# EFFECTS OF CONTINUOUS CONSUMPTION OF ROSELLE (HIBISCUS SABDARIFFA) CALYX EXTRACT (ZOBORODO) IN HYPERTENSIVE AND NON-HYPERTENSIVE CONDITIONS

**BY**

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## DECLARATION

I, Emmanuel Oladipo Ajani, hereby declare that this thesis is a record of my own research work and has not been submitted for the award of any degree to any university. Information and excerpts from published works of others have been gratefully acknowledged in the text.

Emmanuel Oladipo Ajani

17/09/98

Date

## CERTIFICATION

This thesis entitled "Effects of continuous consumption of roselle (Hibiscus sabdariffa) calyx extract ("Zoborodo") in hypertensive and nonhypertensive conditions" by Emmanuel Oladipo Ajani, meets the regulations governing the award of the degree of Master of Science (M.Sc) in Biochemistry of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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## DEDICATION

This thesis is dedicated to God the Father, Son and Holy Ghost, who inspite of all odds accomplished His words concerning me.

## QUOTATION

"The gent who wakes up and finds himself a success hasnt been asleep".

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# ABSTRACT

The $LD_{50}$ of rose lie ealyx extract was determined in mice. Different doses of the extract (125-2000mg/kg body weight) were administered to different groups of normotensive and spontaneously hypertensive rats continuously for 60 days. The body weight (b.wt), blood pressure (systolic and diastolic), water and urinary volume and colouration were periodically monitored during the period of administration of the extract, serum glucose, cholesterol, creatinine, uric acid and proteins were also measured after 30 and 60 days of continuous administration of the extract.

Roselle calyx extract ("zoborodo") was observed to significantly ($P<O.05$) lower both systolic and diastolic pressure in both normotensive and hypertensive rats at all tested doses. The reduction of blood pressure in both groups positively correlated with body weight.

Continuous administration of the extract at lOOOmg/kg b.wt was observed to lead to sudden death in spontaneously hypertensive rats but not in normotensive Wistar-Kyoto rats. Water intake and urinary output was found not to be significantly different in the hypertensive control (untreated) group from the normotensive control (untreated group). The water intake was however, observed to be significantly ($P<O.05$) lowered in all the treated groups (hypertensive and normotensive) compared with the control (untreated), while the urinary output was significantly higher in the treated hypertensive group compared with the control.

After 60 days of continuous administration of the extract, there was a significant ($P<O.05$) increase in serum uric acid at all tested doses in both treated hypertensive and normotensive groups compared with the controls. The serum cholesterol was significantly lower while the serum glucose was significantly higher (at doses > 500mg/kg b.wt) in all the treated rats compared with the control after 60 days of continuous treatment.

Though a trend seems to be established, the serum albumin was not significantly ($P>O.05$) different in the treated groups compared with the control. The serum total protein, however, was found to be significantly ($P<0.05$) higher after 60 days treatment period in the hypertensive group treated with 500mg/kg b.wt dosage compared with the control.

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CHAPTER ONE

# INTRODUCTION

## 1.1 General Introduction

Roselle calyx water extract [zoborodo] sweetened with sugar and often flavoured with fruit juice or spices such as ginger has become a common drink both at homes and social gatherings especially in the northern parts of Nigeria. In sudan, it has been dunk hot by the local people and cold by Europeans, but this later version is now common among the local people also (Pery, 1980).

The arabic name used for the extract "Karkadeh", a name not too different from "Karkade" as it is called in S w i t z e r l a n d has spread widely. Its popularity in the West, at least in the first quarter of the twentieth century, when cooking depended more on the housewife's ingenuity, can be detected from printed recipes (WHO/FAO, 1973). The original habitat of the plant was traced to West Africa (Brouk, 1975; Samy, 1987) where the greatest variation in types are seen. Three basic varieties of the plant have been identified: The red varieties, Rico;the pale green type, Altissima; and the intermediate type, intermedius (Dalziel, 1948;Duke, 1981). Differences are largely related to the extent to which red pigmentation is present in various parts of the plant and appear to reproduce true to type.

The epicalyx and calyx are usually bright red but they may alternatively be whitish or green according to the sub-variety. The fleshy succulent calyces are edible and for this reason, the whole fruit must be harvested and the calyces seperated off for preparation of the drink. The calyces are also used as flavouring for various sauces or for the preparation of jelly or preserve (Brouk, 1975). Chemical analysis carried out on roselle calyx revealed the presence of nutrients such as proteins, carbohydrates, fats, vitamins such as ascorbic acid, riboflavin, thiamin and mineral elements such as Ca, P, and S

(Subramania and Nair, 1972).

A long standing tenet of nutrition holds that people with diets rich in fruits and vegetables enjoy better health than people eating few fruits and vegetables. Consequently, research has sought the components or compounds responsible for this apparent health benefit. Hertog et al; (1993) reported on the role of flavonoids and quercetin in particular in the prevention of coronary heart disease. The favonoids had been found to possess anti-inflammatory, anti-microbial, and anti-neoplastic activities. Some are oestrogenic, others are antithyroidal or mutagenic. (Rastogi and Mehrota, 1991, Das et al, 1994). Some of these actions of flavonoids are thought to be due to their properties as metal chelators, free redical scavengers and chain breakers (Kandaswani and Middleton, 1994).

The numerous roles associated with flavonoids can perhaps account for the wide medicinal and non-medicinal uses associated with roselle calyx exrtract. Medicinally, roselle leaves are emollient and are used as diuretrics, sedative and refrigerant. Fruits are considered anti-scorbutic (Chopra et al, 1956). Infusion of calyces is considered diuretic, choleretic, intestinal antiseptic, mild laxative, cooling, refreshing and considered useful in bilious condition in heart and nerve diseases, high blood pressure and calcified arteries (Chopra et al 1956; Wealth of India, 1969). Seed decoction is reported to be useful in dysuria, dyspesia, and debility (Nardkani, 1964). Tender leaves and stalks are eaten as salad and as port herb and are useful for seasoning curries (Duke, 1981).

Though the antihypertensive effect or roselle calyx infusion in traditional medicine have been reported (Dalziel 1948; Harbone, 1977), information on this are scattered and have not been supported by experimental data on blood pressure. Coupled with this is the fact that there have not been any documented information on the biochemical/clinical effect of the infusion particularly as it affects certain biochemical parameters associated with hypertension and antihypertensive therapy.

Natural colourants have been used in food over a long period of time and have been accepted for such use without supporting toxicological evidence in much the same manner as vegetables and cereal products (WHO/FAO, 1973). Natural colourants, just as food additives has been suggested, should be subjected to safety criteria such as acute toxicity studies, short term and long term toxicity studies, carcinogenicity studies and metabolic studies (WHO/FAO, 1978). A WHO/FAO committee report (1964) on anthocyanin revealed that informations on the colourant are available for only one food colour product namely grapeskins. However, anthocynins are known to be a complex class of colouring meterial which occur in many plant (at least six major anthocyanin compound have been characterized chemically), hence, no acceptable daily intake (ADI) could be established for the colourant. Quercetin, another pigment identified in roselle, was observed in a mutagenic studies to show positive effects in strains of Salmonella typhimurium, however, the purity of the material tested was not reported, hence the available toxicological data were inadequate to evaluate this material (WHO/FAO, 1978). It has been recognised that, because of poor stability of some natural colourants, their decomposition during storage may produce toxic impurities, hence naturalness per se does not assure safety.

Considering the popularity of roselle calyx water extract (zoborodo) as a beverage in Nigeria, and the fact that there are no documented information on the toxicity of the infusion, there is the urgent need to ascertain the safety of the infusion, vis-a-vis the aclaimed antihypertensive property. Hence this research work is undertaken to:-

(i) determine the level (dose) of the extract that would result in acute toxicity in experimental animals.

(ii) assess the effect of the extract on some physiological parameters associated with hypertension.

(iii) assess the effect of the extract on some biochemical parameters with particular reference to protein, carbohydrate and lipid metabolism.

From the result, one would be in a position to advise on the merits and demerits of the continuous consumption of the extract and possibly the optimum concentration (dose).

CHAPTER TWO

# LITERATURE REVIEW

## *DESCRIPTION OF ROSELLE PLANT*

### 2.1. Botanical Description

The plant, Roselle (Hibiscus sabdariffa). belongs to the general order Malvales, family, Malvaceae and tribe Hibiscae. Its species name Sabdariffa is of Turkish origin (Dalziel, 1948; WHO/FAO, 1973). Prior to the establishment of this name by Linne, there was considerable synonyms for the plant among botanists. English, French and Spanish likened it to the Plant, Rumex scatutus, in English "French Sorrel". This name was latter corrupted to form another English name "rouselle" or "roselle" by which the plant is now most widely known (WHO/FAO. 1973). Among the Hausa in Northern Nigeria, it is commonly reffered to as "Yakuwa" or "Sure" (the seed only) or "Zoborodo" (the fleshy calyx). In the southern part of the country, it is called "Ishapa" among the "Yoruba" while the Igbos refer to it as "Ojo" or "Okworo-Ozo" (Dalziel, 1948).

The plant is closely related to several fibre producing plants including kenaf, (Hibiscus cannabinus) and cotton (Gossypium spp) to okro or gumbol (Hibiscus esculentis) and to some ornamental plants such as the swamp or Rose mallow (Hibiscus moschatus) (Crane and Acuna, 1954). It is basically an annual herb, in some environment Old root stocks may give limited perennial growth. Varieties selected for their edible qualities are sub-shrubs, about 0.5-3.0 metres high branching near the base. The flowers are 300 - 500mm in diameter and have five petals. They are larger than the calyx but smaller than the leaves. The petals carry a generally purplish-brown spot near the base, which appear as an eye in the flower. They open for one day only, for about three hours, closing rapidly about mid-day, the petals then becoming twisted. The colour of the calyx varies according to the sub-variety but is always paler towards the base (WHO/FAO, 1973). (Fig 2.1).



Fig: 2.1: Roselle (Hibiscus sabdariffa)

Source: Wealth of India (1959)

## 2.1.2 Distribution And Growth

The plant has been noted to have probably originated in West Africa where the greatest variations in types of roselle are to be seen. (samy, 1987, Edmond, 1990).

The crop has an overall growing period of 4-6 months. While the plants can be propagated from cuttings, they are best grown from seed. They grow best on good soils but will tolerate moderately fertile sands and loamy sands. A temperature range between 20 and 30 $^{0}$C without prolonged dull weather, available rainfall of 200-600mm and still humid air conditions are most conducive for optimum growth (Harrer, 1952; Fahmy et al 1992; ). The crop can withstand warmer, more humid weather than kanaf but is more susceptible to low temperature damage (Petrini et al, 1990). Flowering usually commences in late September or early October depending on the time of planting (WHO/FAO 1973). The calyces mature rapidly and are ready for picking within 15-20 days of blossoming. Picking is carried out promptly with a sudden snap, while they are still tender enough to break away easily and before any woody tissue forms in the fruit. This encourages continuous flowering which may extend until the end of December. The calyx is then cut off and the seed box squeezed out by hand (WHO/FAO, 1973). Yields of 1-2kg 'fruit' per plant can be obtained from varieties of sabdariffa.

## 2.1.3.0 Varieties

Observation made in a number of West African Countries noted that the red, green and intermediate varieties are equally prevalent (Whitby, 1968; WHO/FAO, 1974). Differences are largely related to the extent to which red pigmentation is present in various parts of the plant and appear to reproduce true to type. The general tendency is for the greenest variants to be tallest, unbranched, and about 4.75 per cent more fibrous than the red ones. The varieties, Altissima, reach a height of 5 metres, and is the type grown for fibre. Dalziel (1948) also reported a branch green type called Bhagalpurrinosis. It was concluded that the genetic factor generally controlling red

coluring was absent in this veriety and that a separate factor was responsible for the crimson eye in the flower. At the other end of the scale was another variety called Rico or Rubber (Dalziel; 1948). These types reach about 2 metres under adequate conditions and has large red calyces. Between the two extremes, an intermediate type exists called Intermedius (Dalziel, 1948).

### 2.1.4 *Chemical Composition*

The chemical constituents present in Hibiscus sabdariffa are widely distributed in the various parts of the plants, namely the flowers, seeds, leaves, stems and roots. It is expected in any case that variations will arise due to differences in ambient conditions during production, harvesting, treatment and storage of the crop. (WHO/FAO, 1973). In the context of this work, the prime concern is the calyx and those factors most likely to influence its acceptability as a drink. Of these, flavour is of prime importance.

**Flavour**: The flavour characteristics of roselle has been reported to be due mainly to the organic acid content (WHO/FAO, 1973). The presence of acids such as hibiscic, citric, malic, lactic and tartaric had all been reported (WHO/FAO, 1973; Khafaga and Koch, 1980; Duke, 1987). Hibiscic and citric acids were noted to be the dominating acids and are responsilbe for the distinctive flavour of the roselle influsion (Khafaga and Koch, 1980).

Hibiscic acid was noted to be peculiar to roselle and not reported elsewhere in nature and was suggested to be a lactone of hydroxycitric acid ($C_6H_6O_7$) with 2 asymmetrical carbon atom (WHO/FAO, 1973). A pH range between 2.57 and 2.80 was recorded on samples in the Sudan (Ibrahim et al 1971). In Senegal, a lower pH range between 2.0 and 2.3 was determined, however, higher values of 2.5 to 3.2 were noted in the dried samples (WHO/FAO, 1973).

**Pigments**: The first detailed analysis concentrated on the pigment of the red varieties of calyx. Anthocyanin monoxide (hibiscin chloride) was the first pigment to be

detected. It was noted to be red in colour. It is a cyanidin chloride with the chemical formula $C_{15}H_{11}O_{16}$ (WHO/FAO, 1973). Subramania and Nair (1974) also reported the *presence of two other anthocyanins; cyanidin -3,5-digluco side and cyanidin -3, (2-*glucose) rutinoside, along with two flavonols: gossypetin -8- glucoside and gossypetin -3- glucoside.

Other pigments that had been reported to be present in roselle calyx include hibiscetin, gossepetin and quercetin (Rao and Seshadri, 1942). Hibiscetin was identified as hibiscetin 1,3,3,4,5,7,8 - heptahdroxy flavone and was confirmed by synthesis (Rao et al, 1944). It was noted to be the main pigment of the flower, while gossypectin (gossytrin), gussypitrin and sabdaretin, were reported by Seshadri and Thukur (1961) as *minor pigments.*

When the anthocyanin content of the calyces of five Hibiscus sabdariffa strains were analysed from flowering to overripeness, it was observed that during calyx growth, the anthocyanin content reached 1.7 to 2.5% of the dry weight in all strains analysed. The maximum content was maintained for 10-15 days after which the content dropped considerably. cyanidin -3- sambubioside was the chief anthocyanin, followed by cyanidin -3- glucoside. The delphinidin 3- glucoside were lacking in the Senegalese strain but amounted to 25-35% in the others. The calyx of the Egyptian strain showed maximum anthocyanin concentration 30-40 days after flowering, of the Indian strain after 35-45 days. The strain from central America and Thailand needed 45-50 days to reach this stage of maturity (Khafaga and Koch, 1980).

**Proteins**: Subramania and Nair (1972) reported a protein concentrations of 1.9,3.3 and 1.6g in 100g of roselle's fruit, leaf and inflorescence respectively.

The calyces of strains from Guinea and Ivory Coast were reported to contain 7.9% and 9.3%proteins respectively. In a broad-based nutrition study of the calyces of an intermediate variety collected in West Africa, eighteen aminoacids were detected.

Aspartic acid was the most common amino acid found, while the other amino acids were less than 1.0% of the total amino acids (Duke, 1987).

**Carbohydrates**: Total carbohydrate concentrations of 12.3,9.2, and 11.1g were reported in 100g of roselle's fruit, and inflorescence respectively (Subramania and Nair, 1972).

The dried petals of the flowers was reported to yield 65% (dry weight) mucilage and the mucilage when hydrolyzed and separated using paper chromatography shows the presence of galacturonic acid, galactose and rhamnose. The pectin content has been shown by Duke (1981) to increase in both quality and quantity with sepal development during maturation and has been noted to give the plant its mucilaginous character.

When the mucilage, pectin, sugar and starch contents of the calyces of five (5) strains of Hibiscus sabdariffa were analyzed from flowering to overripeness, Afry el al, (1980) reported that maximum mucilage content reached 24 to 28% in strain from Central America and Egypt, but only 15% in the Indian strain, 22-25 days after flowering. In the strain from Senegal and Thailand, this peak was found only at an overriped stage. The highest pectin contents, determined after mucilage extraction amounted to 2.4% anhydrouronic acid; esterification reached 40-50% in the mucilage and 30-40% in the pectin. Total sugars reached a maximum of 3-5% around the 30th day after flowering. Starch decreased continually during calyx growth to about 0.2% of the dry weight of the overripe stage.

**Fats**: Fat concentrations of 0.1, 0.3, and 0.1g has been reported in 100g of roselle's fruit, leaf and inflorescence respectively. The seed was reported to contain 22.3% fat and the seed extracted with ether contained 0.7% fat (Subramania and Nair, 1972).

The seeds have been reported to keep for over a year without variation in the oil or fatty acid content. The raw oil also exhibited good stability in both free fatty acid contents and colour over one 120 days storage test. It is easily refinable by conventional methods and is claimed to have good cooking properties being comparable to cotton seed oil and

is added as substitute for crude castor oil. Its density at 15°C is reported to be 0.932, Iodine index 107.5, and saponification index 109 (Duke, 1987). Component fatty acids of the seed lipids were 2.1% myristic-, 35.2% palmitic-, 2.0% palmitoleic-, 3.4% stearic-, 34.0% oleic-, 14.5% linoleic-, and three unusual HBr reacting fatty acids (cis-12,13-epoxy-cis-9-octadecanoic (12,13- epoxyoleic) 4.5%; sterculic 2.9%; and malvalic, 1.3% (Ahmad et al, 1979). Salama and Ibrahim (1979) reported the presence of 61.3% beta - sitosterol, 16.5% campesterol, 5.1% cholesterol and 3.2% ergosterlol (said to be rare in vegetable oil but the most common mycosterol in most fungi including yeast) in the seed oil.

