

AN EVALUATION OF NUTRACEUTICAL COMPONENTS OF *SYZYGIUM  
CORDATUM* FRUITS FOR THE TREATMENT OF GASTROINTESTINAL  
TRACT INFECTIONS



University of Zululand

Masters Dissertation

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CORDATUM* FRUITS FOR THE TREATMENT OF GASTROINTESTINAL TRACT  
INFECTIONS

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## Declaration

The research project presented in this dissertation was conducted in the Department of Biochemistry and Microbiology at the University of Zululand (UZ), from March 2013 - November 2014, under the supervision of Mr J.S Shandu and Prof A. K Basson.

I, Sidney T`solanku Teboho Maliehe declare that this work, aside from the supervisory guidance received, is the product of my own original work and effort.

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This work is dedicated to Minah Mohlomi and my family that showed unconditional love during the research project.

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## Abstract

Gastrointestinal tract (GIT) infections represent high morbidity and mortality rates worldwide annually due to malnutrition, microbial drug resistance and newly emerging GIT infections. Although the pharmacological activities of different parts of *Syzygium cordatum* have previously been determined, the nutraceutical value of its fruits and seeds has not been reported. This study aimed at evaluating the nutraceutical value of the fruits and seeds of *S. cordatum* in pursuant to discovering newer and more cost-effective means to prevent GIT infections. The harvested fruits were separated into pulp and seeds, dried and extracted separately with 100% methanol using the Soxhlet extraction method. Proximate analysis was determined using the Association of Official Analytical Chemists (AOAC) standard methods and the energy content was calculated arithmetically. The extracts were screened for phytochemicals and microdilution assay was used to evaluate antibacterial activity of the fruit extracts against the selected GIT infecting bacteria and assayed for cytotoxicity using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The *in vivo* antidiarrhoeal activity was determined using the castor oil-induced rat model. The pulp and seed extracts revealed carbohydrate content of 63.9 and 60.5 %, respectively and the concentrations of the elements (potassium, calcium, iron, zinc and boron) had a range of 0.05 to 151 mg/L. The pulp and seed extracts contained 327 kJ/g and 347 kJ/g of the energy content, respectively. The pulp extract exhibited  $16.4 \pm 1.8$   $\mu\text{g}/\text{mg}$  of total phenolic content while the seed extract had  $21.4 \pm 1.4$   $\mu\text{g}/\text{mg}$ . Antibacterial property of the pulp extract revealed it to have the lowest MIC of 3.13 mg/ml against *Bacillus cereus* (ATCC 10102), *Staphylococcus aureus* (ATCC 25925), *Pseudomonas aeruginosa* (ATCC 7700) and *Enterococcus hirae* (ATCC 8043) while the seed extract had the lowest MIC value (6.25 mg/ml) against *Bacillus cereus* (ATCC 10102), *Staphylococcus aureus* (ATCC 25925) and *Enterococcus hirae* (ATCC 8043). Fruit-pulp extract gave the median inhibitory concentration ( $\text{IC}_{50}$  = 92  $\mu\text{g}/\text{ml}$ ) and the therapeutic index (0.1 - 0.3). The *in vivo* antidiarrhoeal activity showed the percentage inhibition of 41 for the seed extract and 49 for the pulp extract at 400 mg/kg, respectively. The antibacterial and antidiarrhoeal activities were assumed to be due to the detected phytochemicals and thus promoting *S. cordatum* fruits and seeds as safe, potential nutraceutical sources against GIT infections.

## Abbreviations

AOAC	Association of Official Analytical Chemists
ATCC	American Type Culture Collection
cAMP	Cyclic Adenosine Monophosphate
CV	Cell Viability
CFU	Colony Forming Unit
DMSO	Dimethyl Sulfoxide
g	Grams
GIT	Gastrointestinal Tract
HIME	Human Intestinal Microvascular Endothelial
IC <sub>50</sub>	Median inhibitory concentration
INT	<i>P</i> -iodonitrotetrazodium violet
KJ	Kilojoules
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
ml	Millilitre
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
nm	Nanometer
RSA	Republic of South Africa
SANAC	South African National Aids Council
SAS	South Africa Statistics
TI	Therapeutic Index
TLC	Thin-layer chromatography
UNICEF	United Nations International Children's Emergency Fund
UZ	University of Zululand
UV	Ultra Violet
WHO	World Health Organisation

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## Contribution to the body of knowledge (Manuscripts)

(See Appendix)

### Published manuscripts

- A. Maliehe TS, Shandu JS, Basson AK. (2015). Evaluation of the antibacterial activity of *Syzygium cordatum* fruit-pulp and seed extract against bacteria implicated in gastrointestinal tract infections. *Afr .J. Biotechnol.* 14 (16). 1387-1392.
- B. Maliehe TS, Shandu JS, Basson AK. (2015). The antibacterial and antidiarrhoeal activities of the crude methanolic *Syzygium cordatum* [S.Ncik, 48 (UZ)] fruit pulp and seed extracts. *Journal of Medicinal Plants Research.* 9 (33). 884-891.

### Submitted manuscript

Maliehe TS, Shandu JS, Basson AK, Simelane M.B.C, Lazarus G.G, Singh M; Pharmacodynamic effect and cytotoxicity assay of *Syzygium cordatum* fruit-pulp extract in treatment of gastrointestinal tract infections. *Trop J Pharm Res.*



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## CHAPTER ONE

### 1.0 Introduction

Gastrointestinal tract (GIT) infections are the major cause of high morbidity and mortality rates, especially in developing countries. Approximately more than 1.5 billion episodes of GIT infections, that result in more than 3 million deaths, are reported annually in the developing countries (WHO, 2009). 17-20% of deaths of children under the age of five years are attributable to GIT infections per year (WHO, 2009; WHO, 2013). In the Republic of South Africa (RSA), the South African National Aids Council (SANAC), (2012), reported 50 471 children's deaths under the age of five in 2009 and GIT infections were the second leading cause of these deaths. The increasing incidence and severity of GIT infections is mainly dominant in immunocompromised individuals (Chess and Talaro, 2015). GIT infections still impose minor problems in developed countries despite a tremendous development in sanitation, safety food handling and better nutrition. The high morbidity and mortality rates do not only cause a social problem but also economic devastation (WHO, 2009).

The microbial resistance to current antibiotics used against GIT infections, the high cost and side effects of allopathic medicine have elevated more research for easily accessible and cost effective novel resources to prevent and cure GIT infections (Konno, 2004; Purohit and Vyas, 2004). Epidemiological studies have approved consumption of fruits in preferred amounts (400-500 grams per day) to be adequate to save approximately 2.7 million people from chronic infections including GIT infections worldwide per year (Krebs-Smith *et al.*, 2010). Fruits and seeds provide essential nutrients to humans and have a pivotal role in humans susceptible to GIT infections (Martinko and Madigan, 2006; Narrod *et al.*, 2009). Fruits and seeds possess moderate proteins, fats, carbohydrate, water, vitamins and minerals (Dey, 2010). In addition to the wealth of nutrients, fruits are excellent sources of beneficial phytochemicals such as phenolics, flavonoids, tannins, alkaloids and terpenoids (Waaland *et al.*, 2013). Phytochemicals have been elucidated to directly boost the immune system, modulate detoxifying enzymes, facilitate better nutrient absorption and possess strong antimicrobial, antidiarrhoeal and gastroprotective properties in humans (Downs and Wilson, 2012; Neethirajan *et al.*, 2012).

There is an increasing demand for nutraceuticals in recent years due to the growing concern of society with health and quality of life (Harmayanic *et al.*, 2013). According to Dominguez, (2013), nutraceuticals

are foods that have health-promoting properties such as beneficial nutrients and therapeutic compounds that are effective against infections. Nutraceuticals have human health benefits beyond basic nutrition (Chandra *et al.*, 2013). Nutraceuticals derived from indigenous fruits play a vital role in prevention of GIT infections and promotion of human health. They modulate the biochemical mechanisms in which many nutrients are absorbed much better in the body (Ghosh *et al.*, 2013). The most targeted human system for nutraceuticals science are GIT functions, associated with GIT microflora, immunity, nutrient bioavailability and transit time (Dominguez, 2013). Although fruits and seeds are rich in nutraceutical compounds, they have rarely been used for medicinal purposes (van Wyk *et al.*, 2009). However, the increasing demand for novel nutraceutical compounds has given fruits and seeds (even of the indigenous line) more attention as an alternative source for novel nutraceuticals (Chandra, 2013). This study focused on the evaluation of nutraceutical components from the *Syzygium cordatum* fruits and seeds in order to find newer and more cost-effective means for prevention and treatment of GIT infections and the promotion of good health.

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 Plants

Plants provide essential foods, medicine, clothes, cosmetics, fuel and limitless plant-based natural products to humans (Trivedi, 2007). Plants are important for environmental protection such as the prevention of soil erosion and the purification of air and water. They are vital resources for economic activities such as tourism. Plants are of great importance in the cultural and spiritual behaviour of people in different worlds (Raimondo *et al.*, 2009). The Republic of South Africa (RSA) is rich in plant diversity and endemism with over 30 000 species of higher plants (Raimondo *et al.*, 2009; van Wyk *et al.*, 2009).

