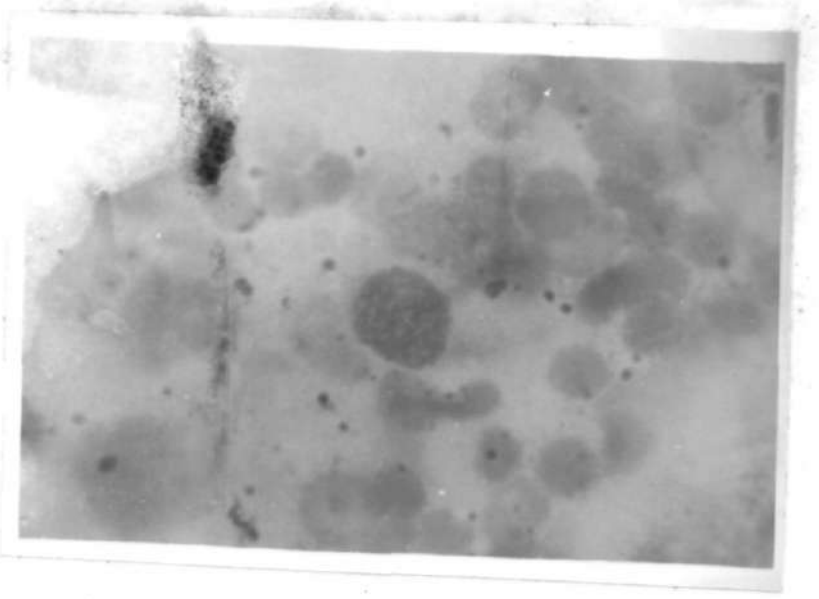


PLATE 1: NORMAL RED BLOOD CELL

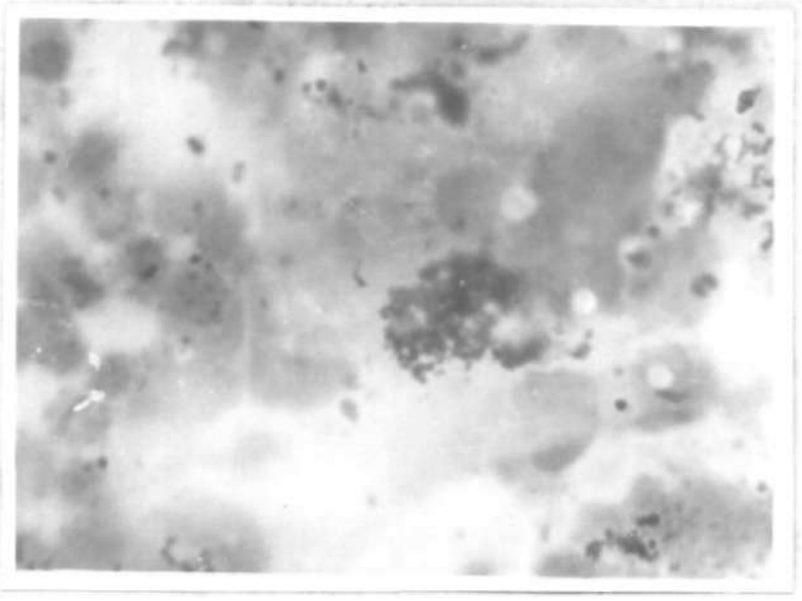


PLATE 2: HAEMOLYSED RED BLOOD CELL BY RICIN

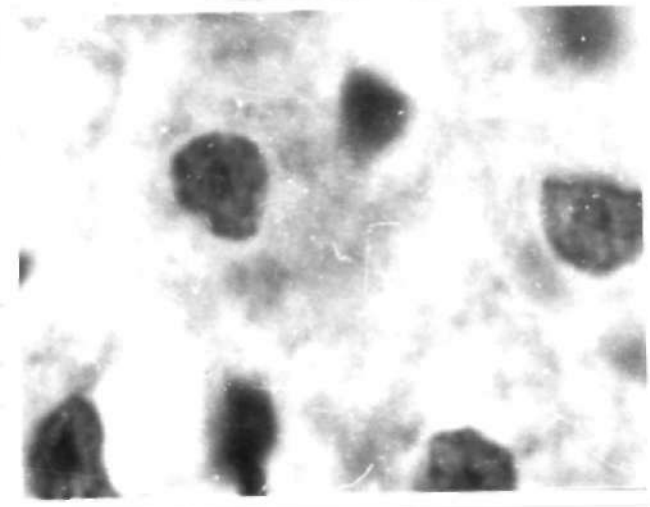


PLATE 3: NORMAL LIVER TISSUE/CELL

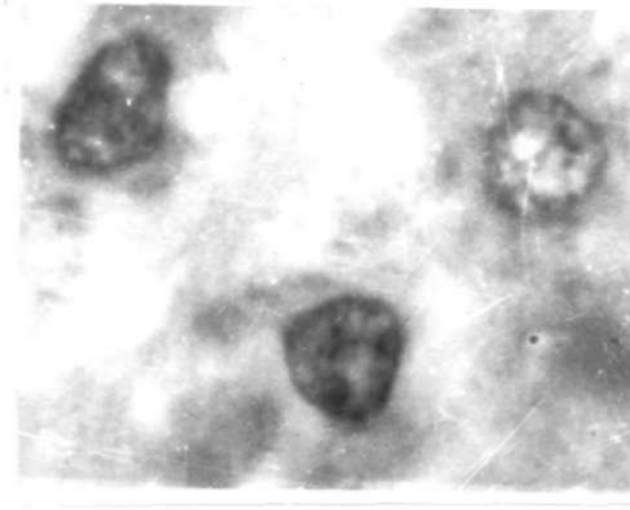


PLATE 4: LIVER NECROSIS CAUSED BY RICIN, PATCHY GREY AREAS, FADING NUCLEOLUS

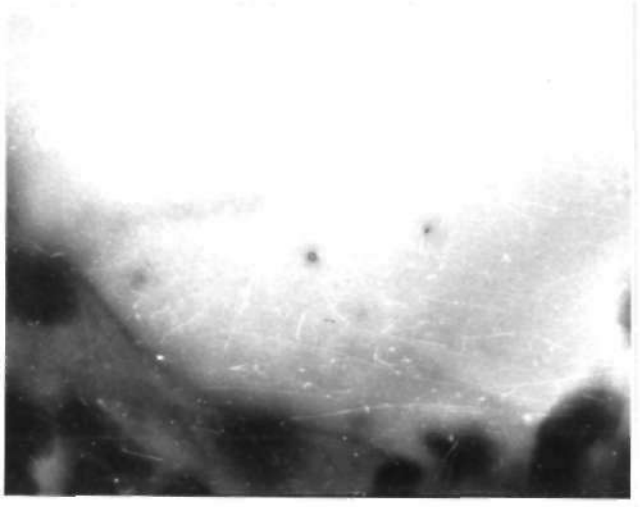


PLATE 5: NORMAL LUNG TISSUES

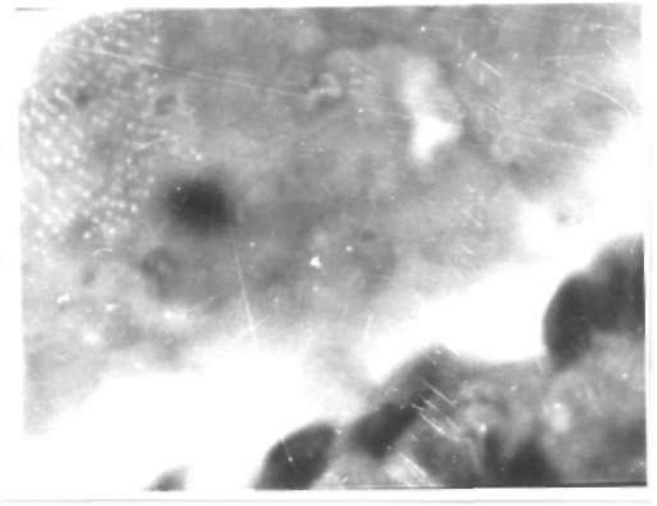


PLATE 6: LUNG OEDEMA CAUSED BY RICIN, ALVEOLAR SPACES FILLED WITH FLUID

the other parts of the plant. It has not been reported to be present in these other parts apart from the seed.

Table 3.12

HAEMOLYTIC EFFECT OF EXTRACTS OF THE DIFFERENT PARTS OF R. COMMUNIS

Plant Part	Effect/Haemolysis
Seed	+
Leaf	-
Stem	-
Root	-

Key + positive

\- negative

3.7.5 RESULTS OF HAEMOLYTIC STUDIES

The wavelength of maximum absorption of the blood sample was determined using the uv spectrophotometer, with distilled water acting as standard. The spectrophotometer was set to scan from the wavelength 180-750nm, with the recorder running. The λmax was then read off the record sheet i.e the peak of maximum absorption shown on the graph drawn. Wavelength of maximum absorbance was 411.6, the absorbance at λmax was 0.869. Working with this wavelength, the following results were obtained on addition of ricin extract Table 3.13).