**Vitamins**: An ascorbic acid concentrations of 14,54 and 14mg were reported in 100g of roselle's fruit, leaf and inflorre-scence respectively (Subramania and Nair, 1972). Dried samples of the calyces from Suriname, East Africa and Java, were reported to contain 52, 30 and 21mg vitamin C per 100g respectively (Duke, 1987), while Karkade (dried calyces minus ovary) contain 0.004 to 0.005% asscorbic acid (Subramania and Nair, 1972).

An attempt to estimate the true vitamin A value of roselle leaves used as vegetables was undertaken by Rao (1967) using chromatography. Values obtained by conventional method of incomplete removal of absorption on calcium carbonate resulted in impurities in the extract. Compared with the alternative of using calcium hydroxide, they differed by as much as 44%. The fruit , leaf and inflorescence were reported to contain 300 ug, 4135 ug and 285 ug B-carotene equivalent per 100g respectively. (Subramania and Nair, 1972)

## 2.2 Uses of Roselle

From the early stage of man's development, his study of plants was intimately carried out and thoroughly applied. This is well examplified in the roselle from its wide range of recorded applications.

## 2.2.1 Medical Uses

The plant, Hibiscus sabdariffa is used for various medicinal purposes in many West African countries. Traditionally, preparations from various parts of the plant such as flower, leaf, calyx and corolla are used as remedy for various illnesses (oliver, 1960).

Aqueous preparations of the plant had been reportedly used as antihypertensive (Oliver, 1960; Sharaf, 1966). The plant has been found to have abundant diuretic effect accompanied by slight diaphoretic action and that it possesses a high intestinal antiseptic action and thus can be used to combat various infectious intestinal diseases (Sharaf, 1966; Duke, 1987). Obiefuna el al, (1992) demonstrated a vaso-relaxant effect of the plant petal extract. The vaso-relaxant effect of the plant was shown to be endothelium dependent which is probably linked to the release of endothelium derived rerlaxant factor, however, not much is known about the mechanism through which Hibiscus sabdariffa relaxes vascular smooth muscle.

The calyces and leaves have been reported to be anti-scorbutic, sedative, refrigerant and hence (an infusion of the calyces called "Sudan tea") is taken in the East of Chad to relieve symptoms of plethora, bronchitis and cough (Dalziel, 1948. Chopra et al, 1956; Duke, 1987). The use of roselle calyces infusion as sedative and refrigerant was reported by Chopra el al, (1956) to be due to the emollient effect which thus causes the cooling of part of the body and hence reduces its metabolic requirement. In native African medicine, roselle infusion are reportedly used to free the flow of urine, as an emollient, a sedative, an aperient and for cleansing wounds. This emollient property has been demonstrated to retard the rate of alcohol absorption and thus lessening the intensity of alcohol effect (Villegas, 1956).

An aqueous extract of the calyx have also been demonstrated to induce an oestrogen-like activity in immature female rats (Ali el al, 1989) and to reduced intestinal motility in intact rat (Ali et al, 1991). Roselle, has been reportedly used in treating aching decayed

teeth, while singed leaves are applied to athlete's foot infections in the West and to poultice abscesses (Sillians; 1953; Duke, 1987). It has also been reportedly useful in decreasing viscosity of blood and stimulation of intestinal peristalsis. (Duke, 1987). The bitter root has been used by the Philippians as an appetizer and tonic (Perry, 1980).

### 2.2.2 Non-Medicinal Uses

The roselle plant appear to have a food industry processing potential which remains largely unexploited. Commercial production of a fibre somewhat coarser than jute but suitable for sacking from the outer part of the stem was developed on a large scale by the Dutch in Java (Watt and Breyer - Brandwilk, 1962). The variety altissima, is normally grown for fibre (Dalziel, 1948). Its calyces are not edible.

The leaves and some times the seedling of the plant are used in cooking as vegetables (Whitby, 1968), while the calyx after the flower fade are reportedly used as a common pot herb or relish soup. They must be gathered at the young succulent stage, since the acid content falls as they become overripe and fibrous (Dalziel, 1948). In Cape Verd, the leaves are pounded into a thin puree which is served over rice (WHO/FAO, 1973). Their sharp taste has led to their inclusion in salads in India where they are also used for seasoning curries (Duke, 1978).

In Nigeria, the seed is used as a substitute for locust bean in dadawa production. The fermented cake being called "dadawa batso", the name applying also to the residue of the seeds after extraction of the oil. This residue is often used for various herbal preparations. (Dalziel, 1948). Prior roasting has been noted, after which the seeds are pounded into meals. The oil is extracted by parching the seed and then placing them for 3-4 days in water made alkaline with ashes. They are then pounded and the oil floats off, or the seeds are crushed and boiled and the oil skimmed off. The resdue is used for food, either in soup or fortified with bean and other articles into cakes etc.

Among the Yorubas in Nigeria, the green types are popular. The calyces are sold either fresh or precooked. They are then further boiled with various soup ingredients, pounded melon seeds being added finally. This soup is served while the calyces still remain whole with yam or maize dough (WHO/FAO, 1974). The seeds are also used in Sudan as a seasoning in the same way as H. cannabinus seeds after fermentation. They are often roasted as a coffee substitute and have been noted to have bitter taste and contain between 13.5 to 18.5% of brownish yellow oil with a sweetish stickly odour, the residual cake being used in animal feeding (Mohammed and Idris, 1991, Salih et al, 1992).

In both French and English speaking areas, they are used as a replacement for French sorrel, as flavouring for jellies, syrups and wine (Duke, 1987) and as a substitute for tea and coffee for people sensitive to excitant (Dalziel, 1948). Traditionally, the calyx has been chewed to alleviate thirst on long desert treks. However, the general attactiveness of the pleasantly flavoured beverage produced, probably at some later stage, as an infusion from it, has perhaps being most widely appreciated (Pery, 1980).

## 2.3. Biochemistry of Roselle Flavonoids

Flavonoids represent a wide variety of natural polyphenolic structures with ubiquitous distributions in plant foods (Herman, 1976; Joe et al, 1995). They occur in beverages such as tea and wine (Hertog, 1993). More than 4000 individual flavonoids have been identified in higher and lower plants (Harbone, 1977). They are hydroxylated, methoxylated, and/or glycosylated derivatives of 2-phenylbenzo (a) pyrene ring which consist of two benzene rings combined by mediation of the oxygen containing pyrene ring. They can be subdivided into six classes of flavonoids and flavonol related compounds: Flavones, flvanones, Isoflavone, flavonols, flavanols and anthocyanins (Joe et al, 1995). Roselle flavonoids can basically be grouped into anthocyanins, flavones and flavonols.

### 2.3.1 Anthocyanins

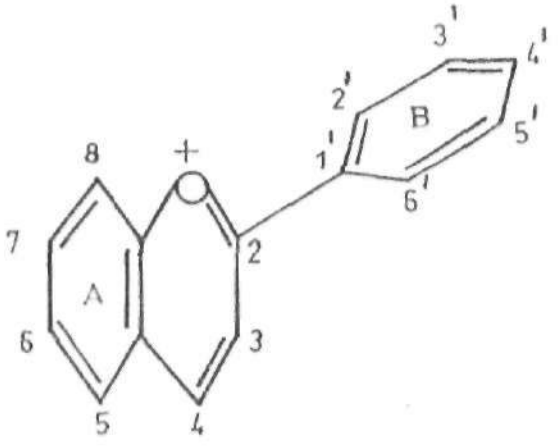
Anthocyanins are natural glycosides. They are water soluble pigments and generally

occur in the aqueous cell-sap (Finar, 1986). All anthocyanins are derivatives of the basic flavylium cation structure (2-phenylbenzopyrylium) (Francis, 1977).(Fig.2.2).

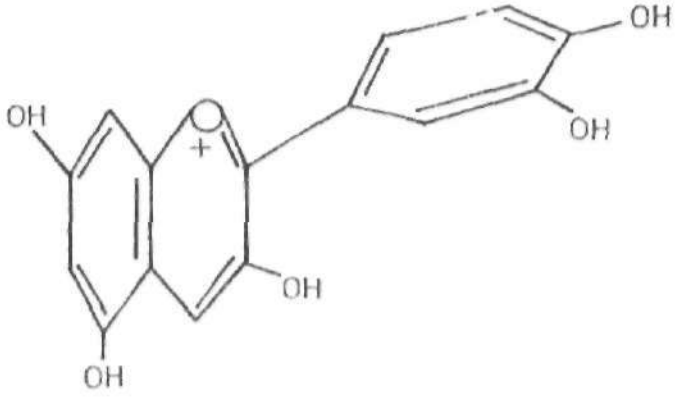
Twenty (20) anthocyanidins are known, but only six: pelargondin, cyanidin, delphinidin, peonidin, petunidin and malvidin are important in foods (Francis, 1977). The commonest ones in roselle are cyanidin and delphinidin. (Fig. 2.2). The sepals of roselle contain delphinidin -3- sambubioside or hibisin, cyanidin -3- sambubioside or gossypicyanin, cyanidin -3- glucoside and delphinidin -3- glucoside (Francis, 1977, Pouget et al, 1990). The different substitution patterns that occur in the B-ring modity the colour of the anthocyanin (Fig.2.3).

Mizukami et al, (1989) reported that in roselle callus cultures, chalcone synthase plays a more important role than phenylalanine ammonia-lyase (PAL) on the anthocyanin biosynthesis and light was identified as one of the regulatory factors.

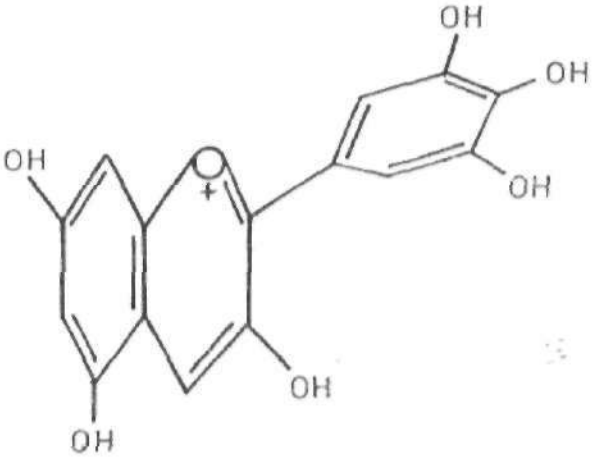
**Stability of Anthocyanins**: Anthocyanins are not particularly stable in solution in food products. They are susceptible to pH changes, oxygen, ascorbic acid and temperature. Anthocyanins changes colour with pH. At pH 1 or lower the compound is almost completely unionised and exhibit the most intense colour. At pH values of 4.5 and above, they are almost colourless (Chichester, 1972). They are unstable in the presence of oxygen and ascorbic acid (Francis 1977). Harbone, (1977) reported that accelerated decoloration of anthocyanins in the presence of ascorbic acids, phenol, sugar derivatives etc. May be caused by actual condensation reactions with these compounds. Even at low pH values (pH 2.0) at which anthocyanins are more stable, the destruction caused by the interaction with ascorbic acids is considerable (Chichester, 1972). Anthocyanins have also been reported to be readily degraded by heat (Francis 1977) and enzymes systems such as phenolases or glycosidases have been reported to decolourze it. (Francis 1977).



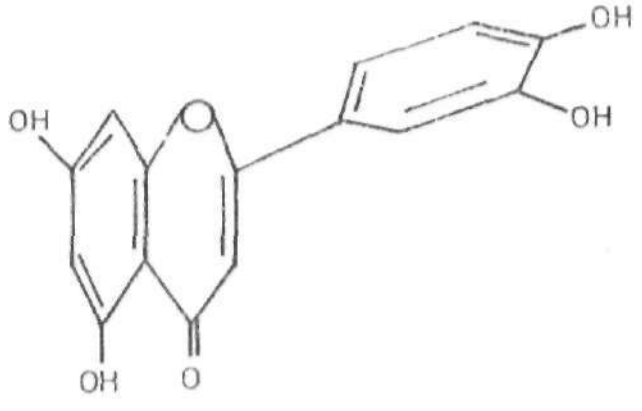

THE FLAVYLIUM STRUCTURE



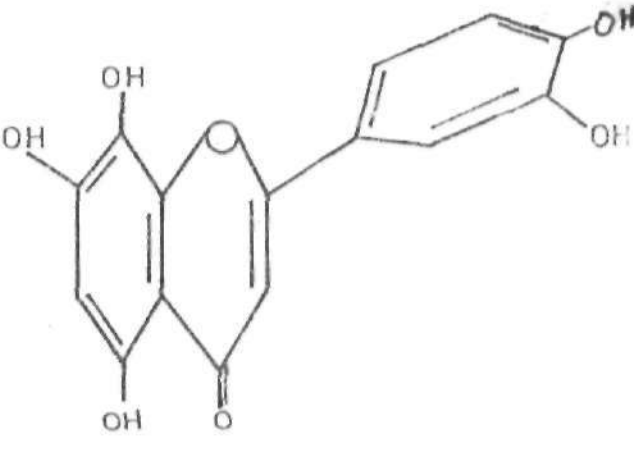

CYANIDIN




DELPHINIDIN




QUERCETIN




GOSSYPETIN

FIG. 2.2 :- STRUCTURE OF SOME ROSELLE FLAVONOIDS.

SOURCE :- FRANCIS ( 1977 )

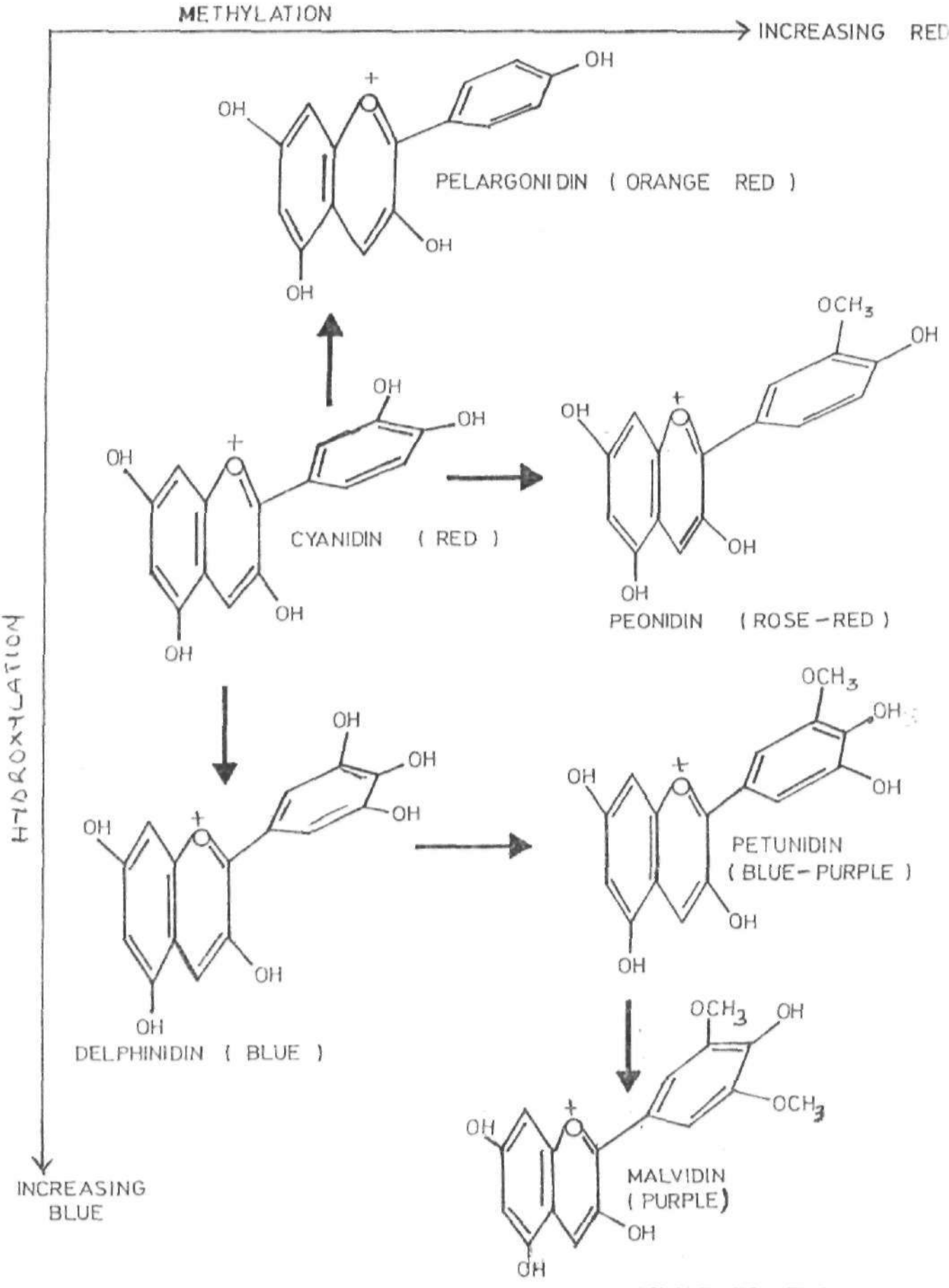



FIG. 2.3:- RELATIONSHIP OF ANTHOCYANIN COLOUR TO THE SUBSTITUTION PATTERN OF THE 'B' - RING.

SOURCE:- WALKER ( 1975 )

### 2.3.2 Flavonols and Flavones

Favonols are very widely distributed both as co-pigment to anthocyanins in petal and also in leaves of higher plants. They occur most frequently in glycosidic combination. Of all flavonols, only three, kaempferol, quercetin and myricetin (corresponding in hydroxylation pattern to the anthocyanin in pelargonidin, cyanidin, and delphinidin respectively) are important in roselle (Harbone, 1977). Flavonol occur as glycosides but the range of different glycosides is less than in the case of the flavonols. They are more resistant to acid hydrolysis (Harbone, 1977).

