THE HISTOLOGIC EFFECT OF FIXATIVES ON THE STAINING PROPERTIES OF *Hibiscus sabdariffa, Sorghum bicolor* and *Garcinia livingstonei.*

BY

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DECLARATION

I declare that the work in the thesis entitled Histological Effect of Fixatives on the Staining properties of *Hibiscus sabdariffa*, *Sorghum bicolour* and *Garcinia livingstonei* has been performed by me in the Department of Veterinary Anatomy under the supervision of Dr. B. I. Onyeanusi, Professor J. O. Hambolu and Professor J. O. Ayo. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

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CERTIFICATON

The thesis entitled Histological Effect of Fixatives on the Staining properties of *Hibiscus sabdariffa, Sorghum bicolor* and *Garcinia livingstonei* by Omokaro, Kenneth Nosa Osagie meets the regulations governing the award of the degree of Master of Science in Veterinary Anatomy of Ahmadu Bello University, and is approved for its contribution to knowledge and literacy presentation.

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DEDICATION

This work is dedicated to my beloved aged parents, Mr. Abel Osazemwinde Omokaro and Mrs. Victoria Imwen Omokaro.

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ABSTRACT

This study describes the effect of the fixatives: Buffered neutral formalin, Heidenhains SUSA fixative, Calcium acetate formalin, Muller's fixative, Helly's fixative and Carnoy's fixative on the tissue staining ability of the extracts of *Hibiscus sabdariffa*, *Sorghum bicolor* and *Garcinia livingstonei.* Tissues were fixed in the fixatives, processed by paraffin wax method and the sections were stained with alcoholic extract of *Hibiscus sabdariffa* and aqueous extracts of *Sorghum bicolor* and *Garcinia livingstonei. Hibiscus sabdariffa* gave a good tissue staining contrast and cellular clarity on both the nucleus and cytoplasm. *Sorghum bicolor* and *Garcinia livingstonei* also gave a good tissue staining contrast particularly for the cytoplasm, hence they are cytoplasmic stains and may be used as substitutes for eosin in routine histological preparations. The effect of the fixatives varied the tissue staining ability of the local stains, with Carnoy's fixative emerging as the fixative of choice in this study.

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CHAPTER ONE

INTRODUCTION

The foundations of histology were laid before the advent of staining methods. Staining techniques, according to Conn (1948) originated from the second half of the 19th century. Early attempts to stain tissues using saffron were neither wholly successful nor followed up by others and specimens were examined unstained. Therefore, tissue components were not selectively highlighted (Conn, 1948).

Generally, histological stains are coloured substances which dye tissues and can be classified according to their origin as natural or synthetic; and according to their physical properties as either acidic, basic or neutral dyes. Dyes can combine with tissues by adsorption, especially when in a state of fine division, to attract and hold other substances on their free surfaces; such stains can usually be differentiated in water or alcohol. While mordanted dye combines with the tissue to form a tissue-mordant-dye complex; this is insoluble in ordinary aqueous or alcoholic solvents, allowing subsequent counterstaining and dehydration to be easily carried out. Fixation assists the interaction of tissues and dyes, proteins are more easily stained after fixation. Formaldehyde and mercuric chloride favour basic dyes, while trichloroacetic acid, picric acid and chromium compounds facilitate the action of acidic dyes. Both acidic and basic dyes are taken up by tissues easily after fixation with ethyl alcohol or acetic acid (Drury *et al*., 1967). An almost innumerable variety of fixatives have been proposed; but only a comparatively small number have come into routine use, each with its own advantages.

In any staining procedure, the specific recommendations in regard to fixation should be followed unless there are definite reasons for some different procedure. There are seven essential steps in handling the tissue before staining begins, namely: fixation followed by

washing, dehydration, clearing, embedding, sectioning and attaching sections to slides (George *et al*., 1981).

Examples of the most commonly employed fixatives in laboratories of microtechnic are as follows: Zenker's fluid, Helly's fluid, Heidenhain's 'SUSA' fluid, Bouin's fluid, Buffered Neutral Formalin, Gendre's fluid, Carnoy's fluid, Muller's fluid (Bancroft, 1975; George *et al*., 1981). The fixative employed in the preparation of tissues for staining is of particular importance (Table I).

TABLE I: TARGET COMPOUNDS SHOWING FIXATIVES OF CHOICE AND THOSE TO BE AVOIDED

Target	**Fixative of Choice**	**Fixative to Avoid**
Proteins	Neutral Buffered Formalin, Paraformaldehyde	Osmium Tetroxide
Enzymes	Frozen Sections	Chemical Fixatives
Lipids	FrozenSections, Glutaraldehyde/Osmium Tetroxide	Alcoholic fixatives, Neutral Buffered Formalin
Nucleic Acids	Alcoholic fixatives, HOPE	Aldehyde fixatives
Mucopolysaccharides	Frozen Sections	Chemical fixatives
Biogenic Amines	Bouin's~, Neutral Buffered Formalin	Chemical fixatives
Glycogen	Alcoholic based fixatives	Osmium Tetroxide

Source: Adopted from Bancroft (1975).

This is because the procedure must be adapted to the tissues target compounds to be examined, and it must be specially designed to bring out and preserve the structures which are desired to be demonstrated.

In view of the prime importance of fixation, the staining procedure is often determined by the method of fixation and vice-versa (Bancroft, 1975). Sometimes the choice of a fixative for tissues influences subsequent histochemical reactions (Culling, 1974). This is because fixatives are chemicals that react with tissue structures to produce new compounds. These compounds are not only capable of withstanding autolysis and putrefaction, but are also capable of preparing the tissue for histochemical reactions. Generally, fixatives which contain acetic acid favour the staining of the nucleus (Avwioro, 2002).

For the purpose of this study, the following six fixatives will be employed: Buffered neutral formalin, Heidenhain's 'SUSA' fluid, Calcium acetate, Muller's fluid, Helly's fluid and Carnoy's fluid (Bancroft, 1975). The compositions of these fluids are shown in Table II.

The search for new histological stains locally from our seemingly untapped plant resource, if successful may enhance the elucidation of structures of the body systems, including research output in histology and histochemistry.

According to Wiam *et al*. (2006), aqueous extract of local dye from *Lawsonia inermis* and ethanolic extract from *Hibiscus sabdariffa* could be used as a substitute for eosin and haematoxylin, respectively in routine histological tissue staining. Egbujo *et al.* (2008) also noted that *Hibiscus sabdariffa* is a good substitute to haematoxylin as a nuclear stain, especially when mordanted with iron or potassium alum.

In view of the current global economic recession and its disastrous effects on the purchasing power of the Naira in the international market in comparison with the United States (US)

Dollar, it has become pertinent to look inwards into our vast local plants and investigate their potentials as suitable nuclear and cytoplasmic stains for histologic purposes. This will effectively reduce the cost of imported chemicals which are gradually becoming out of reach in most laboratories; especially in developing countries including Nigeria. A success in the search for new stains may increase the foreign exchange earnings of the nation.

The general aim of this study was to evaluate the histologic effect of fixatives on the staining properties of extracts of *Hibiscus sabdariffa*, *Sorghum bicolor* and *Garcinia livingstonei*.

Specific objective of the study was:

1. To determine the most appropriate fixative for staining histologic sections with extracts of *Hibiscus sabdariffa*, *Sorghum bicolor* and *Garcinia livingstonei*.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Plant profile

2.2 *Hibiscus sabdariffa*

2.2.1 Plant Classification (Anon a, 2010)

Kingdom: *Plantae*; (Unranked): *Angiosperms*; (Unranked): *Eudicots*; (Unranked): *Rosids*; Order: *Malvales*; Family: *Malvaceae*; Genus: *Hibiscus* and Species: *H. sabdariffa*.

2.2.2 Plant Description

The roselle (*Hibiscus sabdariffa*) according to Vaidya (2000) is a species of *Hibiscus* native to the Old World tropics, used for the production of bast fibre and as an infusion. It is an annual or perennial herb or woody-based subshrub, growing to 2–2.5 m tall. The leaves are deeply three- to five-lobed, 8–15 cm long, arranged alternately on the stems.

The flowers are 8–10 cm in diameter, white to pale yellow with a dark red spot at the base of each petal, and have a stout fleshy calyx at the base, 1–2 cm wide, enlarging to 3–3.5 cm, fleshy and bright red as the fruit matures. It takes about six months to mature (Vaidya, 2000).

2.2.3 Common names of *Hibiscus sabdariffa*

The roselle is known as the *rosella* or *rosella fruit* in Australia. It is also known as *meśta*/*meshta* on the Indian subcontinent, *Tengamora* in Assam, *Gongura* in Telugu, *Mathipuli* in Kerala, *chin baung* in Myanmar, *bissap* in Senegal, Guinea Bissau, Mali, Burkina Faso, Ghana, Benin and Niger, the Congo and France, *dah* or *dah bleni* in other parts of Mali, *wonjo* in the Gambia, *zobo* in Nigeria (the Yorubas in Nigeria call the white variety

Isapa, *karkade* (□□ □□ □□; Arabic pronunciation: [ˈkarkade]) in Egypt, Saudi Arabia, and Sudan, *omutete* in Namibia, *sorrel* in the Caribbean and in Latin America, *Flor de Jamaica* in Mexico, *Saril* in Panama, *rosela* in Indonesia, *asam paya* or *asam susur* in Malaysia. In Chinese it is 洛神花 (*Luo Shen Hua*) . In Zambia the plant is called *lumanda* in ciBemba, *katolo* in kiKaonde, or *wusi* in chiLunda (Mohamad *et al*., 2002a)

2.2.4 Phytochemicals of *Hibiscus sabdariffa*

The plants are rich in anthocyanins and protocatechuic acid. The dried calyces contain the flavonoids, gossypetin, hibiscetine and sabdaretine (Chau *et al*., 2000). The major pigment, formerly reported as hibiscin, has been identified as daphniphylline. Small amounts of myrtillin (delphinidin 3-monoglucoside), Chrysanthenin (cyanidin 3-monoglucoside) and delphinidin are also present (Chau *et al*., 2000; Pau *et al*., 2002).

2.2.5 Uses of *Hibiscus sabdariffa*

According to Chau (2000), the plant is considered to have antihypertensive properties. Primarily, it is cultivated for the production of bast fibre from the stem of the plant. The fibre may be used as a substitute for jute in making burlap. Hibiscus, specifically Roselle, has been used in folk medicine as a diuretic, mild laxative and treatment for cardiac and nerve diseases and cancer (Mohamad *et al*., 2002b).

The red calyces of the plant are increasingly exported to America and Europe, where they are used as food colourings. Germany is the main importer. It can also be found in markets as flowers or syrup in some countries such as France, where there are Senegalese immigrant communities. The green leaves are used like a spicy version of spinach. They give flavour to the Senegalese fish and rice dish *thiéboudieune*. Proper records are not kept, but the Senegalese government estimates national production and consumption at 700 tons (770 short

tons) per year. Also in Myanmar, their green leaves are the main ingredient in making chin baung kyaw curry (Mohammad *et al*., 2008). In East Africa, the calyx infusion, called "Sudan tea", is taken to relieve coughs. Roselle juice, with salt, pepper, asafoetida and molasses, is taken as a remedy for biliousness (Vaidya, 2000).

The heated leaves are applied to cracks in the feet and on boils and ulcers to speed maturation. A lotion made from leaves is used on sores and wounds. The seeds are said to be diuretic and tonic in action and the brownish-yellow seed oil is claimed to heal sores on camels. In India, a decoction of the seeds is given to relieve dysuria, strangury and mild cases of dyspepsia. Brazilians attribute the stomachic, emollient and resolutive properties of the plant to the bitter roots (Pau *et al*., 2002). In Africa, especially the Sahel, roselle is commonly used to make a sugary herbal tea that is commonly sold on the street. The dried flowers can be found in every market. In Thailand, Roselle is drunk as tea, believed to also reduce cholesterol. According to Anon a (2010), it can also be made into wine: Hibiscus flowers are commonly found in commercial herbal teas, especially teas advertised as berry-flavoured, as they give a bright red colouring to the drink. Roselle is associated with traditional medicine, and is reported to be used as treatment for several diseases such as hypertension and urinary tract infections (Mohamad *et al*., 2002b).

2.2.6 Production of *Hibiscus sabdariffa*

China and Thailand are the largest producers and control much of the world supply of *Hibiscus sabdariffa*. According to Mohamad (2002[a]), Thailand invested heavily in roselle production and their product is of superior quality; whereas China's product, with less stringent quality control practices, is less reliable and reputable. The world's best roselle comes from the Sudan, but the quantity is low and poor processing hampers quality. Mexico,

Egypt, Senegal, Tanzania, Mali and Jamaica are also important suppliers, but production is mostly used domestically (Vaidya, 2000).

In the Indian subcontinent (especially in the Ganges Delta region), roselle is cultivated for vegetable fibres. Roselle is called meśta (or meshta, the *ś* indicating an *sh* sound) in the region. Most of its fibres are locally consumed. However, the fibre (and cuttings or butts) from the roselle plant have great demand in various natural fibre-using industries (Mohamad *et al*., 2008).

2.3 *Sorghum bicolor*

2.3.1 Plant Classification

(Watt and Breyer-Brandwijk, 1962).

Kingdom: *Plantae*; Subkingdom: *Tracheobionta*; Superdivision: *Spermatophyta*; Division: *Magnoliophyta*; Class: *Liliopsida*; Subclass: *Commelinidae*; Order*: Cyperales*; Family: *Poaceae*; Genus: *Sorghum*; Species: *S. bicolor* (L.) Moench.

2.3.2 Plant Description

This is a cane-like grass, up to 6 m tall with large branched clusters of grains. The individual grains are small - about 3-4 mm in diameter. They vary in colour from pale-yellow through reddish-brown to dark-brown depending on the cultivar. Most cultivars are annuals, few are perennials (Ricaud *et al*., 1981). Cultivated and most weedy sorghum are non-rhizomatous, culms nodes are either glabrous or shortly tomentose. The inflorescence is contracted. The branches of the inflorescence alternate.

Sorghum bicolor includes all cultivated sorghums as well as a group of semi wild plants often regarded as weeds (Stewart *et al*., 1979; Lipinsky and Kresovich, 1980). Historical records

and archeological data have not been able to clearly state the origin and domestication of *Sorghum bicolor*. Previously 571 cultivars were recognized, but these cross readily without barriers of sterility or difference in genetic balance. Therefore, it makes sense to group them into a single species. Although this makes it difficult for taxonomists to work with the species, it is an advantage to the plant breeder because they can manipulate the genetic make-up of this group to acquire best crops (Asmalu and Endashaw, 1998). Wild species are characterized by distinct ring of long hairs at the nodes, they have loose inflorescence with spreading branches (Denman, 1975). The branches of the inflorescence are whorled. The leaves look much like those of maize, they sometimes roll over. A single plant may have more than two types of leaves. The flower head carries two types of flowers, one type has no stalk and has both male and female parts, and the other flower is stalked and is usually male (Dibb, 1983).

2.3.3 Common names of *Sorghum bicolor*

Sorghum (English); *graansorghum* (Africaans); *mabele* (Pedi, Sotho, Ndebele); amabele (Zambia); *amazimba* (Xhosa); *dawa* (Hausa) (Gill *et al.*, 1977; Morton, 1981) .

2.3.4 Chemistry of *Sorghum bicolor*

Per 100 g, the seed is reported to contain 342 calories, 12.0 g H_2O, 10.0 g protein, 3.7 g fat, 72.7 g total carbohydrate, 2.2 g fibre, 1.5 g ash, 22 mg Ca, 242 mg P, 3.8 mg Fe, 8 mg Na, 44 mg K, 0.33 mg thiamine, 0.18 mg riboflavin, 3.90 mg niacin, and 0 mg ascorbic acid (Wu *et al.*, 1972). The seeds contain butyric, formic, myristic, palmitic, and stearic acids, maltose, emulsine, and are rather rich in vitamin B (Perry, 1980). Cultivars with highly pigmented seeds are rich in condensed catechin tannin, and other phenols (anthocyanins); these are usually the darker bird, insect, and/or fungus-resistant cultivars (Morton, 1981).

Based on 1046 analyses, Miller (1958) reported that the Dry Matter (DM) ranges from 71.0–96.3%, averaging 89.0%. On a zero-moisture basis, Crude Protein (CP) ranges from 8.7–16.8% (mean of 1160 cases, 12.5%), Ether Extract (EE) from 1.4–6.1 (mean of 1159, 3.4%), Crude Fibre (CF) from 0.4–13.4 (mean of 1085 cases, 2.7%), ash from 1.2–7.1% (mean of 1133, 2.2%), and 65.3–85.3% Nitrogen Free Extract (NFE) (mean 79.2%); Ca from 0.01–0.53% (mean of 227 cases, 0.05%), P from 0.10–0.52% (mean of 235 cases, 0.35%), Cu 2–19 ppm (mean of 38 cases = 11), K from 0.28–0.50% (mean of 16 cases, 0.38%), Mg from 0.02–0.25% (mean of 23 cases = 0.19), Fe from 0.000–0.018% (mean of 44 cases = 0.005%), and Mn from 0–27 ppm (mean of 42 cases 16 ppm), S from 0.15–0.21% (mean of 6 = 0.18%), Na from 0.01–0.09% (mean of 9 = 0.05%), Cl from 0.07–0.14 (mean of 7 cases = 0.10%), Co from 0.04–0.73 ppm (mean of 22 cases = 0.3), Zn from 12–19 ppm (mean of 2 = 15), 1.3–8.8 ppm thiamin (mean of 50 cases = 4.6), 0.4–5.7 ppm riboflavin (mean of 168 cases = 1.5), 3.3–24.2 ppm pantothenic acid (mean of 165 = 125), 19.4–92.6 ppm niacin (mean of 171 cases = 48.4), 2.2–10.3 ppm pyridoxine (mean of 46 = 5.9), 528–953 ppm chorine (mean of 13 = 761), and 0.2–5.1 ppm carotene (mean of 66 cases = 1.3).

Palmer and Bowden (1975) reported more than 20 4-demethylsterols, 4-monomethylsterols, and triterpenes. Of 254 analyses of dry roughage, DM ranged from 62.8–94.3% (mean 85.5%). On a zero moisture basis, the dry roughage contained 3.0–17.9% CP (mean of 256 = 7.9), 1.4–3.8% EE (mean of 256 = 2.5), 17.3–38.1% CF (mean of 256 = 26.1), 4.6–18.6% ash (mean of 546 = 8.5%), and 41.0–67.8% NFE (mean 55.0), 0.2–0.8% K (mean of 14 = 1.41%), 0.21–0.4% Mg (mean of 15 = 0.3%), 0.005% Fe, 40–150 ppm Mn (mean of 8115), 0.51–0.74% Cl (mean of 3 = 0.63). One Egyptian variety yielded a red dye substance containing durastantalin ($C_{16}H_{12}O_5$), quercemetrin, a flavanol, and a crystalline substance resembling pyrocatechol (Watt and Breyer-Brandwijk, 1962).

2.3.5 Phytochemicals of *Sorghum bicolor*

The active staining compound in *Sorghum bicolor* had been extracted and found to be a flavonoid (Avwioro *et al*., 2006), which was previously described as apigeninidin, (3-deoxypelargonidin) by Kouda-Bonafos *et al*., (1998).

2.3.6 Uses of *Sorghum bicolor*

Though sorghum is used largely for forage in the US, it is very important in the world's human diet, with over 300 million people dependent on it (Bukantis, 1980). It is grown for grain, forage, syrup and sugar, and industrial uses of stems and fibres. Sorghum grain is a staple cereal in hot-dry tropics, the threshed grain is ground into a wholesome flour. Stalks are used as animal feed and they are important summer fodder where temperatures are high and rainfall is insufficient for corn and they are most important for silage or green soiling, or for hay when grown irrigated in very dry areas. According to Ricaud *et al*. (1981), the pearled grain is cooked like rice or ground into flour. Sorghum, with large juicy stems containing as much as 10% sucrose, is used in manufacture of syrup; sugar can be manufactured from sorghum. Broomcorn is used for making brooms. The seed is used as food, in brewing "kiffir beer", the kiffir corn malt and cornmeal are fermented to make *Leting* (a sour mash); the pith is eaten, and the sweet culm chewed (Watt and Breyer-Brandwijk, 1962). Arubans make porridge and muffins from sorghum meal. Parched seed are used as coffee substitutes or adulterants (Morton, 1981).

2.3.6.1 Folk Medicine

Sorghum is reported to be antiabortive, cyanogenetic, demulcent, diuretic, emollient, intoxicant, and a poison. It is a folk remedy for cancer, epilepsy, flux, and stomach ache (Duke and Wain, 1981). The root is used for malaria in southern Rhodesia (*Zimbabwe*); the

seed has been used for breast disease and diarrhoea; the stem for tubercular swellings. In India, the plant is considered anthelminthic and insecticidal, and in South Africa, in combination with *Erigeron canadense* L., it is used for eczema (Watt and Breyer-Brandwijk, 1962). In China, where the seeds are used to make alcohol, the seed husk is braised in brown sugar with a little water and applied to the chest of measles patients. The stomachic seeds are considered beneficial in fluxes (Perry, 1980). According to Morton (1981), Curacao natives drink the leaf decoction for measles, grinding the seeds with those of the calabash tree (*Cresentia*) for lung ailments. Venezuelans toast and pulverize the seeds for diarrhoea. Brazilians decoct the seed for bronchitis, cough and other chest ailments, possibly using the ash for goitre. Arubans poultice hot oil packs of the seeds on the back of those suffering from pulmonary congestion. According to Grieve's Herbal (1931), a decoction of ca 50 g seed to a liter of water is boiled down to ca 1/2 litre as a folk medication for kidney and urinary complaints.

2.3.7 Production of *Sorghum bicolor*

According to Gills *et al*. (1977) in dry-land conditions, the seeds are normally sown in rows, 75–100 cm apart at the rate of 3–9 kg/ha; higher seed rates used for more humid areas. In good rainfall or under irrigation, the seeds should be closely drilled or broadcast at the rate of 20–35 kg/ha, this resulting in more leaf and less heavy stems, also obviating weeding. Seeds germinate best between 20–30°C (Ricaud *et al*., 1981). They are seeded in rows like corn in most areas (Lipinsky and Kresovich, 1980). Seeding may be as early as March, as in southern Texas; but date of planting depends on use for which crop is intended. In tropics, sorghums may be planted nearly anytime. Crop is sown on well-prepared, firm, moist seedbed. Seed is planted to depth of 1.5–5 cm depending on soil texture and moisture. Later on multiple plantings are often made to equalize forage production throughout the season. In subtropical climates, seeding in late summer or early fall may also be made. Sorghum hybrids are rather

sensitive to low pH and low P and K availability. According to Gills (1977), generally fertilizer with 30–60 kg/ha P and 60–120 kg/ha K is used. Good responses from N fertilizer up to 200 kg/ha have been obtained. Rotational or strip grazing is practiced (Logson, 1974). Weed control by chemicals or mechanical means are important as crop grows slowly in early stages. Cultivate or harrow once after plant emergence and later as required; usually 1–3 cultivations are necessary in tropics. Shallow cultivation is essential to prevent damage to surface roots. Constant rouging is necessary of off-type plants before flowering for both open pollinated and hybrid seed production (Reed, 1976).

2.3.7.1 Harvesting of *Sorghum bicolor*

According to Logson (1974), greatest dry matter yields are obtained at maturity, or when stems are 80–120 cm tall. Such heights are suitable for hay, silage, and green chop; best grazed at 20–30 cm height; best regrowth when 10–15 cm stubble is left. Some sorghum cultivars are very productive, yielding more dry matter than corn. Sorghum is cut when grain is in dough stage, and feeding value of fodder is at maximum (Ricaud *et al*., 1981). Harvesting for green chop or silage is well-suited to mechanical harvesting due to bulk and mass involved. Sorghum for silage should be harvested when seeds are in milk to dough stage. When used for hay, 2–5 harvests may be made per season, each with a potential yield of 2 Militon/hectre (MT/ha) or more. Hay is difficult to cure because of the thick culms, requiring several days in sunshine (Asmalu and Endashaw, 1998). A forage crusher helps reduce the time. Pasturing is cheapest method of harvesting forage. For seed, crop is cut by hand or mower, smaller dwarf types combined. If cut by hand, the heads are dried in heaps, either on the ground or on a threshing floor. If entire plant is cut by hand or binder, it should be stoked and left in field to dry and mature for 10–14 days, and then threshed. Seed is stored

at 12–13% moisture or less. Freshly harvested seed may show dormancy. Hard textured seed may be scarified to improve germination (Gills *et al*., 1977).

2.3.7.2 Yields and Economics

Seed yields may be as low as 200 kg/ha, or as high as 6,000 kg/ha, depending on cultivar and growing conditions; below 2,000 kg/ha is considered not profitable. Average forage yields for silage include; Sorgo, 'Start', 54.3 Mili ton/hectre (MT/ha); 'Honey', 48 MT/Iha; 'Atlas', 42 MT/ha; Sorgo hybrids, 43.4–71.4 MT/ha (Reed, 1976). Sorghum is the fourth most important world cereal grain, following wheat, rice, and corn. Worldwide, grain sorghum is grown on more than 40 million hectares, especially in China, India, and Africa. Grain sorghum hybrids are becoming increasingly available to American farmers as a result of the discovery of cytoplasmic male sterility in 1952. Dibb (1983) compares US yields of 3,300 kg/ha with 900 kg/ha in the developing countries and a reported world record of 21,500 kg/ha. In 1979, the world low production yield figure was 260 kg/ha grain in Botswana, the international production yield, 1,322 kg/ha, and the high, 5,326 kg/ha in Spain (FAO, 1979).

2.4 *Garcinia livingstonei*

2.4.1 Plant Classification (Anderson, 2010)

Kingdom: *Plantae*; Subkingdom: *Tracheobionta*; Superdivision: *Spermatophyta*; Division: *Magnoliophyta*; Class: *Magnoliopsida*; Subclass: *Delleniidae*; Order*: Theales*; Family: *Clusiaceae*; Genus: *Garcinia L.*; Species: *G. livingstonei.*

2.4.2 Plant Description

Garcinia livingstonei is a small tree reaching 18 m in East Africa, pyramidal when young but later spreading, with thick, woody young branches and yellow to red resin. Leaves are usually

3 in a whorl (may be 4 or opposite), variable in shape, but are usually egg- or lance-shaped, generally 60-110 × 30-55 mm, blue-green with whitish veins and waxy. Flowers are in groups of 5-15 in leaf axils on old wood, greenish, whitish or yellow, scented, male and bisexual flowers of different structure (Pooley, 1993). Fruit is an orange berry, 10-40 mm in diameter, with yellowish orange and sticky juice.

The genus *Garcinia* includes some 200, mainly Asian species (Mabberley, 2002). The name commemorates one Laurent Garcin (1683-1751), a French botanist who was active in India, where the genus is particularly diverse. The specific epithet honours the great explorer David Livingstone (1813-1873), who made one of the first records of the tree.

In India and the Far East, some dozens of species of *Garcinia* supply a wide variety of useful and even necessary items. One, *G. mangostana* (the true mangosteen) is so important that it is widely known. Mabberley lists three other Asian species with edible fruits. At least two other species, *G. cambogia* and *G. indica* provide flavourings for curries; the latter is also the source of the edible fat called Goa butter. *G. xanthochymus* and other species yield the resins that are processed into the artists' pigment Gamboge Yellow; the same pigment is also used in dyeing Buddhist priests' robes (Killick and Robson, 1976; Bamps *et al*., 1978; Mabberley, 2002; Balkwill *et al.*, 2004). Curiously, the resident fruit bats in Durban Botanical Garden seem not to eat the fruits of this tree, though they make heavy use of everything else that is edible. *G. mannii* is used in Cameroon as chewing sticks. The only other species indigenous to South Africa is *Garcinia gerrardii,* which has emerald green, opposite leaves, not blue-green leaves in whorls of three or four (Bamps *et al*., 1978).

2.4.3 Common names of *Garcinia livingstonei*

African mangosteen (English); *Afrika-geelmelkhout* (Africaans); *umPhimbi, uGobandlovu* (isiZulu); *mmimbi* (xiTsonga); *mokongono, mokononga* (siPedi) ; *jawa* (Hausa) (Bamps *et al*., 1978).

2.3.4. Phytochemistry

A series of 13 known compounds, including seven benzophenones [guttiferone A (**1**), guttiferone K (**2**), xanthochymol (**3**), guttiferone E (**4**), cycloxanthochymol (**5**), isoxanthochymol (**6**), and gambogenone (**7**)], five biflavonoids [amentoflavone (**8**), 3,8″-biapigenin (**9**), (+)-volkensiflavone (**10**), (+)-morelloflavone (**11**), and (+)-fukugiside (**12**)], and the xanthone derivative alloathyriol (**13**), were identified from the fruits of *Garcinia livingstonei* (Clusiaceae) (Hui *et al.*, 2010).

2.4.5 Uses of *Garcinia livingstonei*

Mangosteens are known for their delicious fruits, and the African mangosteen is no exception to this. Despite the sticky yellow juice, the fruits of this tree are well worth seeking out. The tree is used in traditional medicine, and in particular the powdered root is used as an aphrodisiac. Coates (2002) reports that the fruits can be fermented into a 'pleasant alcoholic beverage'; almost any book on English country wines will give half-a-dozen recipes which could be adapted to make use of these fruits. The wood is susceptible to borers, but has been used as a general-purpose timber (Balkwill *et al*., 2004).

2.4.6 Production of *Garcinia livingstonei*

According to Coates (2002), the African mangosteen easily earns a place in any warm, subtropical garden. The form of the tree resembles a piece of angular modern sculpture, and

the leaves and young branches are an almost unique shade of deep blue-green. The fruits are memorably delightful, and no doubt will cause intense competition between gardeners, neighbours, birds and monkeys, anyone who knows good eating (Duke and Wain, 1981)!

The fact that African mangosteens appear regularly on the Botanical Society plant sale in Durban indicates that they are available as small trees from nurseries, at least in this area. Although it may be harder and possibly less legal to find seeds than nursery-grown plants, Pooley (1993) reported that these trees can easily be grown from seed. Not surprisingly, they are known to be sensitive to cold.

2.5 FIXATION

2.5.1 Purpose of fixation

The purpose of fixation is to preserve a sample of biological material (tissue or cells) as close to its natural state as possible in the process of preparing tissue for examination. To achieve this, several conditions must usually be met (Fox *et al*., 1985; Foss *et al*., 1994). First, a fixative usually acts to disable intrinsic biomolecules – particularly proteolytic enzymes – which would otherwise digest or damage the sample. Second, a fixative will typically protect a sample from extrinsic damage. Fixatives are toxic to most common microorganisms (bacteria in particular) which might exist in a tissue sample or which might otherwise colonise the fixed tissue (Noguchi *et al*., 1997). In addition, many fixatives will chemically alter the fixed material to make it less palatable (either indigestible or toxic) to opportunistic microorganisms. Finally, fixatives often alter the cells or tissues on a molecular level to increase their mechanical strength or stability. This increased strength and rigidity can help preserve the morphology (shape and structure) of the sample as it is processed for further analysis (Basyuk *et al*., 2000). Even the most careful fixation does alter the sample

and introduce artifacts that can interfere with interpretation of cellular ultrastructure. A prominent example is the bacterial mesosome, which was thought to be an organelle in gram-positive bacteria in the 1970s, but was later shown by new techniques developed for electron microscopy to be simply an artifact of chemical fixation. According to Beckstead, (1994), standardization of fixation and other tissue processing procedures take this introduction of artifacts into account, by establishing what procedures introduce which kinds of artifacts. Researchers who know what types of artifacts to expect with each tissue type and processing technique can accurately interpret sections with artifacts, or choose techniques that minimize artifacts in areas of interest (Giannella *et al*., 1997).