Table 3.13

CHANGE IN ABSORBANCE WITH TIME

Time (min)	Samples (absorbance)					
	I	II	III	IV	V	Mean (S.E)
0	0.869	0.869	0.869	0.869	0.869	0.869
5	0.660	0.659	0.654	0.660	0.658	0.658 ± 0.0001
10	0.642	0.640	0.649	0.641	0.645	0.643 ± 0.0001
15	0.628	0.630	0.638	0.628	0.626	0.629 ± 0.0001
20	0.615	0.616	0.619	0.617	0.614	0.616 ± 0.0001
25	0.602	0.599	0.600	0.600	0.604	0.601 ± 0.0001
30	0.594	0.594	0.592	0.595	0.595	0.594 ± 0.00003
35	0.588	0.587	0.588	0.588	0.587	0.588 ± 0.00001
40	0.586	0.585	0.585	0.586	0.586	0.586 ± 0.00001
45	0.586	0.585	0.585	0.586	0.586	0.586 ± 0.00001

Table 3.14

CHANGE IN ABSORBANCE WITH INCREASE IN VOLUME/CONCENTRATION OF RICIN

Vol of ricin Extract (ml)	Sample (Absorbance) I	II	III	IV	V	Mean (S.E)
1.0	0.869	0.869	0.869	0.869	0.869	0.869
0.2	0.831	0.834	0.838	0.832	0.840	0.835 ± 0.0001
0.4	0.792	0.811	0.802	0.794	0.801	0.800 ± 0.0001
0.6	0.772	0.781	0.784	0.774	0.772	0.777 ± 0.0001
0.8	0.760	0.762	0.771	0.759	0.758	0.762 ± 0.0001
1.0	0.745	0.746	0.754	0.739	0.738	0.744 ± 0.0001
1.2	0.729	0.727	0.732	0.727	0.721	0.729 ± 0.0001
1.4	0.716	0.716	0.720	0.708	0.704	0.713 ± 0.0001
1.6	0.701	0.703	0.702	0.700	0.694	0.700 ± 0.0001
1.8	0.695	0.692	0.690	0.691	0.688	0.691 ± 0.0001
2.0	0.688	0.682	0.676	0.678	0.680	0.681 ± 0.0001

The values obtained from the two experiments were plotted out graphically both curves (Graph I and II) sloped from left to right. Steyn (1934) stated that if large amounts of castor beans are eaten, general weakness and collapse may be evident almost immediately. Clinical symptoms are progressive,