### 2.3.3 Metabolism of Flavonoids

Pharmacological activities of flavonoid follows largely from oral ingestion in the diet and knowledge of their metabolism is essential to any understanding of their action in vitro (Harbone, 1977).

Though data on flavonoid metabolism are scattered, quercetin is noted to be largely broken down in vitro, with the production of $CO_2$ from the A ring while three urinary metabolites from the B - ring in rats, rabbits, guinea-pigs and man have been identified, (namely: 3,4- dihydroxyphenyl acetic acid, 4-hydroxy-3- methoxyphenyl acetic acid and M-hydroxyphenyl acetic acid) (Harbone 1977). (Fig 2.4). These reactions occur when quercetin is given orally. When injected, only 3,4- dihydroxy phenyl acetic acid and 4-hydroxy -3- methoxy phenylacetic acids were found in the urine (no M-hydroxyphenyl acetic acid was isolated). It is thus possible that dehydroxylation is a reaction of gut bacteria (Harbone 1977).

Flavones and flavanones are broken down in a similar fashion; the main products detected in the urine being the related phenyl propionic, cinnamic and benzoic acids, instead of the phenyl acetic acids (Harbone 1977).

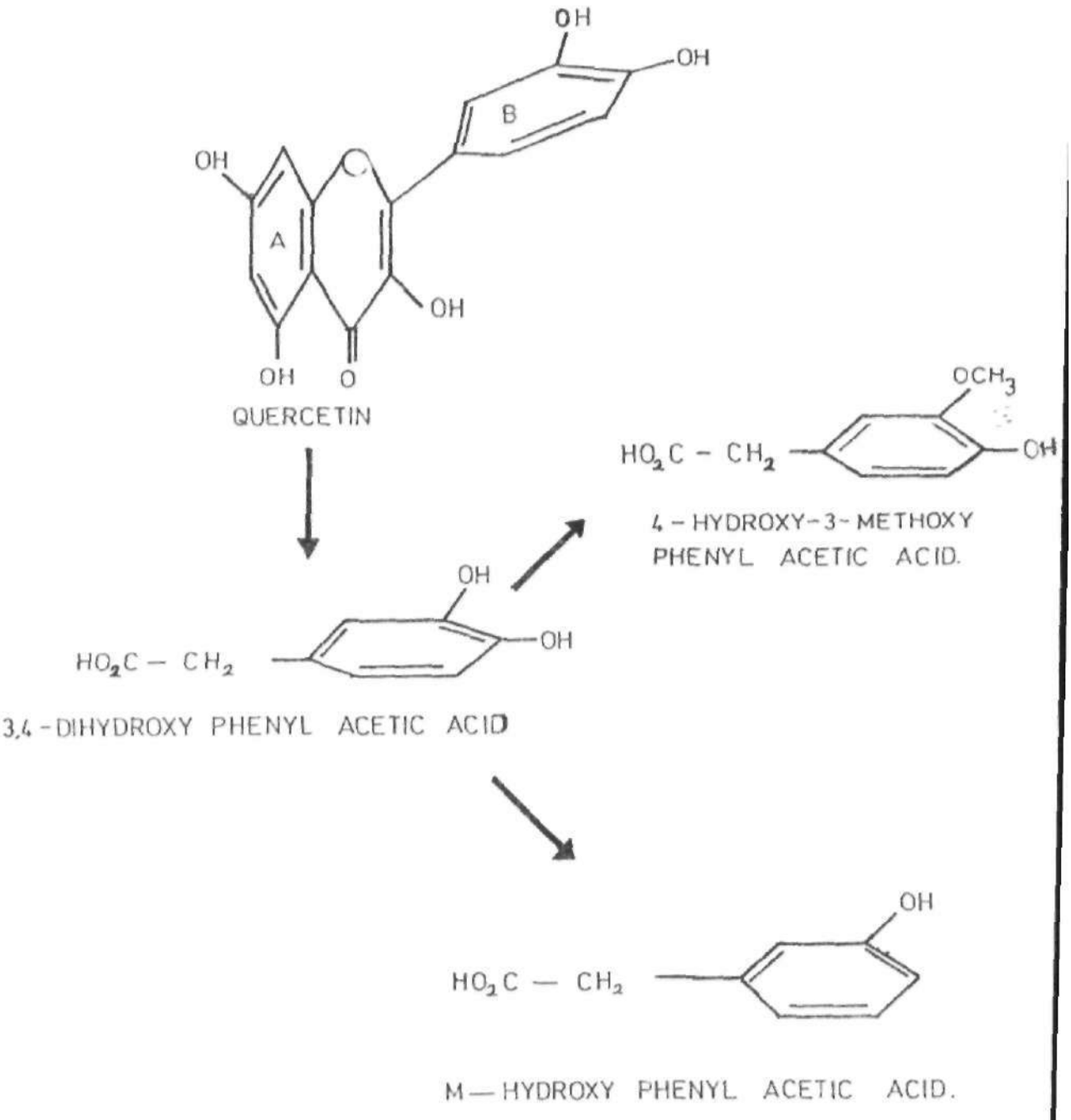



FIG. 2,4:- METABOLISM OF QUERCETIN IN VIVO.

SOURCE:- HARBONE ( 1977 )

The physical chemistry of anthocyanin pigment complexes and the degradation reactions are less well known (Francis, 1977). While Harbone (1977) reported that no colour has been observed in the urine following ingestion of fruits with high anthocyanin content, Sweny and Varghese (1988) reported a pink/red colouration of urine following anthocyanin in beetroot ingestion.

### 2.3.4 Toxicities of Flavonoids

Natural colours have been used in food over a long period of time, and have been accepted for such use without supporting toxicological evidence in such the same manner as vegetables and cereal products. (WHO/FAO, 1964). In practice, it has become increasingly difficult to subject natural colours to the same criteria of evaluating food additives because for most natural colours, their full chemical definitions have not yet being achieved and the closest description often involves specifying the starting material and the method of preparation (Kojima 1978). For example, a full chemical definition of crude anthocyanin extracts would be extremely complex since certain tissues contain a variety of flavonoid components (up to 17 being identified in the skin of red grapes alone) (Markakis, 1974).

Other factors such as difference in soil, climatic condition, age of plant and time of harvest, the nature and proportion of the coloured compound and other component of the same species of the same plant which vary widely have complicated studies on natural colours (WHO/FAO; 1978).

For a "reliable" evaluation of safety - in - use, the following biological information on colours was sought by the Joint Expert Committee on Food additives in 1963 (WHO/FAO; 1964).

i] Acute toxicity Studies

ii] Short-term and long-term toxicity studies (in at least two species).

iii] Carcinogenicity studies (in two species)

iv] Metabolic Studies

The idea was to use the lowest no-toxic effect level (NEL) obtained in these animal studies to estimate an acceptable daily intake (ADI) for man. In such an analysis carried out on anthocyanin, no ADI could be established and no monograph was prepared (WHO/FAO, 1978).

In an attempt to streamline the costly and time consuming testing programmes required on food additives, the United State Food Safety Council (Scientific Committee) have proposed a safety decision - tree - approach (WHO/FAO, 1978) (Fig. 2.5).

Some flavonoids have been documented to have biochemical effects that can translate into toxicities in human particularly if taken in large doses (Jaeger et al, 1988; Lin and Ho, 1995). In vitro, the flavone derivatives, kaempferol and quercetin which are widely distributed in vegetables and other plants have been shown to be mutagenic (Kojima, 1978). In mutagenic studies, quercetin was demonstrated to show positive mutagenic effects in strains of Salmonella typhimurium, however, the purity of the material tested was not reported, hence the available toxicological data were inadequate to evaluate these materials and therefore no adequate dietary intake could be established (WHO/FAO, 1978).

In another experiment, Zloch (1996) documented the demons-tration of mutagenic properties of quercetin in a genotoxicity tests, however, the results of animal experiments and epidemiological studies do not confirm the risk of neoplastic disease in subjects with a normal intake of these substances.

Even though flavonoid generally have been shown to have beneficial effects in cardiovascular diseases because of their antioxidant properties (Kandaswani and Middleton, 1994), one should recognise the complexity of interactions between pro-oxidants and antioxidants. A compound may act as an anti-oxidant in one oxidative challenge system and neutral or even pro-oxidant in another. For example whereas quercetin can sequester and reduce the activity of oxidants including metals (such as iron and copper), with an anion exchange chromatographic method for testing potential

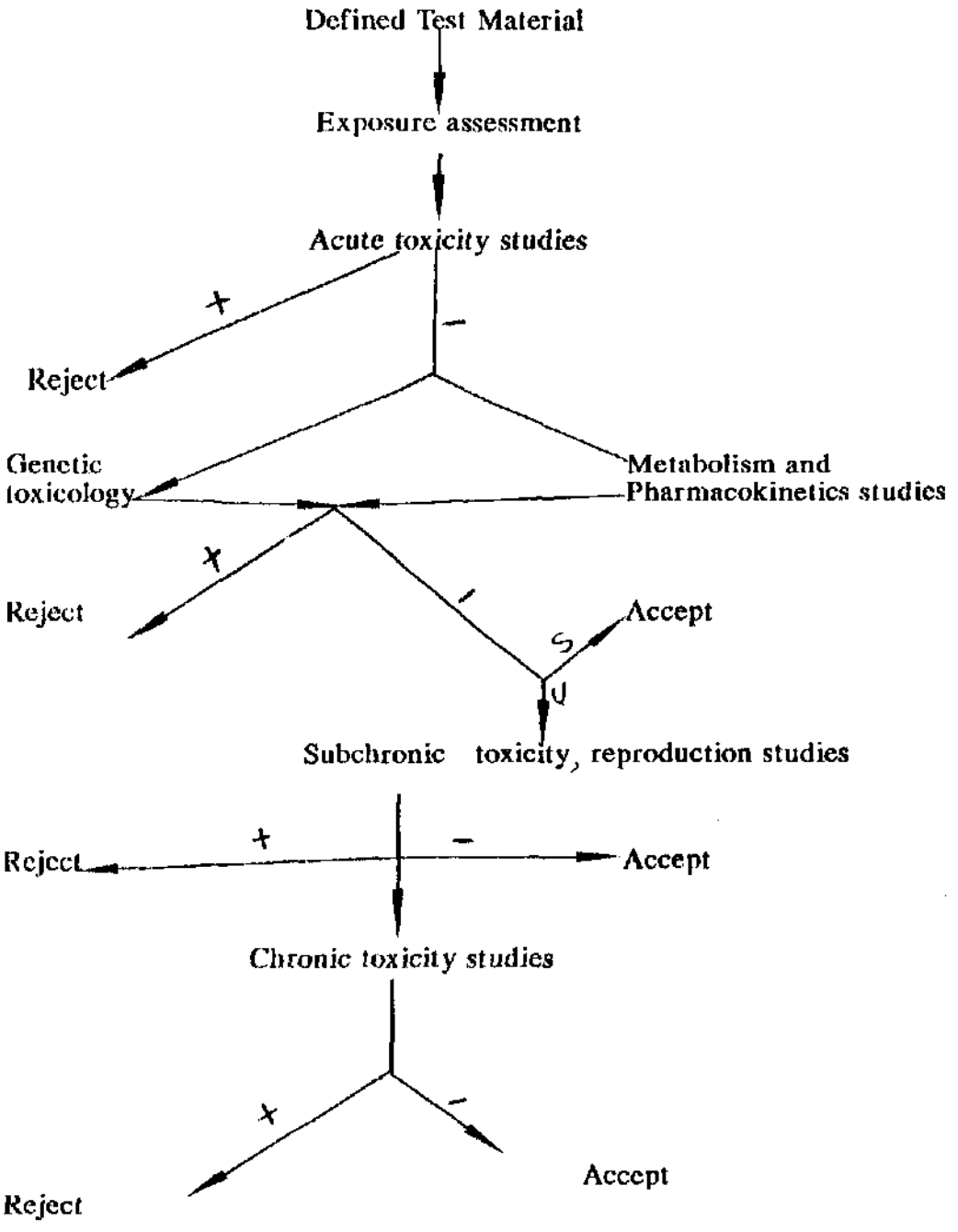



Fig: 25. Safety decision - tree proposed by the Food Safety Council

Source: WHO/FAO ( 1978)

+ = Present socially unacceptable risk

- = Does not present socially unacceptable risk

s = Metabolites known and safe

u = Metabolites unknown and of doubtful safety

inhibitors of low density lipoprotein oxidation, quercetin was found to have pro-oxidant activity (Myara et al, .1993). Hence, one can still reiterate the old maxim that naturalness per se does not assure of safety.

## 2.4 Hypertension and Coronary Heart Diseases

Coronary heart disease (CHD) was defined as a non-fatal myocardial infarction or a fatal coronary event (Ola et al, 1996). It has been identified as a leading cause of death in most industrialised societies and the main modifiable risk factors being hyperlipidemia, hypertension and diabetes mellitus; in subjects with more than one of these factors, the risk is more than additive (Martin et al, 1995).

The term arterial hypertension indicates a chronically elevated systolic and/or diastolic arterial (blood) pressure (WHO/FAO, 1978). The higher the arterial pressure, (systolic or diastolic), the greater the cardiovascular morbidity and mortality. It has been known for over a century, that high arterial pressure lessens life expectancy. The most common cause of death in the patients has been identified to be heart disease. Heart disease includes coronary artery disease, congestive heart failure, and left ventricular failure. Arterial pressure is a substantial factor in the external work of the heart; other things being equal, reduction of a greatly elevated arterial pressure would greatly alleviate or cure heart failure (Pickering, 1972).

Hypertension may be described as mild (diastolic pressure is between 90 and 104mmHg), moderate (diastolic pressure is between 105 and 114mmHg), or severe (diastolic pressure is 114mmHg or above) (Gross, 1979). The term essential hypertension is used to describe the type of hypertension that is seen if no underlying disease is detected while secondary hypertension refer to hypertension occuring as manifestation of known disease (Pickering, 1972). An illiness of which raised arterial pressure is the outstanding feature such as hypertension, may follow two different course which are called the malignant and benign course. The malignant course occurs at any age but is more frequently found in

the young than in the old and is characterized by a rapid decline in renal function. The pressure is usually very high and unless the arterial pressure is reduced at an early stage, the patient dies after a few months or years either of renal failure or cerebral heamorrhage Pickering, 1972). The benign phase usually occurs in rather older subjects in whom the pressures are not as high. The patient's condition remains relatively stable for years and death, when it occurs is either due to heart failure, stroke or intercurrent disease. (Pickering, 1972).

### 2.4.1 Biochemical Basis of Hypertension

Most people with hypertension have the type known as "essential hypertension" with its strong hereditary aspect. In these people, the arterial pressure rises fast especially with each advancing year. Some complex physiologic signal has brought about narrowing of their arterioles with increased resistance to blood flow. The walls of these arterioles are thickened and have an abnormal composition of water and electrolytes (Tobian, 1972). The arterioles of the hypertensive rats contain significantly more water, sodium and calcium than those of rats with "cured" hypertension (Tobian, 1972). This increased content of water and sodium noticed in hypertensive patients have been reported to contribute to the extra narrowing of hypertensive arterioles. The mechanism proposed for this include:

i] The extra water in the wall of the hypertensive arteriole could actually encroach physically on the lumen and decrease its calibre (Tobian, 1972).

ii] The extra water would be expected to decrease the elasticity of the arteriolar wall. This would cause the vessel to dilate less than usual with each pulse wave, which would result in an increased resistance to flow. (Tobian, 1972).

iii] The increased calcium content in arterioles could favour vasoconstriction (Tobian, 1972).

iv] Higher concentration of sodium makes arterial actomyosin more sensitive to calcium and also reduces the binding of calcium by the "sarcoplasmic reticulum" in arteries. Thus, if the intracellular sodium level in hypertensive arterioles is elevated, both of these effects would increase the action of calcium on arterial actomyosin and would favour vasoconstriction (Tobian, 1972).

### 2.4.2 Flavonoid and Coronary Heart Disease

Much of the research work currently going on to unravel the mystery behind the apparent health benefit enjoyed by people with diets rich in fruits and vegetables focuses on dietary antioxidants (Hertog et al, 1993; Joe et al, 1995). This trend is fuelled by our growing appreciation that damaging oxidative precesses are a common biochemical link between otherwise pathophysiological distinct diseases (Ames et al, 1993.

The heart is noted to be the most susceptible of all the organs to premature aging and free radical oxidative stress. Clinical research has clearly documented the role of free radical damage and the progression of numerous degenerative disease, particularly cardiovascular disease. Although highly respective and therefore vulnerable to the effects of oxidative stress, the heart is receptive to the benefits of targeted phytonutrients and antioxidants (Sinatra and Demarco, 1996). Phytonutrients such as the natural flavonoids and carotenoids found in fresh fruits and vegetables such as Vitamins C and E and Beta - Carotene have been noted to have powerful antioxidant effect (Kandaswani and Middleton, 1994). Sustantial epidemiological as well as experimental evidence shows that increasing amounts of Vitamin C, E and B-Carotene in ones diet are preventive for heart diseases (Sinatra and Demarco, 1996).

A small study on elderly men demonstrated a significant inverse association between intake of those flavonoids most commonly consumed in Netherlands and mortality due to coronary diseases (Hertog et al, 1993). Similar findings were reported by Knekt et al, (1996) in a Finland population. Vegetables, fruits and red wine were the major sources of flavonoids in the Dutch study carried out by Hertog (1994), and all these were

documented to be associated with a low risk of coronary heart disease.