#### 2.2 Plants as sources of food

Fruits, seeds, nuts, leaves, tubers and roots have been the earliest and most vital source of food known to mankind (van Wyk, 2005; Madison, 2008; Bille *et al.*, 2013). Over 7000 plant species are known to be edible, worldwide (Lim, 2012). Fruits and seeds are constituents of a healthy diet (Hung *et al.*, 2004). Fruits and seeds are a high-value food and contain macro and minor nutrients (Narrood *et al.*, 2009). The United Nations General Assembly (UN) and the World Health Organization (WHO) in consortia, promote health and the prevention of infections through encouragement of the consumption of adequate fruits and seeds in the human diet (UN and WHO, 2012). Epidemiological studies have also approved the consumption of fruits in preferred amounts (400-500 grams per day) to be adequate to save approximately 2.7 million people from chronic infections worldwide per year (Krebs-Smith *et al.*, 2010). However, the recommended fruit intake is still below the required level especially in developing countries (Kanungsukkasem *et al.*, 2009). Although most researchers' reports show the need to increase the supply of fruits and seeds in diet (Krebs-Smith *et al.*, 2010), the nutrient composition of most fruits and seeds, especially of those of less commercial value, is not well known and available to the public (Lim, 2012; Shajib *et al.*, 2013).



KwaZulu-Natal (RSA) has more than 119 of the edible fruit trees (Young and Fox, 1982). Most of the wild fruits and seeds are found and harvested in the wild. Wild fruits and seeds often substitute the exotic fruits and seeds, especially in periods of food shortage due to their nutritional quality (van Wyk *et al.*, 2007; Cofer, 2008). A large number of the known locally available wild fruits and seeds have significant compositions of essential nutrients (Lim, 2012). Thirty two percent of the South African population live in rural areas and rely extensively on the remarkable diversity of wild fruits and seeds across the seasons especially during starvation periods. In some cases, one species of a fruit tree is of vital importance for the survival of the whole community (van Wyk *et al.*, 2007; SAS, 2014; WHO, 2014).

### **2.2.1 Dietary components of fruit pulps and seeds**

The specific nourishment demands of human beings are fulfilled through a combination of nutrients. Nutrients supply the energy needed for metabolic reactions in the body (Mahan and Escott-Stump, 2004). The body gets its energy mainly from carbohydrates, proteins and lipids. The pulp of fleshy fruits is the primary food source for many people (Gallagher, 2014). The digestible carbohydrates are important in food as a major source of energy (Belitz *et al.*, 2009). Worldwide, carbohydrates account for more than 70% of the caloric value of the human diet (Rubestein *et al.*, 1978). Proteins are a source of stored carbon, nitrogen and sulphur containing chains of 20 different amino acids (Gallagher, 2014). Amino acids are the key nutritional components of foods (Faurie *et al.*, 2003). Amino acids function in growth, the maintenance of body tissues, and the formation of enzymes, hormones and antibodies (Smartt and Nwokolo, 1996). Amino acids contribute towards organoleptic properties, maintain acid-base balance and supply energy to the cells and tissues. Free amino acids are widely distributed in fruits (Manay and Shadaksharaswamy, 2001). Fruit-pulp contains moderate concentrations of protein (Srilakshmi, 2008). The amino acid content in fruit-pulp normally ranges from 0.2-1.3% (Pearson, 1962). The nutritive importance of lipids is based on their role as good energy molecules, sources of fatty acids and vitamins. Lipids contribute to the fruit's attributes such as texture, flavour, nutrition and caloric density. Lipids have a protective role on the cuticle layers on the fruit surface and cell membranes (Will *et al.*, 1981). Lipids contain two and a quarter times the calories found in an equal dry weight of proteins or carbohydrates. Lipid composition of most fruit-pulp is less than 1% (Pearson, 1962). Plants have the ability to accumulate organic acids in the cell vacuoles in considerable amounts required for operation of the tricarboxylic acid (TCA) cycle (Harborne, 1998). Fruits also contain free organic acids (Manay and

Shadaksharaswamy, 2001). Malic and citric acids are predominant in most fruits (Belitz *et al.*, 2009). Fruits are commonly rich in ascorbic acid (Vitamin C) as well (Colfer, 2008). Acids contribute to the flavour of fruits in the free, or combined, form as salts or esters. Organic acids possess some antibacterial properties and are medicinally important (Ray and Bhunia, 2008).

Fruits are rich in fibre and calories (Brown, 2011). The dietary fibre is composed of cellulose, hemicellulose, lignins, pectins and a variety of gums and mucilages (Alasalvar and Shahidi, 2013). The dietary fibre regulates normal bowel functioning, reduces the post prandial hyperglycaemic response and lowers serum cholesterol. Dietary fibre lowers the risk of constipation and incidence of colon cancer. The dietary fibre holds water; softens stools and decreases stool transit time in the intestines. The major part of the edible portion of fresh fruits consists of water of approximately 75-95% in most fruits (Pearson, 1962; Alasalvar and Shahidi, 2013). The large variation of water content occurs within a species since individual cells contain varying water content. Dried fruits contain about 13-22% of water. The utilization and maintenance of energy to build and maintain the body requires the involvement of vitamins and minerals. The vitamins and minerals function as co-enzymes, co-catalysts and buffers during the metabolism processes (Mahan and Escott-Stump, 2004). The healthy immune system, reproduction and optimum cell metabolic rate in humans depends upon 72 trace elements. The elements required by the body in greater amounts include; magnesium (Mg), sodium (Na), zinc (Zn), copper (Cu), potassium (K), iron (Fe), and calcium (Ca) (Belitz *et al.*, 2009). Fruits contain these nutritive elements in high concentrations. The concentration of the elements in fruits depends upon the soil of the cultivated area and the atmospheric conditions (Bukhari *et al.*, 2013).

Over 70% of the world's food supply is derived directly from the seeds of field crops (Rubenstein *et al.*, 1979). The major food seeds are those of the cereal (maize) and legume (beans). Seeds are rich in nutrient constituents. Most seeds contain large and characteristic quantities of polymeric reserves (Bewley *et al.*, 2013). Seeds contain a supply of carbohydrates, lipids and proteins, with minor amounts of phosphate-rich phytin (Bryant 1985). The main reserves in seeds are represented by lipids and carbohydrates (Bradbeer, 1988). The seeds store lipids as triacylglycerols of which most of them are oils (Bewley *et al.*, 2013). Some seeds contain high concentrations of glycolipids, phospholipids and sterols. Starch is the most common, major carbohydrate accumulated in the endosperm and cotyledons (Murray, 1984). Proteins comprise the most abundant nitrogenous storage in seeds with endosperm accounting for approximately 8-20% of seed weight. The stored proteins, albumins, globulins and

prolamins are sequestered in protein storage vacuoles (Bewley *et al.*, 2013). Phytin is a good source of phosphate and mineral elements (Gallagher, 2014). The main stored macro and micro nutrients provide the carbon skeleton and energy for embryo growth during seed germination. The stored molecules provide a major component of the essential dietary nutrients for most humans when consumed. Due to the lack of commercial importance of most indigenous fruits and seeds, minor research has been conducted on the nutritional value of the indigenous fruits and seeds (Lim, 2012). The increasing interest in novel sources for food and the over manipulated genetic diversity within domesticated fruit trees have elevated a shift into wild fruits and seeds as alternative resources for attenuation of the burden imposed by starvation and malnutrition (Gallagher, 2014). In this study, the abundantly found indigenous fruits and seeds of the species *Syzygium cordatum* were evaluated for their nutritional value.

## **2.3 Plants as medicine**

Plant parts, as extracts and in various forms have been used in previous years as medicine for treatments of infections such as GIT infections caused by pathogenic microorganisms and metabolic disorders (Brahmachari, 2012). The World Health Organisation (WHO) has estimated that more than 80% of people in the developing nations rely extensively on plant extracts for their primary healthcare (WHO, 2002). Trivedi, (2007), reported that over 300 million people living in the developing states utilise medicinal plants on a regular basis. Easy accessibility, efficacy of treatment, margin of safety without toxic effects and cost effectiveness are the main reasons for the constant use of plant extracts as their primary medicine (Fennell *et al.*, 2004; Konno, 2004; El-Mahmood and Doughari, 2008; Ahmed *et al.*, 2013; Gakunga *et al.*, 2013). Out of 50 000 plant species found worldwide, only 1% has been phytochemically screened. This implies that there is still a great potential from plant species for discovery of novel chemotherapeutic compounds against GIT infections (Ahmad *et al.*, 2006).

### **2.3.1 Therapeutic value of fruit pulps and seeds**

According to Alasalvar and Shahidi, (2013), phytochemicals are naturally occurring and biologically active compounds that are chemically derived from plants. Phytochemicals are non-nutritional bioactive chemicals that help plants to survive biotic and abiotic environmental stress (Brahmachari, 2012).