2.5.2 Fixation process

Cross *et al*. (1990) stated that fixation is usually the first stage in a multistep process to prepare a sample of biological material for microscopy or other analysis. Therefore, the choice of fixative and fixation protocol may depend on the additional processing steps and final analyses that are planned (Mayers, 1970). For example, immunohistochemistry utilizes antibodies which bind to a specific protein target. Prolonged fixation can chemically mask these targets and prevent antibody binding. In these cases, a 'quick fix' method using cold formalin for around 24 hours is typically used (Sung *et al*., 2000).

2.5.3 Types of fixation

There are generally three types of fixation process;

2.5.3.1 Heat Fixation: After a smear has been allowed to dry at room temperature, the slide is gripped by tongs or a clothespin and passed through the flame of a Bunsen burner several times to heat-kill and adhere the organism to the slide (Werner *et al*., 2000).

2.5.3.2 Perfusion: Fixation via blood vessel. The fixative is injected into the heart with the injection volume matching cardiac output. The fixative spreads through the entire body, and the tissue does not die until it is fixed (Cross *et al.*, 1990). This has the advantage of preserving perfect morphology, but the disadvantages that the subject dies and the cost is high (because of the volume of fixative needed for larger organisms).

2.5.3.3 Immersion: The sample of tissue is immersed in fixative of volume at a minimum of 20 times greater than the volume of the tissue to be fixed. The fixative must diffuse through the tissue in order to fix, so tissue size and density, as well as the type of fixative must be taken into account. Using a larger sample means it will take longer for the fixative to reach the deeper tissue (Beckstead, 1994).

2.5.3.4 Chemical Fixation

According to Fox *et al*., (1985), this is a process whereby structures are preserved in a state (both chemically and structurally) as close to living tissue as possible. This requires a chemical fixative which can stabilise the proteins, nucleic acids and mucosubstances of the tissue by making them insoluble. Chemical fixatives of choice are as shown in Table I.

2.5.4 Fixation and Its Effect on Tissue Nucleic Acids

2.5.4.1 Fixation

As early as 400 BC Hippocrates discussed the biological effects of mercury and alcohol as a fixative (Feeney-Burns, 1985; Fox *et al*., 1985). However, curiosity about the histological structure of tissues began only with the invention of the microscope. Even then, the early microscopists were preoccupied with improving their scopes and cared little about the specimens, for anything visible was impressive by the sheer novelty, once attention was directed toward it by a lens (Feeney-Burns, 1985; Fox *et al*., 1985). A century and

nanomicron magnifications later, the importance of the quality of the specimen for accurate validity of the data were realized. With the advent of technology for developing monoclonal antibodies that would recognize specific antigens, the staining techniques were perfected for *in situ* identification of proteins using tissue sections (Leong *et al.*, 1996). Several reviews have discussed extensively the effects of fixation and fixatives on immunohistochemical detection of antigenic proteins.

2.5.4.2 Pre-fixation Parameters

Ideally, tissues should be fixed immediately and completely from the living state. In animal experiments, this can be achieved by *in vivo* perfusion. This is not possible for human tissues for obvious reasons. Most tissues are removed surgically, the tissue is relatively anoxic for some period because of anaesthesia and the placement of surgical clamps and ligatures to stop bleeding. The transient anoxia, the changes in local pH, and other environmental factors may lead to increased transcription of some genes (Foss *et al.*, 1994; Werner *et al.*, 2000). However, the variable factors of prefixation are primarily determined by the nature of the surgery and as such cannot be manipulated.

2.5.4.3 Pre-fixation Time

By definition the prefixation time refers to the time lapse from the surgical excision of the specimen to the fixative (Godfrey *et al.*, 2000). Significant biochemical alterations occur in tissues within 10 minutes after anoxia (Kingsbury *et al.*, 1995; Labat-Moleur *et al.*,1998). Hence, the prefixation time should be kept at the minimum to minimize ribonucleic acid (RNA) and protein degradation, especially for tissues with high levels of ribonucleases (RNases) and proteases, for example, pancreas, gall bladder, and skin (Hipfel *et al.*, 1998). Any surgical procedure *ex vivo* including grossing of the specimen and sectioning to smaller

pieces should be performed immediately to prevent loss of enzymes, mitochondrial damage, decrease in histological mitosis, and possible transcription of apoptotic factors because of the ensuing anoxia (Williams *et al*., 1999). A loss of 30 to 50% of mitotic figures because of delay in fixation by 2 to 6 hours has been reported (Cross *et al*., 1990). This progressive fall in mitotic index has been implicated to introduce errors in the grading of breast carcinoma.

2.5.5 Effect of Formaldehyde Concentration, pH, and Temperature on Tissue Fixation

The overall rate of formalin-induced modification of deoxyribonucleic acid (DNA) is dependent on the concentration, temperature, and pH of the fixative. It has been shown that when reacted at 5°C below the melting temperature, the denaturation rate of DNA increases with increasing concentration of formaldehyde (McGhee and Von, 1977). Formaldehyde fixation at room temperature results in poor preservation of high-molecular weight DNA, the size of the extracted DNA being directly related to the fixation temperature. Up to 30% of nucleic acids may be lost during fixation (Cross *et al*., 1990; Cross and Start, 1996). Several studies suggest that tissues fixed in formalin at 4°C exhibit the least amount of degradation of the nucleic acids (Tokuda *et al*., 1990; Yagi *et al*., 1996; Noguchi *et al*., 1997). This should be kept in the physiological range, between pH 4-9. The pH for the ultrastructure preservation should be buffered between 7.2 to 7.4. Hypertonic solutions give rise to cell shrinkage and can result in cell swelling and poor fixation. Increasing the temperature will increase speed of fixation. However care is needed not to cook the specimen (Ryter, 1988; Friedrich *et al*., 2000).

Prolonged tissue hypoxia reduces pH in tissues locally decreasing the yield of quantifiable nucleic acids (Kingsbury *et al*., 1995). So also, formaldehyde fixation at acidic pH or in the presence of formic acid causes degradation of tissue nucleic acids. As compared to neutral

buffered formalin, tissues fixed in formaldehyde solution at pH 3.0 had a greater number of artificial mutations (Kingsbury *et al*., 1995). It has been suggested that a higher rate of depurination at lower pH can favour misincorporation of non-complementary nucleotides (cytosine in place of thymine) at apurine and apyrimidine sites (Douglas and Rogers, 1998). Although there have been several reports of successful amplification of DNA from neutral buffered formalin-fixed tissues, the PCR products are generally 500 bp or less (Yagi *et al*., 1996; Noguchi *et al*., 1997).

The low-molecular weight DNA may be the result of scission of phosphodiester backbone of DNA (Douglas and Rogers, 1998). Interestingly, secondary fixation in paraformaldehyde at high pH has been shown to drastically improve the signal of *in situ* hybridization of both low abundant (zinc finger transcription factor) and high abundant (actin) mRNA in human mammary gland tissue (Basyuk *et al*., 2000).

2.5.6 Alternative Fixatives

A method to overcome the problems of formaldehyde is to use an alternative fixative that is better suited for the preservation of nucleic acids and proteins. Some of the alternative fixatives are discussed below.

2.5.6.1 Glutaraldehyde

Like formaldehyde, glutaraldehyde is another cross-linking agent for tissue fixation (Douglas and Rogers, 1998). An aqueous solution of glutaraldehyde (glutaric dialdehyde) is a complex mixture consisting of approximately 4% free aldehyde, 16% monohydrate, 9% dihydrate, and 70% hemiacetal. Using its aldehyde functional groups, glutaraldehyde reacts primarily with the e-amino groups of amino acids and nucleic acids. The coefficient of diffusibility of 4% glutaraldehyde at 4°C is approximately half that of 4% formaldehyde solution (Bancroft and

Stevens, 1996; Leong *et al.*, 1996). Although widely used as a fixative for standard electron microscopy, the slow penetration and the need for periodic purification to maintain the functional aldehyde levels have greatly limited its use as a biological fixative. However, it has been shown that 1% glutaraldehyde at pH 7.0 better preserves high-molecular weight DNA as compared to 10% formalin (Douglas and Rogers, 1998).

2.5.6.2 Genipin

Recently, a naturally occurring cross-linking agent, genipin, has been shown by Noguchi et *al*. (1997) to react spontaneously with amino acids to form a dark blue pigment. After fixation with genipin, the resistance of the tissue against collagenase degradation increased significantly. It has been suggested that genipin may form intramolecular and intermolecular crosslinks with cyclic structure within collagen in biological tissues. Although no apparent cytotoxicity was observed, the effect of genipin fixation on tissue nucleic acids is not known (Sung *et al*., 2000).

2.5.7 Organic Solvents or Alcoholic Fixatives

2.5.7.1 Ethanol and Methanol

Several studies have indicated that non-cross linking alcoholic reagents always yielded superior results as nucleic acid fixatives than aldehydes (Bancroft and Stevens, 1996; Leong *et al*., 1996; Giannella *et al*., 1997; Noguchi *et al*., 1997). Ethanol and methanol preserve nucleic acids better because they bring about little chemical change. Physical and chemical measurements have shown that DNA is largely collapsed in ethanol (65% v/v) and methanol. When the denatured DNA is rehydrated, there is substantial reversion to the original form (Bancroft and Stevens, 1996). The presence of salts is essential for maximum precipitation of nucleic acids from alcohol. This does not interfere with the subsequent use of nucleases, but

there may be shrinkage and hence not suited for morphological studies (Leong *et al*., 1996). One hundred percent ethanol and methanol are known to be excellent fixatives for preserving both high-molecular weight DNA and RNA. The low molecular weight and rapid tissue penetration of the alcohol is thought to contribute to the uniform tissue fixation and minimal loss of tissue components (Giannella *et al*., 1997; Noguchi *et al.*, 1997).

2.5.8 Mixtures of Fixatives

Since no single fixative is ideal for the preservation of all tissue components, mixtures of fixatives have been tried in an attempt to compensate the shortcomings of one by another.

2.5.8.1 Carnoy's Fixative

Ethanol, 60%; Chloroform, 30%; and Glacial Acetic Acid, 10%: According to Foss *et al*. (1994), isolated studies suggest Carnoy's fixative is best for optimal preservation of nucleic acids in tissues. When compared to neutral buffered formalin (NBF) fixation, RNA was found to be easily extractable from Carnoy's-fixed mouse liver and well preserved with only a slight degradation of high-molecular weight RNA (Foss *et al*., 1994). Formaldehyde vapor post-fixation reduces the loss of cellular RNA from the Carnoy's-fixed tissue that may occur during treatments before *in situ* hybridization (Urieli-Shoval *et al*., 1992). It has been suggested that at centres without access to frozen-tissue banking, fixing tissues in alcohol-based fixatives should be considered as an alternative for preserving the tissue molecular integrity, at least for those limited tissues on which subsequent RNA/DNA analysis may be of interest (Giannella *et al*., 1997).

2.5.8.2 Methacarn (Methanol, 60%; Chloroform, 30%; and Glacial Acetic Acid, 10%) Shibutani *et al*. (2000).

Substitution of methanol for ethanol in Carnoy's fixative gives methacarn, which has been shown to be an excellent fixative for preserving tissue RNA. Using cultured cell lines and rodent tissues, Shibutani *et al*. (2000) have shown that not only the extraction efficiency but also the integrity of the extracted total RNA from methacarn-fixed tissues are comparable to that from unfixed frozen cells. Furthermore, the concentration of contaminating genomic DNA is very low in methacarn-fixed tissues. In contrast to formalin-fixed tissues, 300- to 700-bp fragments of both abundant mRNA and low copy number RNA species could be amplified from methacarn-fixed tissues (Shibutani *et al*., 2000). It has been hypothesized by Borrebaeck (1998) that the methacarn fixation causes precipitation of ribosomal proteins in the cells inactivating endogenous RNases and/or masking the mRNA from RNase action. Methacarn fixation is superior to NBF in retaining antigen immunoreactivity and does not require antigen retrieval. It has been recommended for prospective immunohistochemical studies and assessing mitotic indices in tissues (Beckstead, 1994; Borrebaeck, 1998). Using methacarn-fixed, paraffin sections from fetal and infant breast, immunostaining with a panel of antibodies to cytoskeletal proteins and kappa-casein, it has been possible to define in detail the chronological evolution of the major cell types in the human breast from 16 weeks of intrauterine life to two years of age, in both sexes (Beckstead, 1994; Borrebaeck, 1998).

2.5.8.3 Acetone

Acetone has been used as a fixative in the acetone-methylbenzoate-xylene (AMeX) technique (Sato *et al*., 1990). The method involves overnight fixation of tissues in acetone at -20°C, then clearing in methylbenzoate and xylene before paraffin embedding. The product has been shown to preserve good morphology while retaining the immunoreactivity for labile proteins

such as lymphocyte membrane antigens. Significantly, AMeX-processed tissues yielded good quality spooled high-molecular weight DNA suitable for molecular analysis. In addition, specific signals (c-*myc* mRNA and albumin mRNA) comparable to RNA from fresh-frozen tissues could be detected by dot-blot hybridization using RNA isolated from AMeX-fixed tissues (Sato *et al*., 1991; Sato *et al*., 1992; Pavelic *et al*., 1996) .

2.5.8.4 HOPE Fixation

Recently, Olert *et al.* (2001) reporteda novel method of fixation that is compatible forcomplete pathological analysis, including retaining relevant positive features for both immunohistochemistry and molecular pathology. The HOPE technique (Hepes-glutamic acid buffer-mediated organic solvent protection effect) of Olert *et al.* (2001) consists of incubation of fresh tissues in a protecting solution, comprising a mixture of amino acids at pH 5.8 to 6.4. The protection solution is thought to penetrate the tissues by diffusion. This is followed by incubation in acetone at 0 to 4°C for dehydration. It is suggested that the protective solution also helps in keeping the capillaries open for the infusion of acetone, thus accelerating dehydration (Oliver and Jamur, 1999). Finally the specimens are directly transferred into low-melting paraffin and subsequently embedded in paraffin. Essentially, HOPE-fixed sections have been shown to exhibit formalin-like morphology and provide an excellent preservation of proteins and antigenic structures for differential analysis by immunohistochemical and/or enzyme histochemical techniques. More importantly, HOPE-fixed specimens yielded sufficient amounts of good quality DNA and RNA even after a period of five years (Olert *et al*., 2001). The DNA and RNA were suitable for further molecular analysis by PCR, RT-PCR, and *in situ* hybridization. Absence of cross-linking and greater yield of extractable nucleic acids suggest that HOPE fixation offers a distinct alternative fixation method for projects that aim at establishing tissue banks (Foss *et al*., 1994).

2.5.9 Other Fixatives

The use of additional fixatives that incorporate picric acid (Bouin's solution), or mercuric chloride (B5, Zenker's, Helly's, and Ridley's solutions) or tannic acid to increase tissue penetration, or that contain phenol or heavy metal solutions such as zinc sulfate to improve protein precipitation have been validated in immunohistochemical studies, but there is paucity of data in their use as nucleic acid preserving agents (Urieli-Shoval *et al.*, 1992). Several studies have shown that B5 is a poor fixative for nucleic acid preservation (Leong *et al.*, 1996).

2.5.10 Microwave (MW) Irradiation

Electromagnetic waves with a frequency between 300 MHz and 300 GHz are classified as microwaves (Login and Dvorak, 1994). Microwave (MW) irradiation for tissue fixation was first introduced by Mayers (1970), who reported that direct exposure to microwaves produced satisfactory fixation in both mouse and human post-mortem tissues. MW fixation depends on the chemical environment around the specimen during irradiation, duration of microwave exposure, and sequence of microwave irradiation and other chemical or physical fixation methods. Thus, five microwave fixation methods have been defined. 1) Stabilization wherein specimens are subjected to microwave irradiation *in situ* or when immersed in a physiological saline solution in an attempt to preserve structures without the superimposed effects of a chemical fixative. 2) Fast or ultrafast primary microwave chemical fixation in which specimens are irradiated by microwave energy in a chemical environment for a short period of time, ranging from milliseconds to seconds. 3) Microwave irradiation followed by chemical fixation involves continued immersion of specimens in a chemical fixative such as formaldehyde for minutes to hours after MW irradiation to improve uniformity of fixation. 4) Primary chemical fixation followed by MW irradiation to facilitate crosslinking of fixatives

within the specimen. 5) Microwave irradiations in combination with freeze fixation limits freezing artifacts. In general, irradiation times less than 60 seconds, final irradiation temperature between 50 to 55℃ and solution volume less than 50 ml in containers with at least one dimension that is approximately 1 cm are recommended for good MW fixation (Login *et al*., 1998).

The major advantage of microwave stabilization is that there are no chemicals involved that would extract important components from the tissue. However, significant disadvantages in this method include shrinkage, sponginess of tissue, and breakdown of red blood cells (Leong, 1991).

According to Boon et al. (1988), fast primary MW fixation can be used to enhance diffusion of fixation reagents into the tissue, and to accelerate the chemical process by which the fixative cross-links with the protein of the tissue. The reduced exposure to cross-linking chemicals makes it an attractive alternative method of fixation for ultrastructural and genetic studies. However, simply microwaving tissue in formalin often produces disappointing results because the outside of the tissue fixes so rapidly and well that it effectively prevents further diffusion of fixative into the central part of the biopsy. Hence, a hybrid procedure consisting of first soaking tissue blocks in formalin for a few hours at room temperature followed by microwave irradiation for 1 to 2 minutes at 55℃ has been recommended. Excellent immunostaining has been achieved using this hybrid method of fixation (Login and Dvorak, 1994a). The speed with which microwaves can accomplish fixation of both large and small biopsy specimens is a major asset. The MW fixation method considerably reduces the processing time with one-step dehydration and one-step clearing before paraffin infiltration (Leong, 1991; Ruijter *et al*., 1997).

Household MW ovens provide an economical method for accelerated sample preparation. However, they have severe limitations in terms of safety and reproducibility. The magnetrons with variable warm-up time causes inconsistent production of MW power resulting in non-uniform MW fields. In addition, the MWs have restricted penetration ability that is further limited by the poor heat-conducting properties of the tissues (Login and Dvorak, 1994[b]). In an attempt to overcome these limitations, experimental tools have been developed to standardize and calibrate MW ovens for tissue fixation. The neon bulb calibration tool maps regions of high and low power in a MW oven. Agar-saline-Giemsa tissue phantoms help determine the MW irradiation conditions that will uniformly heat a tissue (Login *et al.*, 1998). Using hepatitis B virus DNA sequences and the type I human procollagen gene as markers and liver tissue as a target, Hsu *et al.* (1991) have shown that microwave fixation in phosphate-buffered saline is superior to routine formalin for the preservation of excellent quality of genomic and viral DNAs for nucleic acid hybridization analysis. However, the morphological preservation of various tissues depends on generation of an optimal temperature for each tissue, ranging between 70°C and 85°C. Heating above or below these temperatures creates various artifacts such as vacuolation and changes in chromatin pattern (Hsu *et al.*, 1991).

2.5.11 Classification of fixatives:

Fixatives may be classed as precipitant (P) or non-precipitant (NP) according to their effect on tissue protein (George *et al.*, 1981; Werner *et al.*, 2000). Primary fixatives that are precipitant include; chromium trioxide, ethanol, mercuric chloride, methanol, picric acid and non-precipitant fixatives are acetic acid, formaldehyde, osmium tetroxide and potassium dichromate (George *et al.*, 1981).

2.5.12 Tissue Processing - Dehydration, Clearing and Infiltration

The aim of tissue processing is to remove water from tissues and replace with a medium that solidifies to allow thin sections to be cut. Biological tissue must be supported in a hard matrix to allow sufficiently thin sections to be cut, typically 7 μm (micrometres; 1000 micrometres = 1 mm) thick for light microscopy and 80-100 nm (nanometre; 1,000,000 nanometres = 1 mm) thick for electron microscopy. For light microscopy, paraffin wax is most frequently used. Since it is immiscible with water, the main constituent of biological tissue, water must first be removed in the process of dehydration. Samples are transferred through baths of progressively more concentrated ethanol to remove the water (Werner *et al*., 2000). This is followed by a hydrophobic clearing agent (such as xylene) to remove the alcohol, and finally molten paraffin wax, the infiltration agent, which replaces the xylene. Paraffin wax does not provide a sufficiently hard matrix for cutting very thin sections for electron microscopy. Instead, resins are used. Epoxy resins are the most commonly employed embedding media, but acrylic resins are also used, particularly where immunohistochemistry is required (Drury *et al*., 1967). Thicker sections (0.35 μm to 5 μm) of resin-embedded tissue can also be cut for light microscopy (Fox *et al*., 1985). Again, the immiscibility of most epoxy and acrylic resins with water necessitates the use of dehydration, usually with ethanol.

2.5.12.1Embedding

After the tissues have been dehydrated, cleared, and infiltrated with the embedding material, they are ready for external embedding. During this process the tissue samples are placed into molds along with liquid embedding material (such as agar, gelatine or wax) which is then hardened (Beckstead, 1994). This is achieved by cooling in the case of paraffin wax and heating in the case of the epoxy resins (curing). The acrylic resins are polymerised by heat,

ultraviolet light or chemical catalysts. The hardened blocks containing the tissue samples are then ready to be sectioned.

Formalin-fixed, paraffin-embedded (FFPE) tissues may be stored indefinitely at room temperature, and nucleic acids (both DNA and RNA) may be recovered from them decades after fixation, making FFPE tissues an important resource for historical studies in medicine (Oliver and Jamur, 1999).

Embedding can also be accomplished using frozen, non-fixed tissue in a water-based medium. Pre-frozen tissues are placed into molds with the liquid embedding material, usually a water-based glycol, OCT, TBS, Cryogel or resin, which are then frozen to form hardened blocks.

2.5.12.2Sectioning

Sectioning can be done in a variety of ways. Vertical sectioning perpendicular to the surface of the tissue is the usual method. Horizontal sectioning is often done in the evaluation of the hair follicles and pilosebaceous units (Drury *et al*., 1967). Tangential to horizontal sectioning is done in Mohs surgery and in methods of CCPDMA (Ruijter *et al*., 1997).

For light microscopy, a steel knife mounted in a microtome is used to cut 7-micrometer-thick tissue sections which are mounted on a glass microscope slide. For transmission electron microscopy, a diamond knife mounted in an ultramicrotome is used to cut 50-nanometer-thick tissue sections which are mounted on a 3-millimeter-diameter copper grid. Then the mounted sections are treated with the appropriate stain (Bancroft and Stevens, 1996).

2.5.13 Frozen Sections

Frozen tissue embedded in a freezing medium is cut on a microtome in a cooled machine called a cryostat. Small pieces of tissue (5 x 5 x 3 mm) are placed in a cryoprotective embedding medium - OCT, TBS or Cryogel - then snap frozen in isopentane cooled by liquid nitrogen. Tissue is then sectioned in a freezing microtome or cryostat. Sections are then fixed in one of the following fixatives: absolute acetone for 10-15 minutes, 95% ethanol for 10-15 minutes or absolute acetone for 10minutes followed by 95% ethanol for 10minutes (Ryter, 1988).

2.5.13.1Advantages of frozen sections

- They give a better preservation of antigenicity
- Require minimal exposure to fixative
- They are not exposed to the organic solvents

2.6 STAINS

According to Bancroft, (1975) biological tissue has little inherent contrast in either the light or electron microscope. Staining is employed to give both contrast to the tissue as well as highlighting particular features of interest. Where the underlying mechanistic chemistry of staining is understood, the term histochemistry is used. Hematoxylin and Eosin (H&E) are the most commonly used light microscopic stains in histology and histopathology. Hematoxylin, a basic dye, stains nuclei blue due to an affinity to nucleic acids in the cell nucleus; eosin, an acidic dye, stains the cytoplasm pink. Uranyl acetate and lead citrate are commonly used to impart contrast to tissue in the electron microscope.

2.6.1 Special staining

There are hundreds of various other techniques that have been used to selectively stain cells and cellular components. Other compounds used to colour tissue sections include safranin, oil red O, Congo red, fast green FCF, silver salts, and numerous natural and artificial dyes that were usually originated from the development of dyes for the textile industry (George *et al*., 1981).

2.6.2 Histochemistry and Autoradiography

Histochemistry refers to the science of chemical reactions between laboratory chemicals and components within tissue. A commonly performed histochemical technique is the Perls Prussian blue reaction, used to demonstrate iron deposits in diseases like hemochromatosis (Bancroft, 1975).

Histology samples have often been examined by radioactive techniques. In historadiography, a slide (sometimes stained histochemically) is X-rayed. More commonly, autoradiography is used to visualize the locations to which a radioactive substance has been transported within the body, such as cells in S-phase (undergoing DNA replication) which incorporate tritiated thymidine, or sites to which radiolabeled nucleic acid probes bind in *in situ* hybridization. For autoradiography on a microscopic level, the slide is typically dipped into liquid nuclear tract emulsion, which dries to form the exposure film. Individual silver grains in the film are visualized with dark field microscopy (Bancroft and Stevens, 1996).

2.7 Pre- and Post-histology Artifacts

Artifacts are structures or features in tissue that interfere with normal histological examination. These are not always present in normal tissue and can come from outside

sources (Culling, 1974; Bancroft, 1975; George *et al.,* 1981). Artifacts interfere with histology by changing the tissues appearance and hiding structures. These can be divided into two categories:

Pre-histology artifacts are features and structures that have been introduced prior to the collection of the tissues. A common example of these include: ink from tattoos and freckles (melanin) in skin samples (George *et al.,* 1981).

Post-histology artifacts can result from tissue processing. Processing commonly leads to changes like shrinkage, color changes in different tissue types and alterations of the structures in the tissue. Since these are caused in a laboratory, the majority of post histology artifacts can be avoided or removed after being discovered. A common example is mercury pigment left behind after using Zenker's fixative to fix a section (George *et al.,* 1981).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Reagents

Ethanol absolute, normal saline solution, xylene, picric acid, formaldehyde, acetic acid, hydrochloric acid, sodium bicarbonate, haematoxylin, eosin, Canada balsam, egg albumin, paraffin wax 63^{o}C. All reagents used were of analytical grade and were prepared in all glass distilled water.

3.2 Fixatives

TABLE II: SIX COMMON FIXATIVES AND THEIR COMPOSITIONS

S/No.	Fixative	Formula
1	Buffered neutral formalin	Formalin 100.0 ml Na_2HPO_4 (anhydrous) 6.5 g $NaH_2PO_4.H_2O$ 4.0 g Distilled water 900.0 ml
2	Heidenhain's SUSA fluid	$HgCl_2$ 4.5 g NaCl 0.5 g Formalin 20.0 ml Trichloracetic acid 4.0 ml Distilled water 80.0 ml
3	Calcium acetate formalin	Formalin 10.0 ml Calcium acetate (monohydrate) 2.0 g Distilled water 100.0 ml
4	Muller's fluid	$K_2Cr_2O_7$ 2.5 g Na_2SO_4 1.0 g Distilled water 100.0 ml

5	Helly's fluid	$K_2Cr_2O_7$	2.5 g
		$HgCl_2$, to saturation	5-8 g
		Distilled water	100.0 ml
6	Carnoy's fluid	Alcohol, absolute	60.0 ml
		Chloroform	30.0 ml
		Glacial acetic acid	10.0 ml

Source: Adapted from Bancroft (1975).

3.3 Plant Material and Extraction

3.3.1 *Hibiscus sabdariffa*

The calyx of *Hibiscus sabdariffa* (Sobo) was purchased in Maiduguri Monday Market and was identified at the University of Maiduguri, Department of Botany, Faculty of Sciences and was compared with voucher specimen deposited at the department. The calyx was thoroughly washed and dried in the laboratory for four days. The dried calyx was pounded to a fine powder using pestle and mortar and then stored in a dry cylinder. The powdered calyx was extracted with 75% ethanol using Soxhlet apparatus for six hours. The solution obtained was concentrated in the oven at a temperature of 45^{o} C, and further dried into a fine powder at a temperature of 50^{o} C (Wiam *et al*. 2006).

The same procedure was adopted for *Garcinia livingstonei* and *Sorghum bicolor* but here aqueous extraction was carried out using a Soxhlet apparatus for six hours.

Table III: The composition of *Hibiscus sabdariffa, Garcinia livingstonei* and *Sorghum bicolor* solutions used in this study.

S/No.	Composition of staining solutions	*Hibiscus sabdariffa*	*Garcinia livingstonei*	*Sorghum bicolour*
1.	Powdered extract (g)	5	5	5
2.	Potassium alum (g)	5	-	-
3.	Mercuric oxide (g)	0.15	-	-
4.	Distilled water (ml)	100	100	100
5.	Absolute ethanol (ml)	30	-	-
6.	Formaldehyde (drops)	-	2	2

3.3.1 Preparation of *Hibiscus sabdariffa* solution

Five grams of the dried powered extract obtained was dissolved in 30ml of absolute ethanol. 5 g of alum salt was dissolved in 100 ml of distilled water and boiled. The extract previously dissolved in ethanol was then added to the boiling water. 0.15 g of mercuric oxide was added and boiled for ten minutes. The solution was cooled under tap water and stored in brown bottle until further use (Wiam *et al*., 2006).

3.3.2 *Garcinia livingstonei* solution

Five grams of *Garcinia livingstonei* extract was dissolved in 100 ml of distilled water. Two drops of formaldehyde was added to prevent the growth of mould. The solution was filtered twice with Whatmans No. 1 filter paper before use.

3.3.3 *Sorghum bicolor* solution

Five grams of sorghum extract was dissolved in 100 ml of distilled water. Two drops of formaldehyde was added to prevent the growth of mould. The solution was filtered three times with Whatmans No. 1 filter paper before use.

3.4 Tissue Samples/ Histological Processing

Table III above shows the composition of the various fixatives used in this study. The heart and kidney tissues used for this study were obtained from a male rabbit. The harvested tissues were preserved in fixatives immediately after harvest.

3.4.1 Calcium acetate fixative.

The heart and kidney were fixed in calcium acetate for 48 hours and thereafter dehydrated through ascending grades of ethanol (70%, 90% and absolute ethanol) and cleared in xylene. The tissues were pre-impregnated in xylene paraffin wax in the oven and embedded in pure paraffin wax (Drury *et al*., 1967). Tissues were sectioned at 7 µm thickness and were stained with Haematoxylin and Eosin (H&E), Sorghum and Haematoxylin (S&H), Sobo alone, and *Garcinia livingstonei* + Sobo (G&S) for light microscopic examinations. The same procedure was adopted for Helly's fixative, Buffered Neutral Formalin and Heidenhains SUSA fixative.

3.4.2 Carnoy's fixative

The samples were fixed in Carnoy's fixative for 8 hours, thereafter dehydrated through ascending grades of ethanol (90% and absolute ethanol) and cleared in xylene. The tissues were pre-impregnated in xylene paraffin wax in the oven and embedded in pure paraffin wax (Drury *et al*., 1967). Tissues were sectioned at 7 µm thickness and were stained with

Haematoxylin and Eosin (H&E), Sorghum and Haematoxylin (S&H), Sobo alone, and *Garcinia livingstonei* + sobo (G&S) for light microscopic examinations.

3.4.3 Muller's fixative

The samples were fixed in Muller's fixative for 6 weeks. Thereafter the tissues were dehydrated through ascending grades of ethanol (70%, 90% and absolute ethanol) and cleared in xylene (Drury *et al*., 1967). The tissues were pre-impregnated in xylene paraffin wax in the oven and embedded in pure paraffin wax. They were sectioned at 7µm thickness and stained with Haematoxylin and Eosin (H&E), Sorghum and Haematoxylin (S&H), Sobo alone, and *Garcinia livingstonei* + Sobo (G&S) for light microscopic examinations.

CHAPTER FOUR

4.0 RESULTS

4.1 Microscopic features

When all the animal tissues were examined under a light microscope, they all, irrespective of sexes exhibited similar histological characteristics.

4.1.1 Heart

Generally, the heart wall has three basic layers; the endocardium, myocardium and epicardium. The *tunica intima* of the heart is the endocardium, the tunica media of the heart called the myocardium and is thickest in the ventricular walls. The myocardium was made up of cardiac muscle, the structure that meets the unique functional requirements of the heart.