As intriguing as these flavonoids findings are, Matthew and Stephen (1996) reported that, the epidemilogical evidence is not entirely consistent. For example, in the artherosclerosis risk in a community studied, consumption of apples was not related to thickness of the carotid artery wall (Hertog et al, 1993). Thus it was concluded that, it may be that the contribution of a particular food to cardiovascular health depends on the composition of the diet. The data of Knekt et al, (1996) showed a relatively low intake of vitamin C in the Finish diet and since quercetin may lead to sparing of vitamin C, it may be most important in population with marginal Vitamin C intake. (Mathew and Stephen, 1996).

For proper interpretation of this information about flavonoids, one need to know the biochemical composition of flavonoids, their pharmacological and range of physiological effects, their potential for toxicity and their established health benefits. In a variety of in vitro assays, flavonoids have been found to possess anti-inflammatory, anti-allergic, anti-thrombotic, venotonic, anti-microbial, and anti-neoplastic activities. Some are oestrogenic, others are antithyroidal and certain flavonoids can be mutagenic (Das et al, 1994). On a molecular level, flavonoids can modify the actions of a host of enzymes, including those involved in cell respiration, replication, drug metabolism and immune function (Mathew and Stephen, 1996; Zloch, 1996).

### 2.4.3 Low - density Lipoprotein and Atherogenesis

Lower density lipoprotein, in the form of low density lipoprotein (LDL) and very low density lipoprotein (VLDL), are hypothesized to be involved in the pathogenesis of atherosclerosis after oxidation (Steinberg et al 1989). Various experimental studies have firmly established that elevated plasma concentrations of LDL are associated with accelerated atherogenesis (Goldstein and Brown, 1977; Tyroler, 1987). Newman and Zilversonit, (1962) noted that the cholesterol that accumulates in atherosclerotic lesions

originates primarily in plasma lipoprotein, including LDL. A scheme outlining current concepts of the mechanism of oxidative modification of LDL is shown in fig 2.6.

The cell must somehow generate oxidising potential and one possibility would be the genration of free radicals by the cycloxygenase system or alternatively by lipoxygenases (Steinberg, et al; 1989).

As shown in fig 2.6 several pathways can be envisioned. In one, the lipids in the cell are oxidised first, after which they transfer into the LDL in the medium and initiate chain reactions that oxidise the lipids in the LDL extensively. A second pathways could involve the direct oxidation of LDL lipids during LDL - cell contact. A third pathways postulates the generation of superoxide anions (or other activated oxygen) within the cell, followed by release into the medium where the oxidation of LDL lipids then occurs (Steinberg et al, 1989). Though there has being various conflicting report on the role of superoxide anion, whatever the details of the initiating steps may be, once the LDL contains fatty acid lipid peroxides, there follows (especially in the presence of metal ions) a rapid propagation that amplifies dramatically the number of free radicals and leads to extensive fragmentation of the fatty acid chains. The fragment of the oxidised fatty acid attach covalently to apoprotein B, masking lysine $\varepsilon$-amino groups. Acetyl LDL receptor recognizes the modified apoprotein B moiety, not the lipid moiety, of oxidised LDL. Finally, it should be noted that LDL cholesterol is also oxidised during LDL modification which could enhance its cytotoxicity and atherogenicity (Steinberg et al, 1989).

### 2.4.4 Role of Flavonoids in Inhibition of Low - Density Lipoprotein Oxidation

Flavonoids have been studied in relation ot their improvement of vascular fragility, increasing cellular permeability and vitamin C sparing activities (Kuhnau, 1976). Severl more roles however, have been documented for dietary flavonoids in preventing coronary artery diseases.

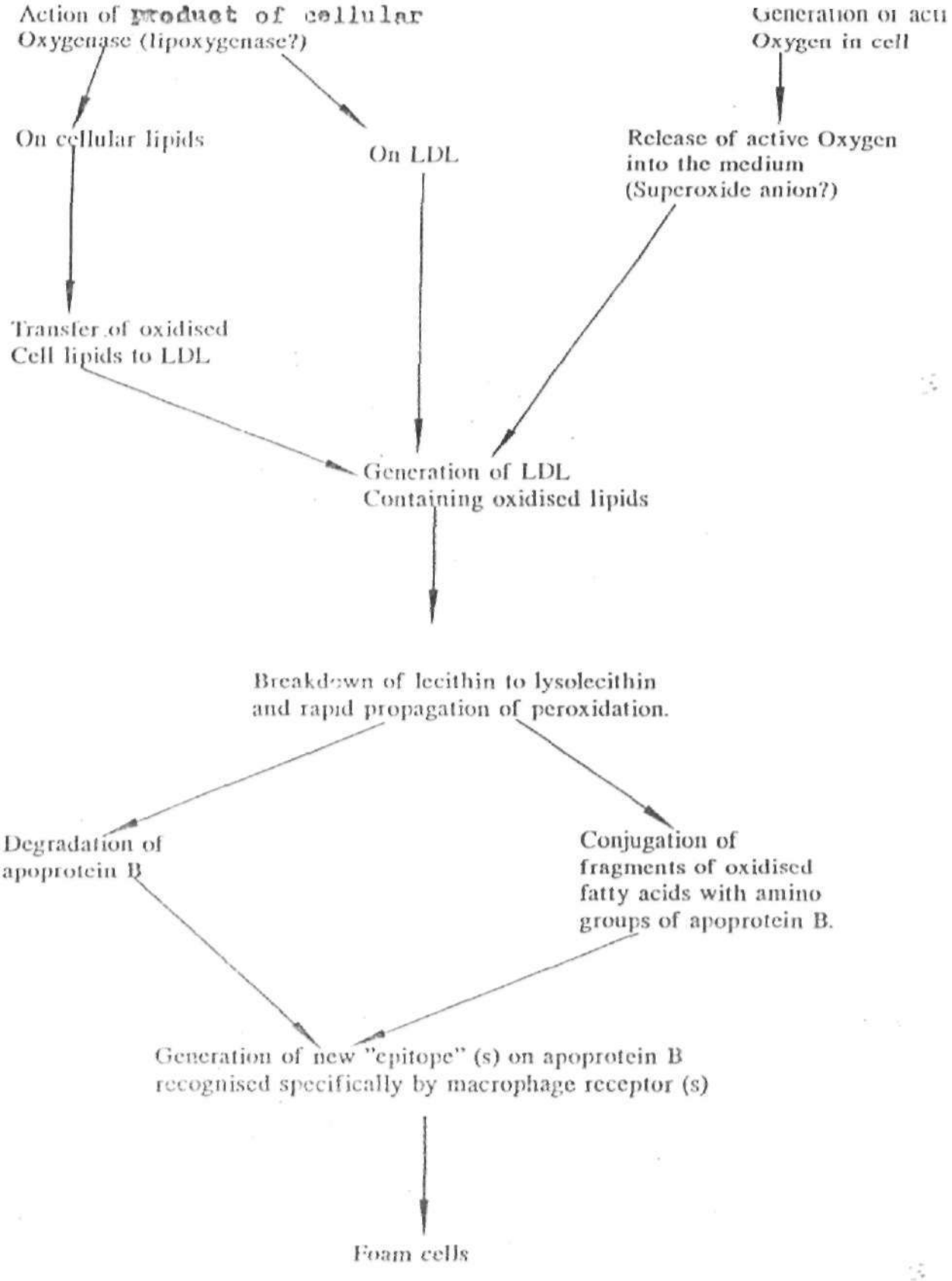



Fig: 2.6: Mechanisms thought to lead to the oxidative modification of LDL by cells.

Source: Steinberg, et al, (1989)

Low density lipoprotein oxidation by free radicals plays a central part in the development of atherosclerosis (Herman 1976, Steinberg et al, 1989; Joe et al, 1995). Antioxidants, may thus delay the onset of atherogenesis by reducing chemically and enzymatically mediated peroxidative reactions and down regulate thrombotic tendencies (Frankel et al, 1993). Flavonoids are scavengers of superoxide anions (Robak and Gryglewski, 1988), Singlet oxygen (Hussain et al, 1987) and lipid peroxyradicals (Sorata et al, 1982) and they can sequester metal ions through liganding (Takahama, 1985; Joe et al, 1995). Flavonols and flavones inhibit cyclo-oxygenase, leading to lower platelet aggregation and reduced thrombotic tendencis. (De Whalley et al, 1990; Laughton, et al, 1991; Kinsella et al, 1993).

Apart from inhibiting the cytotoxicity of oxidised LDL in vitro (De Whalley et al, 1990), it is possible that quercetin and other flavonoids reduce the rate of formation of oxidised LDL and thus inhibit cyclooxygenase which may reduce thrombosis (Laughton et al, 1991). Flavonoids possibly act through both mechanisms; the combination could explain why the association between flavonoid intake and mortality from coronary heart disease was stronger than the association with incidence of myocardial infarction. (Hertog et al, 1993).

Natural flavonoids can donate hydrogen or react with superoxide anion, hydroxy radicals and lipid peroxyradicals in vivo. One mechanism may involve complexation of the natural polyphenolic components with $Cu^{2+}$ to reduce it to a $Cu^{+}$ complex which in turn may reduce hydroperoxides. Alternatively, during LDL lipid peroxidation, the natural polyphenols may act as self regenerating reducing components (Frankel et al; 1993). Thus it was suggested that, if potent antioxidant phenolic components are routinely ingested by the regular consumption of red wine, they may collectively reduce oxidation of lipoprotein (De Whalley et al, 1990; Joe et al, 1995) and reduce thrombotic phenomena and thereby contribute to the amelioration of artherosclerosis and mobidity from coronary artery disease. This account for the reason why a low coronary heart disease was reported in people that drink red wine (Frankel et al, 1993). However, we

need to know more about the pharmaokinetics of flavonoids and the absorption and metabolism of phenol. (Ueno et al, 1983).

CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Chemicals/Reagents

Sodium citrate, sodium tungstate, thiourea, glacial acetic acid, o-toluidine, and bromocresol green were obtained from BDH, Chemical Ltd, Poole, England. Brig- 35, picric acid, and standard solutions of human serum albumin, total protein, glucose, cholesterol, creatinine, and uric acid were obtained from Sigma Chemical Company, U.S.A. All other chemicals are of analytical grade.

### 3.1.1 Instrument and Apparatus

Indirect blood pressure meter, letica. LE 5007, Freeze dryer, Lyovac GT3 (BDH London), spectrophotometer model 6100, Jenway, Triple beam balance, Series 700, Oral intubator and florence flask. All other instruments and apparatus used were routine laboratory apparatus and instruments.

## 3.2 Experimental Subjects

Eighteen (18) albino mice aged 4-6 weeks and weighing between 16.4 and 24.1g, thirty (30) adult normotensive wistar-kyoto rats weighing between 175.1 and 232.5g and fifteen (15) spontaneously hypertensive rats weighing between 161.4 and 235.0g were obtained from the Animal Facility Centre of the National Institute for Pharmaceutical Research and Development, Idu, Abuja. All the animals were acclimatized for 5 days before starting the experiment. The animals were fed ad libitum on standard rat feeds obtained from National Institute for Trypanosomiasis Research, Vom. The composition of the feeds were: carbohydrates (60%); protein, (20.5%); fats, (7.0%), vitamins and mineral elements (2.5%). They were also allowed free access to clean drinking water.

The rats were divided into nine groups and placed in cages that allowed for free movement and the cages were labelled as WKyC (Wistar-Kyoto Control); WkyT1 (treated 1,125mg/kg b.w); WkyT2, (Wistar-Kyoto treated 2, 250mg/kg b.w); wkyT3,(Wistar-kyoto treated 3, 500mg/kg b.w); WkyT4, (Wistar-kyoto treated 4, 1000 mg/kg b.w); WkyT5, (Wistar-kyoto treated 5, 2000mg/kg b.w); SHRC, (Spontaneously hypertensive rat control) and two treatment groups of spontaneously hypertensive rats (SHRT1, 500 mg/kg b.w and SHRT2, 1000mg/kg b.w).

Two rats from each cage were sacrificed after 30 and 60 days of continuous treatment with the roselle calyx extracts. The blood samples were collected and some visceral organs such as liver, kidney and heart were grossly examined.

### 3.2.1 Collection of Blood Samples

The rats were put under anaesthesia with few drops of choloroform in an enclosed container. The rats were then opened up and the blood collected by cardiac puncture.

### 3.2.2 Preparation of serum

The blood was centrifuged after allowing to stand for about 30 minutes to obtain the serum. The serum samples were stored in pyrex sample bottles in the freezer at-20$^0$C until used for analysis.

### 3.2.3 Preparation of Roselle Calyx Extract

The roselle calyces obtained from Wuse Market, Abuja were extracted using the method of Obiefuna et al, (1992).

**Procedure:** Five hundred grams of the oven dried (60$^0$C) calyces were boiled in 3000cm$^3$ distilled water for 15 minutes. This was filtered using a muslin cloth and the filtrate concentrated using a rotary evaporator at 30$^0$C. The low temperature of 30$^0$C was

used so as to prevent anthocyanin degradation and hydrolysis. The concentrate was converted to powder by using a freeze-dryer. The dried powder were kept in hermetically sealed brown containers and stored in a desiccator. On the day of the experiment, a fresh solution of the extract was prepared using distilled water. The precentage yield of the extract was 5.5%.

### 3.2.4 Administration of the Extract

Concentrations of 125mg/kg, 250mg/kg, 500 mg/kg, 1000mg/kg and 2000mg/kg body weight of the extracts were prepared daily using distilled water. The required volume of the extract was administered orally using an oral intubator as a single dose daily for 60 days.

### 3.2.5 Measurement of Blood Pressure

The blood pressure was monitored with an indirect blood pressure meter using the tail cuff method (Agostino et al; 1969).

**Principle:** The indirect blood pressure meansurement is basically sphingomanomeric. A controlled external pressure is applied over the artery of the tail at the same time as the pulse is detected. The pulse signal detection is carried out by means of transducer based on a flexible tempered copper-beryllium open ring. This ring is provided with extensiometric gauges forming a complete bridge and its flexibility allows a good fit around the animals tails irrespective of its diameter. The pulse signals is permanetly monitored on an analogic display making it possible to ensure the carrying out of the measurement with a proper pulse level.

**Procedure:** The measurement process involved several steps, performed authomatically by the instruments. The first step is to determine the initial pulse signal level. Once a constant pulse signal level is detected, the cuff pressure is increased until the pulse signal

disappears and then decreased until the pulse signal level recovers completely. The current cuff pressure is constantly measured by a pressure transducer.

The cuff pressure when the pulse signal level goes down until it disappears gives the systolic blood pressure (SP), while the cuff pressure when the pulse signal level recovers its initial level gives the diastolic pressure (DP). At each intervals, the blood pressure was measured eight times in each rat and the average value was calculated.

### 3.2.6 Measurement of Body Weight of Experimental Subjects

Triple beam balance with a steel pan was used to measure the body weight of all the experimental animals used. The weights were recorded in grams.

### 3.2.7 Monitoring of Urinary Colouration and Volume

Metabolic cages to which graduated water bottles were attached were used to measure the volume of urine output/per day and its colouration as well as volume of water intake. An animal each was kept in a metabolic cage for ten (10) days. Along with the test animal, another metabolic cage was set up for the control. The daily water intake and urine output were noted and recoreded.

### 3.2.8 Acute Toxicity Test

The $LD_{50}$ was determined by intraperitoneal administration using the method of Lorke (1983).

**Procedure:** After an initial five days of adaptation, five groups of mice consisting of three animals per groups were administered with doses of 10, 100, 500, 1000 and 5000 mg/kg body weight of the roselle calyx extracts. Another group of mice consisting of three animals were kept as control.

The animals were observed frequently on the day of treatment during normal working hours and the nature and time of all adverse effects were recorded. Observations were carried out for 14 days and the experiment was then terminated.

## 3.3 Biochemical Analysis

### 3.3.1 Serum Cholosterol Determination

Serum cholesterol was determined using the colorimetric method of Zlatkis and Boyle (1958).

**Principle**: The method applied the salkowski reaction in which cholesterol reacts with ferric ions in acetic acid followed by sulfuric acid leading to the formation of disulphonic acids which is red in colour.

**Procedure:** Into glass stoppered centrifuge tubes were transferred 0.1ml of serum samples and 4.9ml of 10% ferric chloride in acetic acid (1:2v/v) was then added to each tube. The contents of the tubes were mixed well with a retsch mixer and allowed to stand for exactly 15 minutes for the protein to flocculate. The contents were then centrifuged for 5 minutes at 650g. To 2.5ml of the clear supernatant was added 1.5ml of concentrated sulfuric acid, the mixture was allowed to stand for 25 minutes and the absorbance was read at 560nm.

To prepare a calibration curve, a standard solution of 5 mmol/L cholesterol was diluted serially to produce concentrations of 1,2,3, and 4 mmol/L. These were then treated as for the test and the absorbance read at 560nm.

### 3.3.2 Serum Creatinine Determination

Serum creatinine was determined using the Jaffe reaction method. (Brod and Sirota, 1948).

**Principle:** The method involves the reaction of creatinine with picrate in alkaline solution at room temperature producing a red colour within a few minute which is then measured spectrophotometrically.

**Procedure:** Exactly 1ml serum was diluted with 1.5ml water and the protein precipitated by adding 0.5ml sodium tungstate and then 1ml sulfuric acid added dropwise with shaking. The mixture was allowed to stand for 10 minutes at room temperature and then centrifuged at 650g for 5 minutes. Into 3ml of the pipetted supernatant was added 1ml picric acid followed by 1ml sodium hydroxide solution. At the same time a complete reagent blank was put up using 2.5ml water. The absorbance was read after standing at room temperature for 15 minutes at a wavelengh of 500nm.

For a calibration curve, 5mg/dl standard solution of creatinine was serially diluted to produce concentrations of 1,2,3, and 4mg/dl. To each of the prepared standard solutions was added 0.5ml tungstate and then 1ml sulfuric acid. The mixture was allowed to stand for 10 minutes at room temperature and then centrifuged. Into 3ml of the pipetted supernatant was added picric acid and sodium hydroxide as for the test and the absorbance was read at 500nm.