Phytochemicals protect plants against herbivores, environmental stress and invading pathogens including insects, bacteria, fungi and viruses. The site of biosynthesis of phytochemicals is restricted to one taxonomic group within a plant kingdom (Buchanan *et al.*, 2000). However, the accumulation of phytochemicals can be found in any plant tissue (Wink *et al.*, 2010). The concentrations of phytochemicals in tissues depend on the season of the year, climate, habitat, altitude and soil composition (Eisenman *et al.*, 2013).

The potential pharmacological effect of fruits and seeds depends on their bioavailability (absorption in human GIT, metabolism and excretion) of phytochemicals (Arup *et al.*, 2012; Alasalvar and Shahidi, 2013). Thousands of phytochemicals have been harvested and identified in fruits and seeds and their by-products for the treatment of infections and for the maintenance and improvement of good health (Chandra *et al.*, 2013; Waaland *et al.*, 2013). However, a large portion of phytochemicals remain unknown (Alasalvar and Shahidi, 2013).

Phytochemicals have been revealed to boost the immune system, modulate detoxifying enzymes, facilitate better nutrient absorption and for their strong antimicrobial, antidiarrhoeal and gastroprotective properties in humans (De la Rosa *et al.*, 2010; Downs and Wilson, 2012; Neethirajan *et al.*, 2012). Among the phytochemicals, sulphides, carotenoids, flavonoids, glucosinolates, phytoestrogens, isothiocyanates, phytosterols, cardiac glycosides, saponins, alkaloids, tannins, phenols, terpenes, dietary fibres, vitamin A, B, C and E, folic acid, potassium and selenium have been identified in numerous fruits and seeds in various compositions (McWilliams *et al.*, 2009; Heldt *et al.*, 2011; Waaland *et al.*, 2013). A large number of fruits and seeds contain all of the phytochemicals with the exception of carotene, protease and saponins. Some fruits and seeds contain essential oils as well (Srilakshmi, 2008).

### **2.3.2 Mechanism of phytochemicals as antimicrobial agents**

Antimicrobial agents are chemotherapeutic substances used to treat infections caused by microorganisms. The antimicrobial compounds act to alter the structure and function of the microbial cells (Black, 2008). Antimicrobial compounds impose bacteriostatic, bactericidal and bacteriolytic effects on exponentially growing bacterial cultures (Martinko and Madigan, 2006). Five general modes of antimicrobial activities namely; disruption of cell membrane function, inhibition of cell wall synthesis,

inhibition of nucleic acid synthesis, inhibition of protein synthesis and antimetabolite activity are employed by antimicrobial compounds (Black, 2008). Phytochemicals exert the same mode of action as antimicrobial agents (Saleem *et al.*, 2010). Phytochemicals found in fruits and seeds also affect the metabolic reactions by exhibiting an antispasmodic effect, increasing colonic water reabsorption, decreasing electrolyte secretion, suppressing GIT motility and delaying intestinal transit (Ahmad *et al.*, 2006; Chollet and Gleason, 2012). The actions of these phytochemicals, coupled with their antimicrobial activity, explains the benefits of fruits and seeds as the sources of the therapeutic agents for treatment of GIT infections.

Although fruits and seeds are excellent sources of therapeutic phytochemicals, fruits and seeds have rarely been used as medicine (van Wyk *et al.*, 2009; Kossah *et al.*, 2011; Srividhya *et al.*, 2013). However, with more than 70% of microorganisms causing infections having resistance to some antibiotics, the prohibitive costs of treatments consequent upon this resistance and the side effects of allopathic medicine, the use of plant extracts and research for novel plant products with potency against GIT infections have gained more momentum recently (Purohit and Vyas, 2004; WHO, 2004; Brahmachari, 2012). Different parts (roots, bark and leaves) of the species *S. cordatum* (Figure 1) have been used as traditional medicine against GIT infections with the exception of its fruits and seeds (Scott *et al.*, 1996). In this study, *S. cordatum* fruits and seeds were evaluated for their pharmacological properties against GIT infections.

## 2.4. Botanic description and location of *S. cordatum*



Figure 1: A picture of *S. cordatum* showing its leaves and ripe fruits taken at UZ

The genus, “*Syzygium*”, is derived from the Greek word “syzygos” which means “paired” (Grant *et al.*, 1998). It is due to the leaves and twigs that grow at the same point. The specific name is the Latin name “*cordatus*” which means “heart-shaped”. It is in accordance with the heart-shaped base of the leaves. The genus “*Syzygium*” consists of more than 1100 species. The genus *Syzygium* normally refers to trees of the *Myrtaceae* family.

*Syzygium cordatum* is commonly known as Water-berry (English), Water-bessie (Afrikaans), uMdoni (Zulu), Mukute (Shona) and Montlho (North Sotho) (Scott *et al.*, 1996; van Wyk *et al.*, 2009). *S. cordatum* is an evergreen and a medium to large sized tree (Drummond and Moll, 2002). It grows to 8-15 metres in height with a dense spreading rounded crown (Young and Fox, 1982). The stem is thick, dark brown fissured breaking up into irregular sections. The leaves are simple, dark-green, broad and circular, with a bluish-green colour. The leaves have a distinctive arrangement in opposite pairs near the ends of the branches, each pair at right angles to the next. Its flowers are cream-colour to pinkish with stems that are produced in clusters on the tips of the branches (Young and Fox, 1982; van Wyk *et al.*, 2009). *S. cordatum* trees grow rapidly in swamps, near fresh-water streams, in mountain grasslands, bushvelds and in places with high rainfall (van Der Spuy, 1971; Young and Fox, 1982). *S. cordatum* trees are known as a sign of the availability of underground-water. *S. cordatum* are fire resistant and impervious to cold but not frost (Grant *et al.*, 1998). *S. cordatum* trees are native to RSA and are widely distributed in the eastern and north eastern parts of the country. They are found in the Eastern Cape, KwaZulu-Natal and across the northern part of South Africa (Orwa *et al.*, 2009; van Wyk *et al.*, 2009). In Kwazulu-Natal, *S. cordatum* trees are signals of areas that are good for sugarcane plantations (Grant *et al.*, 1998; Orwa *et al.*, 2009).

### 2.4.1 *Syzygium cordatum* fruits



Figure 2: A picture showing ripe *S. cordatum* fruits taken at UZ.

The fruiting season is usually from October to June in the RSA (Drummond and Moll, 2002). The fruits of this family are used for both food and medicine in different parts of the world (Ayyana and Subash-Baru, 2012; van Wyk and van Wyk, 2013). *S. cordatum* trees produce sweet fruits. The fruits (Figure 1 and Figure 2) are found in clusters. They are oval, glabrous, shiny, fleshy, up to 2 cm in length, red or red to purple-black in colour and they are one seeded berries. The fruits are tipped with a calyx of 3-4 mm long (Drummond and Moll, 2002; van Wyk and van Wyk, 2013). The seeds are about 2.8 cm thick (Downs and Wilson, 2012). The ripened fruits are eaten raw. The fruits are eaten by people, birds, monkeys, bush pigs and bush babies (Grant *et al.*, 1998). *S. cordatum* fruits rank fourth (after *Sclerocarya birrea*, *Englerophytum magalismsontanum* and *Strychnos pungens*) as a preferred delicious fruit among indigenous South Africans (De Lange *et al.*, 2005). *S. cordatum* fruits are often fermented to produce potent intoxicating beverages (Young and Fox, 1982). Jam and jelly are also manufactured from these fruits (Palmer and Pitman, 1972). However, the nutraceutical value of *S. cordatum* fruits has not been recorded previously.

#### 2.4.2 Traditional uses of *S. cordatum* parts

The trees of the family and genus of *Syzygium cordatum* are rich in pharmacologically important volatile oils (Scott *et al.*, 1996). *S. cumini*, *S. aromaticum*, *S. jambolanum* and *S. jambos* are the most studied species of this family for pharmacological purposes (Ávila-Peña *et al.*, 2007). The *Syzygium spp* have been utilised in the treatment of diseases such as haemorrhage, dysentery and gastrointestinal disorders. The *Syzygium spp*, are also used as sedative, anticonvulsant, antihypertensive, inhibitors of histamine release and antimicrobials (Bhargava *et al.*, 1968; Kurokawa *et al.*, 1998; Djadjo-Djipa *et al.*, 2000).