The *tunica adventitia* of the heart, the epicardium (also called visceral pericardium), was surrounded by a potential space, the pericardial cavity which was enclosed by a fibrous sac, the pericardium (the parietal pericardium). The parietal pericardium was loosely fixed to the surrounding mediastinal structures. The papillary muscles of the ventricles were extensions of the myocardium which, via the *chordae tendinae*, stabilise the cusps of the mitral and tricuspid valves.

The photomicrographs below show the myocardium with its corresponding staining ability. This is based on the local stain and the type of fixative used while processing the tissue. Attempt was made to stain the nucleus and cytoplasm with ethanolic extracts of *Hibiscus sabdariffa,* aqueous extracts of *Sorghum bicolor* and *Garcinia livingstonei* was also used to stain the cytoplasm. At various magnifications, the results shown below were observed while studying the heart.

Sobo alone (*Hibiscus sabdariffa*) stain, (Plate I – IX); Haematoxylin and Sorghum stain, (Plate XXII – XXXIII); Garcinia and Sobo stain, (Plate XLVII – LVIII) and the conventional Haematoxylin and Eosin stain (Plate LXIX – LXXX).

4.1.2 Kidney

In the cortex, numerous renal corpuscles were visible. The corpuscles tended to be arranged in parallel rows at right angles to the capsule, separated by cortical radial arteries from which they derive their blood supply. Most of the cortical parenchyma surrounding the renal corpuscles consist of proximal and distal convoluted tubules.

Renal corpuscles appeared as dense rounded structures, the glomeruli, surrounded by narrow Bowman's spaces. It was evident that the tubules making up the bulk of the parenchyma between the renal corpuscles differed from one another in diameter, shape and staining intensity.

Attempt was made to stain the nucleus and cytoplasm with ethanolic extracts of *Hibiscus sabdariffa,* aqueous extracts of *Sorghum bicolor* and *Garcinia livingstonei* was also used to stain the cytoplasm. At various magnifications, the results shown below were observed while studying the kidneys.

The photomicrographs below emphasize the renal corpuscle and the glomerulus with its corresponding staining ability. This was based on the local stain and the type of fixative used while processing the tissue. Some of the fixatives produced a very significant Bowman's space due to shrinkage effect on the tissue on preservation. At various magnifications, the results were observed while studying the kidney;

Sobo alone (*Hibiscus sabdariffa*) stain, (Plate X – XXI); Haematoxylin and Sorghum stain, (Plate XXXIV – XLVI); Garcinia and Sobo stain, (Plate LIX – LXVIII) and the conventional Haematoxylin and Eosin stain, (Plate LXXXI – XCII).

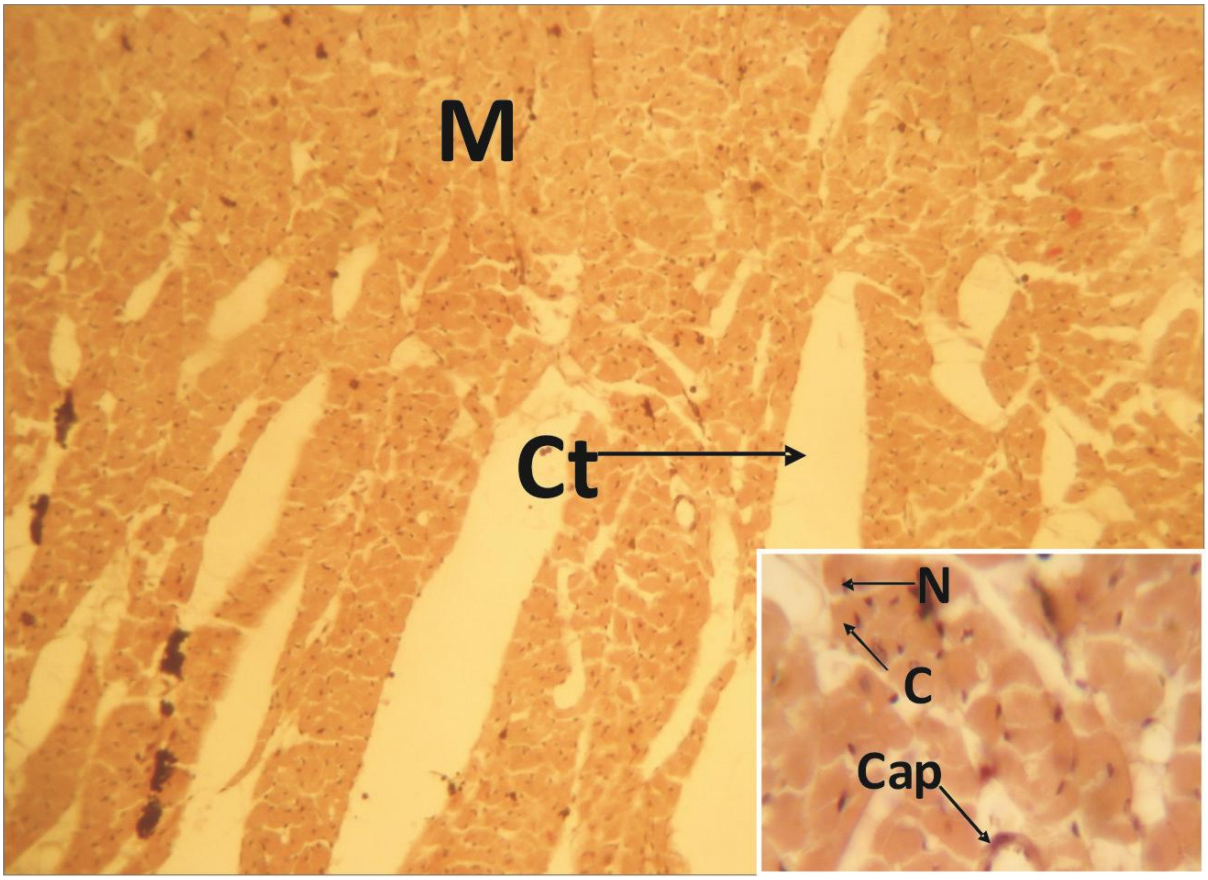


Plate I: Heart (Heidenhain's SUSA Fixative), Haematoxylin and Eosin (H and E).

At a lower magnification of x 100, the muscle fibres (M) and connective tissues (Ct) between the muscle fibres of the heart are clearly differentiated with H and E stain. At a higher magnification of x 400, the nuclei within the sarcoplasm are evident. With Heidenhain's SUSA fixative, Haematoxylin stains the nuclei (N) purplish while Eosin stains the cytoplasm (C) pinkish.

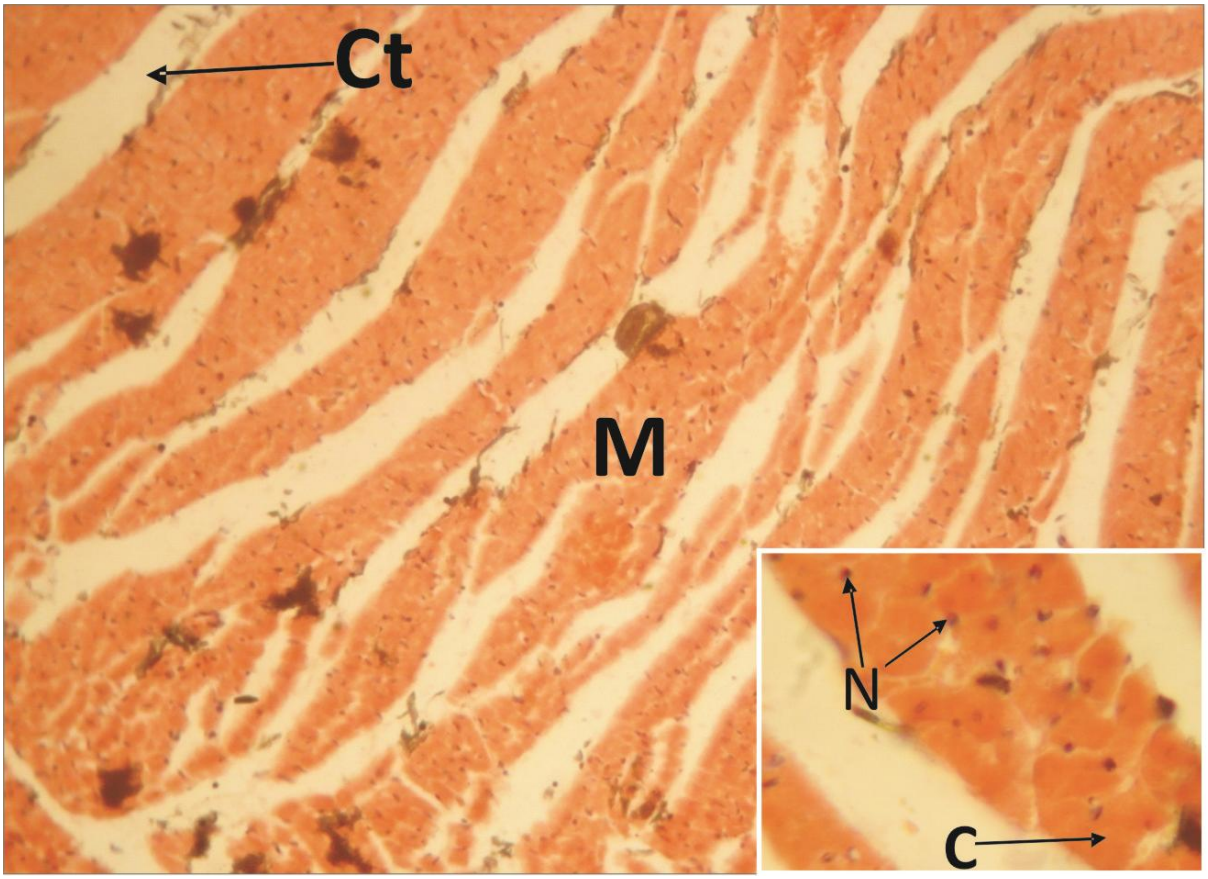


Plate II: Heart (Helly's Fixative), Haematoxylin and Eosin (H and E).

At a lower magnification of x 100, the muscle fibres (M) and connective tissues (Ct) between the muscle fibres of the heart are clearly differentiated with H and E stain. At a higher magnification of x 400, the nuclei within the sarcoplasm are evident. With Heidenhain's SUSA fixative, Haematoxylin stains the nuclei (N) purplish while Eosin stains the cytoplasm (C) with an orange colouration.

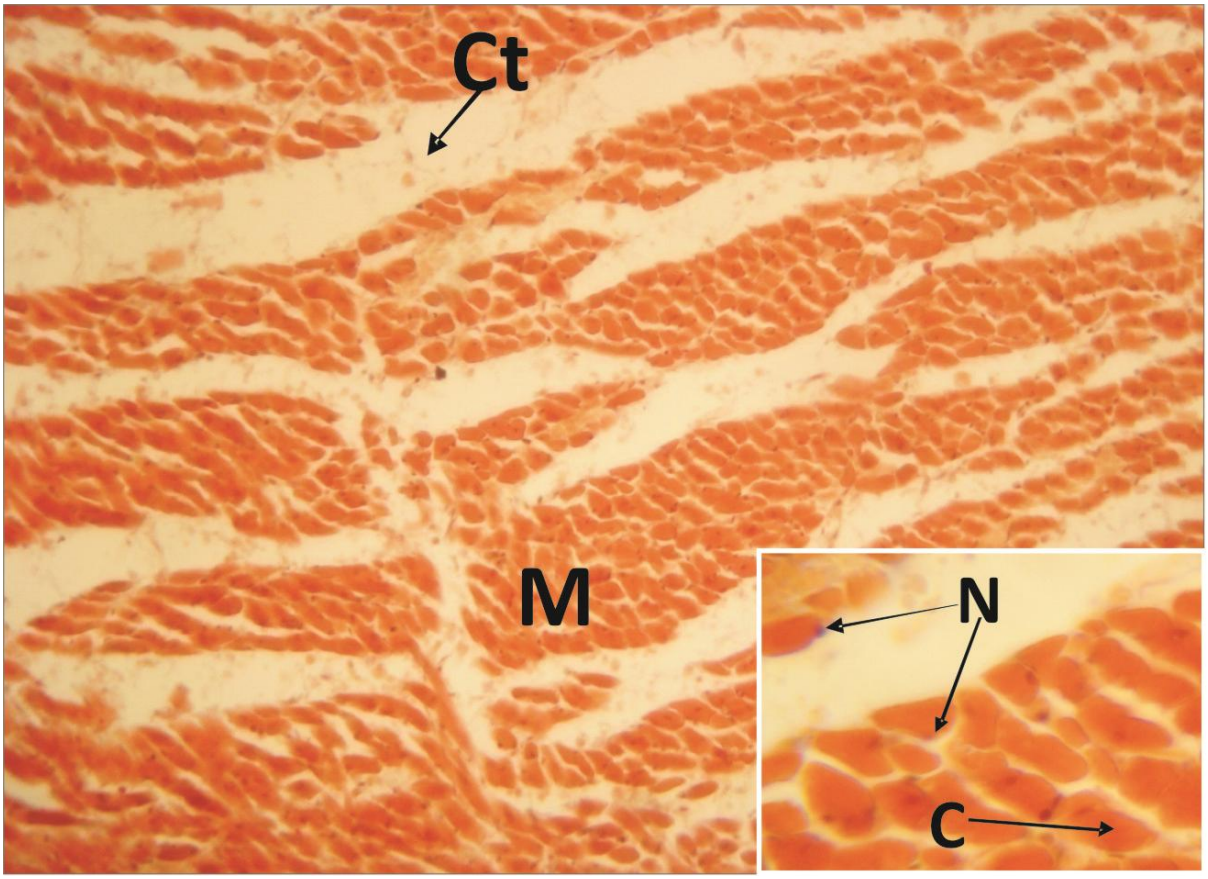


Plate III: Heart (Muller's Fixative), Haematoxylin and Eosin (H and E).

The photomicrograph at x 100 illustrates the muscle fibres (M) and connective tissues (Ct), with the muscle fibres having an orange colour. At a higher magnification of x 400, the nuclei within the sarcoplasm are evident. With Muller's fixative, Haematoxylin stains the nuclei (N) purplish while Eosin stains the cytoplasm (C) with an orange colouration.

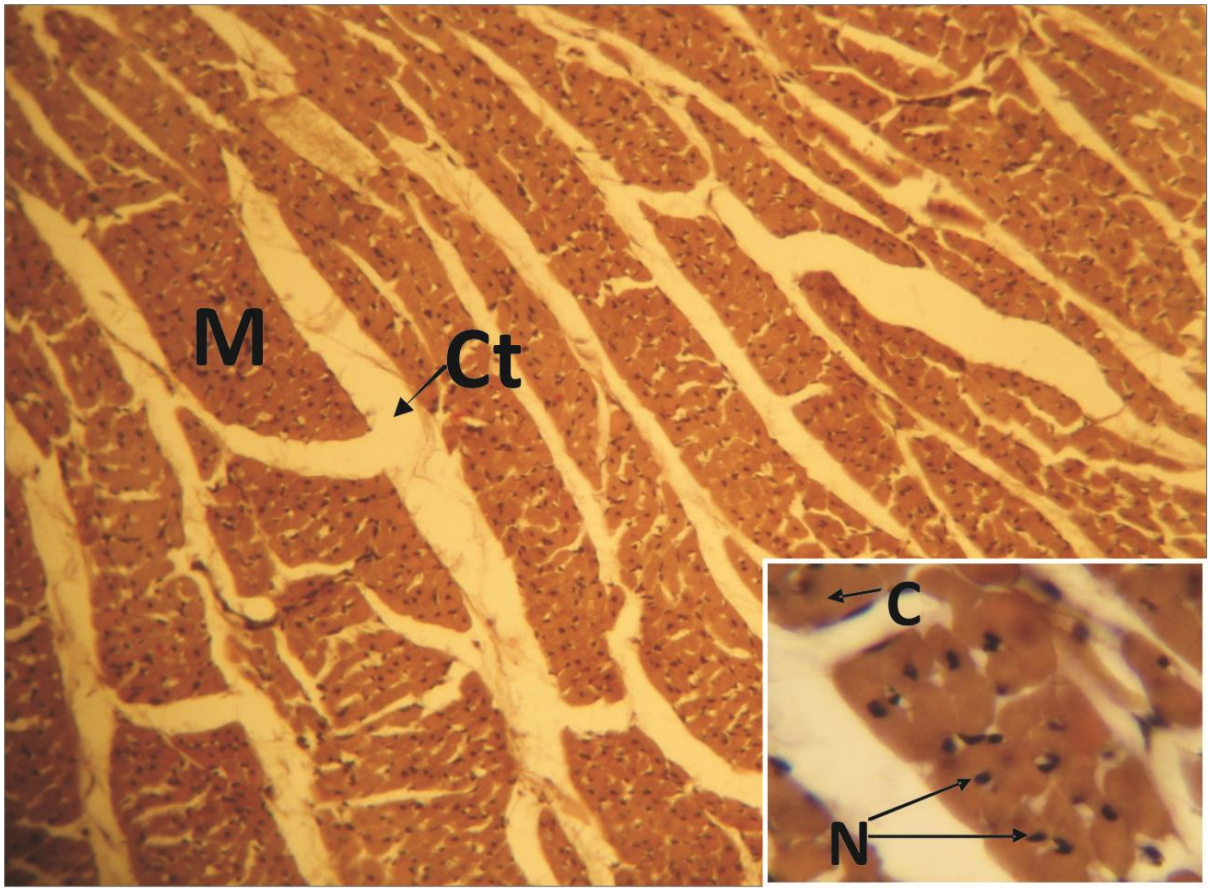


Plate IV: Heart (Carnoy's Fixative), Haematoxylin and Eosin (H and E).

The photomicrograph at x 100 illustrates the muscle fibres (M) and connective tissues (Ct), with the muscle fibres having a brownish colour outline. At a higher magnification of x 400, the nuclei within the sarcoplasm are evident. With Carnoy's fixative, Haematoxylin stains the nuclei (N) purplish while Eosin stains the cytoplasm (C) brownish instead of pink due to the effect of the fixative.

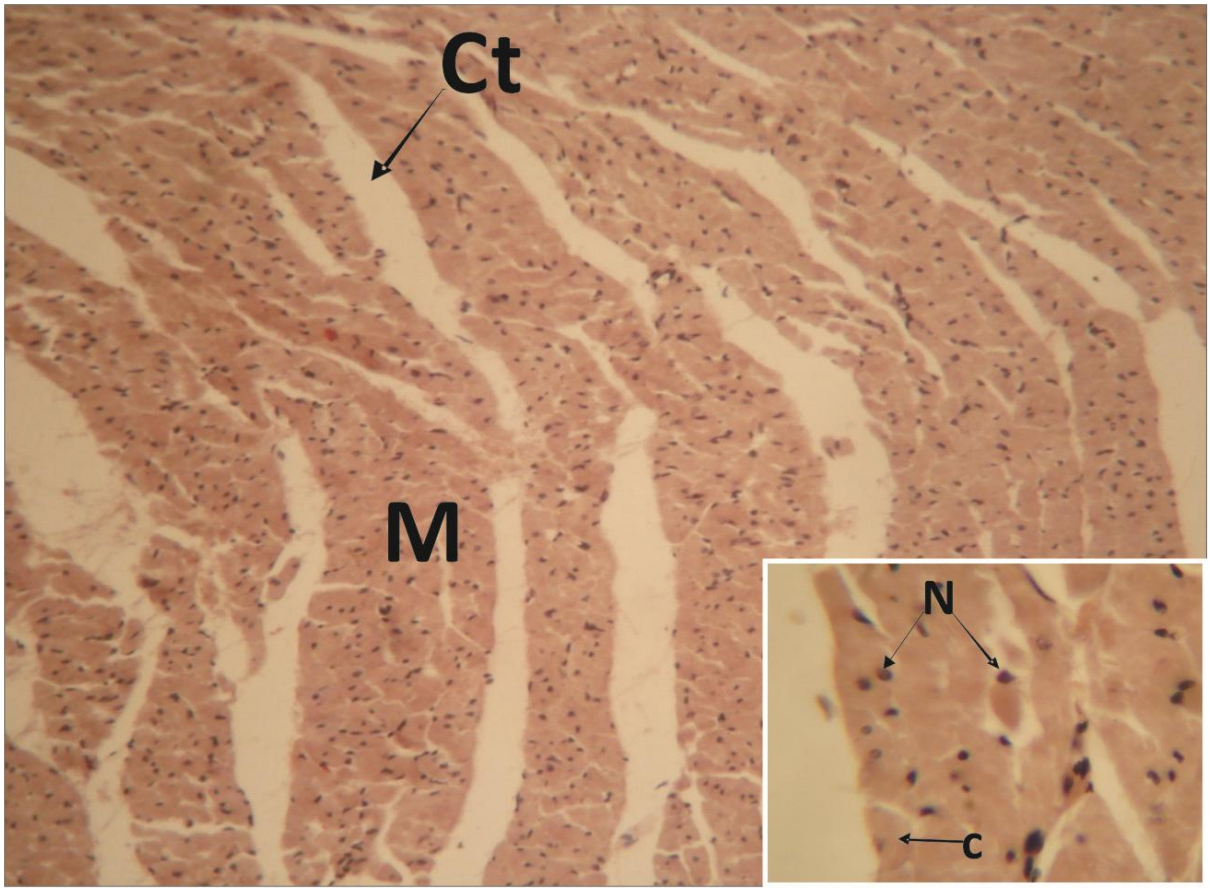


Plate V: Heart (Buffered Formalin Fixative), Haematoxylin and Eosin (H and E).

The photomicrograph at x 100 illustrates the muscle fibres (M) and connective tissues (Ct), with the muscle fibres having a purple colour outline. At a higher magnification of x 400, the nuclei within the sarcoplasm are very distinct. With Buffered Formalin fixative, Haematoxylin stains the nuclei (N) purplish while Eosin stains the cytoplasm (C) pinkish.

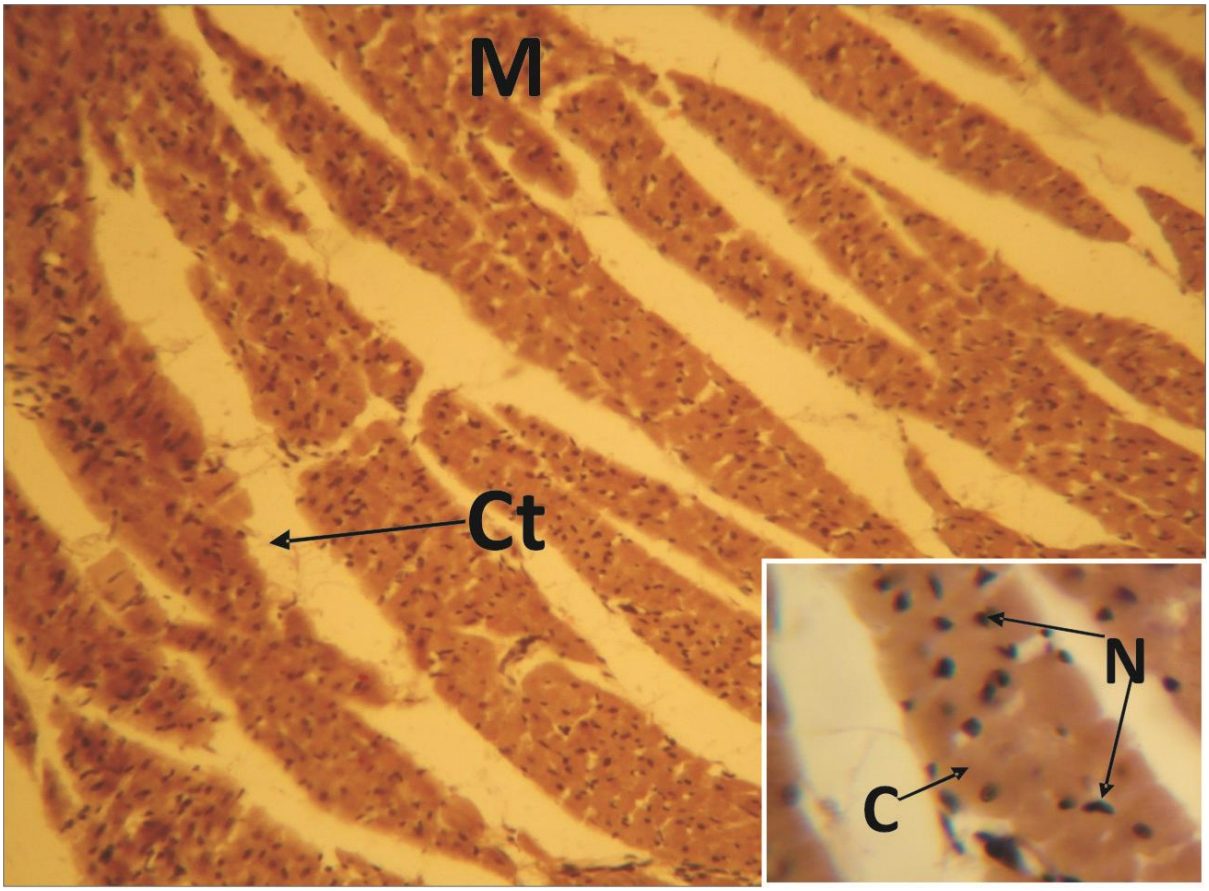


Plate VI: Heart (Calcium acetate Fixative), Haematoxylin and Eosin (H and E).

The photomicrograph at x 100 illustrates the muscle fibres (M) and connective tissues (Ct), with the muscle fibres having a brownish colour outline. At a higher magnification of x 400, the nuclei within the sarcoplasm are evident. With Calcium acetate fixative, Haematoxylin stains the nuclei (N) purplish while Eosin stains the cytoplasm (C) brownish instead of pink due to the effect of the fixative similar to Carnoy's fixative.

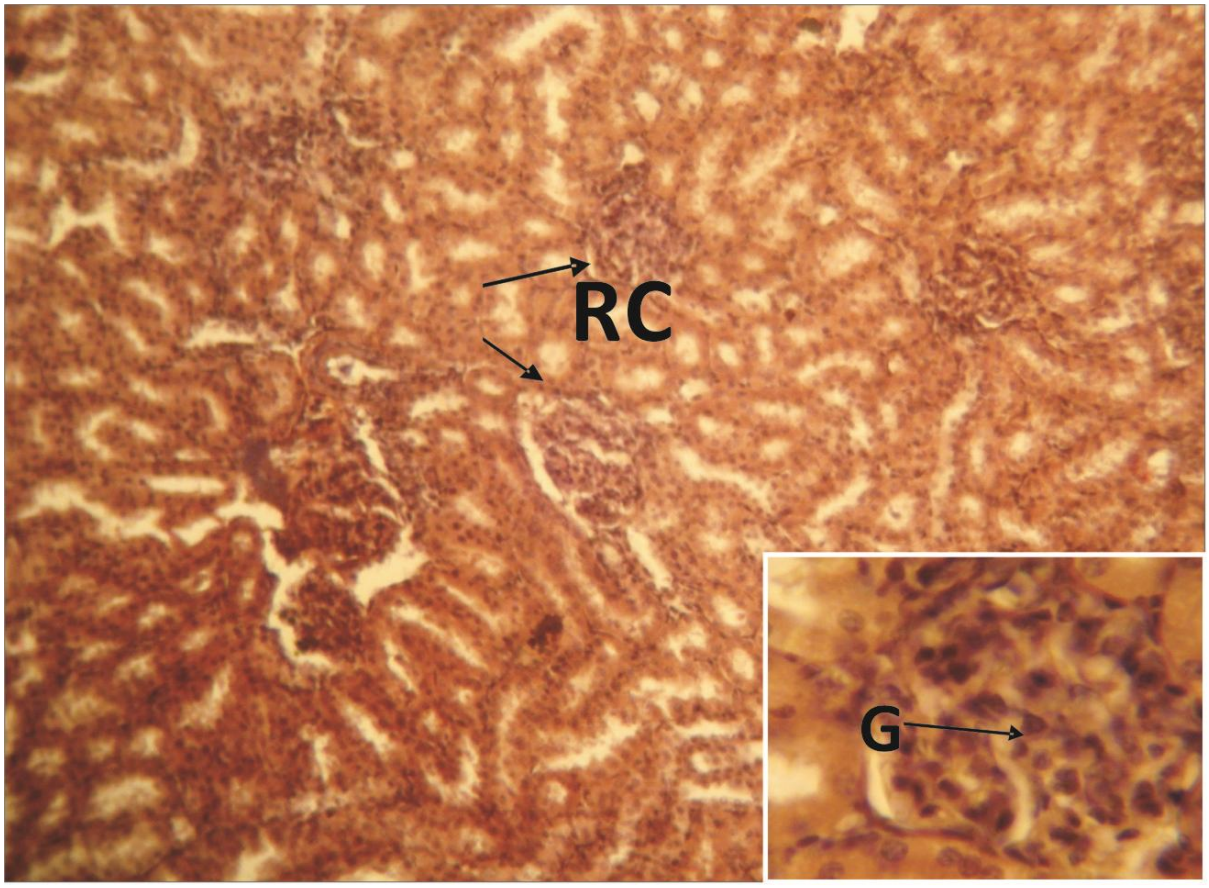


Plate VII: Kidney (Heidenhain's SUSA Fixative), Haematoxylin and Eosin (H and E).

The renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were clearly demonstrated. The cells of the glomerular nuclei within the glomerulus (G) stained purplish with Haematoxylin while Eosin stains the cytoplasm (C) brownish instead of pink due to the effect of the fixative. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be clearly seen within the kidney cortex (Cx).

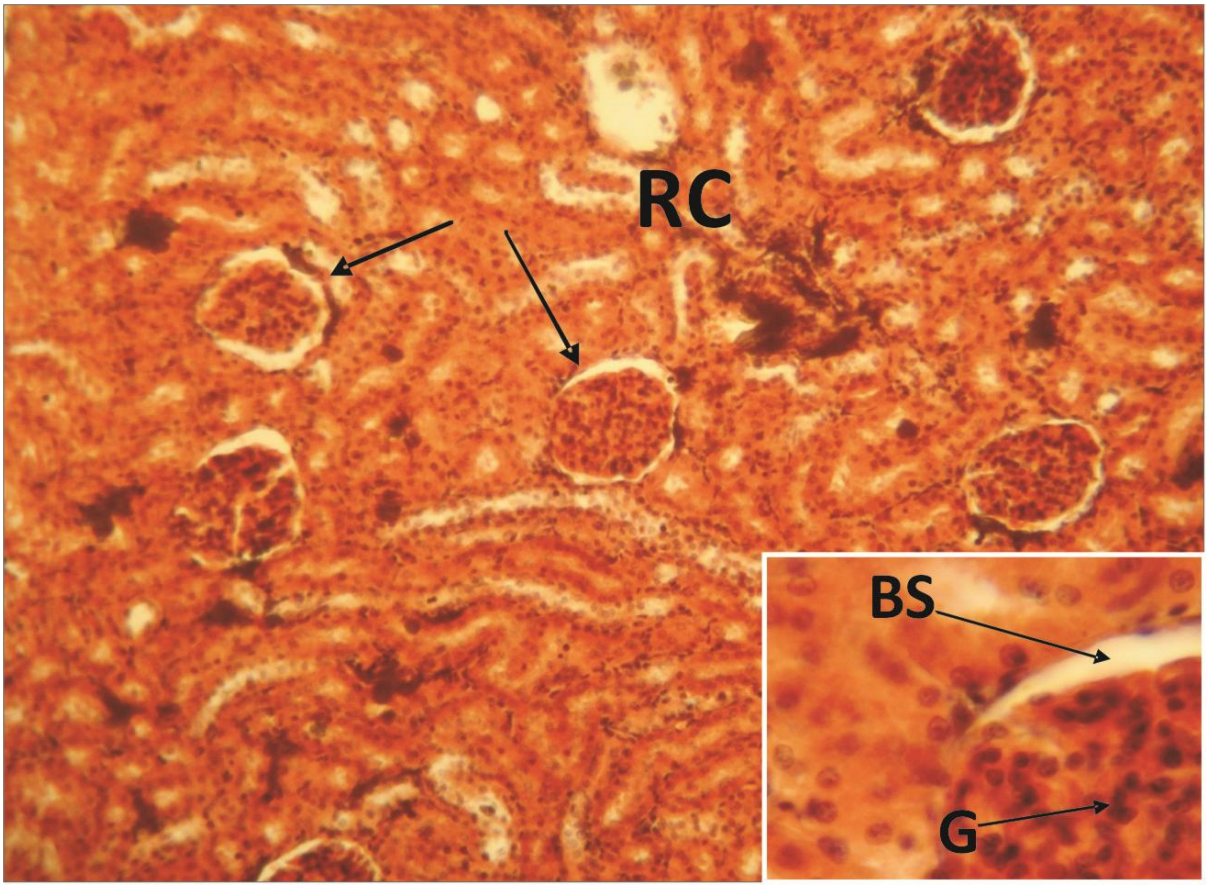


Plate VIII: Kidney (Helly's Fixative), Haematoxylin and Eosin (H and E).

The renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were clearly demonstrated. The cells of the glomerular nuclei within the glomerulus (G) stained purplish with Haematoxylin while Eosin stains the cytoplasm (C) brownish instead of pink due to the effect of the fixative. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be clearly seen within the kidney cortex (Cx).

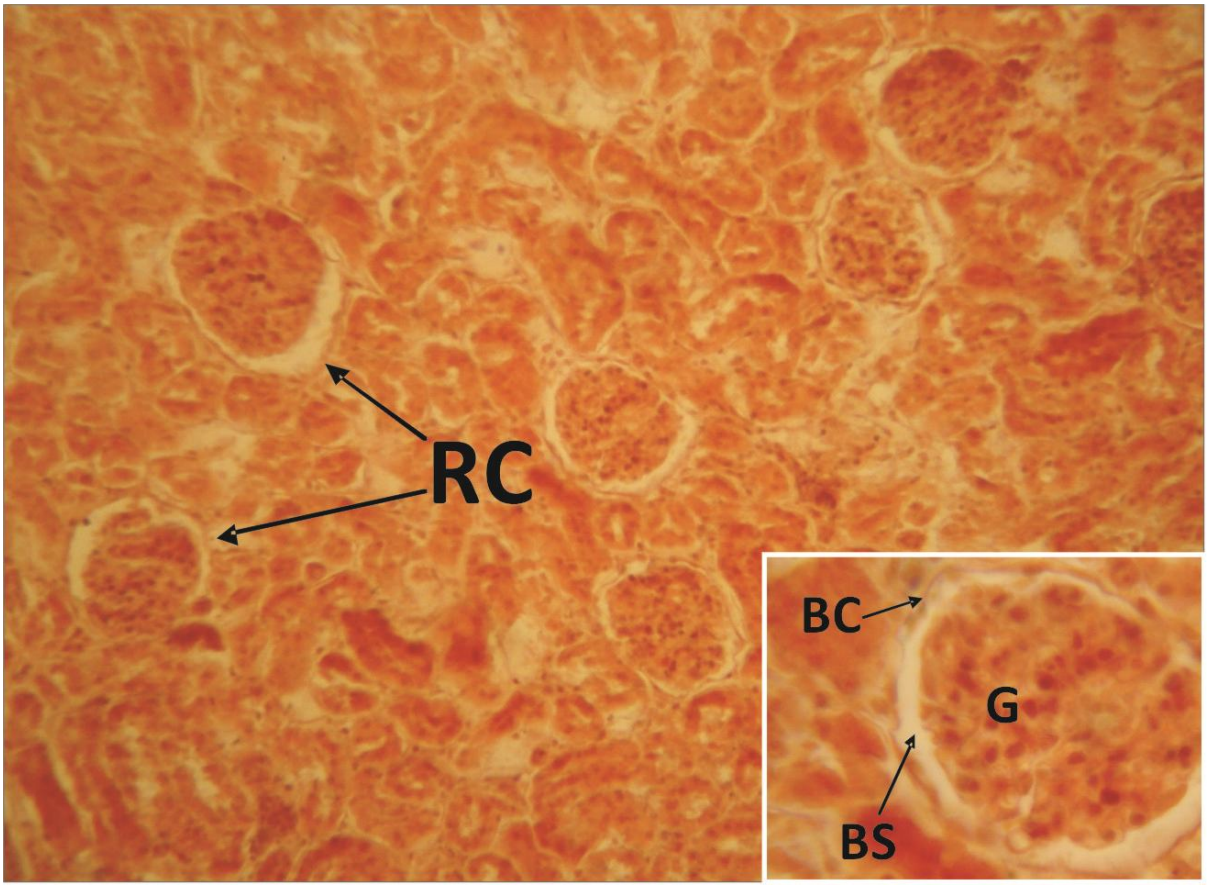


Plate IX: Kidney (Muller's Fixative), Haematoxylin and Eosin (H and E).

The renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were clearly demonstrated. The cells of the glomerular nuclei within the glomerulus (G) stained purplish with Haematoxylin while Eosin stains the cytoplasm (C) with an orange colour instead of pink due to the effect of the fixative. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be clearly seen within the kidney cortex (Cx).

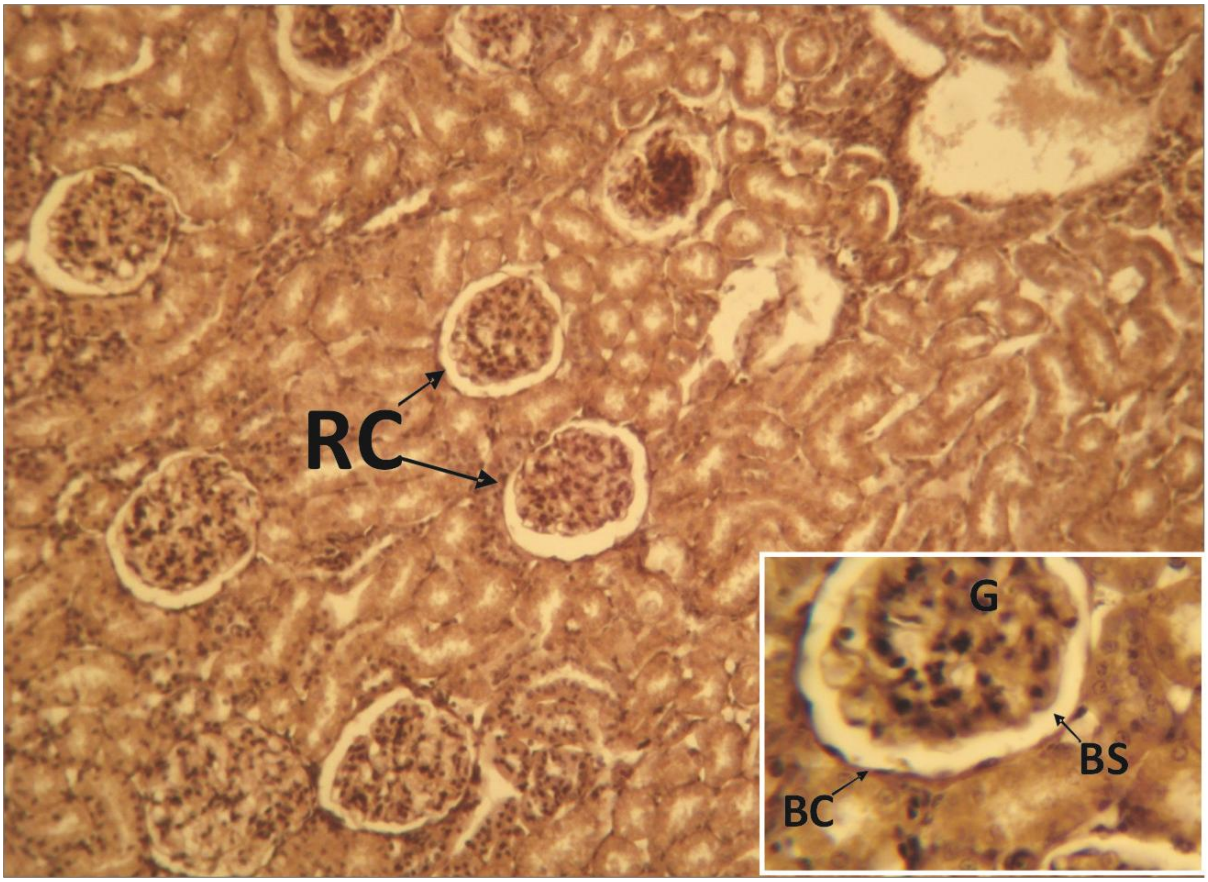


Plate X: Kidney (Carnoy's Fixative), Haematoxylin and Eosin (H and E).

The renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were clearly demonstrated. The cells of the glomerular nuclei within the glomerulus (G) stained purplish with Haematoxylin while Eosin stains the cytoplasm (C) greyish instead of pink due to the effect of the fixative. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be clearly seen within the kidney cortex (Cx).

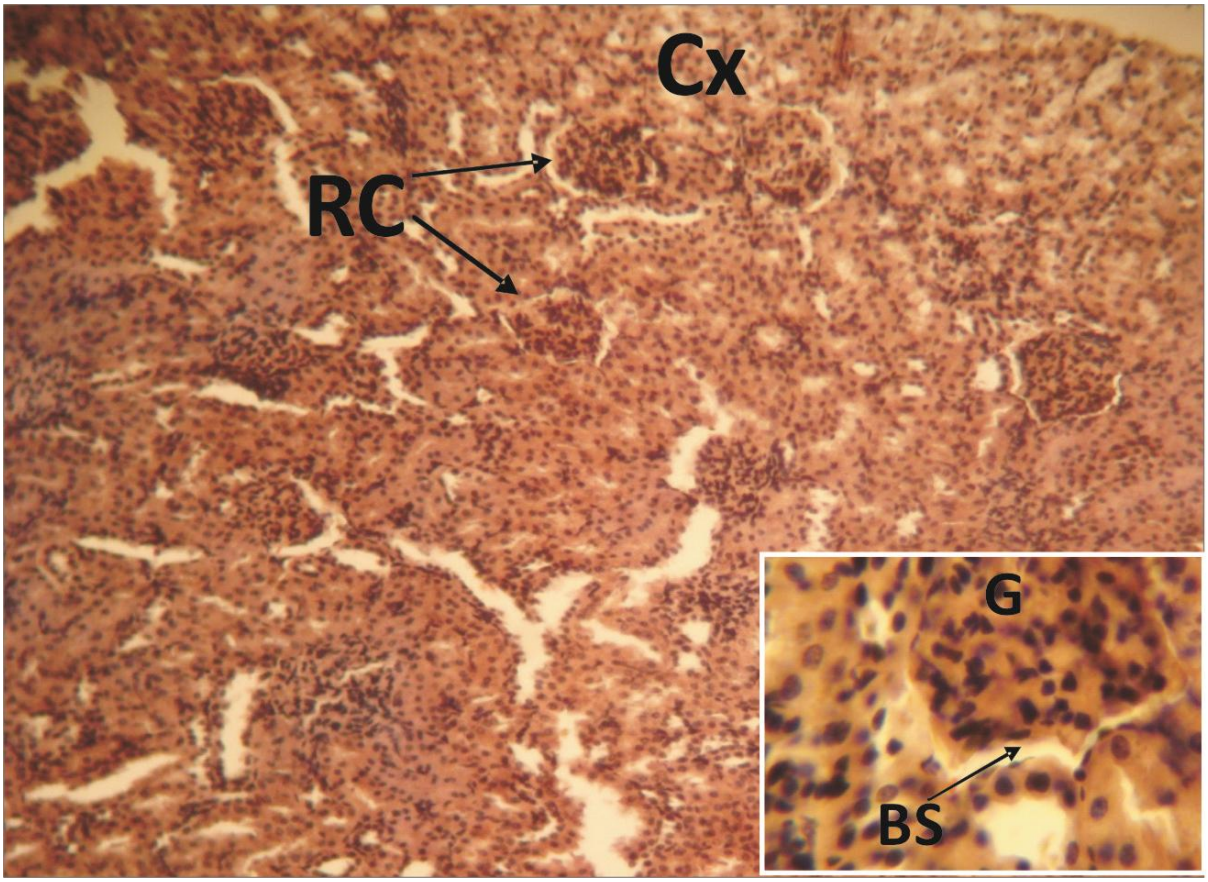


Plate XI: Kidney (Buffered Formalin Fixative) Haematoxylin and Eosin (H and E).

The renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were clearly demonstrated. The cells of the glomerular nuclei within the glomerulus (G) stained purplish with Haematoxylin while Eosin stains the cytoplasm (C) pinkish. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be clearly seen within the kidney cortex (Cx).

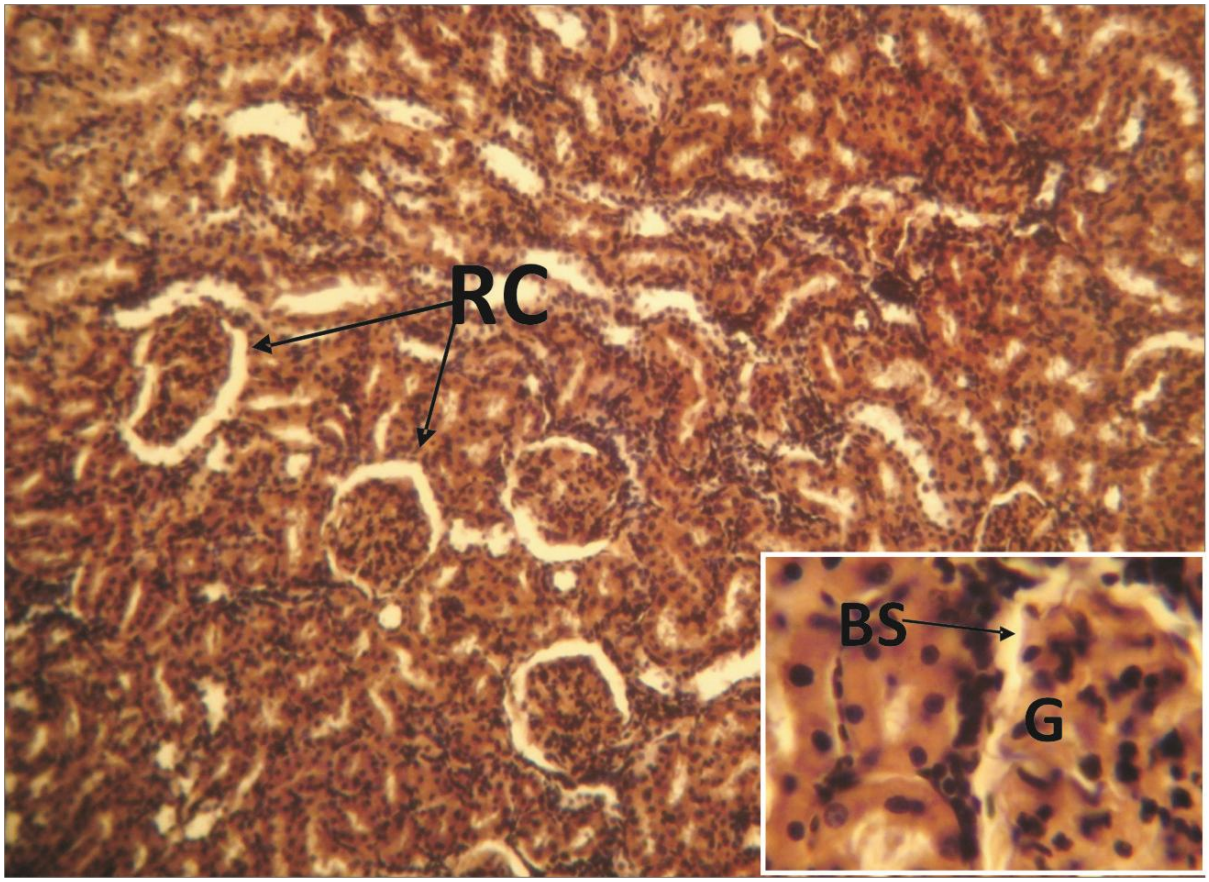


Plate XII: Kidney (Calcium acetate Fixative), Haematoxylin and Eosin (H and E).

The renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were clearly demonstrated. The cells of the glomerular nuclei within the glomerulus (G) stained purplish with Haematoxylin while Eosin stains the cytoplasm (C) pinkish. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be clearly seen within the kidney cortex (Cx).

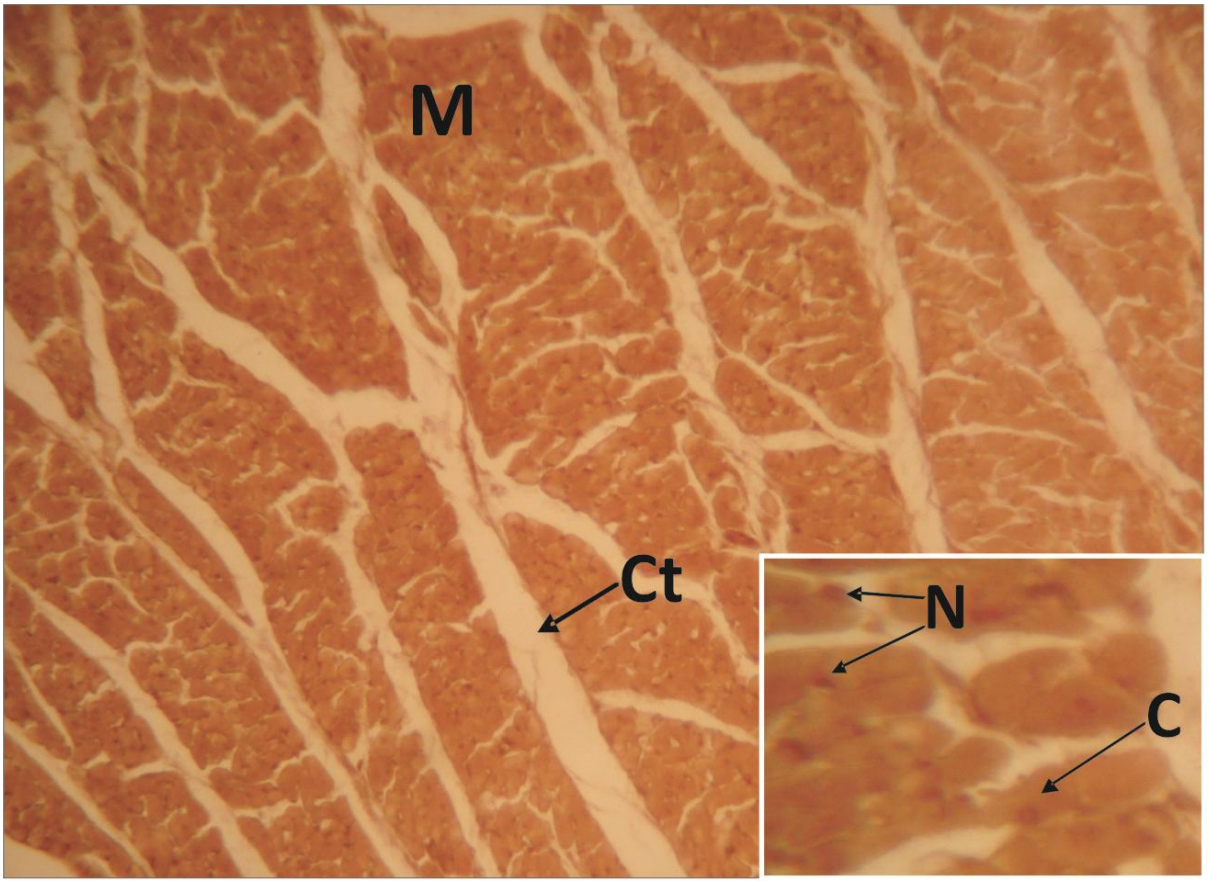


Plate XIII: Heart (Carnoy's Fixative), Sobo alone.

At a lower magnification of x 100, the muscle fibres (M) and connective tissues (Ct) between the muscle fibres of the heart are clearly differentiated with Sobo stain. At a higher magnification of x 400, the nuclei within the sarcoplasm are evident. With Carnoy fixative, Sobo alone stains the nuclei (N) dark brown while the cytoplasm (C) has a lighter shade of brownish colouration.

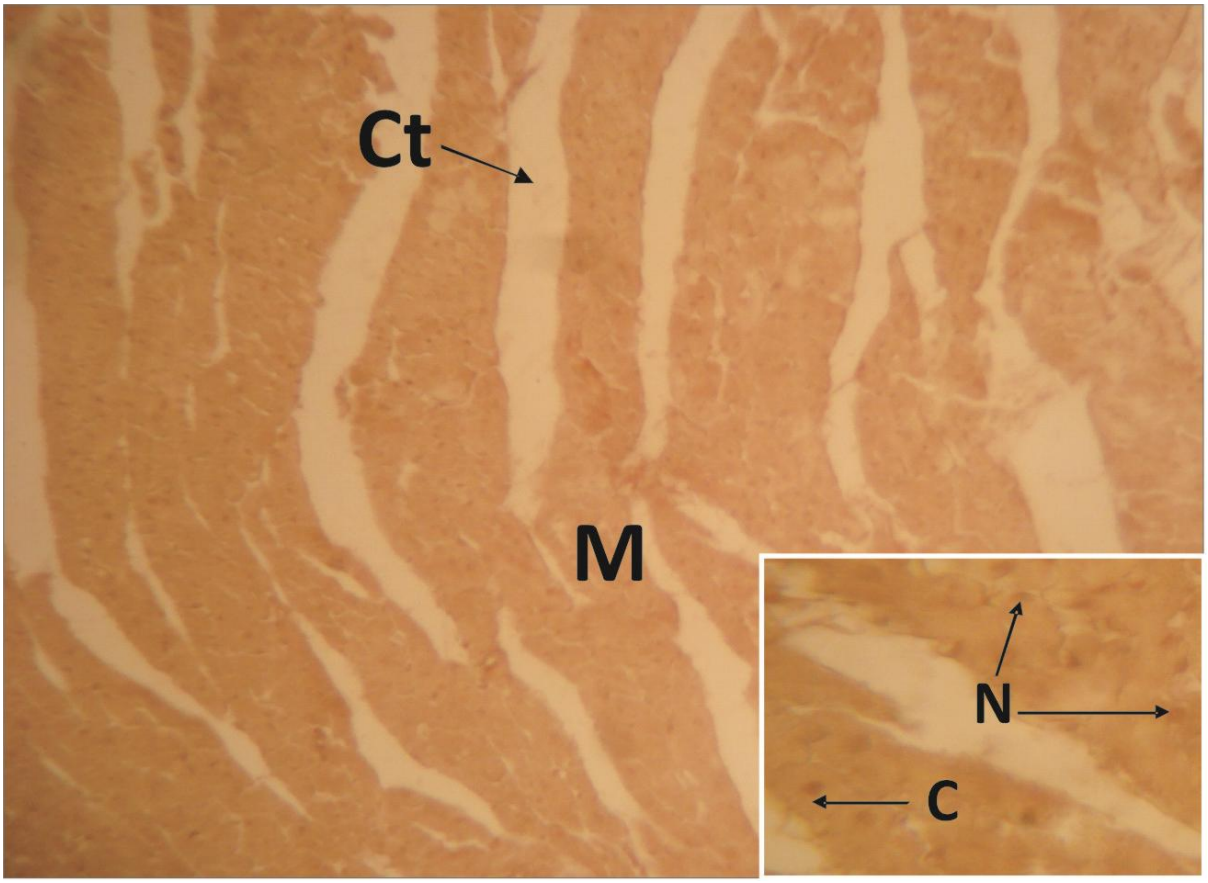


Plate XIV: Heart (Buffered Formalin Fixative), Sobo alone.

The connective tissues (Ct) and muscle fibres (M) preserved with Buffered formalin gave a light brown colouration with Sobo alone stain at x 100. The insertion of x 400 reveals the dark brown stained nuclei (N) and greyish cytoplasm (C).

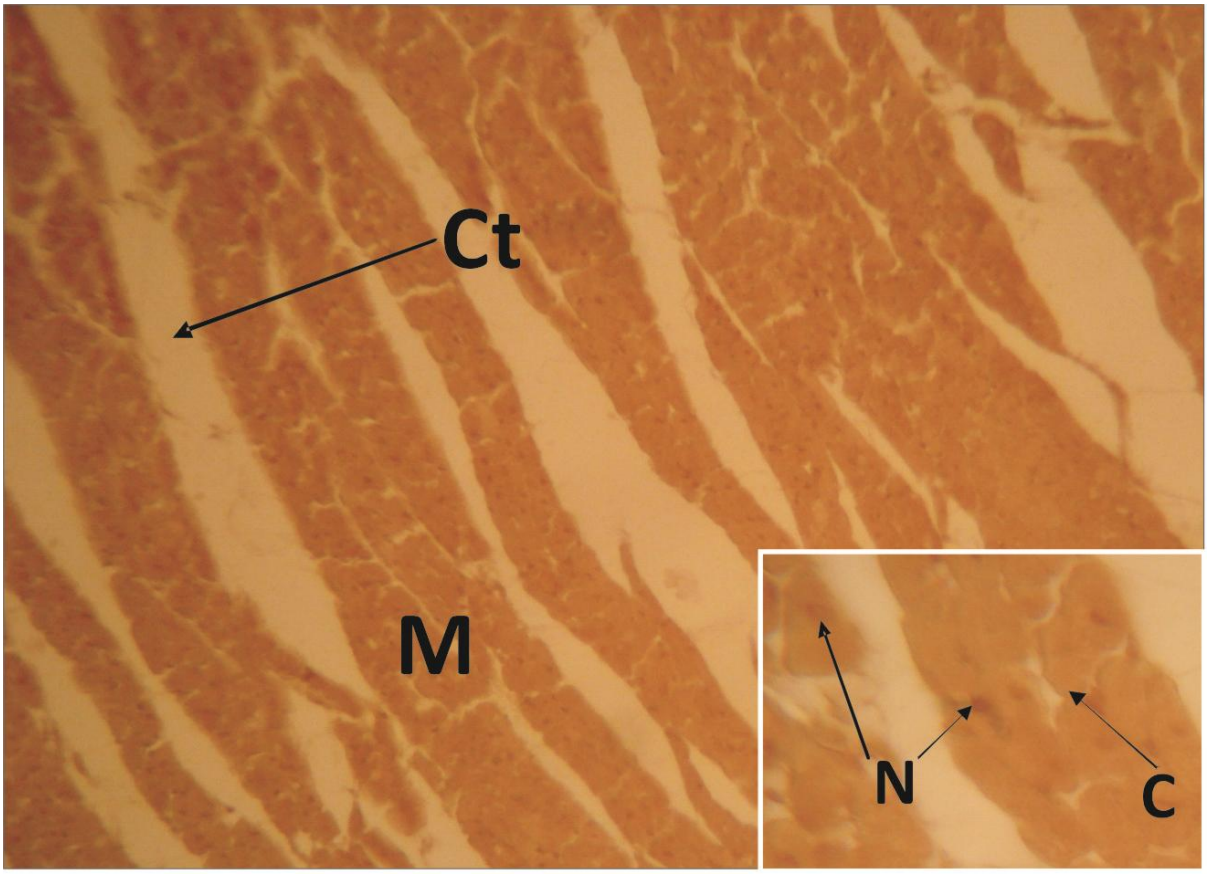


Plate XV: Heart (Calcium acetate Fixative), Sobo alone.

The connective tissues (Ct) and muscle fibres (M) at a magnification of x 100 were distinctly stained upon preservation with Calcium acetate formalin. At a magnification of x 400, the insert show a dark brown stained nuclei (N) and a light brown cytoplasm (C).

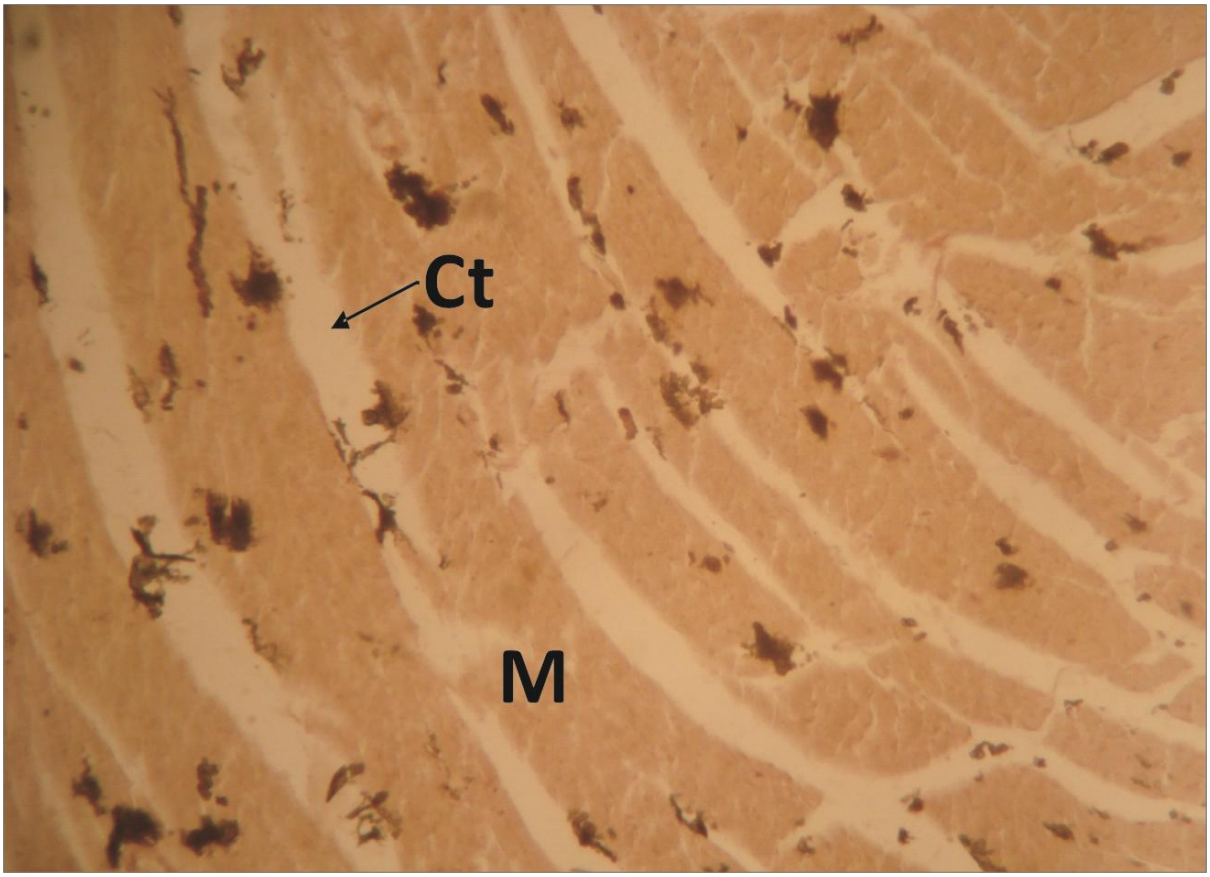


Plate XVI: Heart (Helly's Fixative), Sobo alone.

The connective tissues (Ct) and muscle fibres (M) were clearly stained and well differentiated upon preservation with Helly's fixative. Sobo alone, stained the tissue with different shades of brownish colouration.