### 3.3.3 Serum Glucose Determination

Serum glucose was determined using the 0-toluidine method (Hyvarinen and Nikkila 1962).

**Principle:** The method is based on the fact that glucose produces on heating with 0-toluidine in acetic acid solution, a green colouration whose intensity is proportional to glucose concentration.

**Procedure:** Into a centrifuge tube was meansured out 0.9ml trichloroacetic acid solution followed by 0.1ml serum using a micropipette. The contents were mixed with retsch mixer and then centrifuged at 650g for 15 minutes.

Exactly 2ml of 0-toluidine reagent was added into 0.5ml of the centrifugated sample in a test tube. The test tube was then placed in a boiling water bath for 8 minutes after which the tube was allowed to cool under a stream of tap water.

To estimate the amount of glucose in the serum 5.5mmol/L standard glucose concentrations was treated as for the test and absorbance measured along with the sample at 600nm against distilled water using a spectrophotometer.

**Calculation:** The serum glucose concentration (mmol/L)

$$= \frac{Au \times Cs}{As}$$

where

Au = Absorbance of sample.

As = Absorbance of standard.

Cs = Concentration of standard.

### 3.3.4 Serum Uric Acid Determination

Serum uric acid was determined using the phosphotungstic acid method (Caraway, 1955).

**Principle:** This method relies on development of blue colour (tungsten blue) as phosphotunstic acid (PTA) is reduced by urate in alkaline medium. The colour is read spectrophotometrically at wavelength of 650nm. Removal of protein is obligatory for this method to avoid turbidity and quenching of the absorbance.

**Procedure:** Into 1.0ml of Serum was added 9.0ml of precipitating reagent with mixing. The mixture was centrifuged at 650g for 5 minutes. Into two separate test tubes was then added 5.0ml of clear supernatant and 5ml of water (blank). Exactly 1.0ml of 10% $Na_2CO_3$ solution was added into each of the tubes and the contents mixed very well with a retsch mixer. Exactly 1.0ml of dilute phosphotungstic acid was then added and the content mixed very well. The solution was allowed to stand exactly 30 minutes at room temperature. The absorbance was then measured within the next 20 minutes using a spectrophotometer set at 700nm.

For a calibration curve, 5mg/dl standard solution of uric acid was serially diluted to produce concentrations of 1,2,3, and 4 mg/dl. They were then treated as for the test and the absorbance measured at 700nm.

### 3.3.5 Serum Albumin Determination

Serum albumin was determined using the dye binding (bromo- cresol green) method. (Basil et al, 1970).

**Principle:** The addition of albumin to a solution of bromocresol green in a 0.075M succinate buffer, pH 4.20 results in an increase in absorbance at 628nm. The absorbance concentration relationship is linear for samples containing up to 6g/dl albumin. The use of a non-ionic surfactant (Brij-35) reduces the absorbance of the blank (prevent turbidity and provides linearity).

**Procedure:** To 5.0ml of working dye solution was added 35 Microlitre of serum or working standard solutions. The solution was mixed using a retsch mixer and allowed to stand for 10 minutes at 628nm after adjusting the instrument to zero absorbance with the working dye solution.
For a calibration curve, from a standard albumin solution of 5g/dl, standard working solutions of 1,2,3, and 4g/dl was prepared by serial dilutions. The albumin concentration of the serum was obtained from an absorbance versus concentration plot.

### 3.3.6 Serum Total Protein Determination

Serum total protein was determined using the Biuret method. (Gornall et al, 1948).

**Principle:** This method is based on the fact that polypeptide and protein peptide bonds produce, when reacted with $Cu^{2+}$ ions in an alkaline medium, a complex compound of violet colour, whose colour intensity is proportional to protein concentration in the

medium. Only the peptide part of the protein molecule give this reaction.

**Procedure:** Into a clean test tube was transferred 0.1ml of blood serum while 0.1ml of 9.0% sodium chloride was transferred to another tube as blank. Exactly 5ml biuret reagent was added to each tube and the contents mixed with a retsch mixer. The tubes were then set aside for 30 miunutes to allow the colour to develop and the absorbance read on a spectrophotometer at a wavelength of 560nm. The concentration of blood serum protein was then determined from a standard curve.

To prepare the standard curve, standard concentrations of 1,2,3, and 4g/dl of standard total protein were prepared from a 5g/dl standard total protein solution. The standards were then treated as for the test and the absorbance meansured at 560nm.

# CHAPTER FOUR

## RESULTS

### 4.1 Acute Toxicity Investigation

Following intraperitoneal administration of roselle calyx extract in mice, no observable behavioural changes were noticed in all the mice administered with 10 and 100mm/kg body weight (b.wt) dosage. A lethargic condition with decreased locomotory activity was however, observed in all the mice groups administered with 1000, 3,000 and 5000mg/kg b.wt dosage of the extract and this was dose dependent. While the group of mice administered with 1000 and 3000 mg/kg b.wt dosage recovered fully within 10 minutes, the group administered with 5000mg/kg b.wt fully recovered after 30-40 minutes of appearance of symptoms (Table 4.1).

No death of any of the mice was recorded within 14 days of observation. The $LD_{50}$ is thus estimated to be greater than 5000mg/kg b.wt.

### 4.2 Effect of Roselle Calyx Extract Administration on Body Weight and Blood Pressure of Normotensive and Hypertensive Rats.

The results in Table 4.2 represent the effect of roselle calyx extract on the body weight and blood pressure (systolic and diastolic) of hypertensive and normotensive rats.

**TABLE 4.1: Result of $LD_{50}$ Investigation of Roselle Calyx Extract in Mice**

| DOSAGE OF EXTRACT INTRODUCED (mg/kg b.wt) | WEIGHT OF MICE (g) | MORTALITY* | OTHER OBSERVABLE TOXICOLOGICAL SIGNS |
|---|---|---|---|
| 10 | 16.9 | 0/3 | No observable behavioural changes. |
| 10 | 18.2 | | |
| 10 | 17.9 | | |
| 100 | 18.7 | | No observable behavioural changes. |
| 100 | 18.2 | 0/3 | |
| 100 | 14.0 | | |
| 1000 | 20.2 | | Lethargic condition with relative decreased locomotory activity |
| 1000 | 18.5 | 0/3 | |
| 1000 | 24.5 | | |
| 3000 | 16.4 | | Lethargic condition with relative decreased locomotory activity |
| 3000 | 18.6 | 0/3 | |
| 3000 | 23.2 | | |
| 5000 | 17.3 | 0/3 | Lethargic condition with relative decreased locomotory activity |
| 5000 | 24.0 | | |
| 5000 | 16.2 | | |

* Number of animals which died/Number of animals used.

The initial body weight and blood pressure of the spontaneously hypertensive rats selected for treatments, SHRT1 and SHRT2 were observed not to be significantly ($p > 0.05$) different from each other and were not different from the mean body weight and mean blood pressure of the control group, SHRC. The initial body weight and blood pressure of the normotensive groups selected for treatment, WKyT3 and WKyT4 were also observed not to significantly ($p > 0.05$) differ from each other but the blood pressure (systolic and diastolic) were significantly lower than the blood pressure of the hypertensive groups.

After seven days of continuous administration of the extract, the mean systolic blood pressure of the SHRT1 was significantly ($p < 0.05$) reduced from the initial value of 153 mmHg to 143 mmHg. No significant different was however, observed in the body weight and diastolic pressure compared with the initial values. The systolic and diastolic pressure of the SHRT1 was also not significantly different from the corresponding values in the SHRC after 7 days of continuous treatment. A significant reduction in blood pressure from an initial value of 155/89mmHg before treatment to a value of 133/71mm Hg after 7 days of continuous administration of the extract was also observed in the SHRT2 administered with 1000mg/kg b.wt dosage of the extract. No significant difference ($p > 0.05$) was observed in the body weight of the SHRT2 compared with the initial value before treatment nor was the observed body weight significantly different from the body weight of the SHRT1 and the control group, SHRC. However, the systolic and diastolic pressure of the SHRT2 was significantly lower than the SHRC.

The body weight and blood pressure of WKyT3 and WKyT4 were also observed not to differ significantly from the values before treatment. When compared with the corresponding values in the WKyT3, the weight and blood pressure of the WKyT4 were not significantly different. No significant ($p > 0.05$) difference was also observed between the systolic and diastolic pressure of SHRT2, WKyT3 and WKyT4. The systolic and diastolic pressure of WKyT3 and WKyT4 were however, significantly lowered ($p < 0.05$) compared with the corresponding values in SHRTC and SHRT1.

After 21 days of continuous treatment with the extract, there was a significant reduction in the body weight of the hypertensive rats administered with 500mg/kg b.wt dosage of the extract, SHRT1 (from the initial value of 200.8 to 184.8g). The blood pressure was also significantly reduced from an initial value of 153/91 mmHg to a value of 135/70mmHg after 21 days of continuous treatment. Compared with the corresponding values in SHRC, both the systolic and diastolic pressure were significantly lowered. No significant difference was however observed between the body weight of the SHRT1 and SHRC.

Between the seventh and twenty first day of continuous administration of the extract, the death of all the hypertensive rats treated with 1000mg/kg b.wt dosage of the extract, SHRT2 were recorded. All the animals were noted to have died within 20-30 minutes of the appearance of symptoms (extreme muscular weakness, lethargy and anorexia) and gross examination of their vital organs post mortem did not show any variation from normal.

With continuous administration of the extract for 21 days, a significant reduction in the blood pressure of WKyT3 from an initial value of 132/75 mmHg to a value of 125/66 mmHg was observed. The blood pressure of the WKyT4 also significantly reduced from an initial value of 129/72 mmHg to a value of 119/64 mmHg. No significant difference was however, observed in the body weight of the WKyT3 and WKyT4 compared with the initial values. When compared with the hypertensives, the body weight and diastolic pressure of the WKyT3 and WKyT4 were not sigifiantly different from the SHRT1, through their systolic pressure were significantly lowered. Both the systolic and diastolic pressure of the WKyT3 and WKyT4 were significantly lowered than the corresponding values in SHRC, however, their body weight were not significantly different from each other. Again, the body weight, systolic and diastolic pressure of WKyT3 and WKyT4 were not significantly ($p > 0.05$) different from each other.

A close association was observed between the diastolic pressure and body weight in both the hypertensive and normotensive groups before and during treatment. A positive correlation of 0.17, 0.29, 0.61 and 0.72 were recorded in the WKyT3, WKyT4, SHRT1, and SHRT2 respectively between the body weight and diastolic pressure (Table 4.3).

Table 4.2: Comparison of the Effect of Roselle Calyx Extract Administration on Body Weight and Blood Pressure of Normotensive and Hypertensive Rats.

| ANIMAL | Initial Value (Day 1) | | | Day 7 | | | Day 21 | | |
|---|---|---|---|---|---|---|---|---|---|
| GROUP | WEIGHT (G) | BLOOD PRESSURE (mm Hg) | | WEIGHT (g) | BLOOD PRESSURE (mm Hg) | | WEIGHT (G) | BLOOD PRESSURE (mm Hg) | |
| | | SYSTOLIC | DIASTOLIC | | SYSTOLIC | DIASTOLIC | | SYSTOLIC | DIASTOLIC |
| SHRC | 189.8±16.9 $^{a}$ | 151±3 $^{a}$ | 87±12 $^{a}$ | 193.5±14.8 $^{a}$ | 154±1 $^{a}$ | 91±10 $^{a}$ | 200.5±12.3 $^{a}$ | 153±12 $^{a}$ | 89±7 $^{a}$ |
| SHRT1 | 200.8±8.3 $^{a}$ | 153±4 $^{a}$ | 91±4 $^{a}$ | 191.7±11.1 $^{a}$ | 143±5 $^{ac}$ * | 82±8 $^{a}$ | 184.8±10.7 $^{a}$ * | 135±3 $^{b}$ * | 70±5 $^{b}$ * |
| SHRT2 | 206±17.8 $^{a}$ | 155±10 $^{a}$ | 89±5 $^{a}$ | 182.5±16.2 $^{a}$ | 133±7 $^{bc}$ * | 71±1 $^{b}$ * | ND | ND | ND |
| WKyT3 | 200.8±21.6 $^{a}$ | 132±3 $^{b}$ | 75±5 $^{b}$ | 196.5±22.3 $^{a}$ | 130±2 $^{b}$ | 71±2 $^{b}$ | 191.5±24.2 $^{a}$ | 125±1 $^{c}$ * | 66±3 $^{b}$ * |
| WKyT4 | 195.1±12.3 $^{a}$ | 129±2 $^{b}$ | 72±3 $^{b}$ | 188.1±11.0 $^{a}$ | 125±3 $^{b}$ | 68±4 $^{b}$ | 184.2±13.5 $^{a}$ | 119±3 $^{c}$ * | 64±3 $^{b}$ * |

Number of determination (n) = 5
Results are expressed as mean ± SD
Values in the same column with different letters are significantly different from each other.

Values in the same row with symbol "*" are significantly different from the corresponding initial (Day 1) values.
ND = Not determined.
Statistics = Duncan multiple range test.

Table 4.3: **Association Between Weight and Diastolic Pressure in the Treated Hypertensive and Normotenstive Rats**

| ANIMAL GROUP | INDEPENDENT VARIABLE | DEPENDENT VARIABLE | CORRELATION CO-EFFICIENT |
|---|---|---|---|
| SHRT1 | Weight | Blood Pressure | 0.61 |
| SHRT2 | Weight | Blood Pressure | 0.72 |
| WKyT3 | Weight | Blood Pressure | 0.17 |
| WKyT4 | Weight | Blood Pressure | 0.29 |

## 4.3 Effect of Roselle Calyx Extract Administration on Urine Output in Normotensive and Hypertensive Rats.

To be able to possibly establish a mechanism by which roselle calyx extract lower blood pressure and assess the kidney function during hypertension and antihypertensive therapy, the water intake and urinary output were monitored in the control group and the treated normotensive and hypertensive groups, the result of which is shown in Table 4.4.

The water intake in the hypertensive untreated group, SHRC did not differ significantly ($p > 0.05$) from the normotensive untreated group, WKyC. The water intake of the treated normotensive group WKyT3 was however, significantly ($p < 0.05$) lowered compared with the untreated group,WKyC, so also the water intake of the treated hypertensive group, SHRT1, was significantly lowered compared with the untreated group, SHRC. No signifcant ($p > 0.05$) difference was however, observed between the water intake of the treated hypertensive group SHRT1 and the treated normotensive WKyT3 group. The urinary output of the hypertensive untreated, SHRC, did not differ significantly from the normotensive untreated group, WKyC. No significant difference was also observed between the urinary output of the treated normotensive, WKyT3 and the untreated group, WKyC. The urinary output in treated hypertensive group, SHRT1, was however, significantly ($p < 0.05$) higher than the hypertensive untreated group, SHRC, and also significantly higher

**Table 4.4: Effect of Roselle Calyx Extract Administration on Water Intake and Urine Output in Hypertensive and Normotensive Rats.**

| ANIMAL GROUP | WATER INTAKE ($cm^3$/day) | URINE OUTPUT ($cm^3$/day) |
|---|---|---|
| WKyC | $12.5 \pm 1.3^{a}$ | $4.0 \pm 0.7^{a}$ |
| WKyT3 | $9.8 \pm 1.0^{b}$ | $4.3 \pm 0.6^{a}$ |
| SHRC | $12.4 \pm 1.1^{a}$ | $3.9 \pm 0.3^{a}$ |
| SHRT1 | $10.3 \pm 1.0^{b}$ | $5.0 \pm 0.7^{b}$ |

Number of determination (n) = 10
Results are expressed as mean $\pm$ SD
Values in the same colum with different letters are significantly different from each other.
Statistics = Duncan multiple range test.

than the normotensive untreated group, WKyC and the treated normotensive WKyT3, group.

The urine colouration of the rats (hypertensive and normotensive) studied were observed to be yellowish - brown. The urine colouration of the treated hypertensive and normotensive groups was however, noted to be relatively darker than the untreated group.

## 4.4 Comparison Between Some Serum Constitutents in Spontaneously Hypertensive and Normotensive Wistar Kyoto Rats.

Values obtained for serum glucose, cholesterol, creatinine, uric acid and proteins in hypertensive and normotensive rats are shown in Table 4.5.

No significant difference was observed between the serum glucose, albumin and total protein of the spontaneously hypertensive rats and the normotensive group. The serum cholesterol, creatinine and uric acid level were significantly ($p < 0.05$) higher in spontaneously hypertensive than the normotensive Wistar - Kyoto rat group.

## 4.5 The Effect of Roselle Calyx Extract Administration on Some Serum Constituents of Normotensive Rats.

The result of the administration of roselle calyx extract on serum glucose, cholesterol, creatinine, uric acid and protein concentrations in normotensive rats are as shown in



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**Table 4.5: Serum Cholesterol, Glucose, Creatinine, Uric Acid and Protein Concentrations of Normotensive and Hypertensive Rats (WKyC and SHRC).**

| SERUM CONSTITUENTS | WKyC | SHRC |
|---|---|---|
| Glucose (mmol/L) | $3.05 \pm 0.07^{a}$ | $3.45 \pm 0.27^{a}$ |
| Cholesterol (mmol/L) | $4.0 \pm 0.1^{a}$ | $5.6 \pm 0.1^{b}$ |
| Creatinine ( umol/L) | $110.0 \pm 1.4^{a}$ | $187.5 \pm 1.0^{b}$ |
| Uric Acid (umol/L) | $4.7 \pm 0.2^{a}$ | $11.9 \pm 0.4^{b}$ |
| Albumin (g/d) | $2.15 \pm 0.21^{a}$ | $1.7 \pm 0.61^{a}$ |
| Total Protein (g/dl) | $3.5 \pm 0.6^{a}$ | $3.0 \pm 1.2^{a}$ |

*Number of determination (n)* = 2.
Results are expressed as mean ± SD.
Values in the same row with different letters are significantly different from each other.
Statistics = student t-test.