FIGURE 3·5

GRAPH OF CHANGE IN ABSORBANCE WITH INCREASE IN TIME (TABLE 3.1

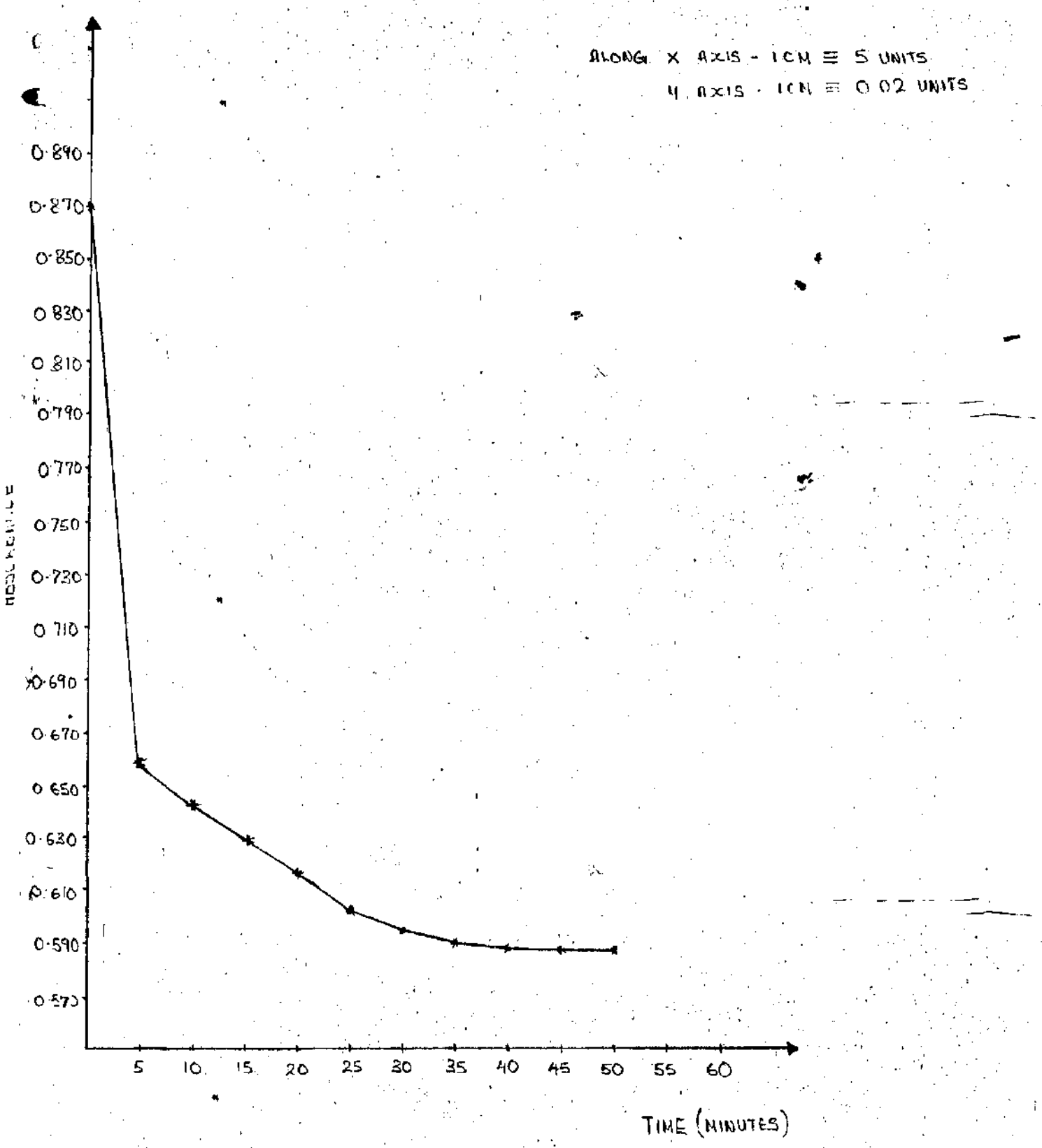
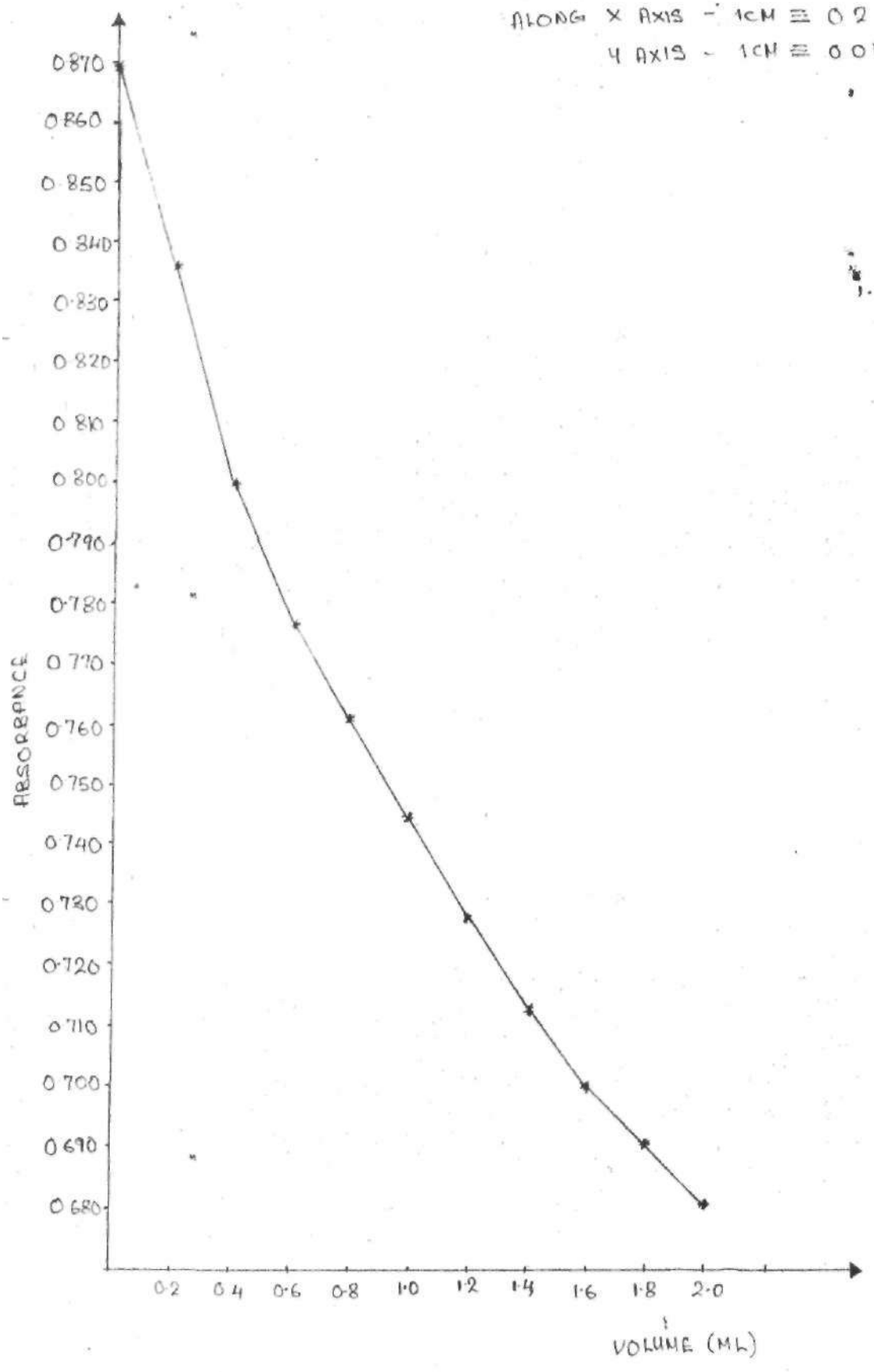