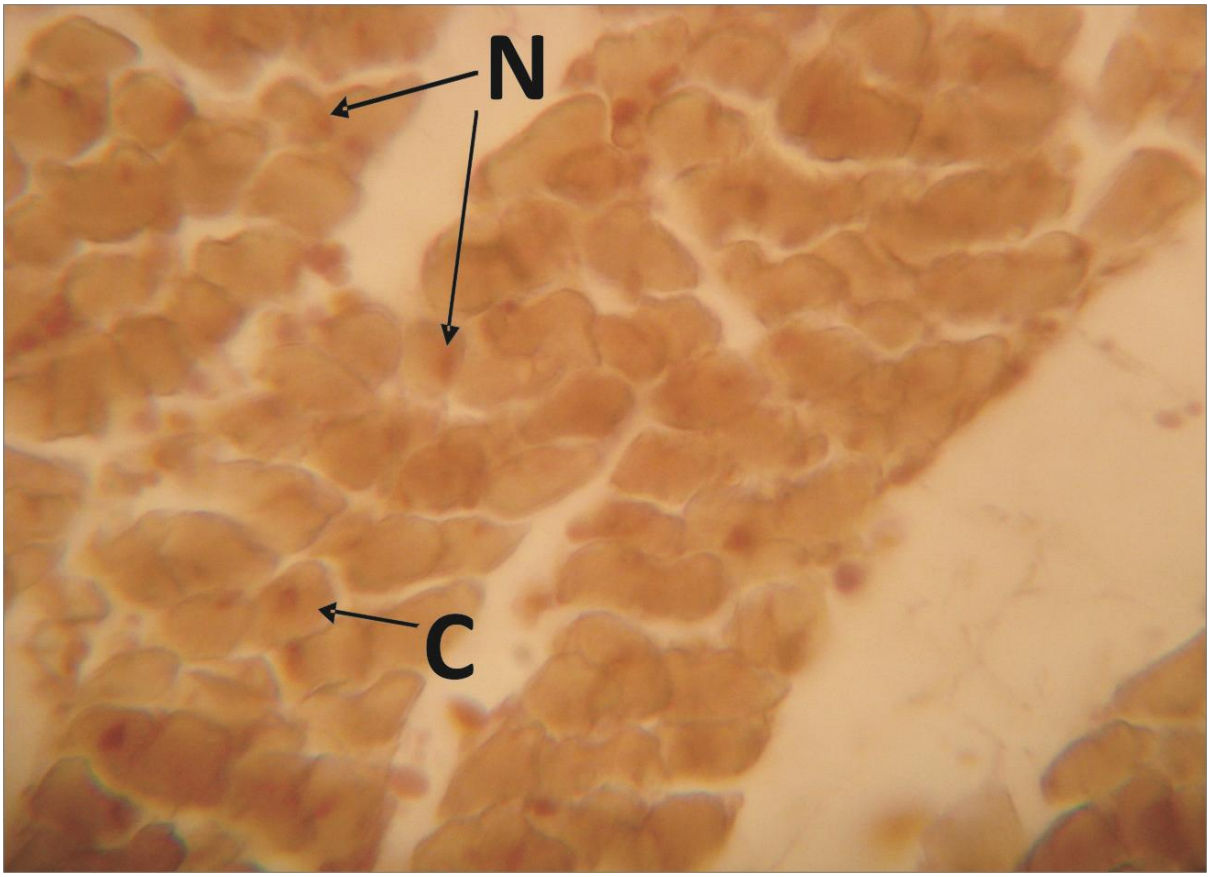


Plate XVII: Heart (Muller's Fixative), Sobo alone.

At a magnification of x 400, the effect of the fixative upon the myocardium when stained with Sobo alone gave rise to a dark brown nuclei (N) and a light brown cytoplasm (C).

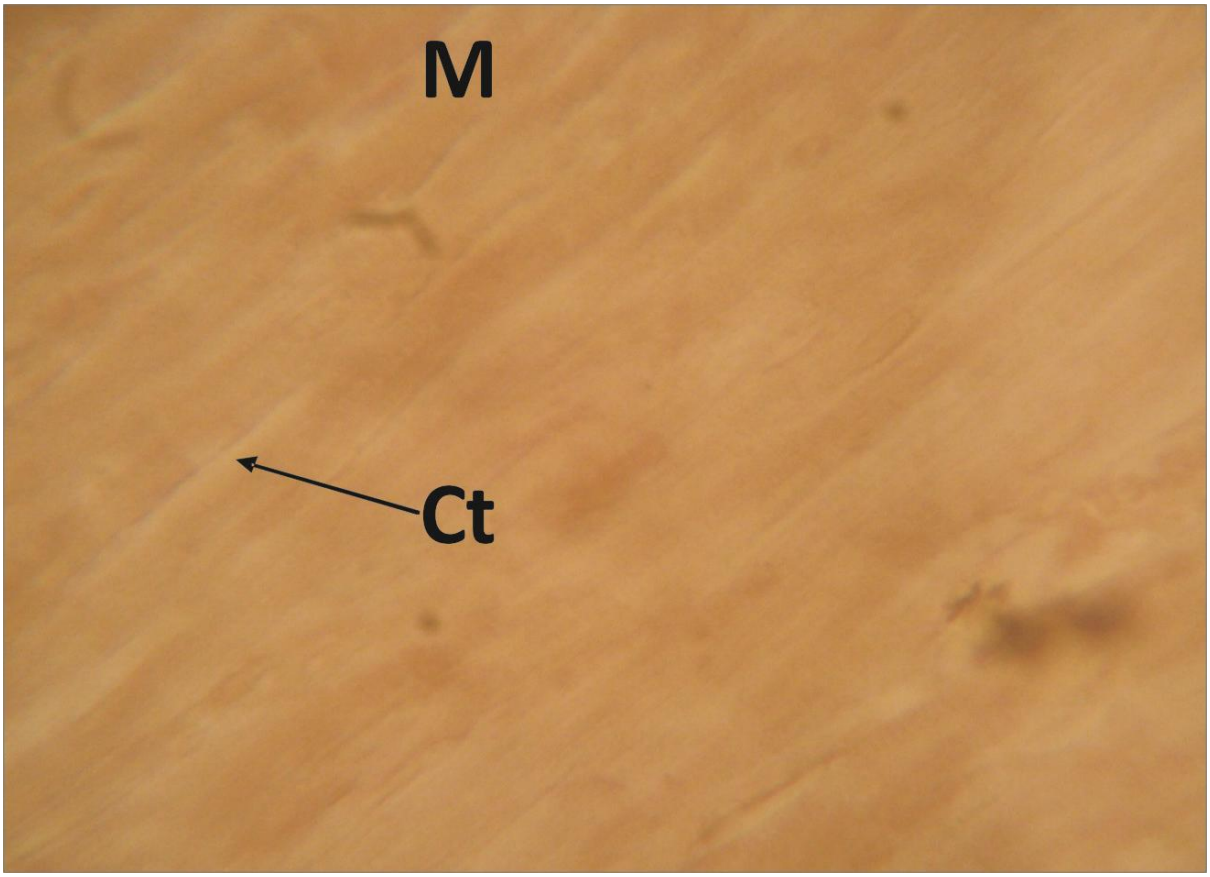


Plate XVIII: Heart (Heidenhain's SUSA Fixative), Sobo alone.

The connective tissues (Ct) and muscle fibres (M) were not well differentiated when stained with Sobo alone due to the effect of the fixative on the tissue.

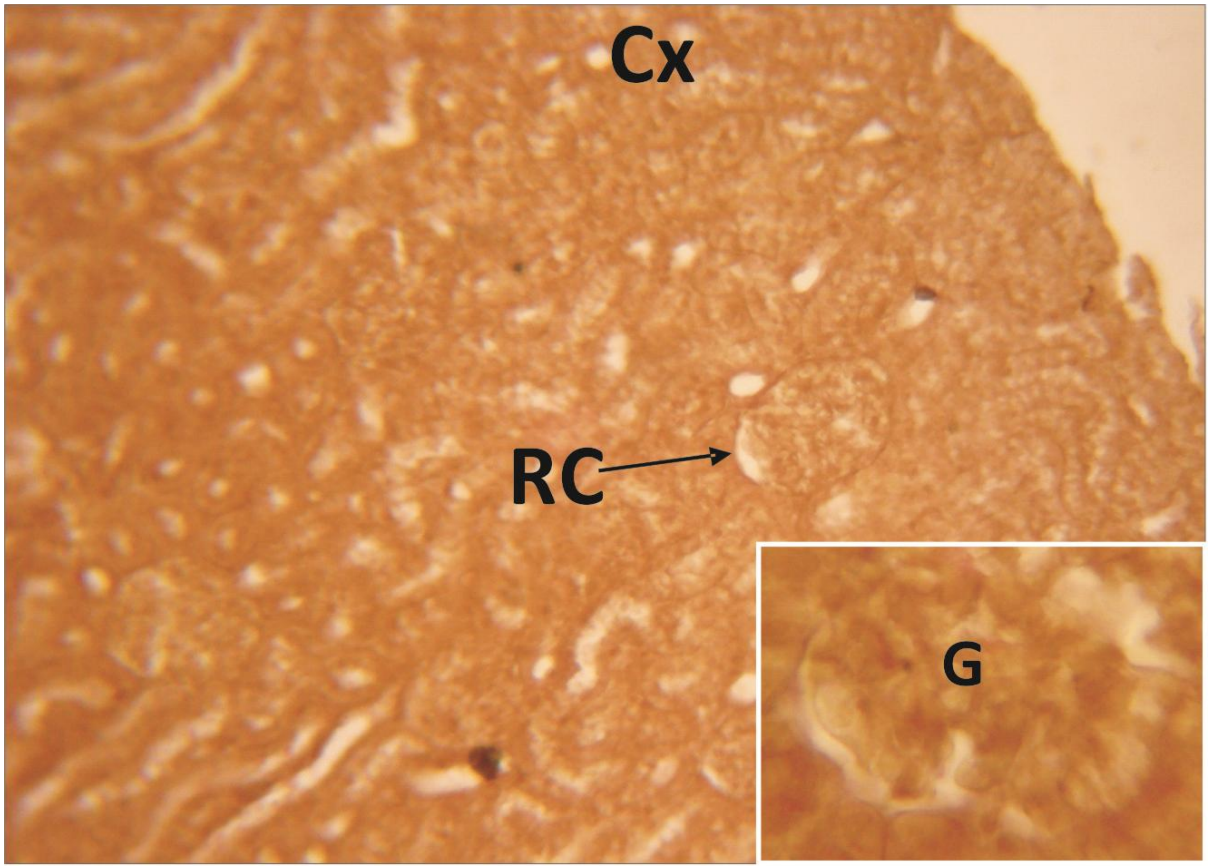


Plate XIX: Kidney (Heidenhain's SUSA Fixative), Sobo alone.

The histological features of the kidney were well preserved in Heidenhain's SUSA fixative, the renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were clearly demonstrated. The cells stained while using Sobo alone had various shades of brownish colouration, the glomerular nuclei within the glomerulus (G) had a dark brown colour. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be clearly seen within the kidney cortex (Cx).

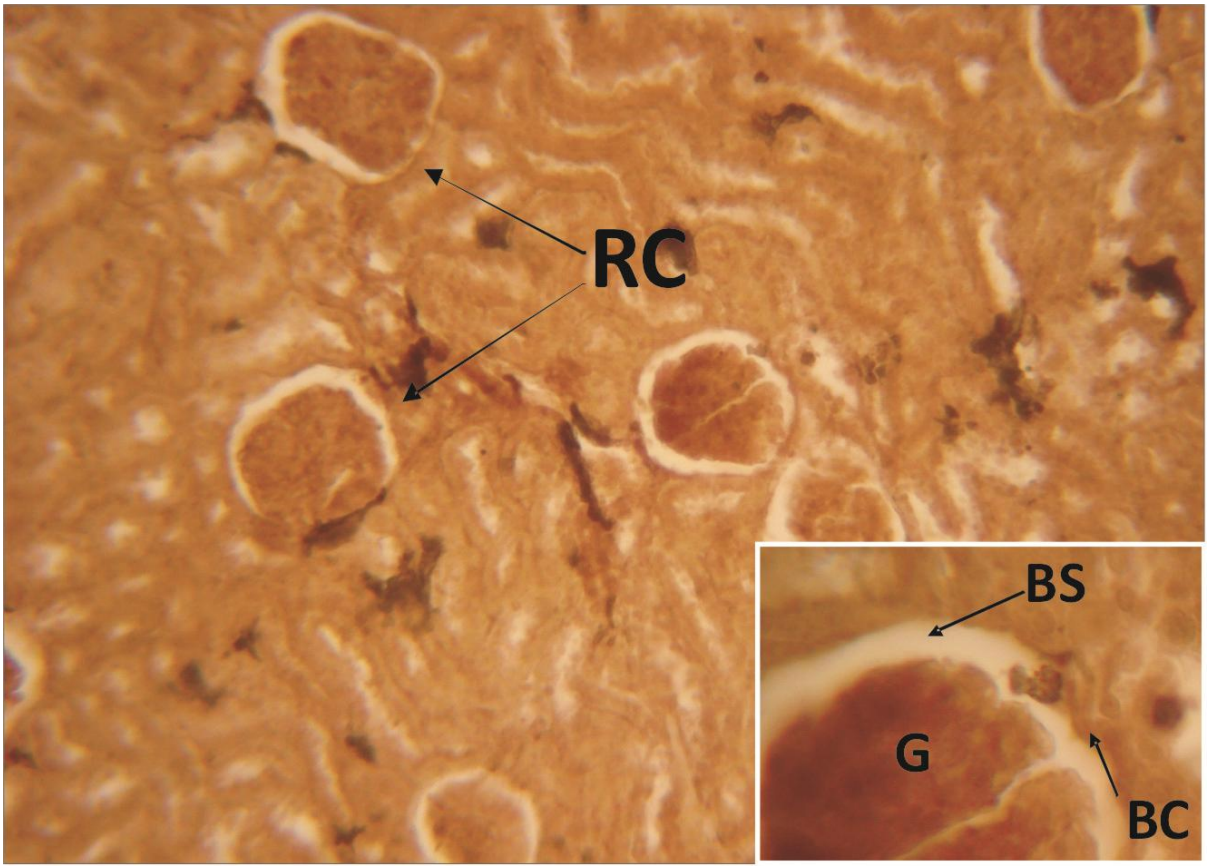


Plate XX: Kidney (Helly's Fixative), Sobo alone.

The histological features of the kidney were well preserved in Helly's fixative, the renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were clearly demonstrated. The cells stained while using Sobo alone had various shades of brownish colouration, the glomerular nuclei within the glomerulus (G) had a dark brown colour. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be clearly seen within the kidney cortex (Cx).

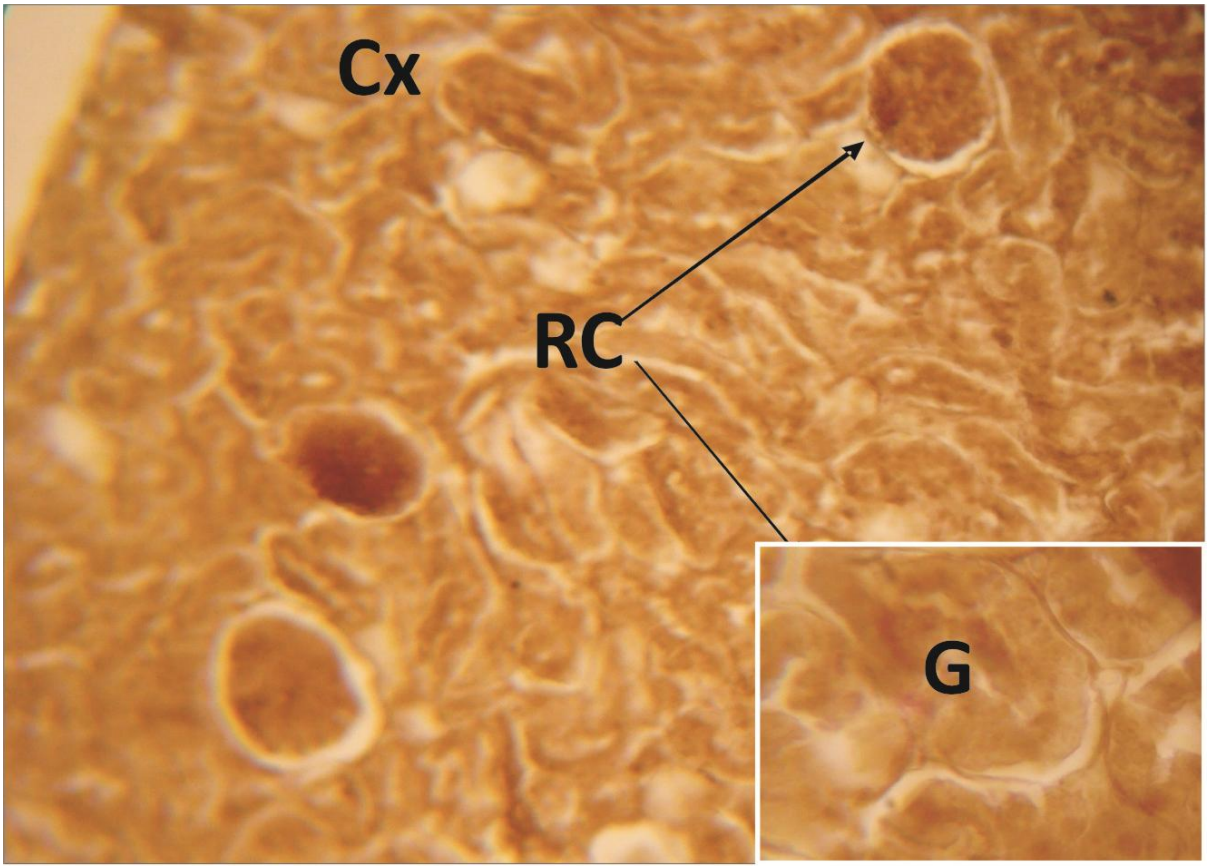


Plate XXI: Kidney (Mullers Fixative), Sobo alone.

The histological features of the kidney were well preserved in Mullers fixative, the renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were clearly demonstrated. The cells stained while using Sobo alone had various shades of brownish colouration, the glomerular nuclei within the glomerulus (G) had a light brown colour. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be clearly seen within the kidney cortex (Cx).

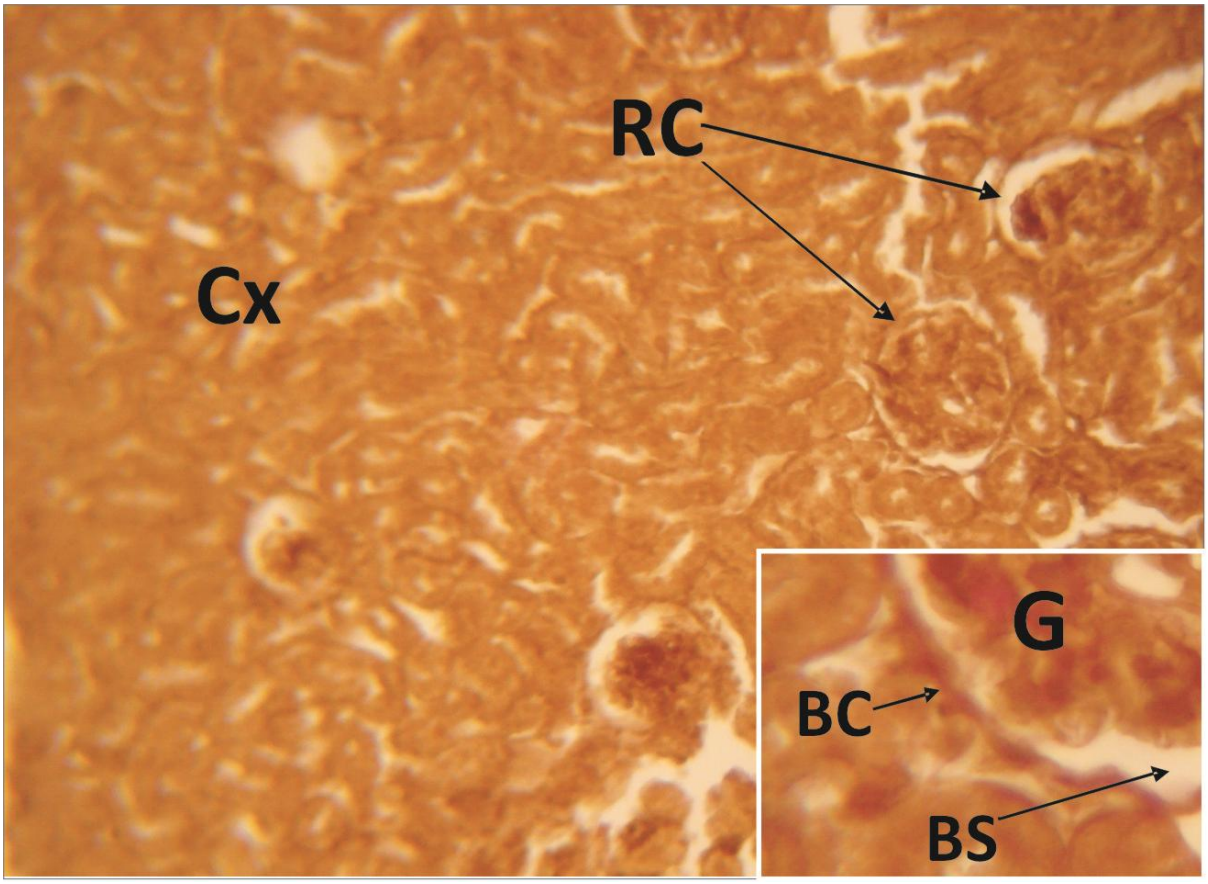


Plate XXII: Kidney (Carnoy's Fixative), Sobo alone.

The photomicrograph suggests that the histological features of the kidney were well preserved in Carnoy,s fixative, the renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were clearly demonstrated. The cells stained while using Sobo alone had various shades of brownish colouration, the glomerular nuclei within the glomerulus (G) had a dark brown colour. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be clearly seen within the kidney cortex (Cx).

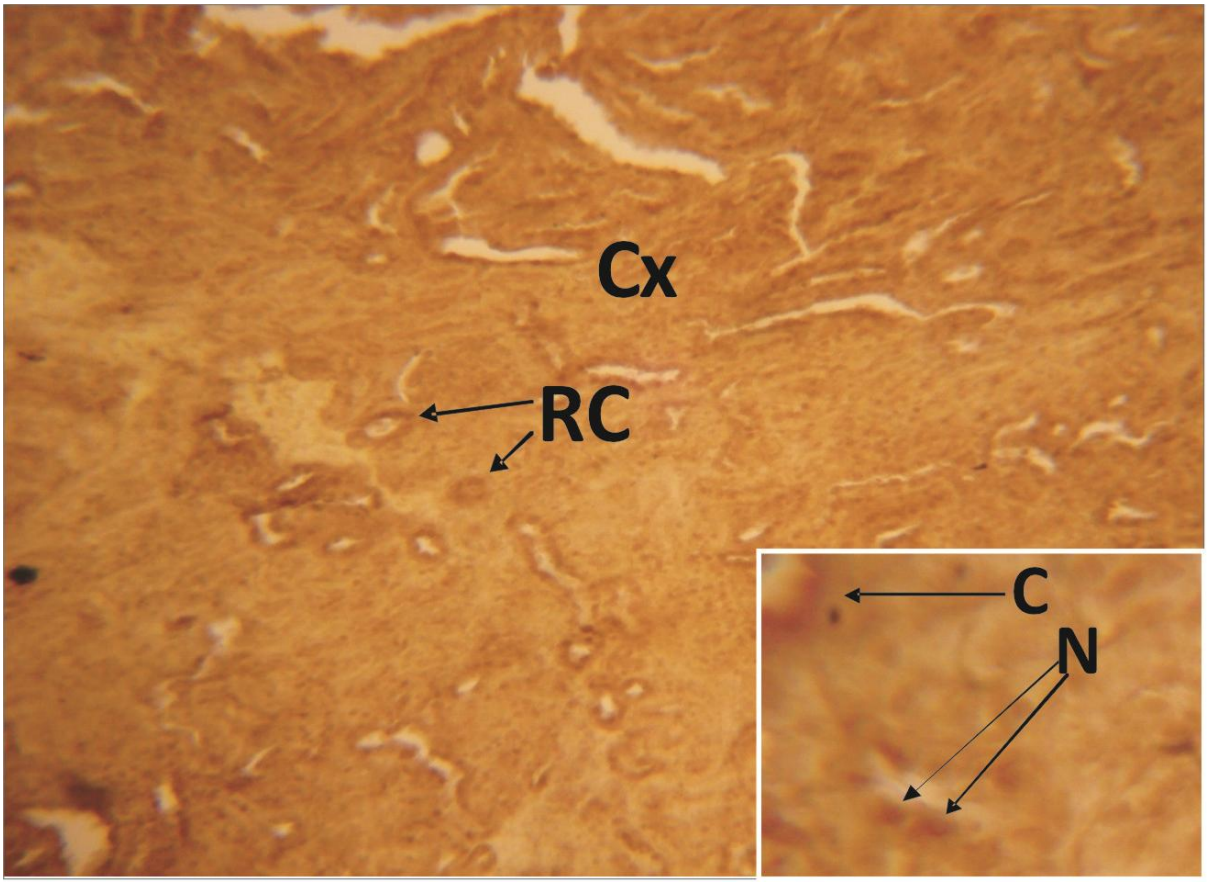


Plate XXIII: Kidney (Buffered Formalin Fixative), Sobo alone.

The histological features of the kidney were not well demonstrated with preservation in Buffered Formalin. The Sobo stain did not highlight features of histologic interest clearly. However, the nuclei (N) stained dark brown while the cytoplasm (C) had a lighter shade of the brownish colouration.

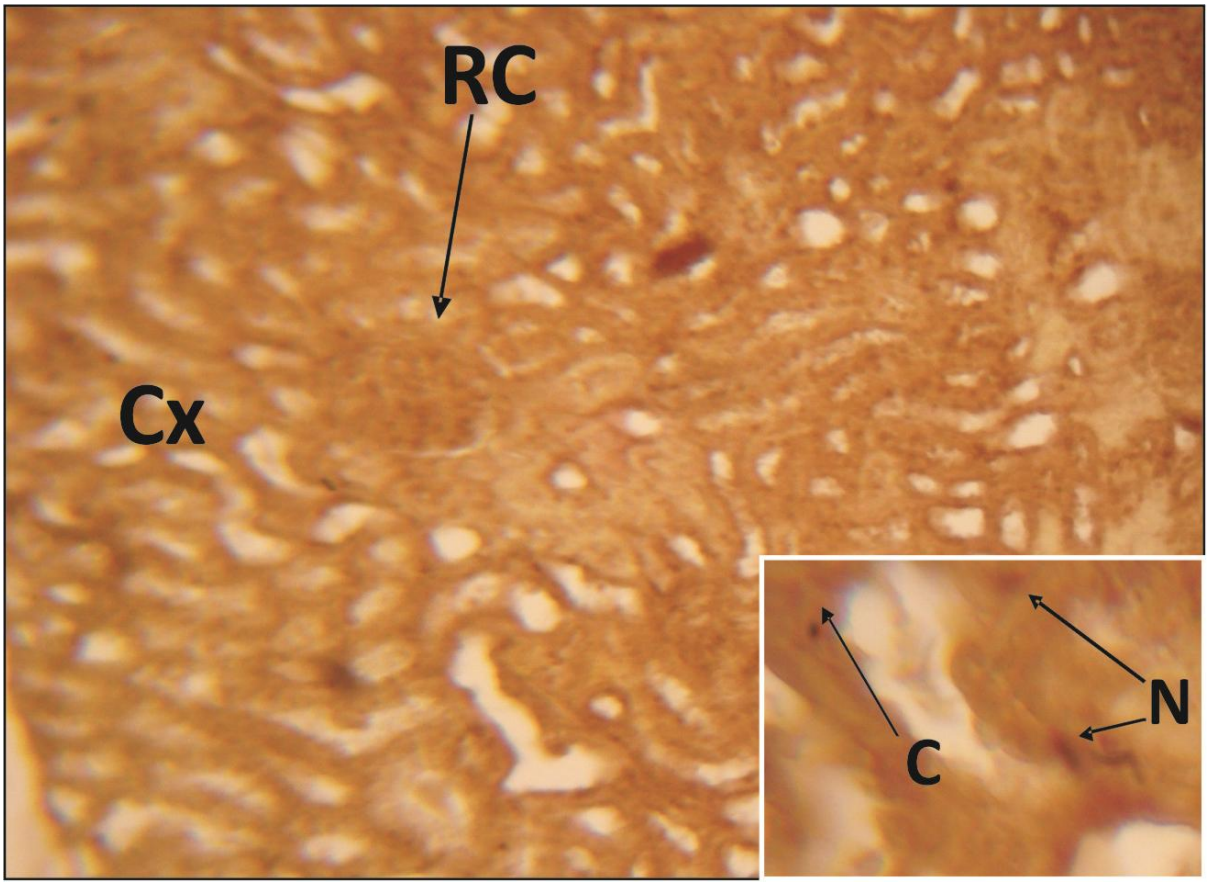


Plate XXIV: Kidney (Calcium acetate Fixative), Sobo alone

The histological features of the kidney were not well demonstrated with preservation in Buffered Formalin. The Sobo stain did not highlight features of histologic interest clearly. However, the nuclei (N) stained dark brown while the cytoplasm (C) had a lighter shade of the brownish colouration.

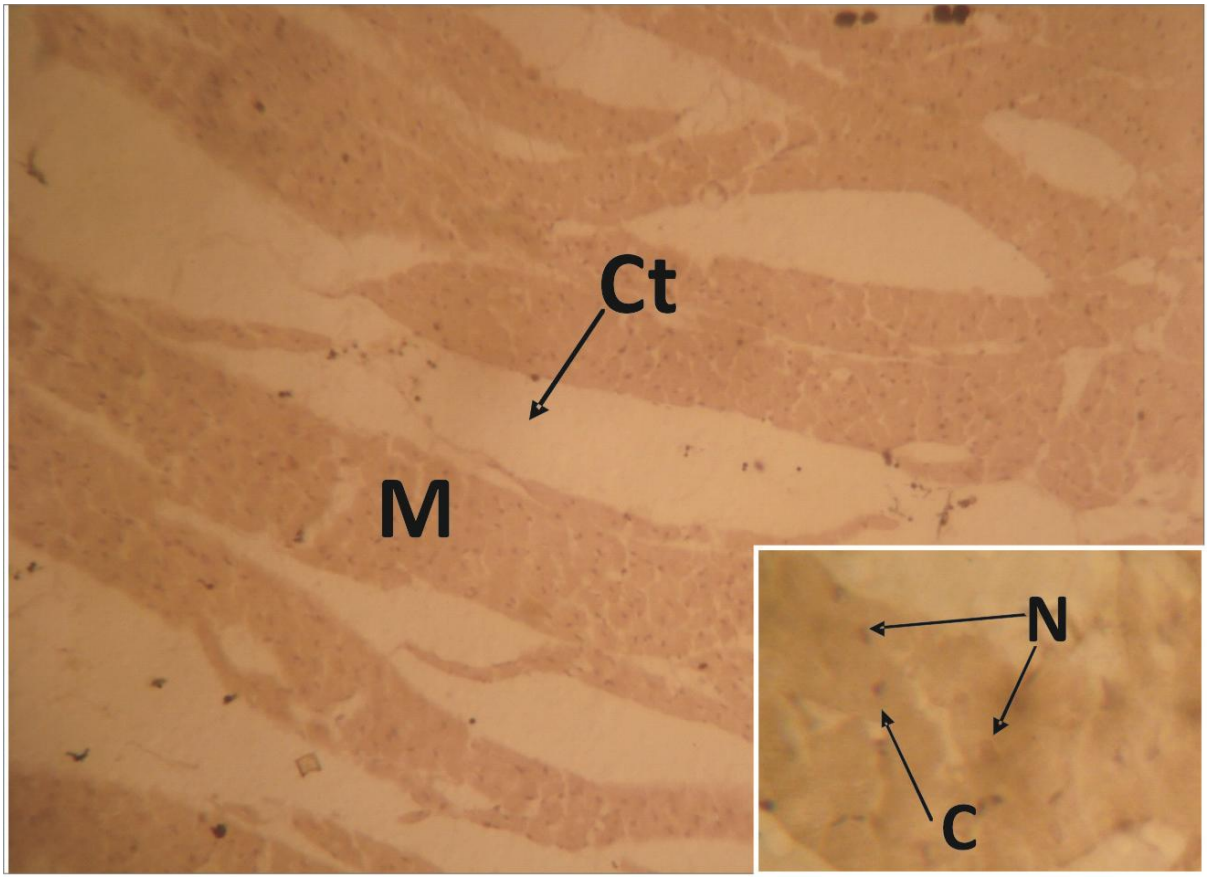


Plate XXV: Heart (Heidenhain's SUSA Fixative), Haematoxylin + Sorghum (H and S).