*S. cordatum* species are used for traditional medicine and charm (van Wyk and van Wyk, 2013). The stem bark, leaves and roots are applied to increase the flow of milk in nursing mothers (Scott *et al.*, 1996). Parts of the tree are traditionally worn by pregnant African women as charms against deformities in children. Unspecified parts are used for stomach complaints, treating wounds, headaches, colds, fever as well as respiratory ailments (Grant *et al.*, 1998). The stem bark is used to treat sexually transmitted infections (De Wet *et al.*, 2013). *S. cordatum* leaf extracts possess antidiabetic and anti-inflammatory properties (van Wyk *et al.*, 2009; De wet *et al.*, 2013). Musabanyane *et al.*, (2005) has indicated that crude *S. cordatum* leaf extracts are effective in treating mild diabetes mellitus but less effective against severe hyperglycaemia. Amabeoku and Deliwe, (2013), found crude *S. cordatum* aqueous extract to have antidiarrhoeal activity. Sibandze *et al.*, (2010), reported that crude *S. cordatum* bark extract with a combination of other plant species (*Breonadia salicina* and *Ozoroa sphaerocarpa*) exert antidiarrhoeal activity. *S. cordatum* bark extracts have been reported to possess antifungal activity and a very strong free radical scavenging activity (Steenkamp *et al.*, 2007). Industrially, the crushed bark is used as a fish poison. *S. cordatum* stem-bark provides a reddish-brown dye for textiles (Grant *et al.*, 1998) and its logs are traditionally woven to make slipways and jetties (van Wyk *et al.*, 2007).



### **2.4.3 Phytochemicals from *S. cordatum***

The exact pharmacological action of the *S. cordatum* extracts is not known. The use of *S. cordatum* extracts as antidiarrhoeal may be explained by the presence of phenolic compounds (Candy *et al.*, 1967). The presence of tannin has been indicated in sapwood and hydrolysis of bark yielded delphinidin (van Wyk *et al.*, 2009). The compounds that were isolated from wood and barks include proanthocyanidines, pentacyclic triterpenoids such as fridelin. The mixture of triterpenes namely; oleanolic acid, ursolic acid, methyl malsinate and methyl corosolate have been isolated from the leaf extracts (Musabayane *et al.*, 2005, 2010). Epifriedelinol, beta-sitosterol, proanthocyanidin, flavonols, vanillic acid, arjunolic acid, gallic acid and a gallicallagic acid complex have been isolated from *S. cordatum*'s parts (Scott *et al.*, 1996). Steroidal triterpenoids, leucodelphinidin and leucocyanidin were found in the bark and leaves. The major essential oil from *S. cordatum* leaf extracts is trimethylpentadecane, a C15 aliphatic methyl ketone (Chalannavar *et al.*, 2011).

### **2.4.4 Toxicity of *S. cordatum* extracts**

Naturally-occurring pharmaceutical compounds from indigenous fruits may have great potential as a solution to prevent GIT infections. However, any plant material used as food or medicine has to be nontoxic (WHO, 1978). Despite fruit-pulp and seed pharmacological benefits, fruit-pulp and seed extracts ought to be screened for their safety before they can be used (Eloff *et al.*, 2014). Changes in toxicity mediated by fruits are often as a result of changes in the ability to detoxify or activate the toxic compounds (Chamber *et al.*, 2015). Although in many cases, toxic fruit-pulp and seed phytochemicals are detected at very low concentrations (generally less than 2% of the dry matter) and have negative physiological effects when absorbed, they still need to be screened to avoid potential toxicity (Makkar *et al.*, 2007). This is due to the fact that fruit extracts may show a therapeutic effect or a toxic effect. Toxic phytochemicals in fruit-pulp and seed extracts can cause damage to immune cells' function, cell lysis and even cell-death. This can directly or indirectly cause more severe occurrences of GIT infections (Mims *et al.*, 2004). Therefore, *S. cordatum* fruit pulp extract was examined for toxicity using colon cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation assay. MTT cell proliferation assay is used to assess the viability and metabolic state of cells.

## 2.5 Gastrointestinal tract (GIT)

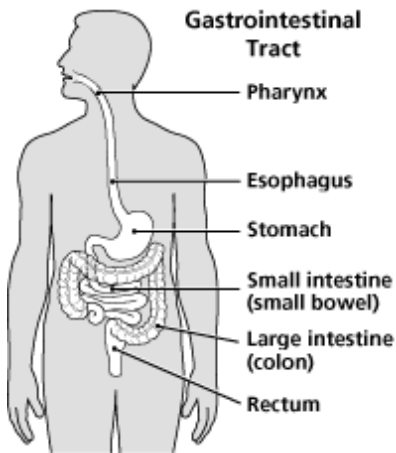


Figure 3: Anatomic structure of GIT (Cleveland Clinic., 2007).

The human GIT (Figure 3) is a multifaceted organ consisting of pharynx, oesophagus, stomach, small intestines (duodenum, jejunum and ileum), large intestines (colon and rectum), glands and accessory structures (Chamber *et al.*, 2015; Lehman *et al.*, 2015). The functions of the GIT are digestion, nutrient absorption, secretion of hormones and enzymes and the excretion of waste from the body (Mill and Bone, 2013). The GIT substantial function is to protect the body from a variety of noxious materials along with the food necessary for the body's maintenance. The functions of the GIT are mediated by intestinal epithelial cells.

### 2.5.1 Etiology of microflora in GIT

The human GIT is sterile at birth (Ashihara *et al.*, 2006). Normally, the microflora acquisition begins during delivery, from the mother's vaginal and faecal microflora and from the environment. The composition of intestinal microbiota is relatively simple in the infant stage but become more complex with age, thus reaching a higher degree of complexity in adulthood (Lim, 2012). The concentrations of bacteria in the stomach and duodenum are usually low due to the low pH ( $\pm$  pH 2). The concentrations of bacteria increase in jejunum and ilium and reach higher concentrations in the colon (Bjeldanes *et al.*, 2009).

The microflora is generally non-pathogenic and some are opportunistic under certain conditions (Lim, 2012). Within limits, the population density of the normal microflora remains stable unless it is disturbed. The normal flora has benign effects only if the host is in good health (Chess and Talaro, 2012). The GIT microflora is important for the normal development of GIT morphology and functioning (Blok *et al.*, 2002). Under normal conditions, the microflora support the ecological balance of GIT microbiota by inhibiting the growth of pathogens and enhancing host defences (Blok *et al.*, 2002; Mill and Bone, 2013; Chess and Talaro, 2015). The pathogens have to compete exclusively with the highly adapted normal flora at the site of the tissues for colonisation before causing an infection (Lim, 2012). The normal microflora of the GIT comprises of microorganisms whose morphology, physiologic and genetic properties allow them to maintain a symbiotic relationship with the host (Lim, 2012). A decreased colonisation of the normal flora often results in the overgrowth of pathogens and infections of the GIT.

The GIT microflora possesses a diverse range of metabolic activities including reductions, hydrolysis and degradations of biomolecules (Bjeldanes and Shibamoto, 2009). The intestinal bacteria have a large metabolic capacity that result in the conversion of dietary macromolecules to metabolites with beneficial or adverse health effects. The intestinal bacteria are important for the maturation and maintenance of the human system (Lim, 2012). The colonisation of the GIT by microflora is largely determined by the host physiological factors (Martinko and Madigan, 2006). The gastric pH, redox potential gradients, digestive secretion (of bile acids, lysozyme, trypsin), diet, immune activity and gut motility all play a role in determining the number of the species diversity of the microflora colonising the GIT (Blok *et al.*, 2002; Chess and Talaro, 2015). The other factors that influence the number and variety of the normal microflora include antibiotics intake, mental stress, starvation and improper dietary habits (Martinko and Madigan, 2006).

### 2.5.2 Nutrition and GIT microflora

Nutrition is the cornerstone of health (Cifuentes, 2013). The health impact of dietary materials on modulating the microbiota and its metabolism towards sustaining a healthy GIT is significant. An individual's resistance to infections is influenced by their nutritional status. Nutritional status strongly influences the type of bacteria that reside in the GIT (Bjeldanes and Shibamoto, 2009). Microorganisms are supported by energy sources of the endogenous origin like mucin. The microbial species get most nutrients and energy supply from dietary residues (Cifuentes, 2013). Diets rich in food from plant sources as opposed to diets rich in food from animal sources favour the presence of bacteria in higher number (Ray and Bhunia, 2008). The alteration of the composition of the normal flora by diverse aforementioned factors gives the true as well as opportunistic pathogens a better chance to multiply and increase susceptibility of humans to pathogens. The undesirable indigenous or transient bacteria grow to higher levels and produce enteric disturbances such as diarrhoea (Ray and Bhunia, 2008). An adequate intake of a balanced diet, on the other hand, does prevent the wide-spread of nutritional-deficiency infections such as GIT infections (Begum *et al.*, 2012).

### 2.5.3 Mechanism of GIT microflora causing infections

GIT infections range from those caused by microbial agents, immunological disorders, biochemical, metabolic and congenital disorders (Boulton *et al.*, 2000). Bacterial gastroenteritis is less frequent and is caused by species of *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, *Vibrio* and *Escherichia coli*. Several Gram-negative microorganisms have been reported to cause gastroenteritis (Hart and Fisher, 1992; Atlas, 1995). Pathogens use either mucosal invasiveness, the production of toxins or both to cause harmful effects on the host (Blok *et al.*, 2002; Martinko and Madigan, 2006). Viruses, bacteria and protozoa have the ability to produce toxins that can be ingested in food and water and cause infection in the GIT (Lim, 2012). Bacterial toxins are classified into exotoxins and endotoxins. Endotoxins are lipopolysaccharides which form part of the cell wall and are secreted into the host by bacteria (Levinson, 2010). Exotoxins are polypeptide molecules that are produced only by Gram-negative rods and cocci. Both endotoxins and exotoxins can cause GIT infections in the absence of the bacterial strains. The invasive pathogenic microorganisms actively penetrate the host's mucous membrane and epithelium after attachment to the epithelial surface. This may be accomplished through production of lytic substances that alter the epithelial cells (Willey *et al.*, 2011). When GIT motility is impaired, the

frequency of microbial overgrowth and infections of enteric pathogenic microorganisms also increases, leading to severe GIT infections (Kasper and Fauci, 2013).