Table 4.6. The serum constituents of WKyC after 30 and 60 days treatment period were not significantly different from the initial values.

No significant difference was found between the serum glucose of all the treated normotensive Wistar Kyoto rats and the untreated group WKyC after 30 days of continuous administration of the extract. After 60 days of continuous administration, the serum glucose of all the treated rats were found to be significantly ($p<0.05$) higher than the untreated group. No significant difference was however, observed between the serum glucose of the treated rats at different dosage levels. The serum cholesterol of all the treated rats were also observed not to differ significantly from the untreated group after 30 days of continuous administration of the extract. Further administration of the extract continuously for 60 days was however, observed to result in reduction of serum cholesterol of the treated rats from the value observed after 30 days and this was noticed to be significant at dosage level of 1000 and 2000mg/kg b.wt (i.e WKyT4 and WKyT5 respectively). When compared with untreated group, the serum cholesterol of the WKyT4 and WKyT5 were noticed to be significantly lowered and were also lowered significantly when compared with the corresponding values observed at all other tested dosage except in WKyT3.

A significantly lowered serum creatinine was observed in WKyT2, WKyT3, WKyT4, and WKyT5 compared with the untreated, WKyC after 30 days of continuous administration of extract and this was noticed to be dose dependent. With continuous administration of the extract up to 60 days, a further reduction was observed in the serum creatinine of all the treated rats. When compared with the observed value after

**Table 4.6: Effect of Roselle Calyx Extract Administration on Serum Glucose, Cholesterol, Creatinine, Uric Acid and Proteins Concentrations in Normotensive Rats.**

| | INITIAL (DAY 1) | AFTER 30 DAYS | | | | | | AFTER 60 DAYS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SERUM CONSTS. | WKyC | WKyC | WKyT1 | WKyT2 | WKyT3 | WKyT4 | WKyT5 | WKyC | WKyT1 | WKyT2 | WKyT3 | WKyT4 | WKyT5 |
| SERUM GLUCOSE (mmol/L | 2.5±0.07$^{a}$ | 3.05±0.07$^{a}$ | 3.80±0.51$^{ab}$ | 3.50±0.41$^{ab}$ | 3.30±0.40$^{ab}$ | 3.70±0.40$^{ab}$ | 3.55±0.07$^{ab}$ | 2.95±0.11$^{a}$ | 3.85±0.49$^{ab}$ | 3.70±0.14$^{b}$ | 3.60±0.14$^{b}$ | 3.85±0.12$^{b}$ | 3.70±0.16$^{b}$ |
| SERUM CHOLESTEROL (mmol/L) | 4.0±0.1$^{a}$ | 4.0±0.1$^{a}$ | 4.4±0.8$^{ab}$ | 4.4±0.1$^{a}$ | 4.1±0.1$^{a}$ | 4.4±0.1$^{a}$ | 4.6±0.1$^{a}$ | 4.0±0.2$^{a}$ | 4.2±0.5$^{a}$ | 3.9±0.1$^{a}$ | 3.9±0.8$^{ab}$ | 3.0±0.1$^{b}$ | 3.1±0.2$^{b}$ |
| SERUM CREATININE (μmol/L) | 110±1.4$^{a}$ | 110.0±1.4$^{a}$ | 105.0±3.7$^{a}$ | 90.5±3.0$^{b}$ | 46.8±1.0$^{c}$ | 44.2±0.8$^{c}$ | 17.0±1.5$^{d}$ | 109.6±1.5$^{a}$ | 98.8±0.1$^{e}$ | 88.6±0.1$^{b}$ | 46.2±0.7$^{c}$ | 30.3±0.2$^{f}$ | 10.5±0.29$^{g}$ |
| SERUM URIC ACID (umol/L) | 4.7±0.2$^{a}$ | 4.7±0.2$^{a}$ | 6.8±0.1$^{b}$ | 7.0±0.9$^{b}$ | 9.2±0.7$^{c}$ | 9.9±0.1$^{c}$ | 11.9±0.5$^{d}$ | 4.8±0.4$^{a}$ | 8.7±0.4$^{c}$ | 11.4±0.8$^{d}$ | 11.4±0.1$^{d}$ | 12.3±0.7$^{d}$ | 16.2±0.3$^{e}$ |
| SERUM ALBUMIN (g/dL) | 2.15±0.2+$^{a}$ | 2.15±0.24$^{a}$ | 2.58±032$^{a}$ | 1.60±0.91$^{a}$ | 2.70±0.68$^{a}$ | 1.70±0.47$^{a}$ | 1.53±0.69$^{a}$ | 2.14±0.61$^{a}$ | 2.63±0.80$^{a}$ | 2.13±0.19$^{a}$ | 2.82±0.24$^{a}$ | 2.29±0.60$^{a}$ | 1.81±0.58$^{a}$ |
| SERUM TOTAL PROTEIN (g/dL) | 3.5±0.6$^{a}$ | 3.5±0.6$^{a}$ | 4.0±0.1$^{a}$ | 3.3±0.8$^{a}$ | 3.7±0.2$^{a}$ | 3.3±0.1$^{a}$ | 3.6±1.0$^{a}$ | 3.4±1.7$^{a}$ | 4.1±0.4$^{a}$ | 3.7±0.2$^{a}$ | 4.1±0.8$^{a}$ | 4.4±0.1$^{a}$ | 4.8±0.4$^{a}$ |

Number of determination (n) = 2
Results are expressed as mean ± SD
The initial serum constituents for all the rats are not significantly different from the corresponding value in WKyC.
Values in the same row with different letters are significantly different from each other.
Statistics: Duncan mutiple range test.

30 days of treatment, all the treated rats were observed to have their serum creatinine significantly lowered. The observed serum creatinine after 60 days were also significantly lowered in all the treated rats compared with the untreated group and this was seen to be dose dependent.

A significantly higher serum uric acid was observed in all the treated rats compared with the untreated group after 30 days of continuous administration of the extract and this was also seen to be dose dependent. After 60 days, a further increase was observed in the serum uric acid of all the treated rats from the observed value after 30 days of continuous administration, and this was significant at all tested doses. The serum uric acid was also higher in all the treated rats compared with the untreated after 60 days of continuous administration of the extract.

The serum proteins (albumin and total protein) were not significantly different in all the treated rats compared with the untreated both after 30 days and 60 days of continuous administration of the extract.

## 4.0 The Effect of Roselle Calyx Extract Administration On Some Serum Constituents Under Hypertensive Condition.

In Table 4.7 is shown the result of the effect of administration of roselle calyx extract on serum glucose, cholesterol, creatinine, uric acid and protein in hypertensive condition.

After 30 days of continuous administration of the extract, the serum glucose of the hypertensive rat, SHRT1 was not significantly ($p>0.0.5$) different from the corresponding value in the control group, SHRC. A significantly ($p<0.05$) elevated glucose level, compared with the control was however, observed in the treated rats after 60 days of continuous administration. No significant difference was also observed in the serum cholesterol of the treated hypertensive and the control group after 30 days of continuous administration of the extract. It was however, observed that the serum cholesterol was significantly reduced after 60 days treatment period from the observed value of 5.8 mmol/L after 30 days treatment to 5.3 mmol/L. The observed value was also significantly ($p<0.05$) different from the value obtained in the control group. A significantly lowered serum creatinine of 108.2 umol/L was observed in the treated group after 30 days of continuous treatment with the extract compared with a value of 187.5 ummol/L in the control group. With continuous administration of the extract, a further significant reduction in serum creatinine to a value of 82.3 mmol/L was observed in the treated group. The serum uric acid was observed to be significantly higher in the treated group than the control after 30 days of continuous treatment with the extract. Continuous treatment with the extract for 60 days, however, did not significantly alter serum uric acid level.

No significant variation was observed in the serum albumin and total protein between the treated and the control group after 30 days of continuous treatment. The serum albumin after 60 days treatment was also not significantly different in the treated group from the control group. However, a significantly higher serum total protein was observed after 60 days in the treated group compared with the control and this was also significantly higher than the value observed after 30 days treatment period.

**Table 4.7: Effect of Roselle Calyx Extract Administration on Serum Glucose, Cholesterol, Creatinine, Uric Acid and Proteins in Spontaneously Hypertensive Rats.**

| SERUM CONSTITUENTS | INITIAL (DAY 1) | AFTER 30 DAYS | | AFTER 60 DAYS | |
|---|---|---|---|---|---|
| | SHRC | SHRC | SHRC1 | SHRC | SHRC1 |
| GLUCOSE (mmol/L) | $3.45 \pm 0.27^{a}$ | $3.45 \pm 0.03^{a}$ | $3.50 \pm 07^{ab}$ | $3.45 \pm 02^{a}$ | $3.55 \pm 01^{b}$ |
| CHOLESTEROL (mmol/L) | $5.6 \pm 0.1^{a}$ | $5.6 \pm 0.1^{a}$ | $5.8 \pm 0.1^{a}$ | $5.6 \pm 0.1^{a}$ | $5.3 \pm 0.1^{b}$ |
| CREATININE ( mol/L) | $187.5 \pm 1.0^{a}$ | $187.5 \pm 1.0^{a}$ | $108.2 \pm 0.1^{b}$ | $188.2 \pm 0.1^{a}$ | $82.3 \pm 3.8^{c}$ |
| URIC ACID (umol/L) | $11.9 \pm 0.4^{a}$ | $11.9 \pm 0.4^{a}$ | $21.9 \pm 0.3^{b}$ | $11.9 \pm 0.4^{a}$ | $23.2 \pm 2.0^{b}$ |
| ALBUMIN (g/dl) | $1.7 \pm 0.61^{a}$ | $1.7 \pm 0.58^{a}$ | $1.95 \pm 0.9^{a}$ | $1.74 \pm 0.3^{a}$ | $2.34 \pm 0.2^{a}$ |
| TOTAL PROTEIN (g/dl) | $30 \pm 1.2^{a}$ | $3.0 \pm 02^{a}$ | $3.4 \pm 0.1^{a}$ | $3.0 \pm 0.3^{a}$ | $4.5 \pm 0.3^{b}$ |

Number of determination (n) = 2
Result are expressed as mean $\pm$ SD
Values in the same row with different letters are significantly different from each other.
The initial serum constituents for all the rats are not significantly different from the corresponding value in SHRC.
Statistics = Duncan multiple range test.

# CHAPTER FIVE

# DISCUSSION, CONCLUSION AND RECOMMENDATION

## 5.1 Discussion

The $LD_{50}$ was investigated so as to determine the amount of roselle calyx extract that would result in acute toxicity. Investigation of the acute toxicity is the first step in the toxicological investigation of an unknown substance. The index of the acute toxicity is the $LD_{50}$ which is regarded as the dose of a drug required to produce a lethal effect in 50% of individuals (Lorke 1983).

The toxicological signs observed at doses of 1000, 3000 and 5000mg/kg b.wt i.p of the roselle calyx extract during $LD_{50}$ investigation is similar to the adverse effects associated with antihypertensive therapy. Other notable adverse effects of antihypertensive drugs that have been reported include fatigue, depression, headache, anxiety and sleep disturbance (Martin et al, 1995).

The water intake and urine output in hypertensive condition is not significantly different from the normotensive condition (Table 4.4). This could suggest that the renal output in hypertension is normal. This is a confirmation of an earlier reported work of Guyton et al, (1972), where it was noted that the renal output of water and salt in patient with essential hypertension is normal. It was observed that during roselle calyx extract

consumption, the urinary output in the hypertensive rats treated with the extract, was significantly higher than the urinary output in the control group and was also higher than the urinary output in the treated normotensive group. This could suggest that consumption of roselle calyx extract (zoborodo) affects the kidney function. This could then imply that the extract has diuretic properties since it increases urinary output. Diuretics have been described as drugs which increase the rate of urine production by the kidneys (Gillies et al, 1991). These drugs have been noted to reduce the volume of extracellular fluid, enhance the urinary excretion of sodium chloride and secondarily increase the volume of urine excreted by the kidney (Guyton et al, 1972). The increased urinary output observed in the treated rats during administration of roselle calyx extract, was not compensated for by an increase in fluid (Water) intake. This could be detrimental since it tends to suggest that animals placed on the extracts are prone to dehydration.

Traditionally, roselle calyx has been reported to alleviate thirst on long desert treks and has been helpful during fasting to sustain the day long fast (Chopra et al, 1956; WHO FAO, 1973). The thirst alleviating characteristics of roselle could perhaps be attributed to its cooling and emollient properties which could possibly explain why the treated rats take lesser water than the control group.

Though no mortality of any of the experimental animals were recorded during the $LD_{50}$ investigation and the $LD_{50}$ was thus estimated to be above 5000mg/kg b.wt, which

indicates its apparent safety (Lorke, 1993), continuous consumption of the extract at concentration of 1000mg/kg b.wt resulted in the sudden death of all the hypertensive rats within twenty one (21) days of treatment. The normotensive rats placed on the same dose and above may have fared well in this trial compared with the hypertensive group because of the abnormal distribution of electrolytes in hypertensive condition (Tobian, 1972). More water, sodium, and calcium had been reported to be found in the arterioles of hypertensive rats than those from rats with "cured" hypertension (Tobian and Binion, 1952), and this have been reported to contribute to extra narrowing of the hypertensive arterioles.

Emmanuel et al, (1959), also reported that the extra narrowing of the arterioles in hypertension is as a result of its diminished intracellular concentration of arteriolar potassium and normal extracellular values. Since all diuretics of the thiazide type enhances the excretion not only of sodium, but also of potassium, a negative potassium balance may develop (Gross, 1979; Gillies et al, 1991). Diuretics was reported by Gillies et al, (1991) to cause a fall in plasma potassium over 1-2 weeks of treatment and patients on high doses were noted to have increased risk of developing serious hypokalemia. Roselle Calyx extract has been reported to enhance membrane stabilization and may thus stimulate $Na^+$ $K^+$ ATPase. The intracellular concentration of potassium is maintained by this $Na^+$ $K^+$ ATPase pump (Adegunloye, et al, 1993; Edward and Treiss, 1995). Continuous consumption of roselle could thus possibly result in loss of plasma potassium. Hence, sudden death of the SHRT2 observed in this study could be

attributed to potassium loss resulting from stimulative activity of the extract on $Na^+$ $K^+$ ATPase. It has been suggested that diurectic induced hypokalemia may increase the risk of coronary heart disease and may increase sudden cardiac death (MRFIT, 1985), and this has been opined to be responsible for fatal complications related to the use of diuretics (Martin et al, 1995). Again a number of blood pressure lowering drugs are excreted via the kidneys so that their use in renal failure will be followed by drug retention and subsequent evidence of overdose (Gillies et al, 1991). The fact that the electrolyte distribution is uneven in hypertensive condition compared with the normotensive could have greatly contributed to the observed effect in the hypertensive, whereas the normotensive group fared well. The possibility of the sudden death being of cardiac origin due to hypokalemia could be supported by the evidence that no gross variation was observed in the vital organs of all the rats when examined after death.

The significant reduction in systolic and diastolic pressure observed in both the hypertensive and normotensive rats administered the roselle calyx extract, confirmed the efficacy of the extract in reducing blood pressure and hence its antihypertensive property.

It can also be said here that the efficacy of roselle calyx extract in reducing blood pressure is dose dependent. This is supported by the observation that the reduction in blood pressure (systolic and diastolic) in SHRT2 (1000mg/kg b.wt) was significant after 7 days treatment period where as the reduction of these two parameters become significant in SHRT1 (500mg/kg b.wt) after 21 days treatment period. Again since the

blood pressure of the normotensive group was found to be significantly lower than the hypertensive group (before administration of extracts) and that after 21 days of continuous treatment with the extract, the diastolic pressure of the SHRT1 was not significantly different from WKyT3 and WKyT4, it may then mean that the blood pressure lowering effect of roselle calyx extract is more pronounced in hypertensive than in normotensive conditions (Table 4.2). This also could account for the reason why the urine output was found to be significantly higher in the treated hypertensive group than the normotensive group treated with the same dosage (Table 4.3). This view is supported by the evidence that the blood pressure lowering effect of some antihypertensives are more pronounced in hypertensive than normotensive animal (Gross, 1979).

The close association and positive correlation observed between the body weight and blood pressure indicates that blood pressure greatly depend on body weight and that roselle calyx extract could be very useful where hypertension is complicated with obesity. A considerable contribution of body mass towards blood pressure has ealier been reported and had been noted to apply to "low pressure" as well as to "high pressure" groups (Kannel et al, 1977).

The observed darker coluration of the urine of the treated rats compared with the control groups could be an indication that flavonoids are not completely broken down but that some are still excreted through the urine. Though this results disagrees with Harbone

(1977), who reported no observation of colour in the urine following ingestion of fruit with high anthocyanin contents, it agrees with Sweny and Vargheze (1988), who reported a Pinkish urine appearance following ingestion of anthocyanin.

### 5.1:1 Biochemical Analysis

Serum creatinine and uric acid were determined so as to serve as indices for kidney function during hypertension and roselle calyx extract consumption.

Creatinine is the end product of creatine metabolism. It is present mostly in skeletal muscle where it is involved in energy storage as creatine phosphate (CP). Creatine phosphate is converted to creatine in the synthesis of ATP from ADP, catalyzed by the enzyme creatine-kinase (CK). In the process, small amounts of creatine are irreversibly converted to creatinine which is removed from circulation by the kidneys. Creatinine is normally elevated when renal function is impaired and is not affected by dietary protein levels (Ronald and Richard, 1991). The significantly higher creatinine concentration in SHRC compared to that in WKyC, may mean that in hypertension, there is impairment of renal function (Table 4.6). This then implies that though urinary output appears to be normal in hypertension, the function of the kidney is not normal (Guyton et al, 1972). Rosanky et al, (1990) reported that high blood pressure can either be a cause or consequence of kidney disease, one can then suggest that the impairment of renal function in spontaneously hypertensive rats was caused by the elevated arterial pressure.