At a lower magnification of x 100, the muscle fibres (M) and connective tissues (Ct), between the muscle fibres of the heart are clearly differentiated. With H and S stain, the muscle fibres had a brownish colouration. At a higher magnification of x 400, the nuclei within the sarcoplasm are evident. With Heidenhain's SUSA fixative, Haematoxylin stained the nuclei (N) purplish while the cytoplasm (C) had a greyish colouration.

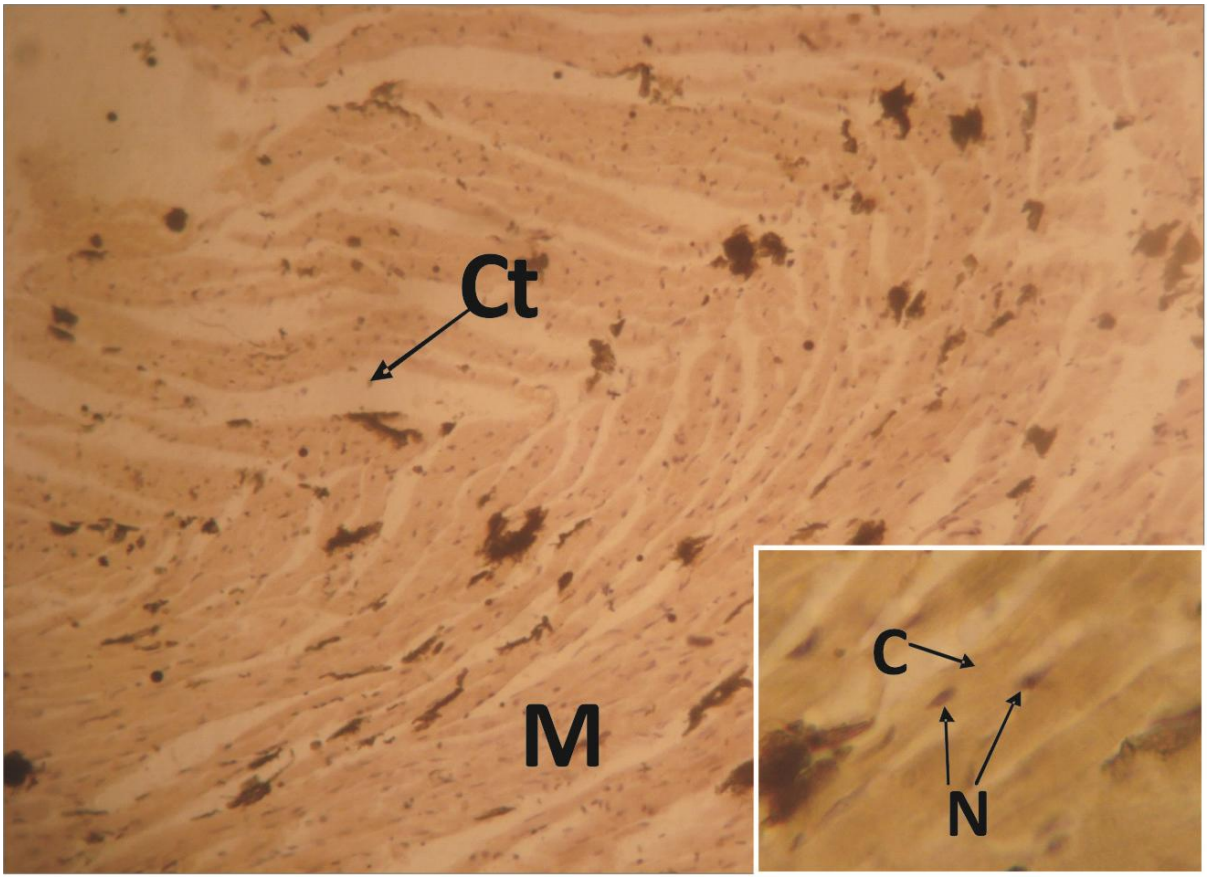


Plate XXVI: Heart (Helly's Fixative), Haematoxylin + Sorghum (H and S).

At a lower magnification of x 100, the muscle fibres (M) and connective tissues (Ct), between the muscle fibres of the heart are clearly differentiated. With H and S stain, the muscle fibres had a brownish colouration. At a higher magnification of x 400, the nuclei within the sarcoplasm are evident. With Helly's fixative, Haematoxylin stained the nuclei (N) purplish while the cytoplasm (C) had a greyish colouration.

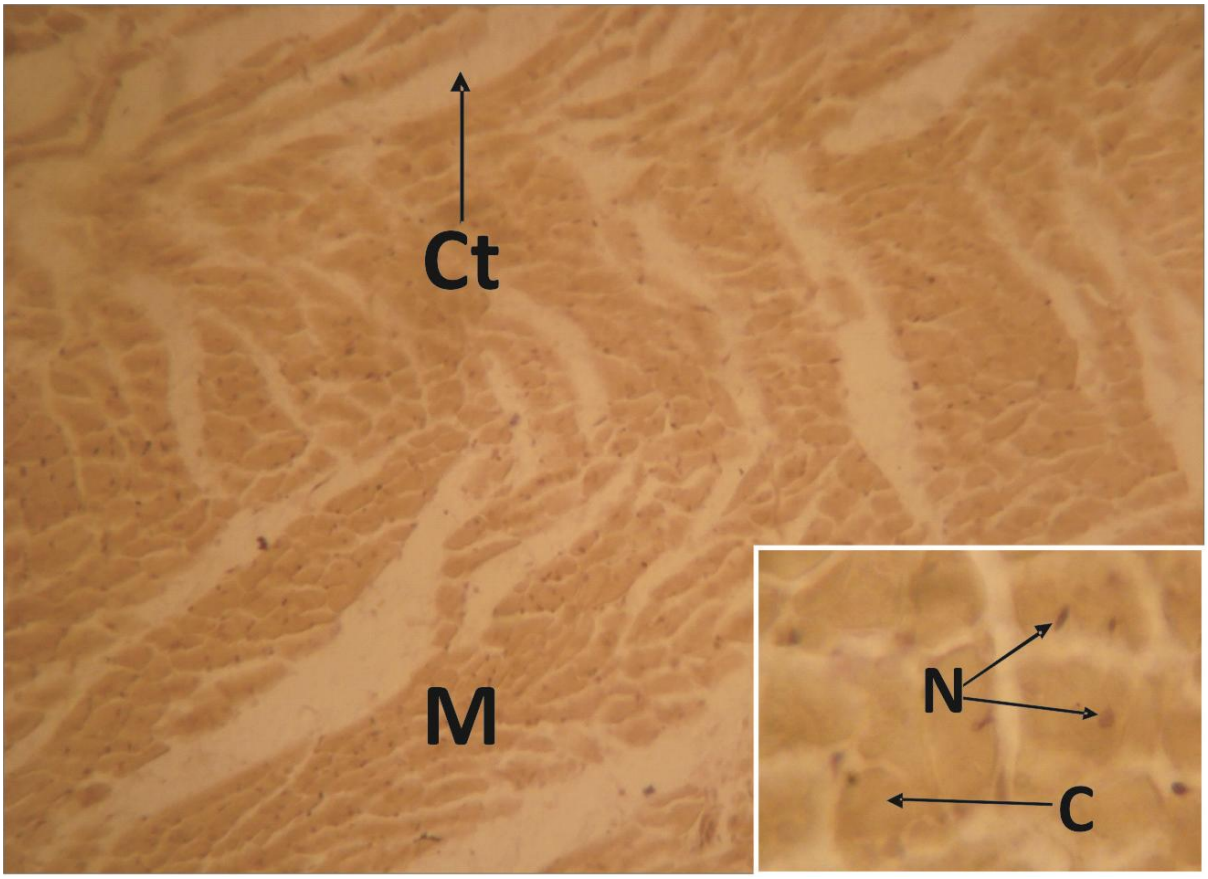


Plate XXVII: Heart (Muller's Fixative), Haematoxylin + Sorghum (H and S).

At a lower magnification of x 100, the muscle fibres (M) and connective tissues (Ct), between the muscle fibres of the heart are clearly differentiated. With H and S stain, the muscle fibres had a brownish colouration. At a higher magnification of x 400, the nuclei within the sarcoplasm are evident. With Muller's fixative, Haematoxylin stained the nuclei (N) purplish while the cytoplasm (C) had a greyish colouration.

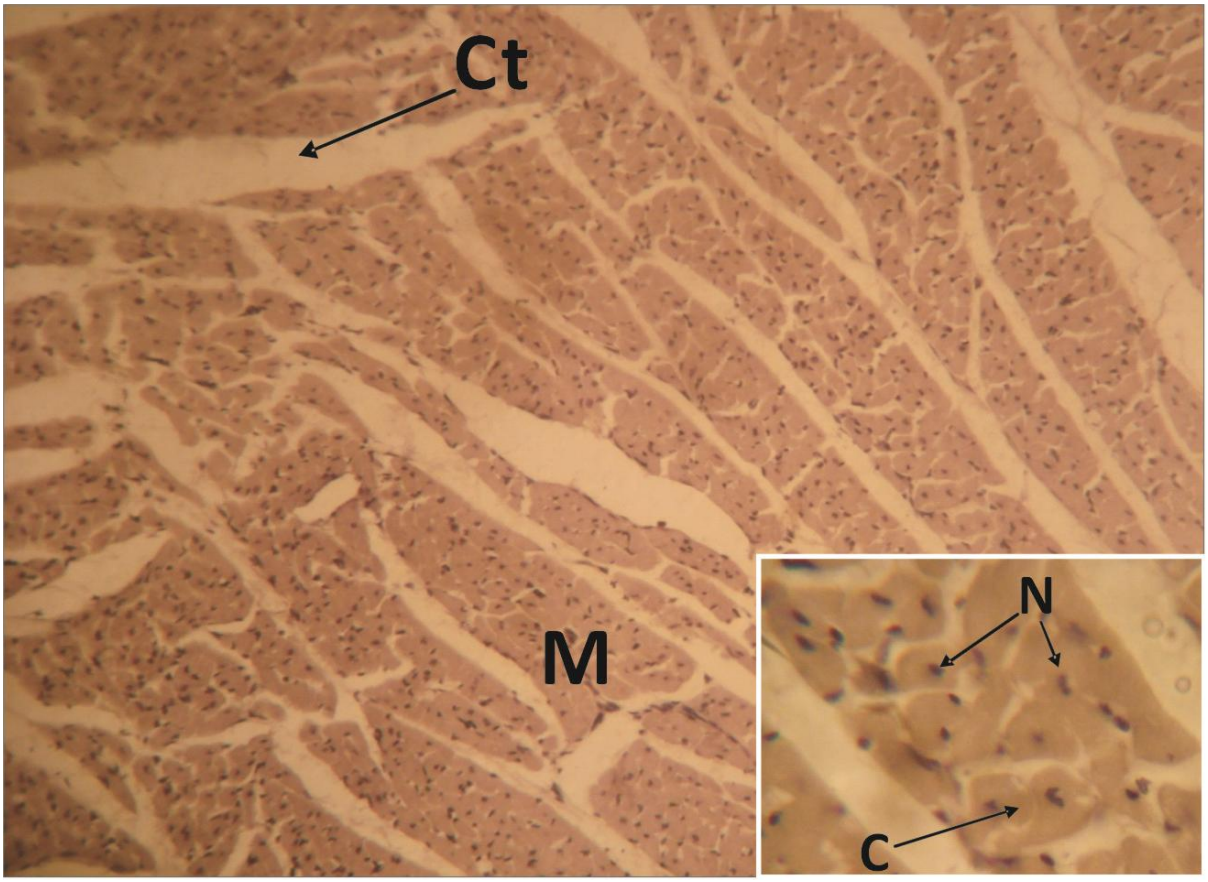


Plate XXVIII: Heart (Carnoy's Fixative), Haematoxylin + Sorghum (H and S).

At a lower magnification of x 100, the muscle fibres (M) and connective tissues (Ct) between the muscle fibres of the heart are clearly differentiated. With H and S stain, the muscle fibres had a light purple colouration. At a higher magnification of x 400, the nuclei within the sarcoplasm are evident. With Carnoy's fixative, Haematoxylin stained the nuclei (N) purplish while the cytoplasm (C) had a greyish colouration.

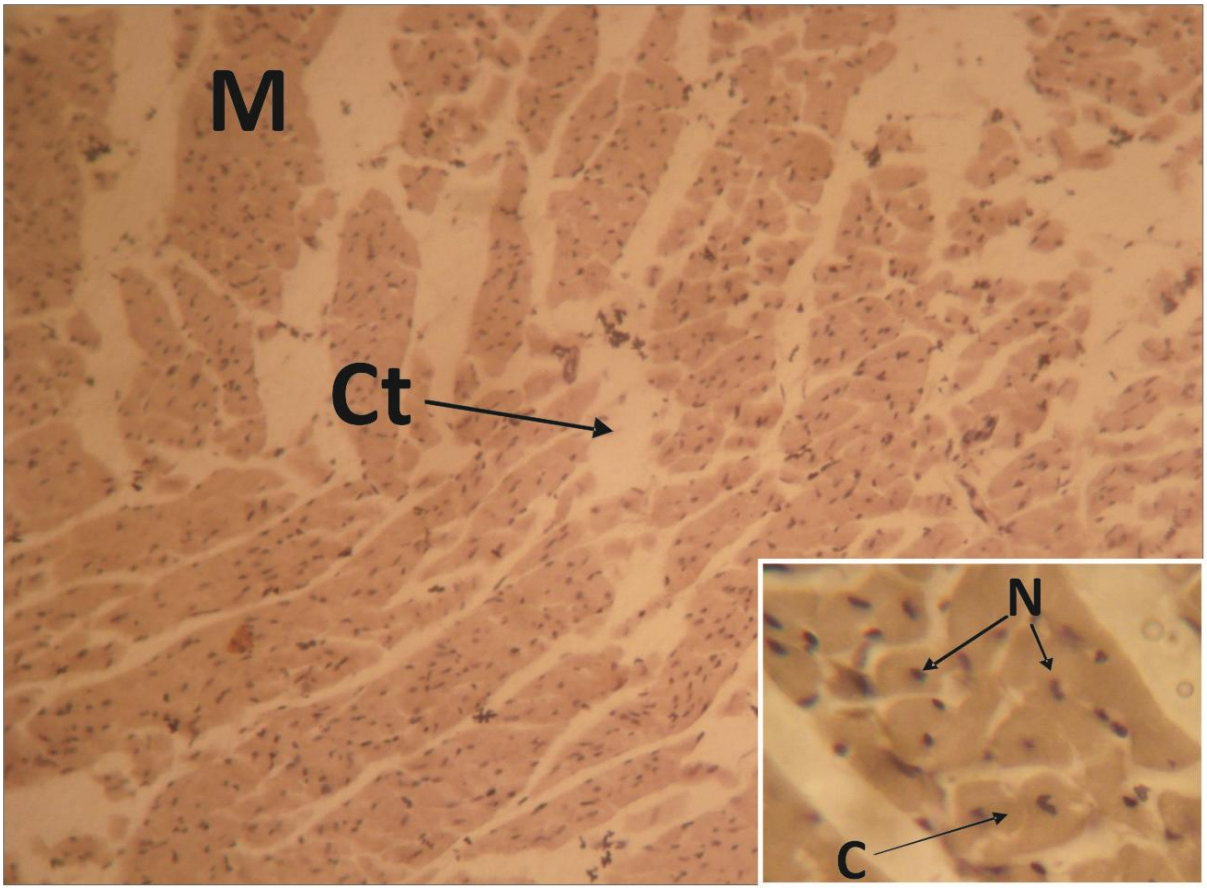


Plate XXIX: Heart (Buffered Formalin Fixative), Haematoxylin + Sorghum (H and S).

At a lower magnification of x 100, the muscle fibres (M) and connective tissues (Ct), between the muscle fibres of the heart are clearly differentiated. With H and S stain, the muscle fibres had a light purple colouration. At a higher magnification of x 400, the nuclei within the sarcoplasm are evident. With Buffered Formalin fixative, Haematoxylin stained the nuclei (N) purplish while the cytoplasm (C) had a greyish colouration.

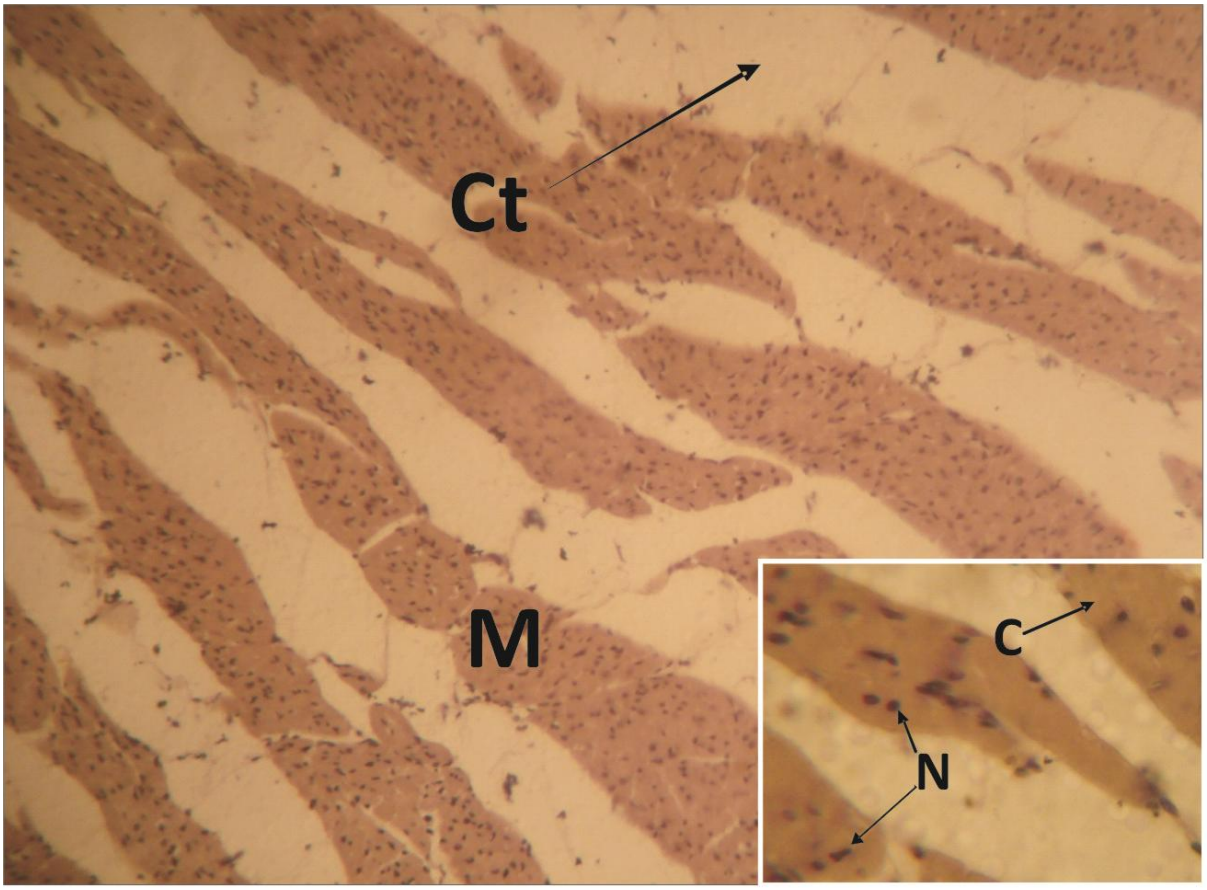


Plate XXX: Heart (Calcium acetate Fixative), Haematoxylin + Sorghum (H and S).

At a lower magnification of x 100, the muscle fibres (M) and connective tissues (Ct) between the muscle fibres of the heart are clearly differentiated. With H and S stain, the muscle fibres had a light purple colouration. At a higher magnification of x 400, the nuclei within the sarcoplasm are evident. With Calcium acetate fixative, Haematoxylin stained the nuclei (N) purplish while the cytoplasm (C) had a greyish colouration.

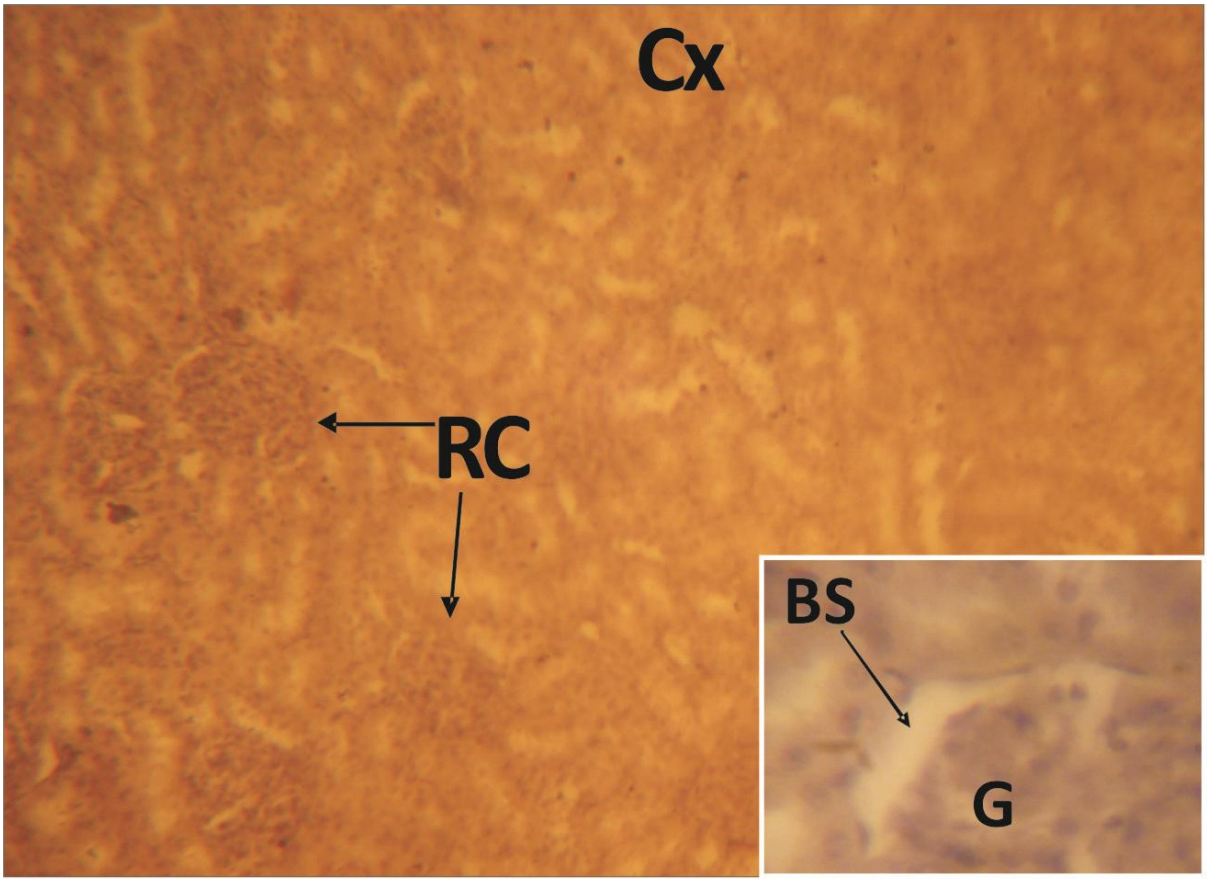


Plate XXXI: Kidney (Heidenhain's SUSA Fixative) Haematoxylin + Sorghum (H and S).

The histological features of the kidney were well preserved in Heidenhain's SUSA fixative, the renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were not clearly demonstrated. The cells of the kidney cortex stained generally brownish while using H and S, the glomerular nuclei within the glomerulus (G) had a purplish colour. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be faintly seen within the kidney cortex (Cx).

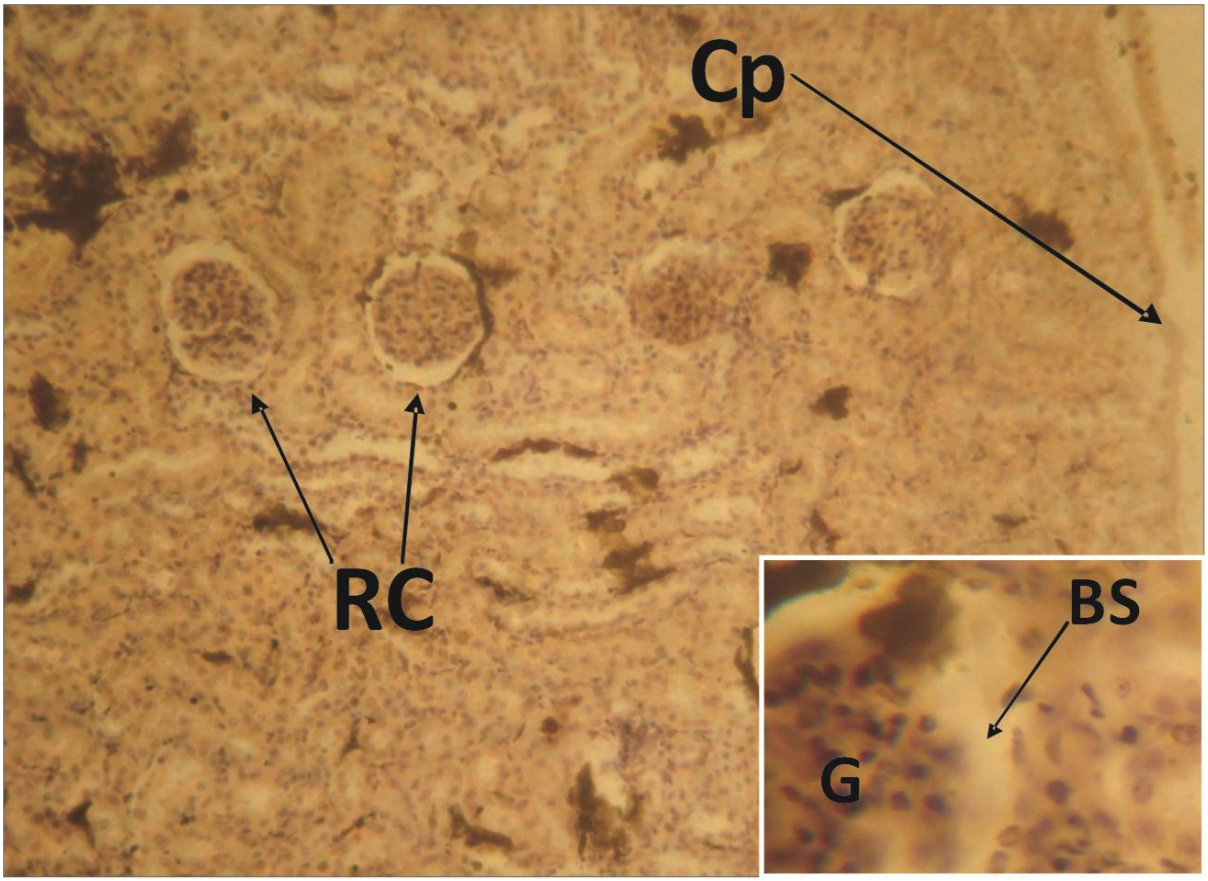


Plate XXXII: Kidney (Helly's Fixative) Haematoxylin + Sorghum (H and S).

The histological features of the kidney were well preserved in Helly's fixative, the renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were not clearly demonstrated. The cells of the kidney cortex stained generally brownish while using H and S, the glomerular nuclei within the glomerulus (G) had a purplish colour. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be faintly seen within the kidney cortex (Cx).

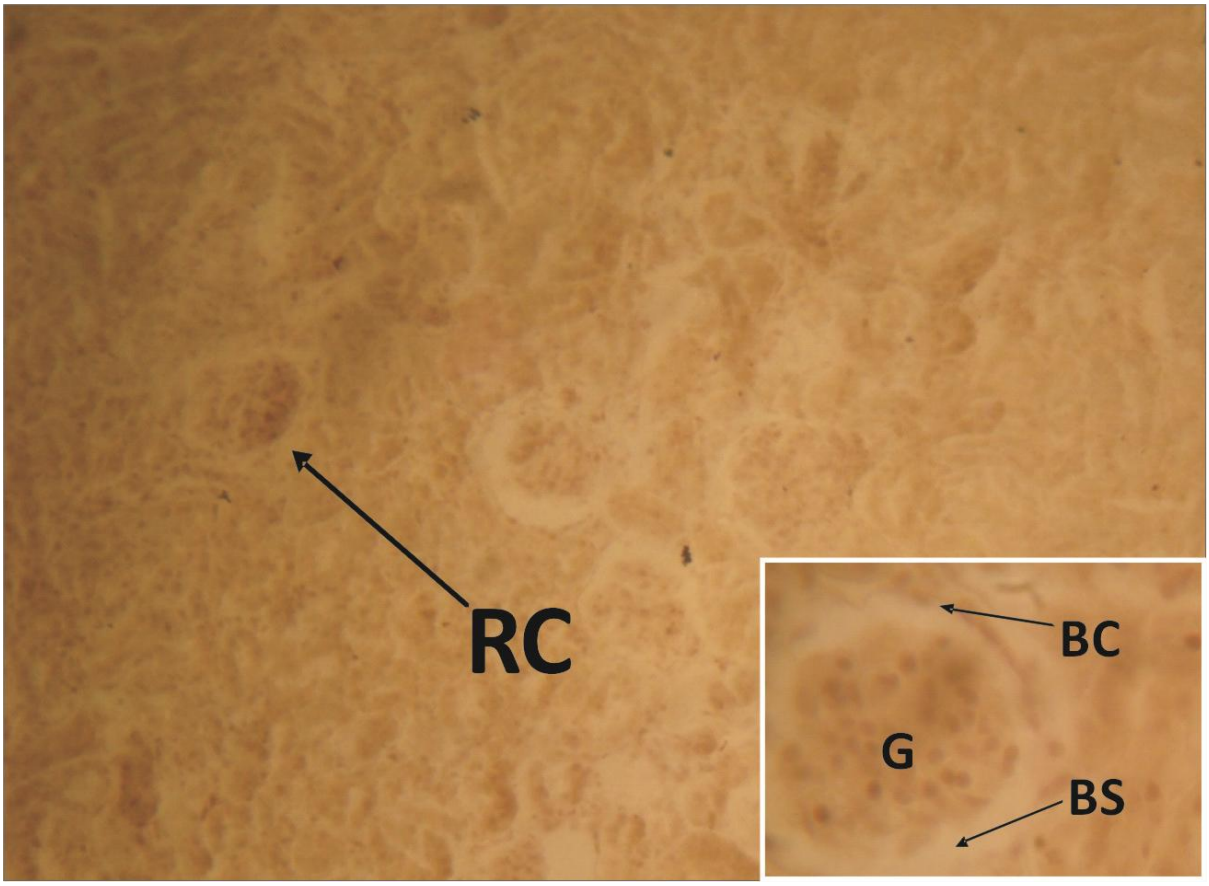


Plate XXXIII: Kidney (Muller's Fixative) Haematoxylin + Sorghum (H and S).