#### **2.5.4 Nutrition and immunology**

Malnutrition is an unhealthy condition that results due to deprivation of essential nutrients in diets and disordered digestion in the body. Malnutrition has an effect on the ability to mount rapid and effective responses. Malnutrition decreases production of immune cells and their functions, cytokine production and the ability of lymphocytes to respond appropriately to cytokines (Cortes *et al.*, 2007; Cervantes *et al.*, 2011). A balanced diet maintains the normal immune function with all immune cells working at full capacity. The immune cells in the mucosa of the GIT need to be well provided with nutrients and functioning to keep human beings healthy (Goulart, 2009).

Nutritional status plays a major role in the determination of the effectiveness of the immune response. Adequate nutrition supports the growth and multiplication of immune cells. The immune system is an important link in the chain of host defence mechanism (Cowan, 2015). The immune cells are attributed with effect functions such as cytokine production, antibody production and cytotoxicity. The cytokine network interaction that is critical to host defence, may be affected by poor nutritional status and some non-specific mechanisms of immunity may be impaired or absent as a result of malnutrition (Cortes *et al.*, 2007; Cervantes *et al.*, 2011; Chess and Talaro, 2015). With the inadequate nutrition, the synthetic process of lymphocytes cells becomes impaired and results in immunodeficiency. Inadequate diet often leads to malnutrition which in turn impairs bodily functions and increases susceptibility to infections (Cortes *et al.*, 2007; Cervantes *et al.*, 2011). Phytochemicals and essential nutrients in fruits and seeds reduce immune suppressions and enhance the activation of immune cells, prevention of malnutrition and GIT infections (Chandra *et al.*, 2013).

### 2.5.5 Diarrhoea

Diarrhoeal infections are major causes of morbidity and mortality worldwide, especially in developing countries among infants, children and aged people (WHO, 2009). The high morbidity and mortality rates do not only cause a social problem but also economic devastation in developing nations. Malnutrition often manifests as diarrhoea (Reinhardt and Fanzo, 2014). Diarrhoea is a gastrointestinal disorder that is characterised by a decrease in the stool consistency and an increase in frequency, fluidity, or volume of the faeces during defecation for a period of days or weeks (Mazzolin *et al.*, 2013). True diarrhoea implies passing of increased amounts (300g per day) of loose stools (Kumar *et al.*, 2001). Acute diarrhoea (that which last less than 4 days) is mostly observed after dietary indiscretions. Chronic diarrhoea (that which may last for 3-4 weeks) is often caused by conditions such as inflammatory bowel diseases, endocrine disorders (hyperthyroidism, diabetic autonomic neuropathy) or radiation colitis (Porth 2005). Chronic diarrhoea is a common symptom in humans infected with immunodeficiency virus (HIV). The most severe form usually occurs in persons with a CD<sup>4+</sup> T-helper cells count of less than 50 cells/ml.

Diarrhoeal infections reflect a wide variety of abnormalities in the GIT that include food intolerance, food poisoning and enteric infections through hepatobiliary activity (Mill and Bone, 2013). The common symptoms of diarrhoeal infections range from mild and self-limiting symptoms (Mims *et al.*, 2004). However, severe diarrhoea may be life-threatening particularly to people who are malnourished or immunocompromised (WHO, 2013). Severe diarrhoea may lead to a disordered gastrointestinal tract (GIT) motility, dehydration, electrolyte imbalance, acidosis and even death (Dyer and Gould, 2011). Diarrhoea often occurs due to the damage of intestinal mucosal cells by invasive microorganisms, exotoxins and endotoxins of microbial origin in contaminated food and water (Cliver, 1990; Kumar *et al.*, 2001; Mims *et al.*, 2004; Smith and Charter, 2010). A vast number of microbial toxins excreted by microorganisms stimulate intestinal secretion and intestinal motility (Eley, 1992). Generally, four mechanisms involved during the diarrhoeal periods are the defective absorption of solutes, increased secretion of solutes, structural abnormalities in the intestine and altered intestinal motility (Cliver, 1990; Mims *et al.*, 2004). About 14 litres of fluid may be lost per day in severe cases of diarrhoea (Kumar *et al.*, 2001). The foul smell from the stools is due to poor fat absorption (Cliver, 1990).

Bacterial pathogens that are associated with secretory diarrhoea are *Vibrio spp*, enterotoxigenic and enterohaemorrhagic *E. coli* (Cross, 2013). These pathogens are generally noninvasive. Inflammatory diarrhoea on the other hand is frequently caused by enteroinvasive *E. coli*, species of *Shigella*, *Campylobacter* or nontyphoidal *Salmonella* (Smith and Charter, 2010). The viral diarrhoea is mostly due to rotavirus and calicivirus. *Entamoeba histolytica*, *Giardia*, *Cryptosporidium* and *Cyclospora spp* are protozoal organisms that cause diarrhoea (Carey, 2009). Severe diarrhoea may lead to disordered GIT motility, dehydration, electrolyte imbalance, acidosis, malnutrition and even death. Diarrhoeal infections are often accompanied by pain, urgency, perianal discomfort and incontinence (Cliver, 1990; Eley, 1992; Dyer and Gould, 2011).

### **2.5.6 Current diarrhoeal treatment**

The competition for nutrients by microorganisms, blockage of the adhesion site, inhibition of toxin formation and blockage of their receptors contribute to antidiarrhoeal activity. Some metabolic reactions do enhance antidiarrhoeal activity by stimulating the synthesis of immunoglobulin A, stimulating the type Th1 response and stimulating the formation of cytokinins 11-12, 11-2 particularly interferon gamma. Thus, diarrhoeal infections can be effectively treated with rehydration solutions, bismuth subsalicylate, antiperistaltic agents and prophylactic antimicrobial agents (fluoroquinolone or macrolide) (Kasper and Fauci, 2013). The most effective antidiarrhoeal agents are the opioid derivatives (loperamide), diphenoxylate, atropine and tincture of opium. Atropine (used in this study as a positive control in *in-vivo* antidiarrhoeal activity) is a tertiary amine belladonna alkaloid. Atropine is a racemic hyoscyamine tropic acid ester of the base tropine (Champe and Harvey, 2009). Atropine has a high affinity for muscarinic receptors located on the exocrine gland, cardiac and smooth muscle ganglia, intramural neurons (Hodgson and Kizior, 2014). It binds competitively at the muscarinic receptors to prevent acetylcholine to bind and thus reversing excessive secretions of fluids and electrolytes and produce ophthalmic cycloplegia (Hollinger, 2008; Champe and Harvey, 2009; Hodgson and Kizior, 2014). Atropine actions reduce the intestinal hypertonicity and hypermotility of the GIT. When appropriate doses are taken, atropine reduces the frequency of bowel movements and relieves abdominal cramps associated with diarrhoea (Lehne, 2004).

Food that provides calories is necessary to facilitate the renewal of enterocytes. Humans infected with diarrhoea are often encouraged to take traditional feeding of fruits (Porth, 2005). Fruit extracts, in a dose-related manner exert an antidiarrhoeal effect (Ashorobi and Umukoro, 2005; Carey, 2009; Maha *et al.*, 2013). *S. cordatum* aqueous leaf extracts have been reported to have antidiarrhoeal activity (Amabeoku and Deliwe, 2013). The combination of the *S. cordatum* extracts with other plant species has also been approved to exert antidiarrhoeal properties (Sibandze *et al.*, 2010). To elucidate the mechanism of the antidiarrhoeal activity of *S. cordatum* fruit-pulp and seed extracts, it was worthwhile to extend the study to their effects on GIT motility.

### **2.5.7 Castor oil**

Castor oil is an effective emollient laxative agent (Priff and Harold, 2005). Castor oil causes a decrease in fluid and nutrient absorption, increases the electrolyte secretion and water and produces alterations in intestinal motility (Lehne *et al.*, 1990). The diarrhoeal activity of castor oil is attributed to its active cathartic triglyceride fraction known as ricinoleic acid (Chambers *et al.*, 2015). Castor oil-induced diarrhoea is as a result of the action of ricinoleic acid formed from the hydrolysis of its triglyceride in the duodenum by pancreatic lipase. The ricinoleic acid stimulates intestinal hypersecretion, hypermotility and decreases gastrointestinal transit time (Schellack, 2004). The antidiarrhoeal activity and antimotility activity of the methanolic *S. cordatum* fruit-pulp and seed extracts were evaluated by employing castor oil induced diarrhoea in rats.