Aministration of roselle calyx extract reduces the creatinine level significantly in both the normotensive and hypertensive rats studied compared with the control group. This reduction was also noted to be dose dependent and also depend on the length of administration of the extract. Based on this results therefore, one can suggest that consumption of roselle calyx extract improves renal function and that renal activity improves with increasing dosage of the extract. It has earlier being reported that diuretic drugs may affect urinary excretion of creatinine and also creatinine clearance (Nancy et al, 1995) hence this results establish that roselle calyx extract is diuretic.

Another serum constituent that can be used in assessing kidney function is uric acid. Uric acid is a major waste product of purine catabolism and has been used in diagonizing decreased renal function. It is synthesized primarily in the liver, a reaction catalyzed by the enzyme xanthine oxidase, transported through the blood to the kidney where it is filtered, partially reabsorbed and partially further secreted before final excretion in the urine (Ronald and Richard, 1991). Breckenridge (1996), noted that hyperuricemia is commonly found in hypertensive patients and this has been reported to be rather due to impaired renal elimination of urate than from its overproduction. This is supported by the present study since a high uric acid level is found in the control hypertensive group compared with the normotensive group. It has been observed that the degree of hyperuricemia correlates with the severity of hypertension which also correlates strongly with the increased body weight (Kinsey, et al, 1961; Klein et al, 1973).

Kinsey et al, (1961), reported that thiazide diuretics administration increased association with hyperuricemia. It was also noted that although serum uric acid levels during diuretics treatment were significantly greater in general, the increase was greater in those with lower diastolic pressure. The results here suggest that consumption of roselle calyx extract increases uric acid level which is dose dependent and also depend on length of administration. This increased uric acid level could be due to changes in extracellular fluid volume which has been reported to affect urate excretion (Steale, 1969). Gillies, et al, (1991) reported that uric acid retention which combined with decreased extracellular fluid volume, causes a rise in plasma uric acid levels. Uricosuric agents, particularly thiazide diuretics acts more distally in the nephron to block reabsorption of uric acid. Uric acid is poorly soluble in water and urate crystals readily precipitate from urine with high concentrations of urate to produce urate kidney stones (Steale, 1969). Patients with high blood uric acid levels have been noted to often deposit urate crystal in soft tissues, especially joints, thus resulting in gout (Gillies et al, 1991; Ronald and Richard, 1991). This has been noted to manifest by painful swollen and inflammed joints.

Cholesterol which may be affected by diet has been reported to increase in renal disease, liver disease and diabetes mellitus (Hag et al, 1995). These diseased condition along with hypertension has been noted to be modifiable risk factors in coronary heart disease (Goode et al, 1995). It has often been said that hypertension and hyperlipidaemia co-exist more often than would be expected by chance (Castelli and Andersion, 1986;

Crique et al, 1986) a fact that is established in the present study since the hypertensive control group was noticed to have a significantly raised serum cholesterol compared with the normotensive control group. Although a common metabolic disorder may be responsible for the association between hypertension and hyperlipidaemia, another possibility is that one may be implicated in the development of the other. (Crique et al, 1986). A significantly raised systolic and diastolic pressure have been reported in subjects who have raised triglycerides and reduced high density lipoproteins (HDL) cholesterol. After 60 days of continuous administration of the extract, serum cholesterol of the hypertensive rats, SHRT1 was significantly lower than the value before treatment. This value was also lowered significantly than the observed value after 30 days of continuous treatment. Again, the Serum cholesterol observed in normotensive WKyT4 and WKyT5 after 60 days of continuous administration of the extract was significantly lowered than the observed values after 30 days. Thus it could be reasoned that continuous consumption of roselle calyx extract lowers serum cholesterol significantly and that this is evident after treatment for about 60 days. Administration of diuretics as a sole agent in the treatment of hypertension has been noted to result in an increase in serum cholesterol and triglycerides (Ames and Hill, 1976), the average increment however, varied from patients to patients (Richard et al; 1976). Long term clinical trials however, indicate that serum cholesterol level are elevated only during the first 6-12 months of diuretics treatment and then decreases to pretreatment level (Amery et al, 1982; Miettinen et al, 1985). This view is supported by this study and it can again be added that this decrease is only evident in the normotensive group at doses of 1000 and

2000mg/kg b.wt which is higher than the dose of 500mg/kg b.tw that produced the significant decrease in the treated hypertensive group. (Table 4.6 and 4.7).

Another serum constituent measured which can serve as a good index of liver function is glucose. The maintenance of blood glucose level below about 11mmol/L has been reported to minimise loss of this energy source from the body (Ellefson, et al, 1976). The serum glucose level in the hypertensive control group did not differ from the normotensive control group (Table 4.5). Since the observed glucose level in the groups were below the normal glucose level of 5.0mmol/L, it appears then that spontaneous hypertension in rats is not complicated by diabetes mellitus. The serum glucose in the treated hypertensive and normotensive groups were significantly higher after 60 days of continuous treatment compared with the level in the control groups, signifying that treatment with this extract raises the serum glucose level. Chronic use of diuretics has been shown to impair glucose tolerance (Gross, 1979; Gillies et al, 1991) and subjects with impaired glucose tolerance may have an increased risk of cardiovascular desease (Fuller et al, 1979). Opinion however, still differ on the role of diuretics in diabetes mellitus (HDFP, 1979; MRFT, 1985; Gillies et al, 1991). In an attempt to find out the interplay between diabetes mellitus and diuretics, Einer et al, (1989), observed that diuretics may cause a decrease in insulin sensitivity which does not disappear with time and may thus elicit the development of diabetes mellitus particularly in people already predisposed to the disaese. Hence, Einar et al, (1989), suggest that hyperinsulinism and insulin resistance may be a risk factor in coronary disease.

The fact that no significant variation was observed in the serum proteins of the hypertensive control group compared with the normotensive control group suggest that hypertension does not significantly alter serum proteins. Within 30 days of treatment with the extract, no significant variation was also observed in the serum proteins of the treated rats from the control group. It was however, noted that in between 30 and 60 days of continuous treatment, a trend was established and the serum total protein of SHRT1 was observed to be significantly higher after 60 days than the control group. It can thus be suggested that follow up programmes with roselle calyx extract may result in significant increase in serum proteins. Though serum proteins has not been reported as an important clinical factor in hypertension and antihypertensive therapy (Gross 1979), the observed significant increase in serum proteins here could possibly have resulted from excessive loss of water through urine associated with the consumption of the extract which was not accompanied by increased water intake. Apart from accounting in large part for the oncotic pressure of blood pressure, albumin has been noted to serve as a circulating reservoir of amino acids which would clear rapidly into the urine if not incorporated into a higher molecular weight protein (Ronald and Richard, 1991). Increase in serum albumin concentrations have been noted to result from severe dehydration in which plasma water leaves the circulation (Ronald and Richard, 1991). Since total protein is a measure of albumin and globulin, an increase in albumin will result in increase in total protein and this could perhaps account for the observed increasing trend in serum proteins. Because water loss through urine is reported here to be higher in hypertensive than normotensive condition during treatment with the

extract (Table 4.3), this could possibly account for the reason why the increase in serum protein is significant in hypertensive condition but not in normotensive condition (during the extract consumption) suggesting that the hypertensive rats administered with roselle calyx extract are much prone to dehydration than normotensive ones.

## 5.3 Conclusion

The first conclusion that can be drawn from this work is that roselle (Hibiscus sabdariffa) calyx extract (Zoborodo) lowers both systolic and diastolic pressure suggesting that it has beneficial effect in hypertensive patients. Further more, the possibility of roselle extract exerting its antihypertensive effect via diuretic activities is established. Also it is reported here that though roselle calyx extract administration would prove effective in hypertension management, especially in essential hypertension in human which has been compared most extensively with spontaneously hypertensive condition in rats (both types of hypertension develop gradually with age, the haemodynamic factors are similar, the main cardiovascular complications occuring in the brain, heart and kidney are similar (Yamori et al, 1974), not withstanding, the risk factors involved in its (Zoborodo) continuous consumption particularly as it affects electrolyte redistribution and other metabolic changes as reported here may be deleterious especially at very high doeses.

## 5.4 Recommendation

Based on the results of this study, the blood pressure lowering and diuretic effects of roselle calyx extract (Zoborodo) is confirmed and thus the extracts could be recommended for hypertension management. It is howerve, advisable to follow the recommendations of the fifth joint National Committee on Detection Evaluation and Treatment of High blood pressure (1993) in its usage i.e beginning treatment with a low dose of diuretic. This is of particular importance since this report provide evidence that at high dosage in both hypertensive and normotensive conditions, deleterious metabolic changes do occur.

Attempt should also be made to determine the serum potassium concentrations during roselle calyx extract consumption, and if the extract is proved to be a non-potassium spairing diurectic, supplement with potassium during its consumption should be encouraged while also the potassium level in the serum should be monitored perodically. Again the effect of continued reduction of serum cholesterol during follow-up programmes should be examined while the role of the observed glucose increase during treatment with roselle calyx extract in diabetes mellitus will also need further investigation.

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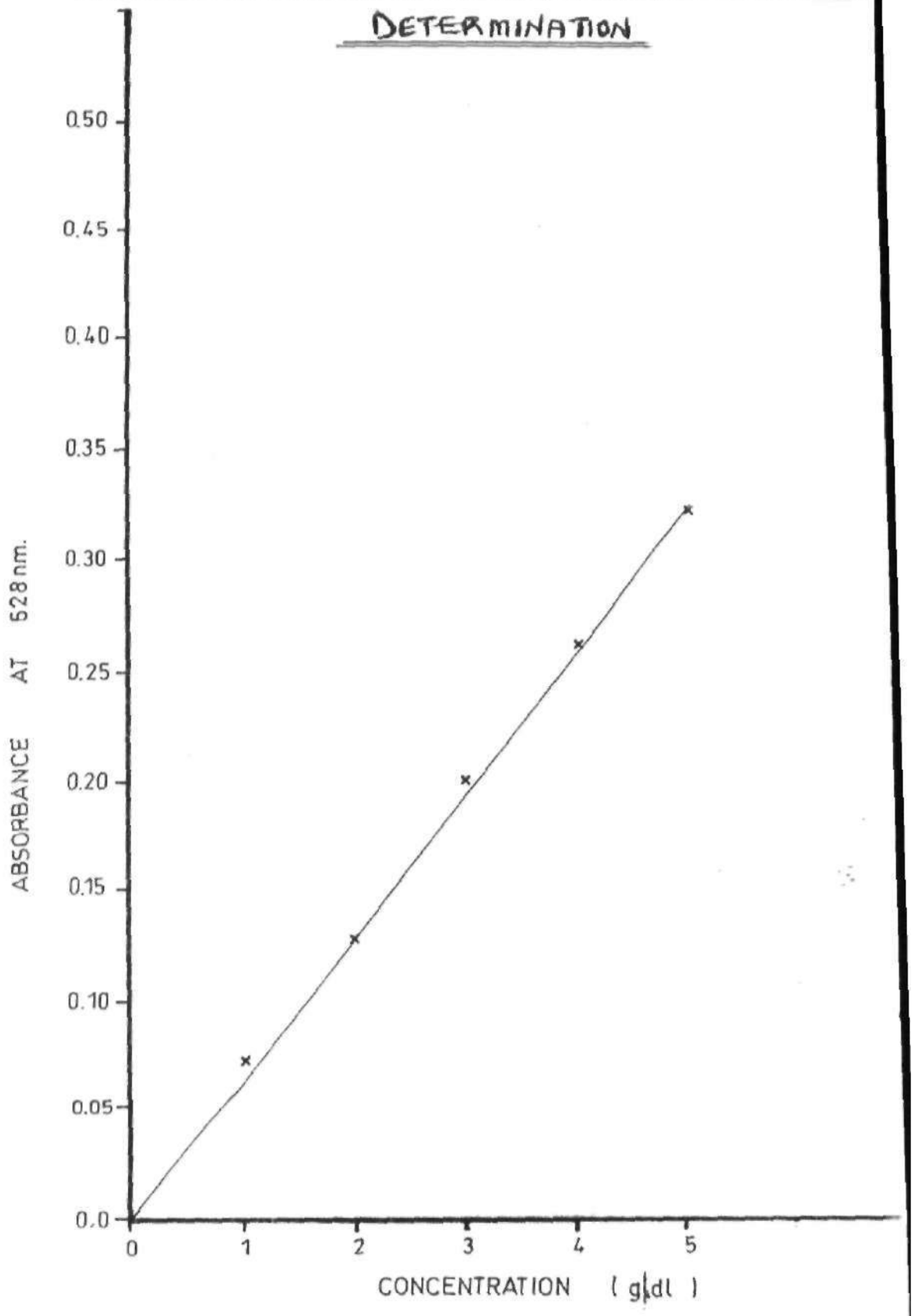
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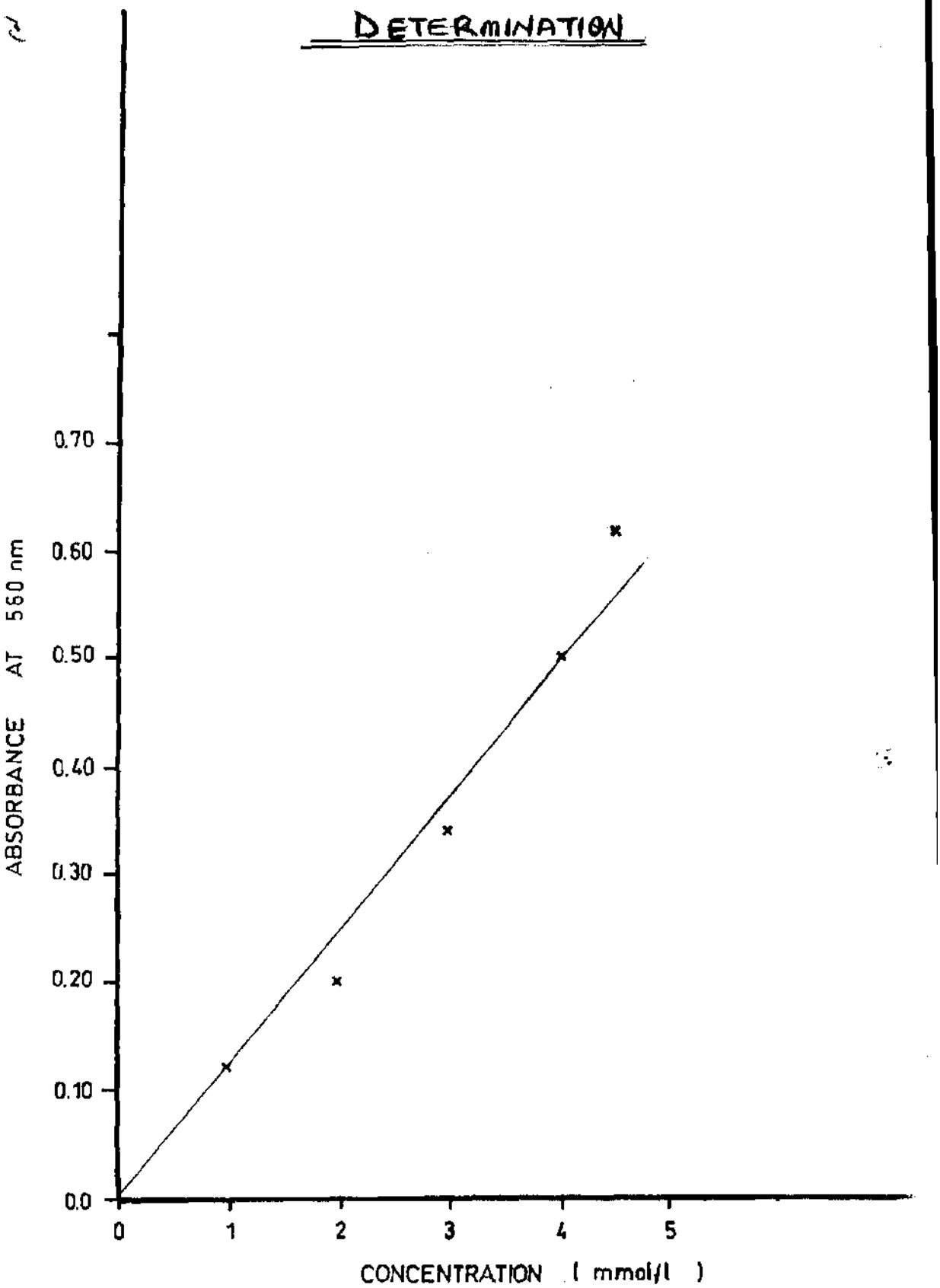
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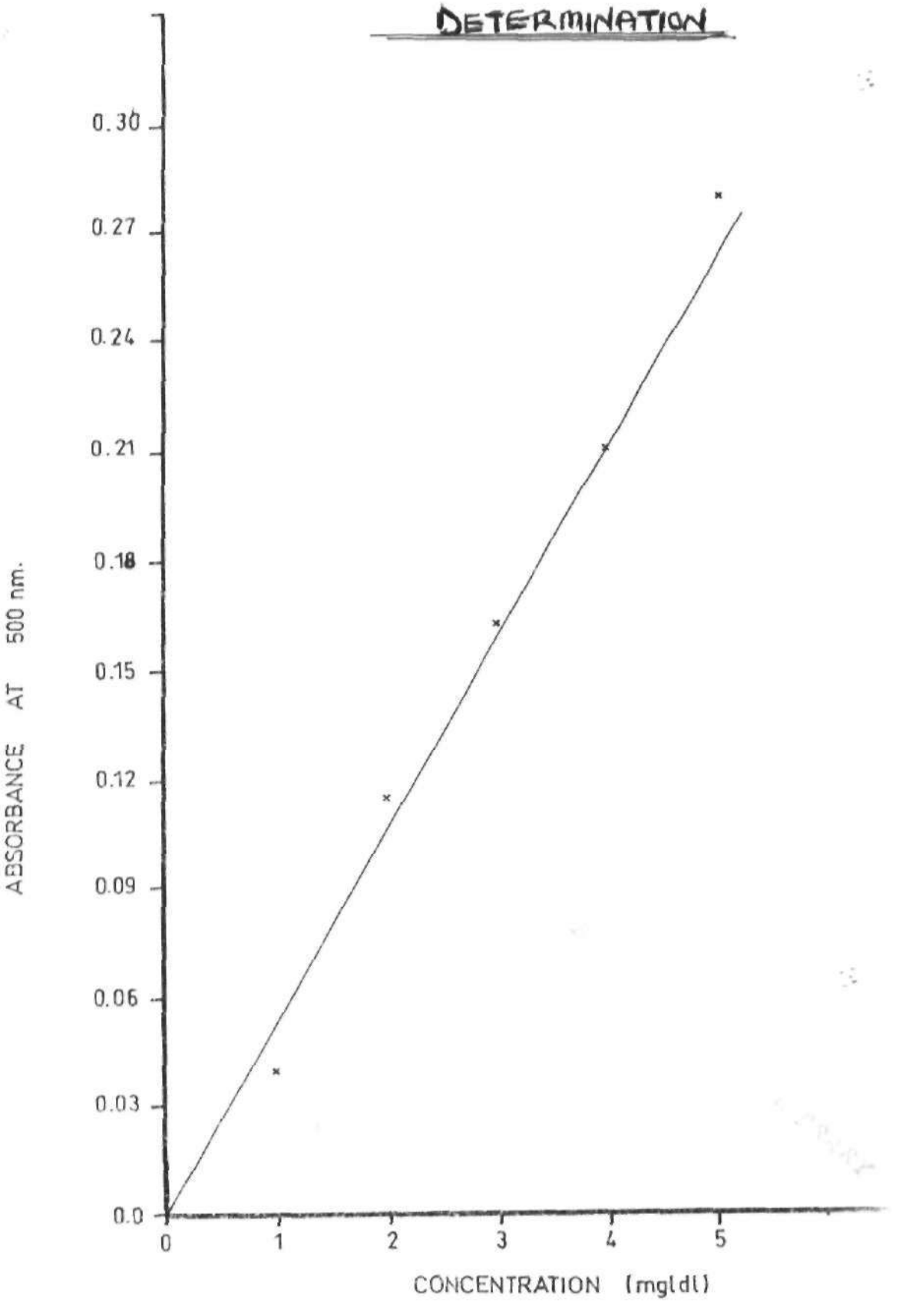
APPENDIX I **CALLIBERATION CURVE FOR ALBUMIN DETERMINATION**