Muller's fixative preserved the histological features of the kidney; the renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were not clearly demonstrated. The cells of the kidney cortex stained generally brownish while using H and S, the glomerular nuclei within the glomerulus (G) had a purplish colour. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be faintly seen within the kidney cortex (Cx).

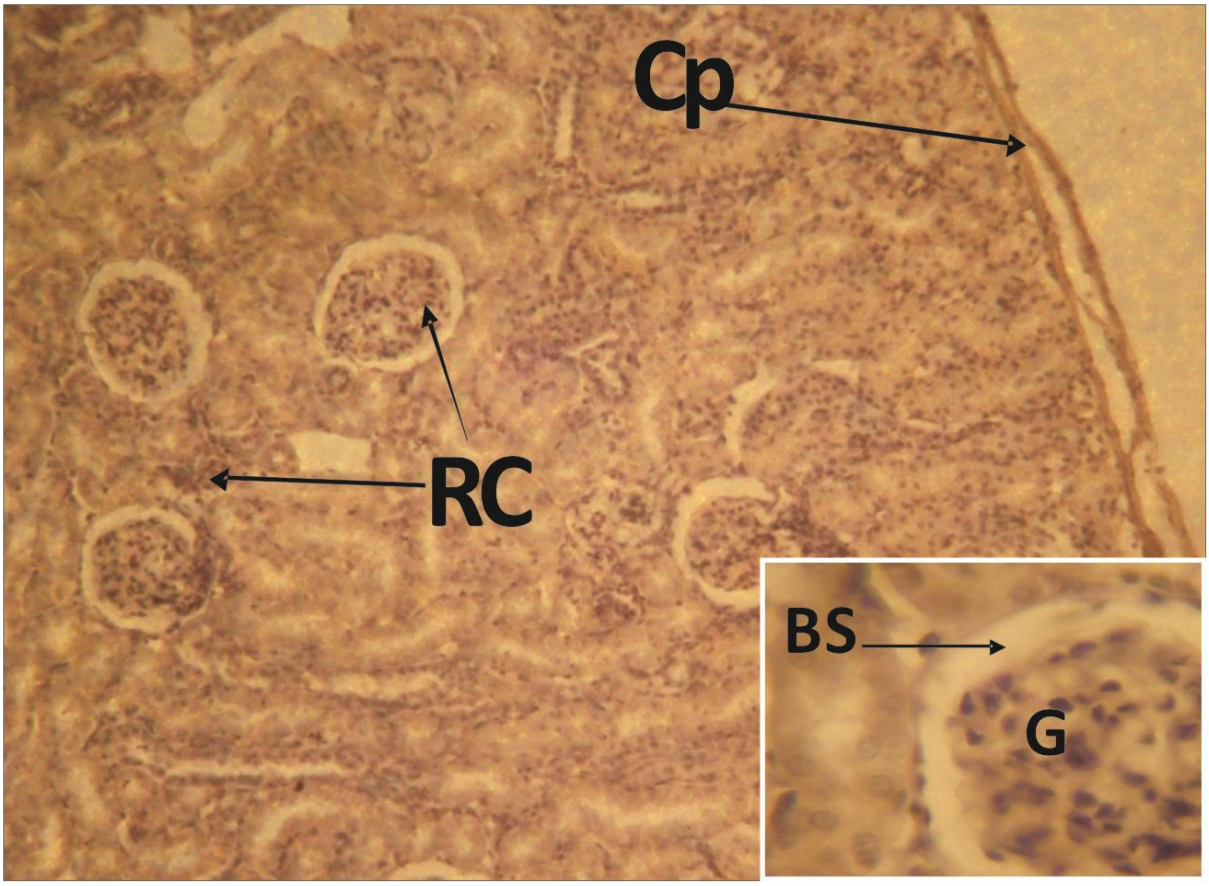


Plate XXXIV: Kidney (Carnoy's Fixative) Haematoxylin + Sorghum (H and S).

The histological features of the kidney were well preserved in Carnoy's fixative; the renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were clearly demonstrated. The cells of the kidney cortex stained generally brownish while using H and S, the glomerular nuclei within the glomerulus (G) had a purplish colour. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be clearly seen within the kidney cortex (Cx).

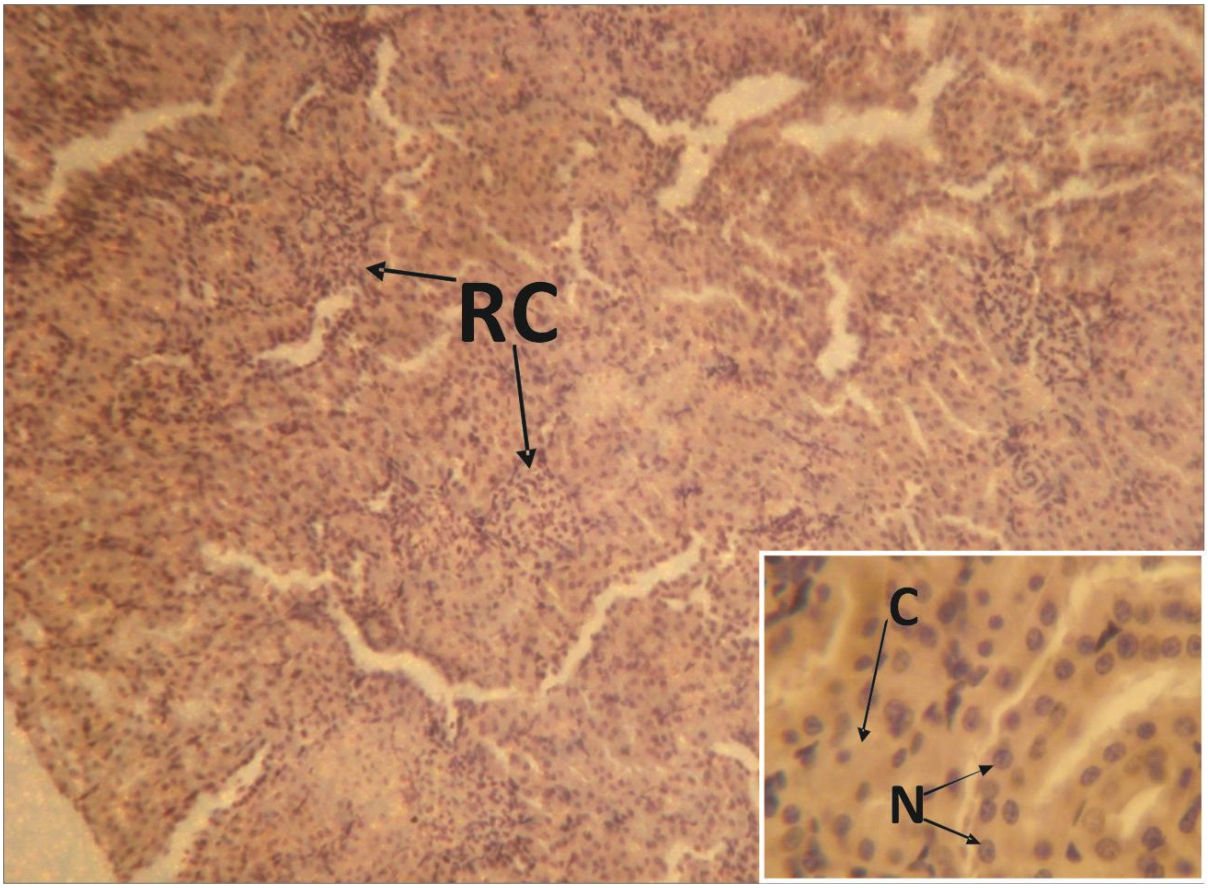


Plate XXXV: Kidney (Buffered Formalin Fixative) Haematoxylin + Sorghum (H and S).

Buffered Formalin fixative preserved the histological features of the kidney; the renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were not clearly demonstrated. The cells of the kidney cortex stained generally brownish while using H and S, the glomerular nuclei within the glomerulus (G) had a purplish colour. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be faintly seen within the kidney cortex (Cx).

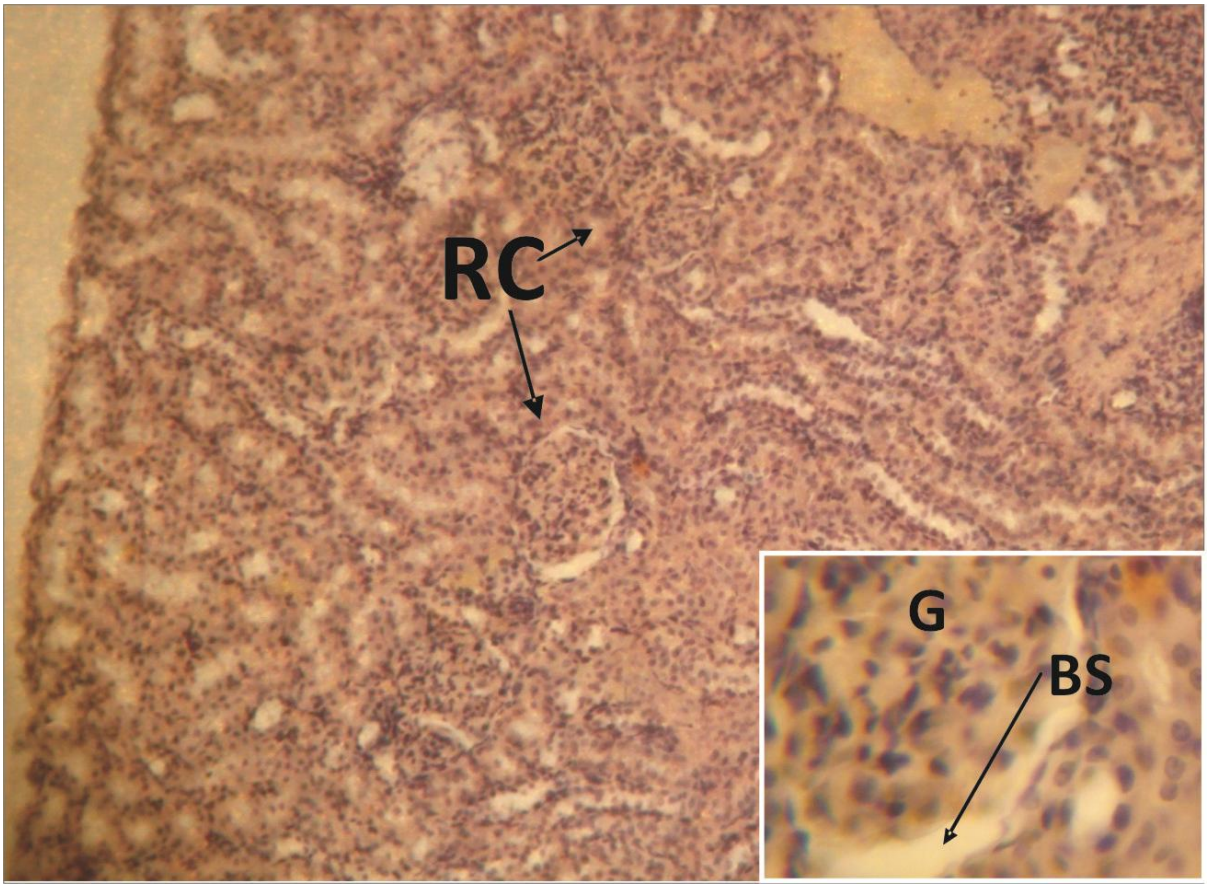


Plate XXXVI: Kidney (Calcium acetate Fixative) Haematoxylin + Sorghum (H and S).

The histological features of the kidney were well preserved in Calcium acetate fixative, but on staining the renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were poorly demonstrated. The cells of the kidney cortex stained generally brownish while using H and S, the glomerular nuclei within the glomerulus (G) had a purplish colour. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be clearly seen within the kidney cortex (Cx).

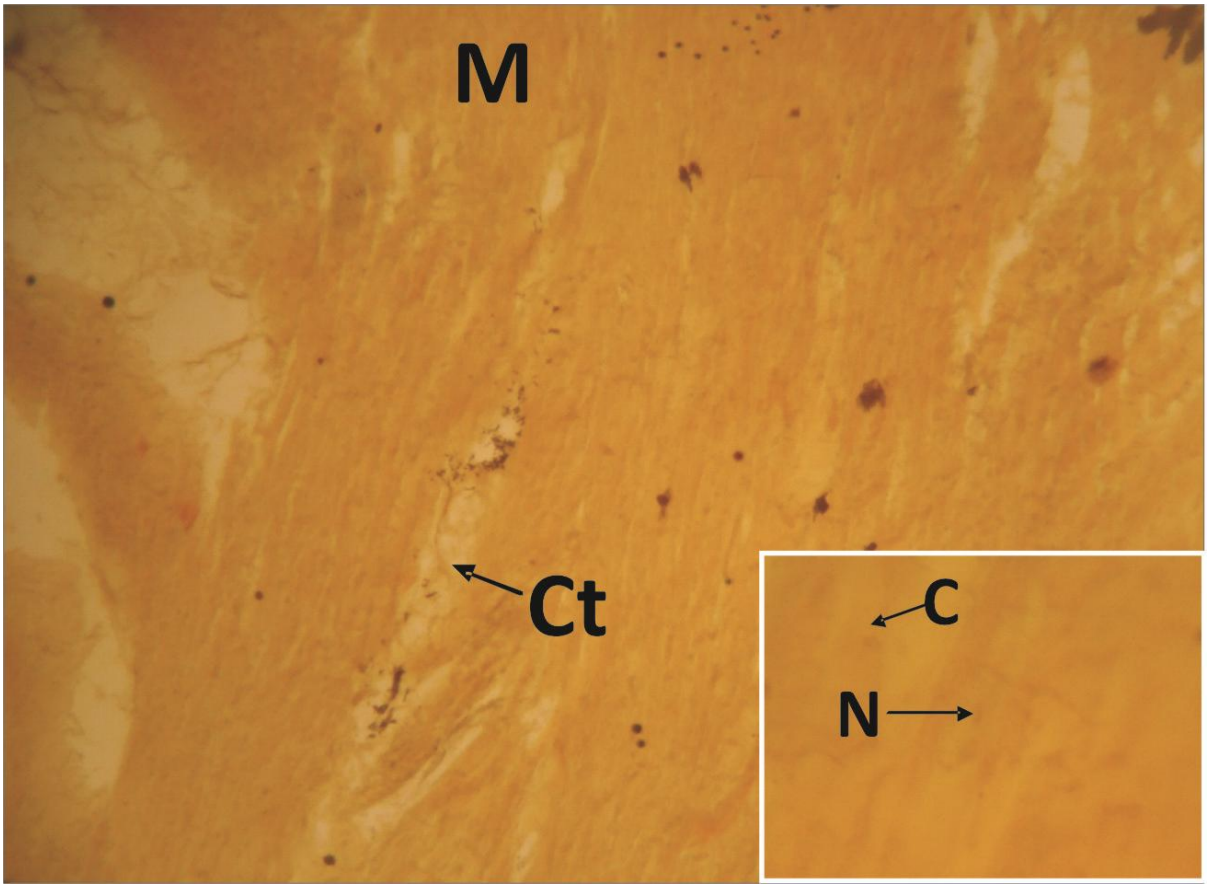


Plate XXXVII: Heart (Heidenhain's SUSA Fixative), Garcinia + Sobo (G and S).

At a lower magnification of x 100, the muscle fibres (M) and connective tissues (Ct) between the muscle fibres of the heart were not clearly differentiated. With G and S stain, the muscle fibres had a yellowish colouration. At a higher magnification of x 400 with Heidenhain's SUSA fixative, Sobo stained the nuclei (N) brownish while the cytoplasm (C) had a greenish-yellow colouration.

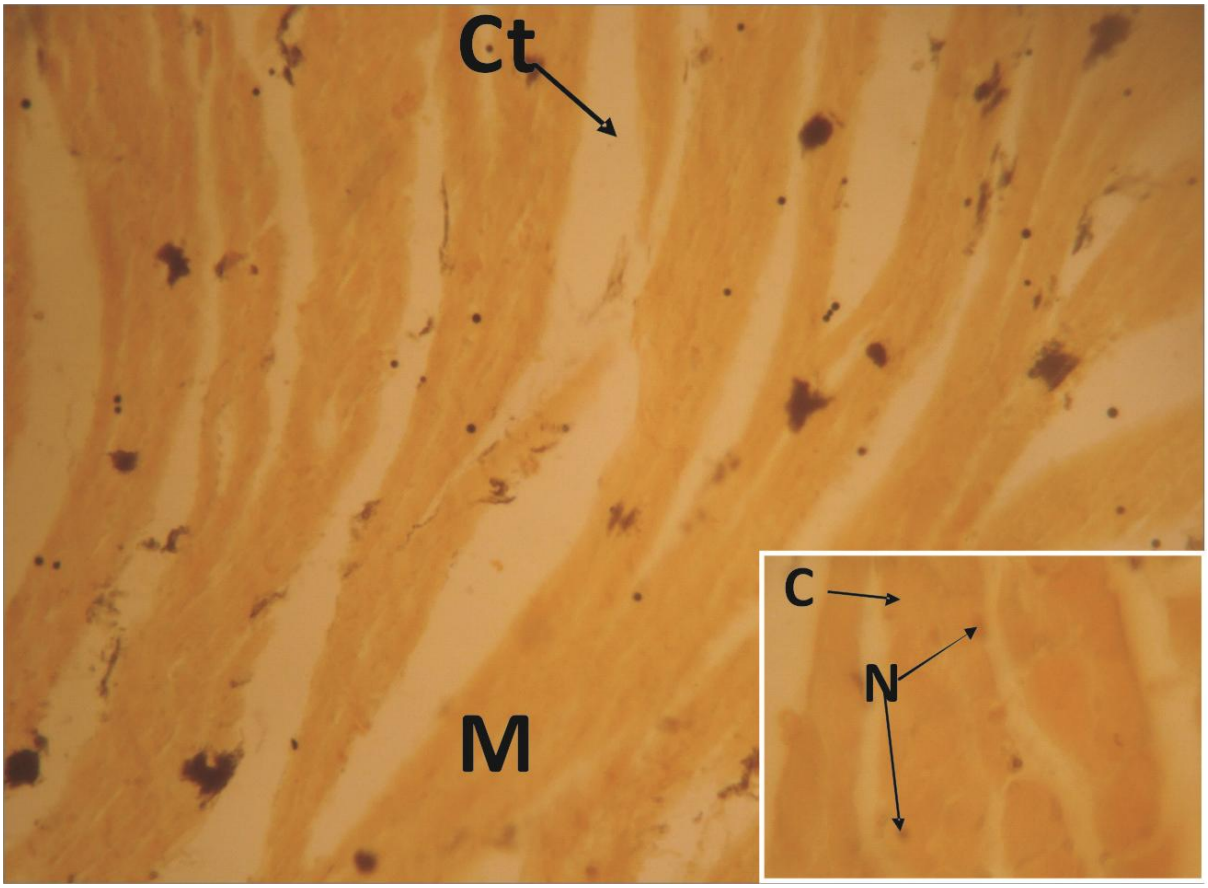


Plate XXXVIII: Heart (Helly's Fixative), Garcinia + Sobo (G and S).

At a lower magnification of x 100, the muscle fibres (M) and connective tissues (Ct) between the muscle fibres of the heart were not clearly differentiated. With G and S stain, the muscle fibres had a yellowish colouration. At a higher magnification of x 400 with Heidenhain's SUSA fixative, Sobo stained the nuclei (N) brownish while the cytoplasm (C) had a yellowish colouration.

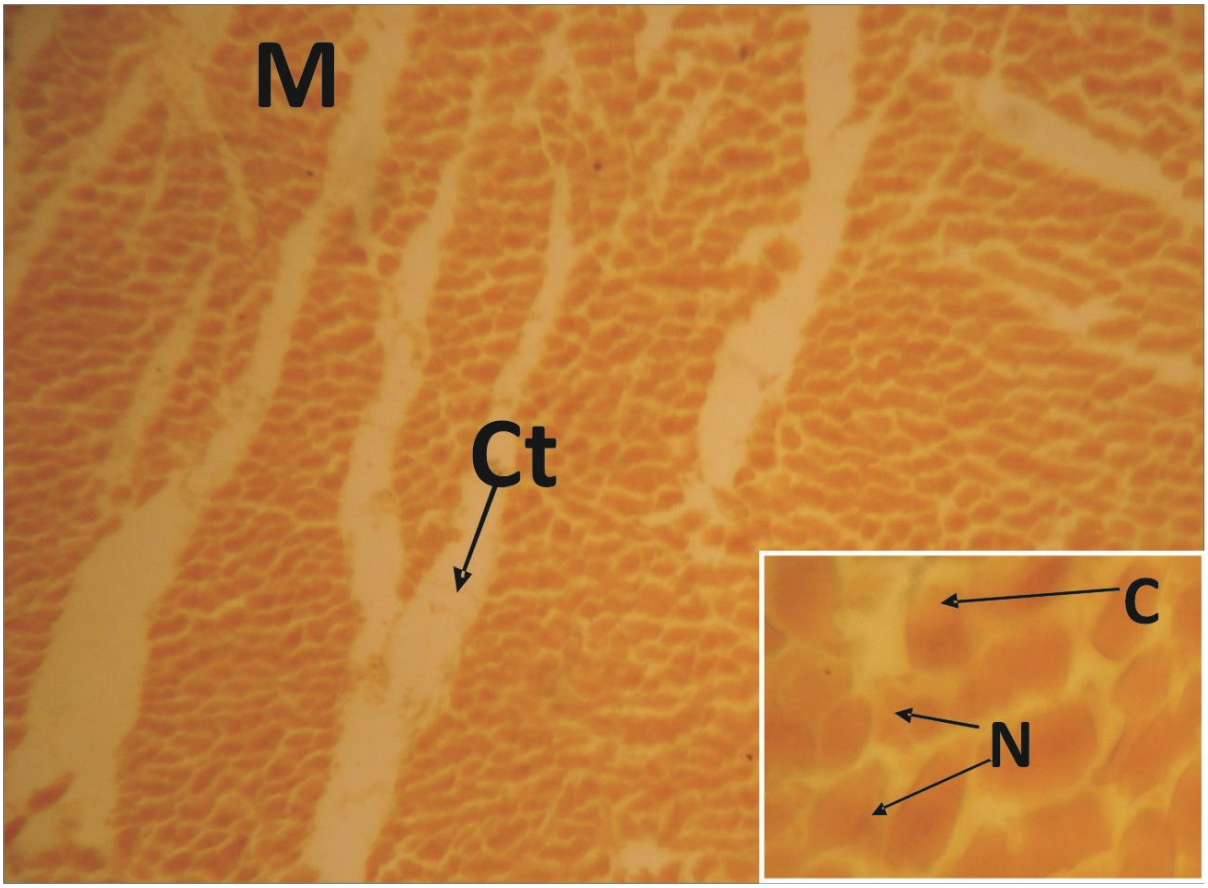


Plate XXXIX: Heart (Muller's Fixative), Garcinia + Sobo (G and S).

At a lower magnification of x 100, the muscle fibres (M) and connective tissues (Ct) between the muscle fibres of the heart were not clearly differentiated. With G and S stain, the muscle fibres had a yellowish colouration. At a higher magnification of x 400 with Heidenhain's SUSA fixative, Sobo stained the nuclei (N) brownish while the cytoplasm (C) had a yellowish colouration.

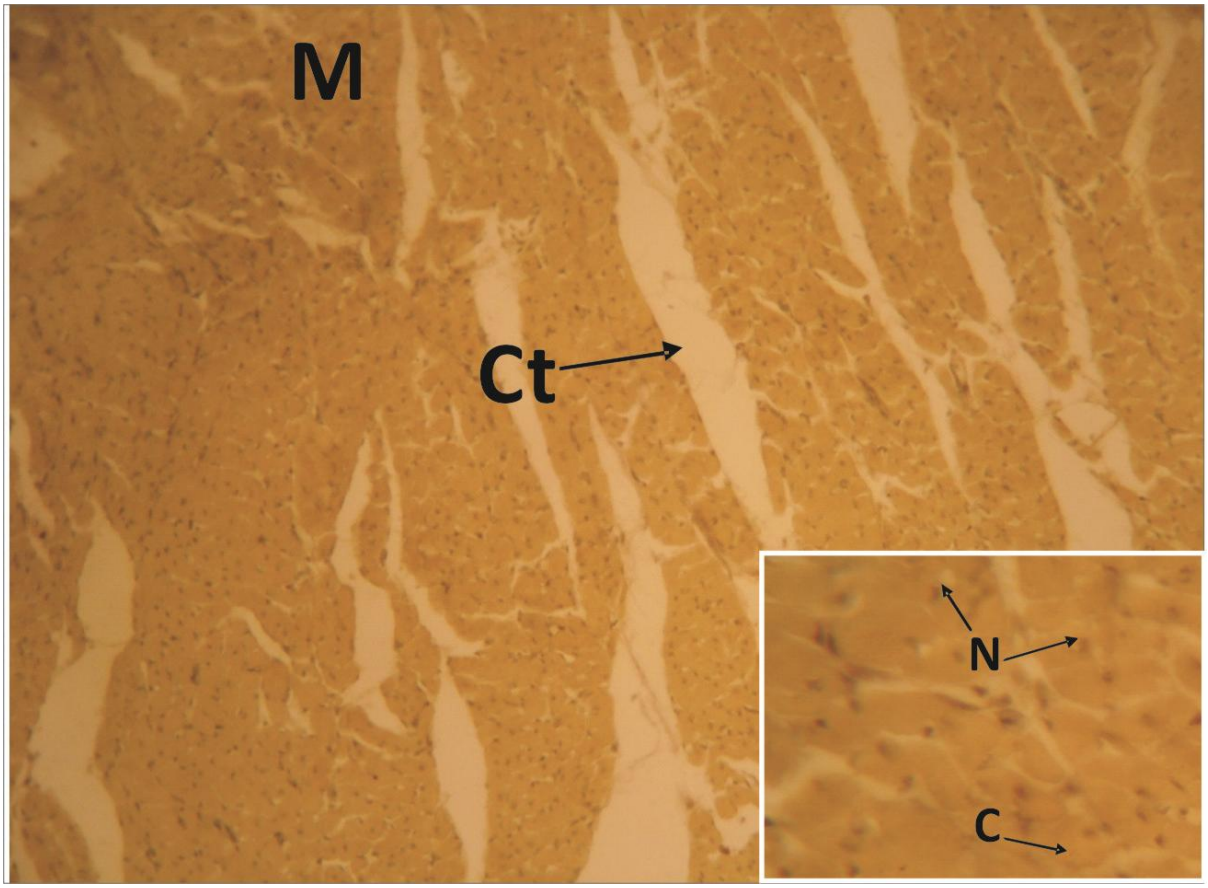


Plate XL: Heart (Carnoy's Fixative) Garcinia + Sobo (G and S).

At a lower magnification of x 100, the muscle fibres (M) and connective tissues (Ct) between the muscle fibres of the heart were clearly differentiated. With G and S stain, the muscle fibres had a greyish colouration. At a higher magnification of x 400 with Heidenhain's SUSA fixative, Sobo stained the nuclei (N) purplish while the cytoplasm (C) had a greyish colouration.

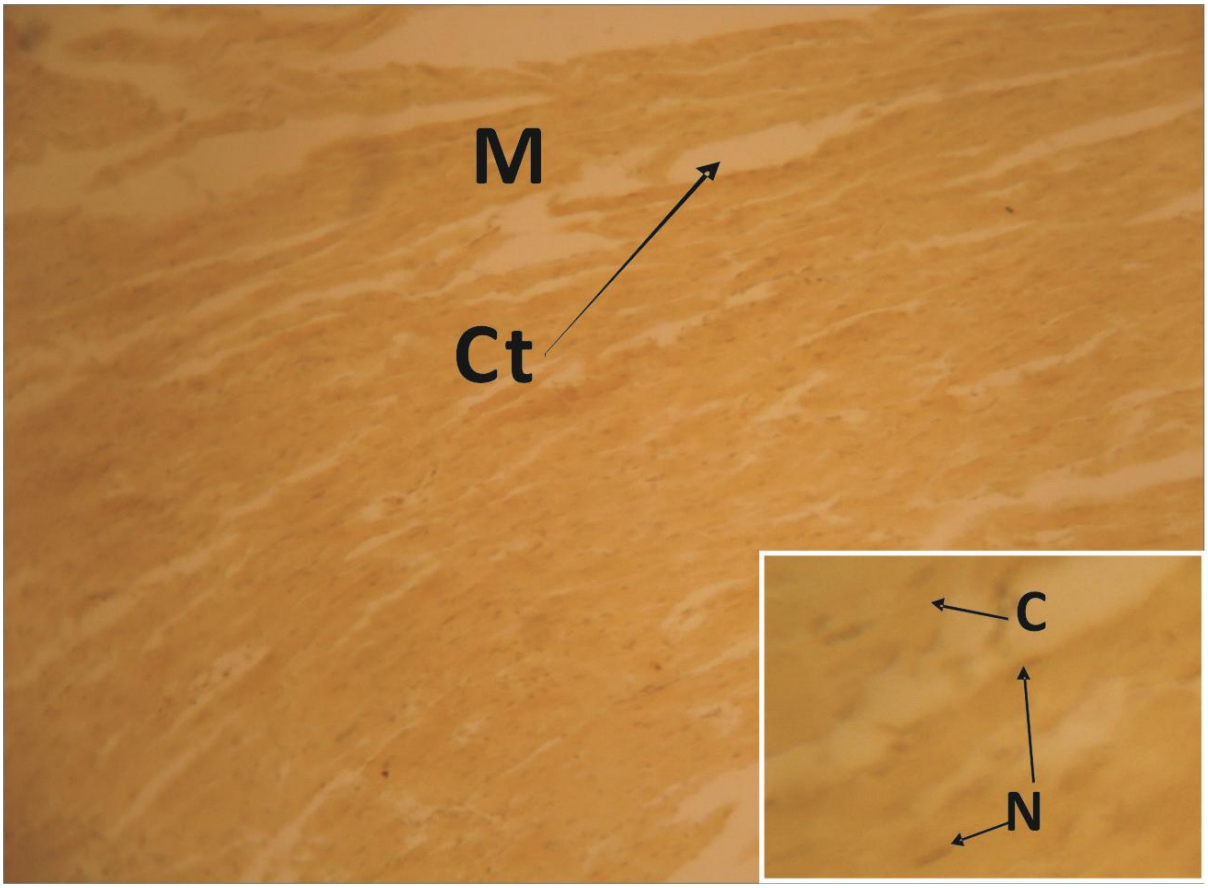


Plate XLI: Heart (Buffered Formalin Fixative), Garcinia + Sobo (G and S).

At a lower magnification of x 100, the muscle fibres (M) and connective tissues (Ct) between the muscle fibres of the heart were not clearly differentiated. With G and S stain, the muscle fibres had a yellowish colouration. At a higher magnification of x 400 with Heidenhain's SUSA fixative, Sobo stained the nuclei (N) brownish while the cytoplasm (C) had a greyish colouration.