## **2.6 GIT bacteria used in this study**

### **2.6.1 *Escherichia coli***

*Escherichia coli* are Gram-negative, facultative, anaerobic, non-spore forming bacilli (Eley 1992; Love *et al.*, 1998). *E. coli* is one of the predominant enteric species in the human GIT (Talano, 2006; Alkhaldi *et al.*, 2012). There are six groups of *E. coli* namely; enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC) and diffusely adherent *E. coli* (DAEC) (Love *et al.*, 1998; Alkhaldi *et al.*, 2012; Lehman *et al.*, 2015). *E. coli* are divided on the basis of serotyping of O antigen (Eley 1992). The four groups of *E. coli* (enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enteropathogenic *E. coli* and enteroaggregative *E. coli*) have a tendency to cause diarrhoeal infections (Cowan and Talaro, 2009). *E. coli* groups are known to be transmitted through contaminated food or water (Eley 1992; Lehman *et al.*, 2015). *E. coli* (ATCC 25922) strain was used in this study.

### **2.6.2 *Vibrio vulnificus***

*Vibrio vulnificus* is a halophilic, Gram-negative, curve-shaped vibrio (Alkhaldi *et al.*, 2012). *V. vulnificus* is mostly found along coastal areas. *V. vulnificus* cause gastroenteritis in immunocompromised individuals who have eaten raw shellfish contaminated with this microorganism (Levinson, 2010). The infective dose from ingestion of *V. vulnificus* is largely unknown (Alkhaldi *et al.*, 2012). *V. vulnificus* is associated with distinctive clinical features such as abdominal pain, vomiting and diarrhoea (Eley, 1992). The environmental isolate (*V. vulnificus* AL 042), isolated by Okoh and Igbinosa. (2010), was used in this study.

### **2.6.3 *Vibrio fluvialis***

*V. fluvialis* is a halophilic, Gram-negative, rod-shaped, oxidase positive bacterium. *V. fluvialis* causes diarrhoea, abdominal pain, fever and dehydration. The mechanisms involved in the pathogenicity of *V. fluvialis* are not well understood. *V. fluvialis* is acquired through contaminated water or seafood (Forbes *et al.*, 2002). The environmental isolate (AL 042), isolated by Okoh and Igbinosa. (2010), was used in this study.

#### **2.6.4 *Salmonella typhimurium***

*S. typhimurium* is a Gram-negative, non-spore forming and facultative anaerobic bacilli (Eley, 1992). *S. typhimurium* are attracted to the dead cells on the villi tips of ileal epithelium and cause a chemotactic response in the intestines (Roth 1998). *S. typhimurium* is a heat stable toxin and causes gastroenteritis-vomiting, diarrhoea and mucosal irritation (Eley, 1992; Cowan and Talaro, 2009; Willey *et al*, 2011). *S. typhimurium* (ATCC 700030) was used in this study.

#### **2.6.5 *Staphylococcus aureus***

*Staphylococcus aureus* is a Gram-positive, nonmotile, catalase-positive bacterium. *S. aureus* is a versatile human pathogen capable of causing sporadic food poisoning, toxic shock syndrome, pneumonia, postoperative wound infection, and nosocomial bacteremia (Alkhalidi *et al.*, 2012). In most cases, *S. aureus* takes temporary residence in the GIT and produces exotoxins (Cowan and Talaro, 2009). *S. aureus* colonises the epithelial cells of intestines through adherence. When *S. aureus* is ingested, the bacterium produce toxins with a wide spectrum of cytolytic activity and cause infections through direct invasion of the tissues followed by symptoms which include abdominal cramps, vomiting, and diarrhoea (Murray *et al.*, 2009; Alkhalidi *et al.*, 2012). *S. aureus* (ATCC 25925) was used in this study.

#### **2.6.6 *Enterococcus hirae***

*Enterococcus hirae* are Gram-positive, catalase negative, facultative anaerobes. *E. hirae* are opportunistic pathogens. (Alkhalidi *et al.*, 2012). *E. hirae* are commensal bacteria in human GIT. *E. hirae* cause infections such as endocarditis, bacteremia, intra-abdominal infections. The symptoms of *E. hirae* infections include diarrhoea, abdominal cramps, nausea, vomiting, fever, chills and dizziness. *E. hirae* (ATCC 8043) was used this study.

### **2.6.7 *Bacillus cereus***

*Bacillus cereus* is a Gram-positive, facultatively anaerobic, endospore forming rod (Lehman *et al.*, 2015). *B. cereus* is widespread in the environment and is often isolated from the soil and vegetation. *B. cereus* cause food-borne sickness (Alkhaldi *et al.*, 2012; Lehman *et al.*, 2015). The route of entry of *B. cereus* is often by the consumption of food contaminated with enterotoxigenic *B. cereus* or with the emetic toxin. *B. cereus* produces two types of toxins, one that causes diarrhoeal type infections and the other causes an emetic infections (Cowan and Talaro, 2009; Lehman *et al.*, 2015). *B. cereus* also causes infections through direct invasion and destruction of tissues (Murray *et al.*, 2009). The symptoms of *B. cereus* diarrhoeal-type food poisoning include watery diarrhoea, abdominal cramps and pain. *B. cereus* (ATCC 10102) strain was used in this study.

### **2.6.8 *Pseudomonas aeruginosa***

*Pseudomonas aeruginosa* is a Gram-negative, rod-shaped bacterium that is mainly found in soil and water. *P. aeruginosa* also resides as a normal microflora in the GIT (Levinson, 2010). *P. aeruginosa* is an opportunistic microorganism and mostly infects people with immunocompromised defence systems. *P. aeruginosa* (ATCC 7700) was used in this study.

## 2.7 Problem statement

Various studies, previously done in the rural districts of RSA, reported high morbidity and mortality rates due to GIT infections. GIT infections ranked as the second most common cause of deaths in the rural areas in RSA. The high rates of morbidity and mortality were directly and indirectly associated with malnutrition (SANAC, 2012). Labadarios *et al*, (2005), stated that the diets of most RSA citizens, living in rural areas, are of low nutrient value and mostly predominated by starchy foods. The consumption of fruits, vegetables and meat products are of low intake in rural areas and leads to malnutrition (Cortes *et al.*, 2007; Cervantes *et al.*, 2011). The species *S. cordatum* is a widely distributed indigenous tree in RSA. *S. cordatum* has been used both for treatment of GIT infections and food purposes (Scott *et al.*, 1996). The study aimed at evaluating the nutraceutical components of *S. cordatum* fruits and seeds in order to find more novel substances to prevent malnutrition and the high prevalence of GIT infections.

## 2.8 Value to the body of knowledge

Fruits and seeds are rarely used for medicinal purposes although there is good evidence that they possess properties to reduce the risk of various infections including GIT infections (van Wyk, 2005; van Wyk *et al.*, 2009). There is also a limited knowledge documented about the nutritive and medicinal qualities of the wild fruits and seeds (Etkin, 1986). The results obtained from this study would add value to the body of knowledge regarding the nutritional and medicinal value of the indigenous *S. cordatum* fruits and seeds. The obtained information can be used to discover various solutions to human problems such as malnutrition and GIT infections in the rural areas in the RSA.

## 2.9 Study – area

The study was conducted in the Department of Biochemistry and Microbiology at the University of Zululand (UZ) main campus. The university is situated at KwaDlangezwa in the north of KwaZulu Natal, South Africa.

## 2.10 Aim

The study aimed at evaluating the nutraceutical components of *S. cordatum* fruit-pulp and seeds for the prevention of malnutrition and GIT infections.

## 2.11 Objectives

1. To collect, identify and extract *S. cordatum* fruit pulps and seeds
2. To determine and quantify major dietary components in *S. cordatum* fruit pulp and seed extracts.
3. To screen and quantify some phytochemicals in *S. cordatum* fruit pulp and seed extracts.
4. To assess the antibacterial activity of *S. cordatum* fruit pulp and seed extracts against bacteria implicated in GIT infections.
5. To elucidate the cytotoxicity of *S. cordatum* fruit pulp extract.
6. To determine the *In vivo* antidiarrhoeal and antimotility activities of *S. cordatum* fruit pulp and seed extracts.

## 2.12 Hypotheses

- (1): *S. cordatum* fruits and seeds possess valuable dietary components.
  
- (2): *S. cordatum* fruits possess medicinal compounds that can be used in the treatment of GIT infections.

## 2.14 Null-hypotheses

- (1): *S. cordatum* fruits do not possess enough of valuable dietary components.
  
- (2): *S. cordatum* fruits do not possess enough of medicinal compounds that can be used in the treatment of GIT infections.

## CHAPTER THREE

### 3.0 Materials and Methods

#### 3.1 Sample collection and treatment

Fresh *S. cordatum* fruits were randomly harvested in summer (February, 2014) from the trees in the main campus of the UZ. The fruits were confirmed at the Department of Botany at UZ. The seeds of *S. cordatum* fruits were manually separated from their pulps (fleshy part). The fruit pulps and seeds were air-dried at room temperature for seven days. The dried *S. cordatum* fruit pulps and seeds were separately ground to a coarse powder using an electric grinder with mesh size of 1.0 mm to increase the surface area for solvents during the extraction process. The ground samples were stored at 4 °C until required for use.