FIGURE 3·6

GRAPH OF CHANGE IN ABSORBANCE WITH INCREASE IN CONCENTRATION / VOLUME (TABLE 3:1



becoming more serious as the case goes on. The ingestion of such large amounts of castor bean could be easily compared to the addition of the ricin to the blood in which case haemolytic effects are seen almost immediately. The absorbance of bolld sample was seen to change on addition of the ricin extract. The greatest change was observed to occur within the first five minutes, which is indicated by the very steep slope of the curve of the graph within this period. Changes thereafter lessening (curve levels out) until a point at which it becomes constant i.e no more change in the absorbance, this occured after 50 minutes (Fig 3.5). The patient either dies or is probably starting recovery at this point.

The haemolytic activity of the ricin extract was also observed to increase as the concentration of extract increased. This means that the chances of recovery in cases of poisoning depends on the amount of the poison taken and the time of exposure before the treatment begins.

No latent period was observed between time of addition of ricin and change in absorbance. This latent period observed in cases were the castor bean is eaten (McCunn et al, 1945) is probably a time lapse between the time the bean is eaten and when it is

absorbed into the blood stream. In the experiment no time lapse was observed due to the direct addition of ricin extract to the blood sample. This shows that treatment should be given as soon as castor bean is eaten before the time lapse elapses and symptoms start because from the experiment it can be seen that the effect of the ricin increases as the time of exposure increases.

CONCLUSION AND RECOMMENDATIONS

Poisoning by castor bean from the plant, Ricinus communis is a common phenomenon in many parts of the world, yet the plant is still used extensively in traditional medicinal practices, orthodox medicine and even in industries. For the first time, a detailed pharmacognostical evaluation of the other parts of the plant apart from the seed which has been investigated by many workers and has a lot of literature on it, has been carried out.

The various data and results from the pharmacognostic study of this plant include the microscopy, chemomicroscopy and phytochemical screening thedetermination of the nature of the chemical constituents; and the toxicological profile i.e the median lethal dose, chronic toxicity and haemolytic studies which can be employed as a means of controlling quality (or standardizing) the various traditional medicine preparations made from the plant. These traditional preparations should also be validated and those that cannot be substantiated should be discarded/discontinued so as to prevent possible future harm that they may cause. Suitable doses for taking this traditional preparations should be recommended and the practitioners should be instructed on how to dispense them.

Some of the data, particularly those of pharmacoprial importance such as moisture content, ash values and extractive values could be useful in the preparation of monograph of the Ricinus communis.

The data obtained from the toxicity studies could be useful in the treatment and maintenance of cases of poisoning by the bean (cake) in both human and veterinary medicine. Health workers should be educated on the way and manner of which the poisoning progresses so as to be able to give prompt or symptomatic treatment.

In the more recent field where ricin is now employed in cancer chemotheraphy, these toxicity datas could be employed in the research of ways to reduce the cytotoxic effects on normal tissue and to increase possibility of full recovery.

Knowledge of the chemical constituents of the various parts of plant such as alkaloids, flavanoids and the different types of reducing sugars will help in the exploitation of the full economic potential of the plant. However, more work is required to be done in order to quantify these compounds, so as to know whether the plant can act as a cheaper source for these compounds.

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