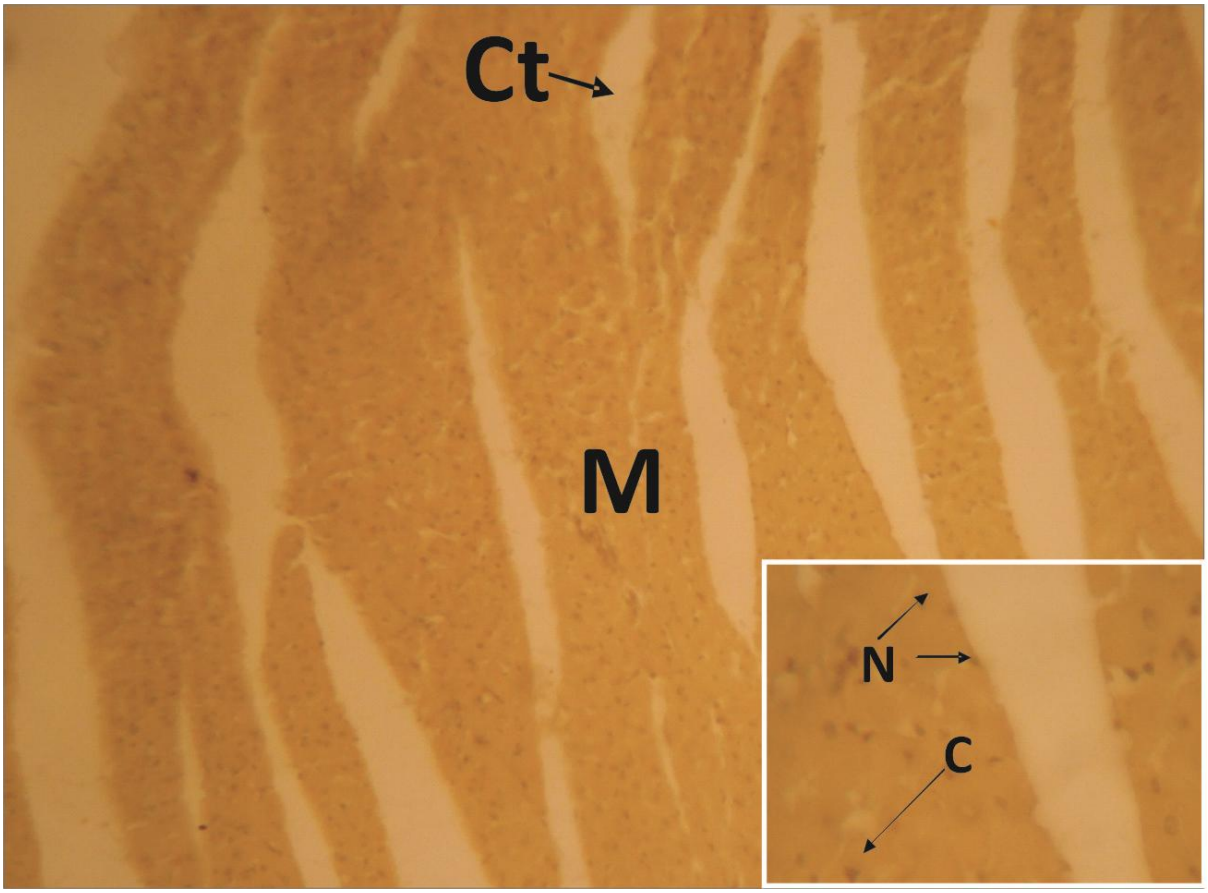


Plate XLII: Heart (Calcium acetate Fixative), Garcinia + Sobo (G and S).

At a lower magnification of x 100, the muscle fibres (M) and connective tissues (Ct) between the muscle fibres of the heart were not clearly differentiated. With G and S stain, the muscle fibres had a yellowish colouration. At a higher magnification of x 400 with Heidenhain's SUSA fixative, Sobo stained the nuclei (N) purplish while the cytoplasm (C) had a greyish colouration.

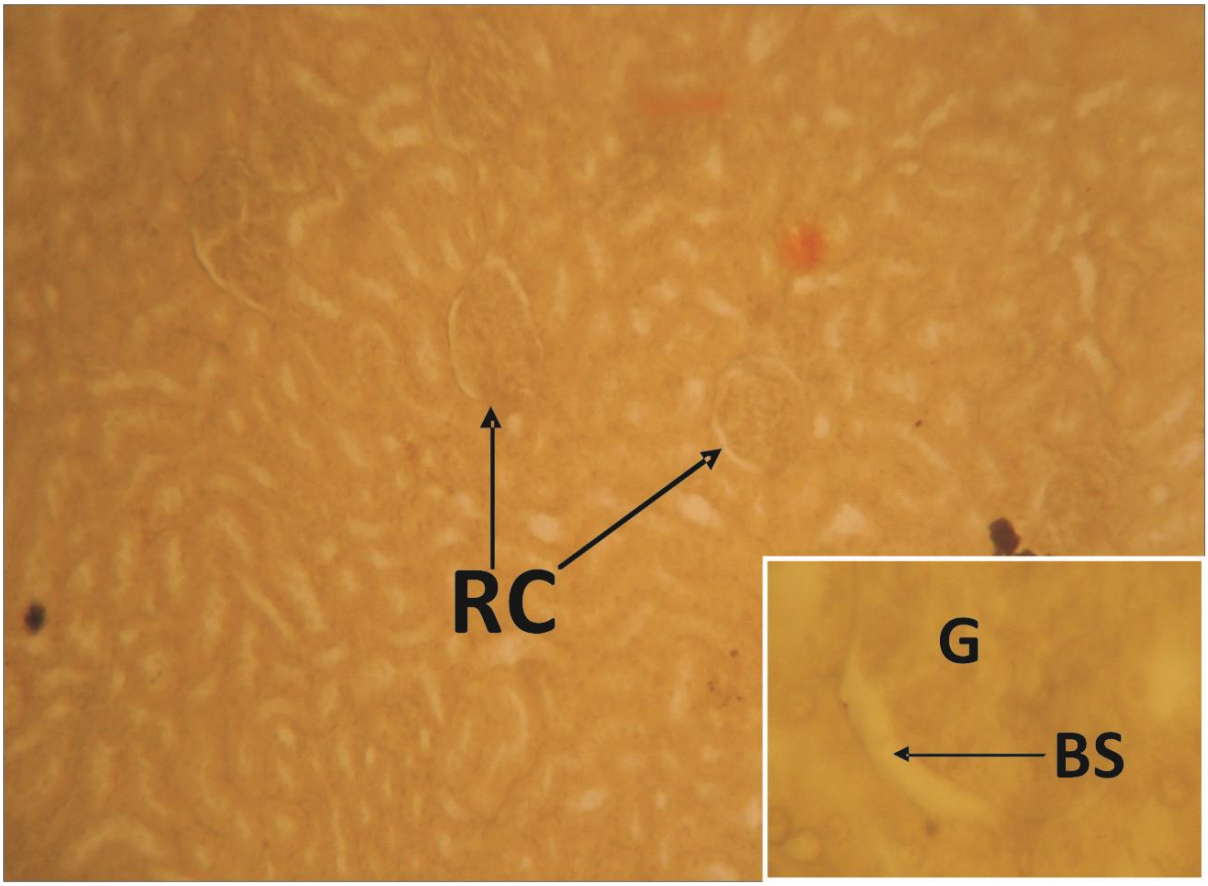


Plate XLIII: Kidney (Heidenhain's SUSA Fixative), Garcinia + Sobo (G and S).

The histological features of the kidney were well preserved in Heidenhain's SUSA fixative; the renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were not clearly demonstrated. The cells of the kidney cortex stained generally greyish while using G and S, the glomerular nuclei within the glomerulus (G) was not distinct. The Bowman's space (BS) and Bowman's capsule (BC) can also be faintly seen within the kidney cortex (Cx).

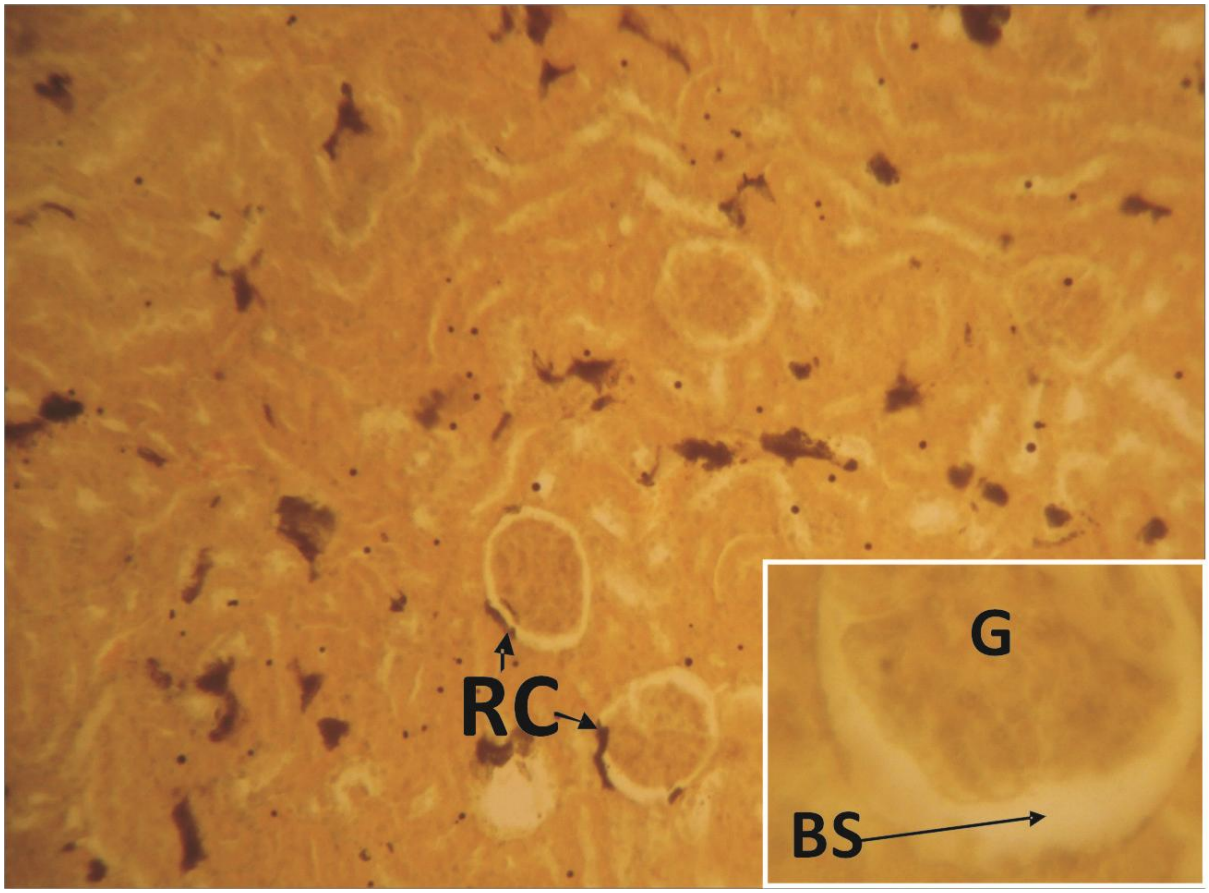


Plate XLIV: Kidney (Helly's Fixative), Garcinia + Sobo (G and S).

The histological features of the kidney were well preserved in Helly's fixative; the renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were not clearly demonstrated. The cells of the kidney cortex stained generally while using H and S, the glomerular nuclei within the glomerulus (G) had a purplish colour. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be faintly seen within the kidney cortex (Cx).

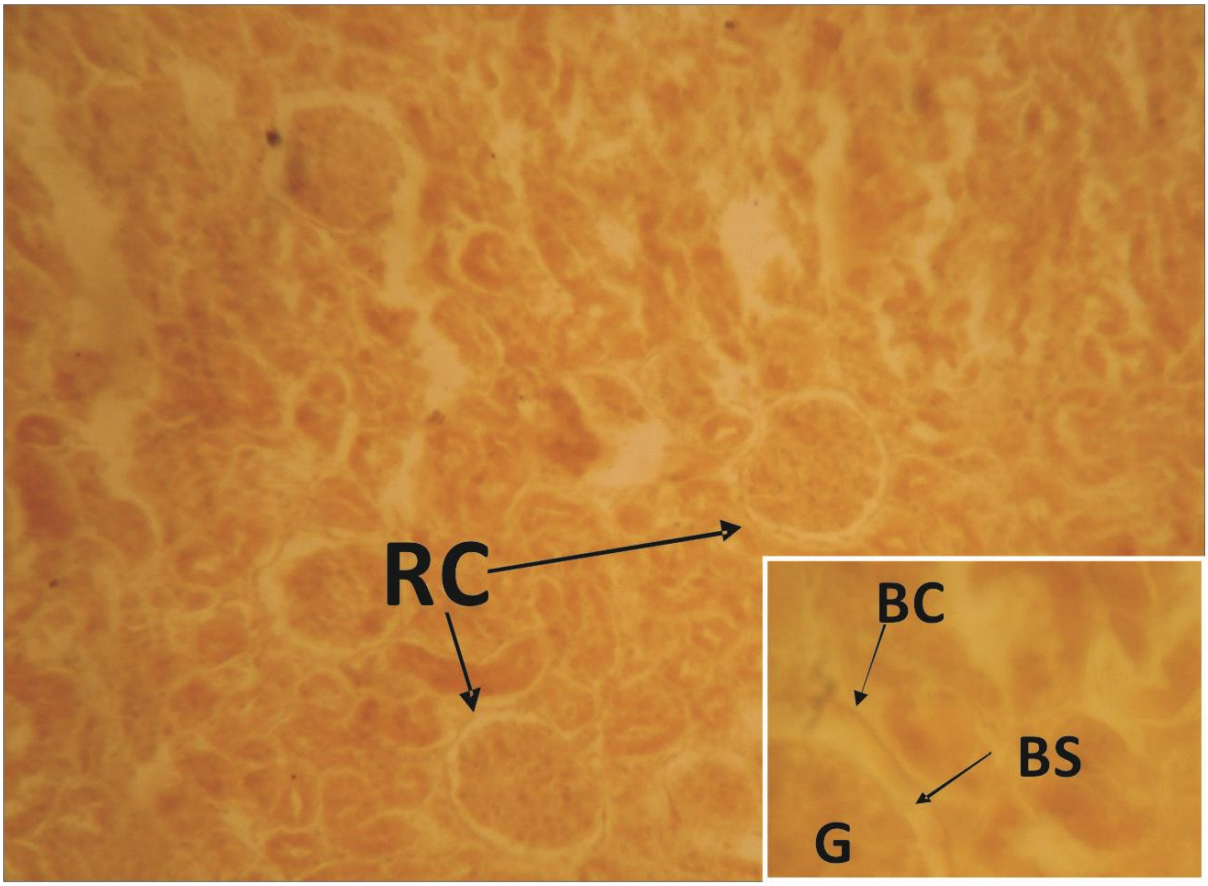


Plate XLV: Kidney (Muller's Fixative), Garcinia + Sobo (G and S).

Muller's fixative preserved the histological features of the kidney; the renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were not clearly demonstrated. The cells of the kidney cortex stained generally yellowishish while using G and S, the glomerular nuclei within the glomerulus (G) had a brownish colour. The Bowman's space (BS) was clear due to the shrinkage effect of the fixative. The Bowman's capsule (BC) can also be clearly seen within the kidney cortex (Cx).

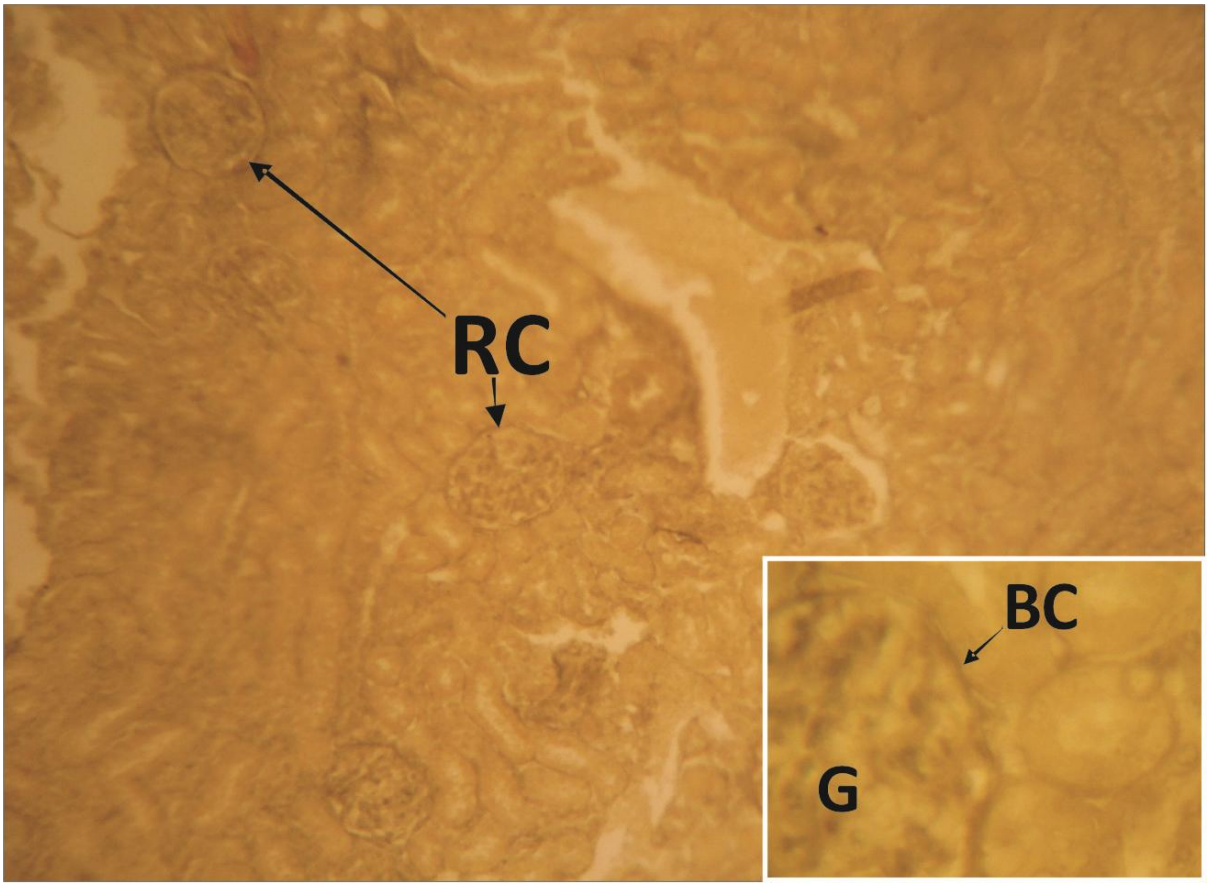


Plate XLVI: Kidney (Carnoy's Fixative), Garcinia + Sobo (G and S).

Carnoy's fixative preserved the histological features of the kidney; the renal corpuscles (RC) at x 100 and the glomerulus at x 400 magnifications were not clearly demonstrated. The cells of the kidney cortex stained generally yellowish while using G and S, the glomerular nuclei within the glomerulus (G) had a greenish colour. The Bowman's space (BS) was absent. The Bowman's capsule (BC) can also be clearly seen within the kidney cortex (Cx).

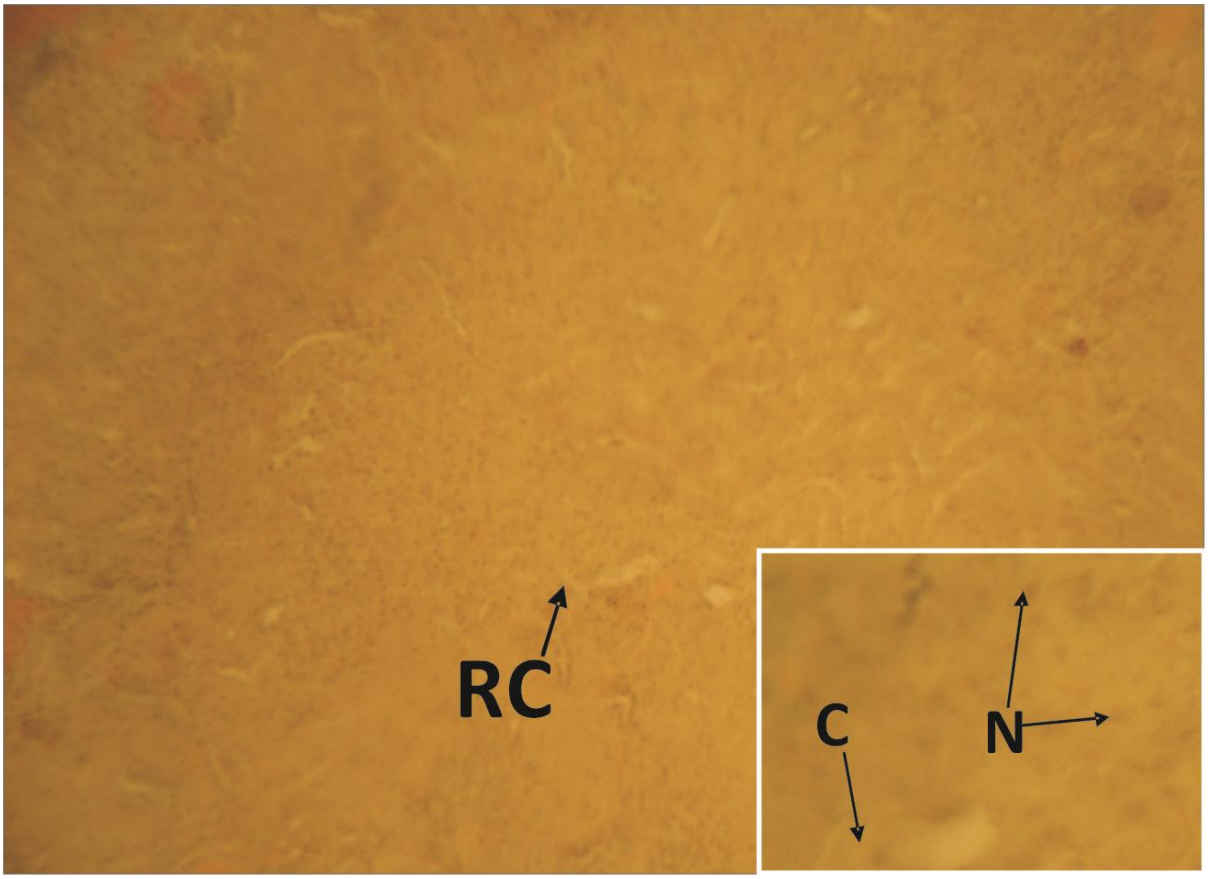


Plate XLVII: Kidney (Calcium acetate Fixative), Haematoxylin + Sorghum (H and S).

The histological features of the kidney were well preserved in Calcium acetate fixative as demonstrated on other sections, however, on staining, the renal corpuscles (Rc) and the glomerulus (G) were not distinctly highlighted. The photomicrograph appeared greenish in colour.

CHAPTER FIVE

5.0 DISCUSSION

This study has attempted to elucidate the histologic effect of fixatives on the staining properties of local stains obtained from plant extracts (*Hibiscus sabdariffa, Sorghum bicolor and Garcinia livingstonei*).

Haematoxylin and Eosin: Myocardium

Haematoxylin and Eosin had been the age-long acceptable control standard in studies related to conventional histologic staining. However, processing the same tissue with different fixatives yielded notable differences. It was observed that the general muscle fibre outline for the myocardium was good, the nuclei were prominent and stained with the usual purplish colouration. However, the muscle fibres had different shades of colouration. Due to the effect of the fixatives the fibres appeared orange in colour with Helly and Muller fixatives, and with shades of brownish colouration using Carnoy, calcium acetate and buffered formalin. The shades of brownish colouration of the muscle fibre of the myocardium decreased in the following order;

Carnoy ⟹ Calcium acetate ⟹ Buffered formalin ⟹ SUSA

In a descending order, the histologic effect of fixatives on Haematoxylin and Eosin stained myocardium is as follows;

Carnoy ⟹ Calcium acetate ⟹ Helly ⟹ Buffered formalin ⟹ SUSA ⟹ Muller.

Kidney

In the kidney study of the same stain, the renal corpuscles were prominent with all the fixatives with the glomerular nuclei appearing purplish. The Bowman's space was evident but more clearly seen with Helly, Muller, Carnoy and Calcium acetate fixative than with SUSA and buffered formalin fixative. The cellular outline was either brownish or orange in colouration, with Helly and Muller fixative it appeared orange while with the others it stained with different shades of brownish colouration.

In a descending order, the histologic effect of fixatives on Haematoxylin and Eosin stained renal cortex is as follows;

Carnoy ⟹ SUSA ⟹ Calcium acetate ⟹ Buffered formalin ⟹ Muller ⟹ Helly.

Hibiscus sabdariffa: Myocardium

While using extracts from the *Hibiscus sabdariffa* alone on sections of the myocardium, it was observed that with the various fixatives, Sobo produced a colour contrast for both the nuclei and muscle fibres. This contrast was of varying intensity. The nuclei appeared dark brown while the muscle fibre appeared light brown.

With Carnoy and calcium acetate fixatives, the nuclei and muscle fibre were distinct, the nuclei stained purplish while the muscle fibre was light brown. However, the nuclei were not as distinct with calcium acetate. Muller and buffered formalin fixatives also gave a colour contrast that was not as good as Carnoy and calcium acetate fixatives, although the nuclei and muscle fibre were also outlined with inferior colour shades. Helly and SUSA fixatives gave the poorest histologic contrast, with Helly's fixative giving a better cellular outline in comparison with SUSA.

In a descending order, below is the cellular colour contrast effect of various fixatives on the myocardium stained with Sobo.

Carnoy ⟹ Calcium acetate ⟹ Muller ⟹ Buffered formalin ⟹ Helly ⟹ SUSA.

Kidney:

Generally, with Sobo alone as stain, the kidney cortex was well outlined with prominent renal corpuscles. The cellular outline was generally light brown in coloration. At a higher magnification of x 400, the numerous nuclei in the glomerulus could be seen; which were generally those of the capillary endothelial cells, mesangial cells and podocytes. The squamous cells lining the Bowman's capsule were not distinct with most tissue preparations. The Bowman's space varied in size depending on the fixative effect on the renal tissue, hence appearing artifactually enlarged due to shrinkage of the glomerulus. Generally, the nuclei appeared dark brown or purplish depending on the fixative, while the cytoplasm or surrounding tissue appeared light brown. The Bowman's space mostly appeared empty.

Carnoy and Helly fixatives gave a good staining effect on the kidney cortex; the renal corpuscle, Bowman's capsule, Bowman's space and glomerular nuclei were distinct in appearance. The Bowman's space was not enlarged with Carnoy fixative, hence no excessive tissue shrinkage occured during processing. However, with Helly's fixative, the Bowman's space was enlarged due to tissue shrinkage. Muller and SUSA also gave a good cellular contrast similar to what was obtained with Helly. However, glomerular shrinkage was less with Muller fixative in comparion with Helly. Calcium acetate fixative outlined the renal corpuscle better than with buffered formalin, the nuclei with both fixatives generally stained purplish.

In a descending order, the histologic effect of fixatives on Sobo stained renal cortex is as follows;

Carnoy ⟹ Helly ⟹ SUSA ⟹ Muller ⟹

Calcium acetate ⟹ Buffered formalin.

With Sobo alone as a histologic stain, Carnoy fixative produced the best cellular color contrast. Sobo was observed here to have both a nuclear and cytoplasmic staining effect with varying shades of brownish coloration. A study conducted by Egbujo *et al*. (2008) on the staining effect of Roselle (*Hibiscus sabdariffa*) on the histologic section of the testis showed that the nuclear staining was enhanced when the extract was mordanted with potassium alum, possibly due to the formation of a mordant and dye complex known as a 'dye-lake'. This is similar to the haematoxylin-mordant complex.

Sorghum bicolor: Myocardium

Haematoxylin has long been identified as a nuclear stain and sorghum (*Sorghum bicolor* L. Moench) as a cytoplasmic stain. In this study, Haematoxylin was used as a primary stain and extracts from sorghum as a counterstain. The myocardium of the heart gave a good histologic contrast with Carnoy, Buffered formalin and calcium acetate fixative; the nuclei were prominent and purplish, while the muscle fibres appeared greyish under high power resolution. SUSA, Muller and Helly fixatives also produced a histologic contrast with similar coloration to the above, but with an inferior cellular contrast.

In a descending order, the histologic effect of Haematoxylin and sorghum stain on the myocardium is as follows;

Carnoy ⟹ Buffered formalin ⟹ Calcium acetate ⟹

Helly ⟹ Muller ⟹ SUSA.

Kidney:

In the renal study using the same stain, the renal corpuscles in the kidney cortex were prominent, the Bowman's space enlarged, the glomerular nuclei purplish, the cytoplasm greyish and the squamous cells were evident. In the medulla, the staining effect on the cellular appearance of the nuclei and cytoplasm were the same, that is, purplish nuclei and greyish cytoplasm.

SUSA, Muller, Helly, calcium acetate and buffered formalin showed various shades of purplish nuclei and greyish cytoplasmic colouration, except for Muller fixative where the glomerular nuclei appeared dark brown.

In a descending order, the histologic effect of Haematoxylin and sorghum stains on the kidney is as follows;

Carnoy ⟹ Calcium acetate ⟹ Buffered formalin ⟹

SUSA ⟹ Helly ⟹ Muller.

In a similar study on the effect of some fixatives on the staining ability of *Sorghum bicolor* extracts on tissue sections (Avwioro *et al*., 2006), it was observed that there was no significant difference in the staining reactions of ethanolic extracts on tissue sections pre-fixed in 10% formol saline, Zenker's fluid, Helly's fluid and Bouin's fluid. However, in this study, there was a significant difference in staining recation with aqueous extracts on tissue sections in different fixatives.

Garcinia livingstonei: Myocardium

In another study, extracts from *Garcinia livingstonei* was used as a cytoplasmic stain or counter stain to Sobo which has a known nuclear staining effect and hence the primary stain. Generally, the heart muscle fibres stained yellowish, while the nuclei stained either purplish or brownish depending on the fixative used. With SUSA, Helly, and buffered formalin, the nuclei appeared brownish while with Carnoy and Calcium acetate, the nuclei appeared purplish. However, with Muller fixative, the nuclei were not prominent.

Also, the muscle fibre outline was good using Carnoy, Calcium acetate and Helly but poor using SUSA, buffered formalin and Muller.

In a descending order, the histologic effect of *Sobo* alone and *Garcinia* stain on the myocardium is as follows;

Carnoy ⟹ Calcium acetate ⟹ Buffered formalin ⟹

Helly ⟹ Muller ⟹ SUSA.

Kidney:

For the renal tissue study, the stains had a positive effect; the renal corpuscles were prominent using Carnoy, SUSA, Helly and Muller fixatives. The glomerular nuclei were not as evident in comparison with Sobo alone as with Haematoxylin and sorghum stains. With Carnoy and calcium acetate fixatives, the glomerular nuclei were stained with shades of greenish colouration. Generally, the Bowman's space was apparent and the cellular outline of the renal cortex yellowish in colour.

In a descending order, the histologic effect of *Sobo* alone and *Garcinia* stain on the renal cortex is as follows;

Carnoy ⟹ SUSA ⟹ Helly ⟹ Muller ⟹

Calcium acetate.

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

The various fixatives produced histologic sections of good quality. Carnoy's fixative, however, gave the best result with the various stains used and is therefore recommended when carrying out studies on any of the local stains used in the present study.

Hibiscus sabdariffa can be a good substitute for haematoxylin as a nuclear stain especially when mordanted with potassium alum. With the various fixatives used in this study, Hibiscus stain, stained the nucleus and the cytoplasm and agrees with the finding of Egbujo *et al.* (2008) and Wiam *et al.* (2006).

With *Garcinia livingstonei* and *Sorghum bicolor* all the tissues picked up the stain and appeared yellowish and brownish respectively. However, only the cytoplasm was stained with the extract. A combination of the extract of *Garcinia livingstonei* and Sobo gave a tissue stain that demonstrated both the nucleus and cytoplasm.

It was concluded that both the extracts of *Garcinia livingstonei* and *Sorghum bicolor* may be used as a substitute for Eosin in daily histological preparations, thereby reducing the cost of purchasing chemicals. However, these local cytoplasmic stains gave a cellular contrast abit inferior to eosin but can however be improved upon.

For *Hibiscus sabdariffa*, a further study is recommended to identify the iso-electric point (pH point) at which both nuclei and cytoplasm of cells could be stained with the best contrast. Also, a study to determine the optimal temperature for its use and compatibility with other cytoplasmic stains can be carried out.

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Names - Uses - Phytochemicals - Production
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