#### 3.2 Extraction

A *Soxhlet* extraction method was performed according to Bii *et al*, (2009), with some modifications. The ground *S. cordatum* fruit pulp and seed samples (100 g each) were separately subjected to the *Soxhlet* extraction using 400 ml of 100% methanol (Univ.AR). The samples were agitated on a mechanical shaker at a speed of 200 rpm at 37 °C for 12 hours. Extractions were repeated three times for each sample. The third extractions were left for 24 hours. The extracts obtained were filtered through Whatman filter paper and concentrated using a Büchi rotary evaporator at 45 °C. The yields of each extract were weighed and re-dissolved in 100 ml of 10% dimethyl sulfoxide (DMSO) to the volume concentration of 100 mg/ml. The extracts were stored at 4 °C until further use. The percentage yields from *S. cordatum* fruit-pulp and seed extracts were calculated using the formula below by Shahid (2012).

$$\% \text{ Yield} = \text{weight of extract (g)} / \text{weight of powdered sample (g)} \times 100$$

### 3.3 Quantitative and qualitative analyses of the dietary components

#### 3.3.1 Carbohydrates (Molisch's test)

Five hundred milligrams of powdered fruit pulp and seed samples were dissolved in 5 ml of distilled water and then filtered. Five drops of Molisch's reagent were added to the filtrates, followed by an addition of 1 ml of concentrated H<sub>2</sub>SO<sub>4</sub>. After 2 minutes, 5 ml of distilled water was added. Red or dull violet colour formation at the interphase of the two layers was an indication of the presence of carbohydrates (Sadasivam and Theymoli 1985).

#### 3.3.2 Starch (Iodine test)

Fruit-pulp and seed extracts (1.0 ml) and standard starch solutions (1 ml) were poured in separate test tubes. Two drops of iodine solutions were added and mixed thoroughly and 2 ml of starch solution was used as a positive control. A deep blue to black colour product indicated the presence of starch (Sadasivam and Theymoli, 1985).

#### 3.3.3 Reducing sugars (Benedict's test)

Five drops of *S. cordatum* fruit pulp and seed extracts were added to 2.0 ml of Benedict's reagent and mixed thoroughly. The mixtures were placed in a boiling water bath for 5 minutes and cooled to room temperature. Glucose was used as a positive control. A brown (red)-colour precipitate indicated the presence of simple sugars (Sadasivam and Theymoli, 1985).

#### 3.3.4 Total carbohydrates (Anthrone method)

The total carbohydrates in crude fruit-pulp and seed extracts were estimated by the anthrone method as described by Yemm and Willis. (1954). Ten milligram of powdered *S. cordatum* fruit pulp and seed samples were extracted with 50 ml of 80% ethanol for 6 hours at 60 °C. The resulting mixtures were filtered and then made up to 100 ml with distilled water. For the estimation, an aliquot of filtered *S. cordatum* fruit-pulp and seed (1.0 ml) were mixed with 10 ml of anthrone reagent. Glucose (1.0 ml) was used as a standard. The test tubes were heated in a boiling water bath for 10 minutes and then cooled. The absorbance was measured at 620 nm in a Beckman spectrophotometer. The amount of glucose in the *S. cordatum* fruit pulp and seed extracts was calculated.



### **3.3.5 Organic acid (Indophenol test)**

Vitamin C (Ascorbic acid) was determined by the indophenol test according to standard method as follows: Nine drops of indophenol solution were added to 5 ml of methanolic *S. cordatum* fruit pulp and seed extracts and thoroughly mixed by shaking. Three millilitre of pure ascorbic acid was used as a positive control and distilled water as a negative control. A change in colour from blue to colourless (or pink) indicated the presence of vitamin C (Gleeson *et al.*, 1992).

### **3.3.6 Proteins (Biuret test)**

The presence of proteins was identified using the Biuret test according to Milio and Loffredo. (1995). Five drops of Biuret reagent were mixed with 2 ml of *S. cordatum* fruit pulp and seed extracts, respectively. The mixtures were warmed at 37 °C for 10 minutes and then cooled to room temperature. Three millilitre of milk was used as a positive control and distilled water as a negative control. A deep violet (or blue) colour was an indication of the presence of protein.

### **3.3.7 Free amino acids (Ninhydrin paper chromatography)**

The amino acids present in *S. cordatum* fruit pulp and seed extracts were identified qualitatively using the Ninhydrin paper chromatography method according to Kay *et al.*, (1956) and Acree *et al.*, (2005) with some modification. An aliquot (0.2 ml) of the test solutions was spotted onto separate filter papers. The filter papers were then dipped into the ninhydrin solution until the solution reached the areas spotted with the test solutions. The filter papers were then dried in an oven at 110 °C for 10 minutes. A blue spot on the filter paper indicated the presence of amino acids.

### **3.3.8 Total proteins content**

Total protein content was calculated according to the formula below by Nielsen. (2009).

$\% \text{Proteins} = (100 - [\% \text{ total ash} + \% \text{ total moisture} + \% \text{ crude fats} + \% \text{ crude fibre} + \% \text{ crude carbohydrates}])$ .

### **3.3.9 Fats content (Ethanol emulsion test)**

The presence of fats or lipids was determined by the Ethanol emulsion test as described by Nielsen. (2009). Three millilitre of *S. cordatum* fruit pulp and seed extracts were added into 3 ml of 96% ethanol and thoroughly shaken for 15 minutes. Afterwards, 2 ml of distilled water was added into both extracts and further shaken for 15 minutes. Cooking oil was used as a positive control. A white, cloudy precipitation (emulsion) was the indication of the presence of fats.

### **3.3.10 Crude fats content (Solvent extraction method (AOAC Method 920.39))**

The ground fruit pulps and seed samples (50 g) were each extracted with 200 ml of hexane for total lipid content for 48 hours. The solid to solvent ratio mixtures were filtered through a Whatman no-1 filter paper. The liquid extracts were evaporated to dryness through a rotary vacuum evaporator at 45 °C and the fat content was calculated as a percentage using the formula below:

$\% \text{ Fat} = \text{mass of fat in crude extract} / \text{mass of original sample}$  (Nielsen, 2009).

### **3.3.11 Ash content (Dry ash method (Association of Official Analytical Chemists (AOAC) Method 900.02))**

The ground fruit pulps and seeds samples (3 g) were ashed at 550 °C. They were heated in a muffle furnace for 12 hours. The ash residues were dissolved in 20 ml HCL, filtered through acid washed filter paper, evaporated to dryness and ignited at 550 °C respectively. The total ash content was calculated in mg of ash per g of air-dried material using the formula below:

$\% \text{ Ash} = \text{weight of ash} / \text{weight of original sample} \times 100$  (Motsara and Roy, 2008).

### 3.3.12 Crude dietary fibre content

The nonenzymatic gravitational method (AOAC Method 993.21) was used to determine the crude fibre contents of *S. cordatum* fruit pulp and seed samples. The fat-free powdered fruit-pulp and seed samples (3 g) were weighed and transferred into a 100 ml Erlenmeyer flask. Two hundred (200 ml) of 37% aqueous sulphuric acid was added and the flasks were connected to the reflux condenser and boiled. The contents of the flasks were rotated at 5 minute intervals for 30 minutes through mixing. After digestion, the contents of the flasks were filtered through a Whatman filter paper in a funnel and washed free of the acid with boiling distilled water. The washed residues were gently transferred back to the Erlenmeyer flask, and 150 ml of 20% aqueous sodium hydroxide solution was added and the flasks connected to the reflux condenser. The samples were again boiled for 30 minutes, rotating the flask at 5 minutes intervals. The contents were again filtered through a Whatman filter paper in a funnel, and then washed free of the alkaline solution with boiling distilled water. The residues were transferred to porcelain crucibles, and the crucibles, together with the contents, were dried to a constant weight at 110 °C and cooled before weighing. The crucibles and their contents were then ignited in a furnace at 550 °C for 60 minutes and then cooled to room temperature. The loss in weight during incineration was equivalent to the weight of crude fibre in the samples and was expressed as percentage crude fibre using the formula below:

% Crude fibre = weight after drying / weight of original sample x 100 (Nielsen, 2009).

### 3.3.13 Moisture content

The conventional oven method (AOAC 926.08) was used to determine the percentage of moisture in *S. cordatum* fruit-pulp and seed samples. The ground *S. cordatum* fruit pulp and seed samples (2 g) were weighed into a crucible and placed into a hot oven at 110 °C. After 8 hours, the samples were taken-off, cooled and weighed. The moisture contents were expressed as percentage moisture using the formula below:

% Moisture (wt/wt) = wt of wet sample-wt of dry sample / wt of wt sample x 100

Whereby; wt is weight in grams (Nielsen, 2009).