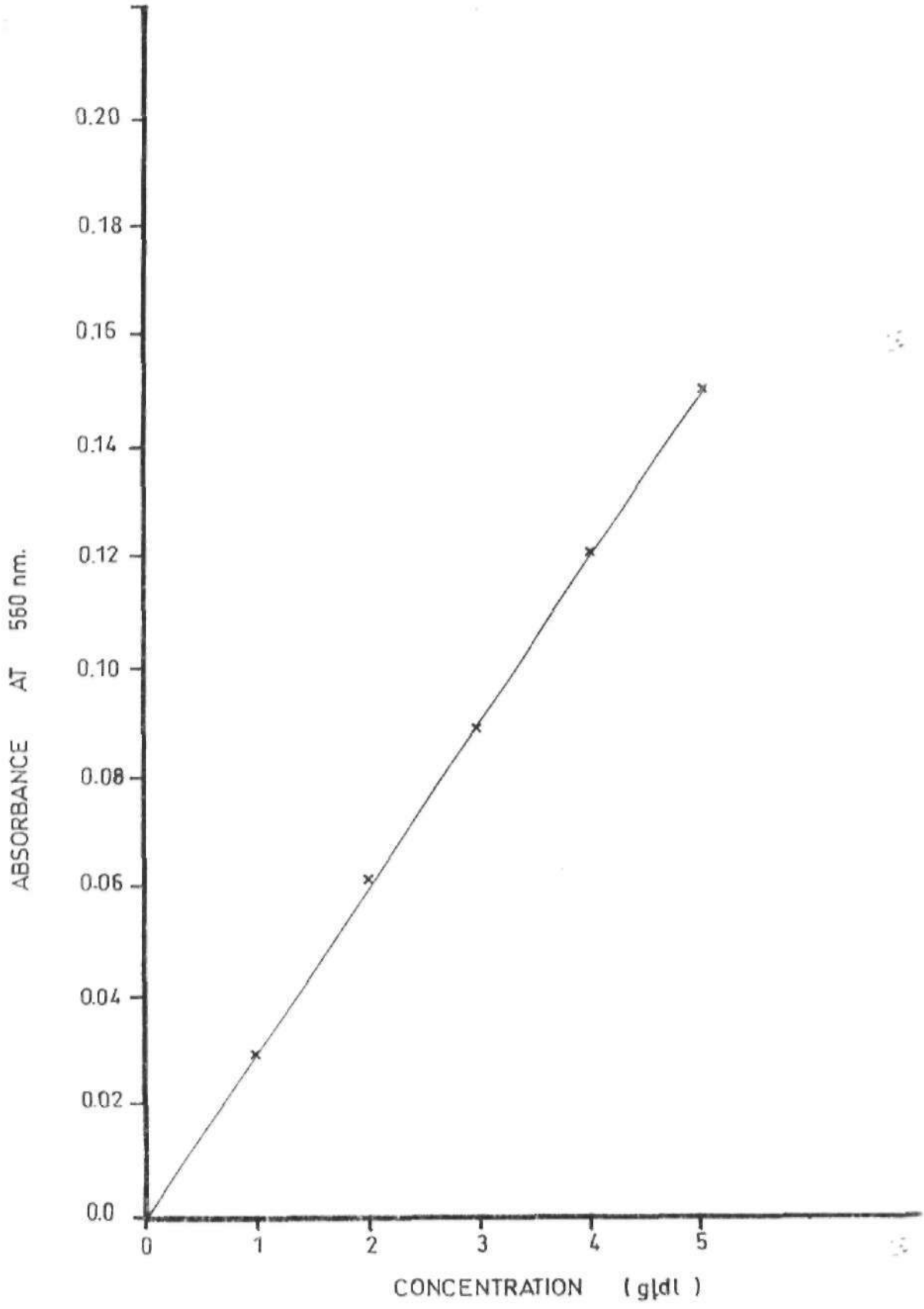
APPENDIX II **CALLIBERATION CURVE FOR CHOLESTEROL DETERMINATION**



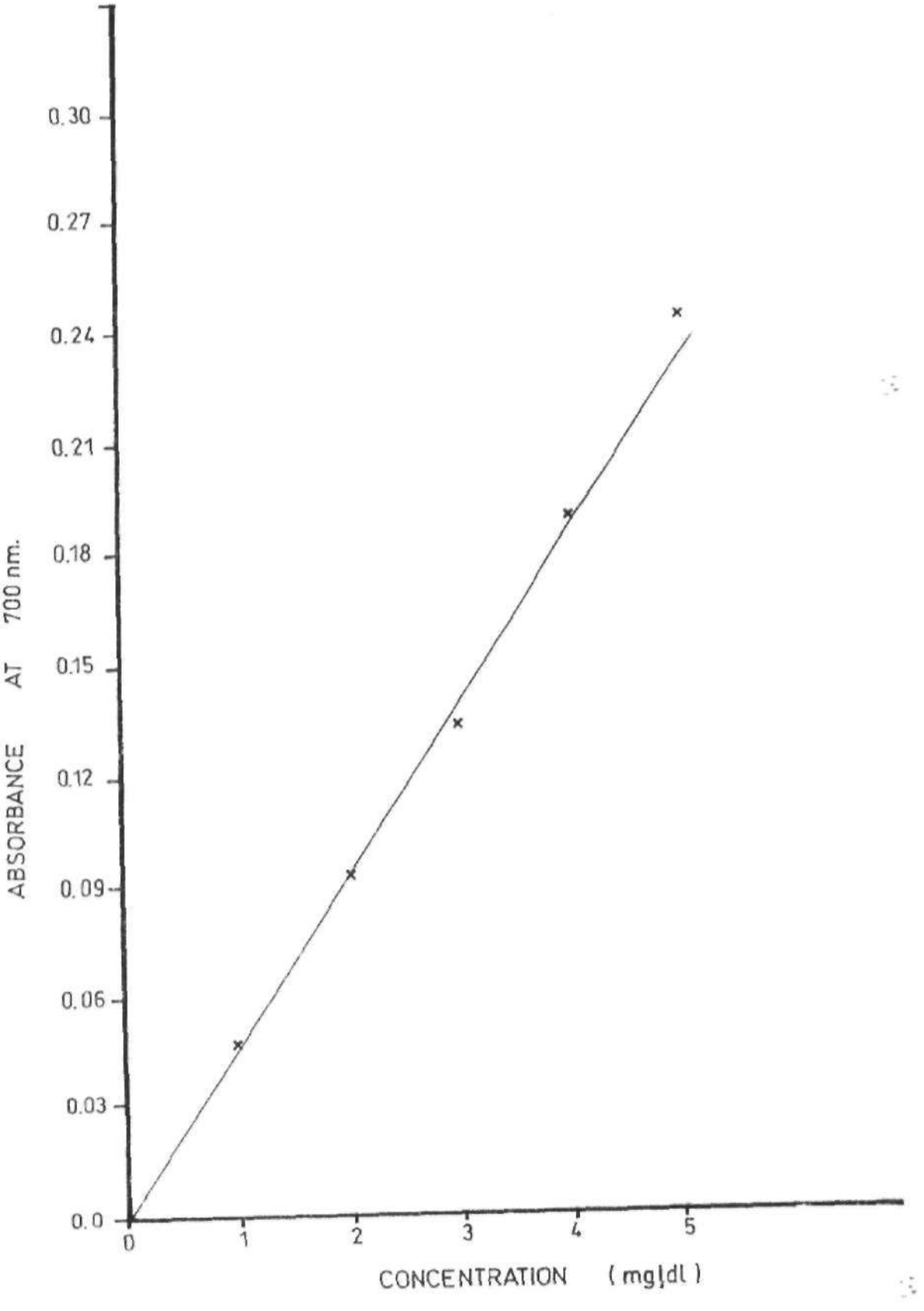
APPENDIX III **CALLIBERATION CURVE FOR CREATININE DETERMINATION**



APPENDIX IV **CALLIBERATION CURVE FOR TOTAL PROTEIN DETERMINATION**



APPENDIX V **CALLIBERATION CURVE FOR URIC ACID DETERMINATION**



APPENDIX Vi

EFF[redacted]IFFA) CALYX EXTRACT ON BLOOD PRESSURE AND BODY WEIGHT IN HYPERTENSIVE AND NORMOTENSIVE RATS

| | Initial Values (Day) 1 | | Day 7 | | Day 21 | |
|---|---|---|---|---|---|---|
| Animal Group | Weight (g) | Blood Pressure (mm Hg) | Weight (g) | Blood Pressure (mm Hg) | Weight (g) | Blood Pressure (mm Hg) |
| SHRc | 187.0 | 152/100±6/7 | 189.0 | 154/95±9/15 | 203.5 | 155/94±7/19 |
| | 161.4 | 148/79±5/14 | 171.7 | 153/100±6/8 | 188.1 | 155/90±4/7 |
| | 200.5 | 150/72±7/9 | 201.8 | 153/75±5/14 | 201.1 | 150/70±7/7 |
| | 200.1 | 156/98±3/3 | 194.0 | 153/95±3/6 | 187.5 | 150/94±7/6 |
| | 200.0 | 150/85±3/10 | 210.0 | 156/88±4/10 | 215.1 | 156/90±9/7 |
| SHRT1 | 209.7 | 155.92±2/5 | 200.4 | 147/88±4/5 | 186.6 | 138/78±6/8 |
| | 206.7 | 146/94±3/4 | 206.0 | 144/85±5/4 | 200.0 | 131/70±4/5 |
| | 190.2 | 153/85±2/7 | 187.1 | 143/75±6/6 | 185.0 | 139/68±3/5 |
| | 263.1 | 154/88±5/9 | 179.5 | 140/72±7/9 | 170.0 | 135/66±3/5 |
| | 194.3 | 155/95±2/4 | 185.2 | 142/89±5/6 | 182.5 | 134/70±4/6 |
| SHRT2 | 235.5 | 171/94±4/5 | 202.4 | 146/72±3/3 | ND | ND |
| | 206.7 | 155/95±4/3 | 192.8 | 135/72±3/8 | ND | ND |
| | 190.2 | 148/85±3/7 | 162.1 | 125/71±4/7 | ND | ND |
| | 203.1 | 157/88±14/5 | 186.7 | 139/70±7/7 | ND | ND |
| | 194.3 | 146/85±3/15 | 160.3 | 122/70±2/4 | ND | ND |
| WKyT3 | 209.1 | 133/74±4/5 | 196.2 | 125/70±4/8 | 193.2 | 125/65±8/15 |
| | 232.5 | 132/79±8/10 | 220.4 | 125/69±4/7 | 218.4 | 125/63±4/8 |
| | 189.5 | 133/70±6/7 | 170.7 | 125/75±4/6 | 165.5 | 123/70±4/5 |
| | 197.7 | 135/70±4/5 | 180.2 | 126/70±3/3 | 170.0 | 126/66±3/4 |
| | 175.1 | 132/80±2/8 | 165.0 | 130/71±4/9 | 160.2 | 124/68±7/10 |
| WKyT4 | 169.3 | 128/73±1/5 | 158.7 | 128/70±6/4 | 153.6 | 121/65±2/7 |
| | 200.2 | 130/63±3/8 | 189.0 | 125/65±3/11 | 190.7 | 114/65±7/6 |
| | 181.5 | 129/70±3/5 | 175.0 | 126/68±4/4 | 166.5 | 119/66±7/1 |
| | 194.6 | 130/76±4/6 | 170.6 | 121/70±7/9 | 165.1 | 118/66±2/3 |
| | 180.0 | 125/72±4/4 | 173.0 | 124/65±3/5 | 170.1 | 120/60±3/8 |

APPENDIX Vii

EFFECT OF ROSELLE CALYX EXTRACT ON WATER INTAKE AND URINARY OUTPUT IN SPONTANEOUSLY HYPERTENSIVE RATS

| DAY | WATER INTAKE ($cm^3$) | | URINARY OUTPUT ($cm^3$) | |
|---|---|---|---|---|
| | SHRc | SHRT1 | SHRc | SHRT1 |
| 1 | 12.0 | 11.0 | 4.0 | 5.0 |
| 2 | 10.5 | 11.0 | 4.0 | 6.0 |
| 3 | 11.0 | 10.5 | 4.0 | 4.5 |
| 4 | 12.0 | 10.5 | 4.0 | 5.5 |
| 5 | 11.0 | 10.0 | 4.0 | 5.5 |
| 6 | 14.0 | 10.0 | 4.0 | 5.5 |
| 7 | 13.5 | 10.0 | 4.0 | 5.0 |
| 8 | 14.0 | 9.5 | 4.0 | 4.0 |
| 9 | 13.5 | 9.5 | 4.0 | 5.0 |
| 10 | 13.5 | 9.5 | 4.0 | 4.0 |

APPENDIX Viii

EFFECT OF ROSELLE CALYCES EXTRACT ON WATER INTAKE AND URINARY OUTPUT IN NORMOTENSIVE RATS.

| DAY | WATER INTAKE ($cm^3$) | | URINARY OUTPUT ($cm^3$) | |
|---|---|---|---|---|
| | WKyC | WKyT3 | WKyC | WKyT3 |
| 1 | 12.0 | 11.5 | 4.0 | 4.0 |
| 2 | 12.0 | 10.5 | 4.0 | 5.0 |
| 3 | 11.5 | 10.5 | 3.0 | 5.0 |
| 4 | 12.0 | 10.0 | 3.0 | 4.5 |
| 5 | 12.0 | 9.5 | 3.5 | 3.5 |
| 6 | 14.0 | 10.0 | 4.5 | 4.0 |
| 7 | 14.0 | 10.0 | 5.0 | 5.0 |
| 8 | 12.0 | 9.0 | 4.5 | 4.0 |
| 9 | 13.0 | 8.5 | 4.5 | 4.0 |
| 10 | 12.0 | 8.0 | 4.0 | 3.5 |

APPENDIX ix

**Preparation of Reagents**

**Biuret Reagent**

In 300ml of distilled water was dissolved 173g of sodium citrate and 100g of sodium carbonate by heating the mixture in a water both.

Separately in another 300ml portion of distilled water was dissolved 17.3g of copper sulphate. Both solutions were poured into a 1000ml graduated flask and made up to the mark with the distilled water.

**O -Toluidine Reagent**

In 95ml of glacial acetic acid was dissolved 0.15g of thiourea followed by addition of 6ml distilled 0- toluidine. This was then mixed with shaking and the prepared solution stored in a brown bottle.

**Succinate Buffer (0.10M, pH 4.0)**

In about 800ml of distilled water was dissolved 11.9g of succinic acid, the pH was adjusted to 4.0 with NaoH and diluted to 1litre with water. The solution was stored at 4°C.

**Phosphotungstic Acid (Folin and Denis)**

In a florence flask contining 800ml of distilled water was dissolved 100g of molybdate free sodium tungstate. This was followed by the addition of 80ml of 85% phosphoric acid. The flask was attached to a reflux condenser and boiled gently for 2 hours. The solution was cooled to room temperature, diluted to 1000ml with water and mixed. It was then stored in a brown bottle.

***Precipitating Reagent***

Into 700ml of water was added 100ml of 10% sodium tungstate. To this was again added 100ml of 0.1m $H_2 SO_4$ and the solution stored in a reagent bottle.