### **3.3.14 Spectrophotometric determination of the selected elements**

The determination of trace elements in *S. cordatum* fruit pulp and seed extracts was done by a spectrophotometer. The concentrations of trace elements ( $K^+$ ,  $Na^+$ ,  $Ag^+$ ,  $Ca^{2+}$ ,  $Zn^{2+}$ ,  $Fe^{3+}$ ,  $Al^{3+}$  and  $B^{3+}$ ) were determined at the wavelength of 320 nm using a spectrophotometer (Spectroquant-Pharo 100).

### **3.3.15 pH**

The pH of the fruit pulp and seed extracts was determined according to the method used by Asagbra *et al*, (2012), with some modification. This was determined by weighing 1 g of fruit pulp and seed methanolic extracts and suspending them separately in 9 ml of 10% Tween twenty. The pH was measured with an Adwa AD12 pH meter.

### **3.3.16 Nutritional value**

The energy values (EV) of the fruit pulp and seed extracts were calculated using the following expression by Rao *et al*, (2012), as kj/g. The formula used was:

Energy value =  $9 \times \% \text{ fat} + 4 (\% \text{ protein} + \% \text{ carbohydrates})$ .

### **3.4 Phytochemical screening**

The extracted crude *S. cordatum* fruit pulp and seed extracts were screened for phytochemicals. The phytochemical screening was done in all the extracts according to Harborne (1973).

#### **3.4.1 Alkaloids (Mayer's test and Dragendroff's test)**

The concentrated *S. cordatum* fruit pulp (2 ml) and seed extracts (2 ml) were individually dissolved in dilute hydrochloric acid (HCl) and sieved respectively. The filtrates were treated with Mayer's reagent. The appearance of a yellow coloured precipitate indicated the presence of alkaloids. The filtrates were treated with Dragendroff's reagent. Occurrence of the red precipitate indicated the presence of alkaloids. Samples were observed for the presence of turbidity or precipitation and were scored: a (+) score was recorded if the reagent produced only a slight opaqueness; a (++) score if a definite turbidity but no flocculation was observed; a (+++) score if a definite heavy flocculation was produced; and a (-) score if neither turbidity nor precipitation was observed (Harborne, 1973).

#### **3.4.2 Cardiac glycosides (Ferric chloride test)**

The concentrated *S. cordatum* fruit pulp (2 ml) and seed extracts (2 ml) were separately hydrolysed with 30% of HCl acid. The solutions were treated with Ferric Chloride solution and immersed in boiling water for about 3 minutes. After the mixtures were cooled, extracts were further treated with sodium nitroprusside in pyridine and sodium hydroxide. The pink to blood red colour was an indication of the presence of cardiac glycosides (Harborne, 1973).

#### **3.4.3 Saponins (Froth test and Foam test)**

The concentrated *S. cordatum* fruit pulp (2 ml) and seed extracts (2 ml) were diluted with distilled water to 4 ml. The mixtures were thoroughly shaken in a test tube for 10 minutes respectively. The occurrence of a layer of foam witnessed the presence of saponins. The formation of foam that persisted for 10 minutes was the indication of the presence of saponins. The saponin content was classified as: (-) = for absence if no froth; (+) = for weak positive if a froth less than 1.0 cm; (++) = for medium positive if froth is 1.2 cm high; and (+++) = for strongly positive greater than 2.0 cm high (Harborne, 1973).

#### **3.4.4 Flavonoids (Alkaline reagent test)**

Pulp and seed extracts (2 ml each) were treated with four drops of sodium hydroxide solution respectively. The occurrence of a deep yellow colour, which became colourless on addition of dilute acid, demonstrated the presence of flavonoids (Harborne, 1973).

#### **3.4.5 Tannins (Ferric chloride test)**

The dry, powdered *S. cordatum* fruit pulp and seed extracts (1 g) were extracted with 2 ml of distilled water and filtered with Whatman filter paper. The water extracts (2 ml) were treated with 15 % ferric chloride test solution (2 ml). The resultant colour was noted. A blue to dark, green or blue-green precipitate indicated the presence of tannins (Harborne, 1973).

#### **3.4.6 Phenols (Ferric chloride test)**

The *S. cordatum* fruit pulp and seed extracts (1 ml each) were treated with 3 drops of iron (III) chloride solutions respectively. Bluish black colour proved the presence of phenols (Harborne, 1973).

#### **3.4.7 Terpenoids (Salkowski test)**

The dry, powdered form of *S. cordatum* fruit pulp and seed extracts (1 g) were extracted with 10 ml of chloroform for 20 minutes and filtered with Whatman filter paper respectively. Three millilitre of concentrated sulphuric acid was then carefully added to both filtrates to form a layer. A reddish-brown colour on the interface was the evidence of the presence of terpenoids (Harborne, 1973).

### 3.4.8 Betulinic acid (Thin-layer chromatography)

An original line of 2 cm from the edge, across the plate was drawn. Betulinic acid was loaded on thin-layer chromatography plate as a standard indicator followed by the loading of methanol extracts of *S. cordatum* fruit pulp and seed, respectively. The thin-layer chromatography plate was placed in a chromatography tank containing a mixture of hexane and ethyl acetate in the ratio of 7:3, respectively, covering about 1 cm of the plate. The chromatography was allowed to proceed until the hexane-ethyl acetate reached the top of the plate. At that point, the chromatogram was removed from the tank and dried using a hot air dryer. It was then sprayed with 5% sulphuric acid-methanol solution. The plate was viewed under ultra violet light at 354 nm. The appearance of a pink colour indicated the presence of betulinic acid (Walker, 1984).

### 3.4.9 Total phenolic content

The total phenolic contents were determined by the Folin-Ciocalteu method according to Makkar *et al.* (1993). An aliquot (0.2 ml) of 500 µg/ml methanolic fruit-pulp and seed extracts were made up to 1.0 ml with distilled water, respectively. 0.5 ml of Folin-Ciocalteu reagent (1N) was added, followed by 2.5 ml of sodium carbonate solution (20%). The mixtures were mixed properly, and then incubated at room temperature for 40 minutes. The absorbance of the blue-coloured complex formed was measured at 725 nm against the appropriate blank. The total phenolic content was determined from the standard curve of tannic acid and expressed as equivalents of tannic acid (µg/mg).

### 3.5 Antibacterial activity

The bacterial strains known to cause GIT infections used in this study included; *Bacillus cereus* (ATCC 10102), *Staphylococcus aureus* (ATCC 25925), *Enterococcus hirae* (ATCC 8043), *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 700030), *Pseudomonas aeruginosa* (ATCC 7700), *Vibrio fluvialis* (AL 019) and *Vibrio vulnificus* (AL 042).

#### 3.5.1 Revival of the selected bacterial strains

The selected bacteria were inoculated into sterile nutrient broth and incubated overnight at 37 °C. Thereafter, 1 ml from each of the bacterial cultures was pipetted into 9 ml of fresh prepared nutrient broth in separate test tubes labelled with corresponding bacteria. The test tubes were then incubated at 37 °C overnight. After an overnight incubation, absorbance of the selected bacterial strains was read in the spectrophotometer (600 nm) for determination of their turbidity. The turbidity of the resulting suspensions was diluted with nutrient broth to obtain an absorbance of 0.132. This absorbance was taken as comparable to 0.5 McFarland turbidity standard. The turbidity was estimated to be equivalent to  $1.5 \times 10^6$  colony forming unit (CFU)/ml (Qaralleh *et al.*, 2012).

#### 3.5.2 Minimum inhibitory concentration (MIC)

A serial microdilution method was adapted as described by Eloff (1998) and Qaralleh *et al.* (2012) to measure the minimal inhibitory concentration (MIC) of the fruit pulp and seed extracts. Ninety six well microplates were used to quantitatively determine the MIC of both extracts. The sterile nutrient broth (50 µl) was added to all the wells of the 96-well microplates and 50 µl of the extracts (50 mg/ml, in 10% DMSO) was poured in the wells in the first rows and mixed well on separate microplates. The extracts' mixtures (50 µl) were removed from all the wells in row A to perform a 3-fold serial dilution down the columns, respectively. The last 50 µl, in the last column was discarded so that the total volume solution of each well was 50 µl. The selected bacterial strains (50 µl) were transferred into the corresponding wells. 10% Tween twenty was used as a negative control while ciprofloxacin (20 µg/ml) was used as a positive control. The plates were covered and incubated at 37 °C overnight. 0.2 mg/ml of P-iodonitrotetrazodium violet (INT) solution was utilised after the incubation period. 40 µL of 0.2 mg/ml INT solution were added to each well and incubated at 37 °C for 30 minutes. A reddish colour which was the result of INT being reduced by the metabolic activity of bacteria to formazan indicated bacterial



activity. The clear colour was the indication of the absence of bacterial activity since the INT was not broken-down to form formazan. The tests were replicated three times and the mean values were reported. The MIC was taken as the lowest concentration of the extracts required to inhibit the bacterial growth.

### **3.5.3 Minimum bactericidal concentration (MBC)**

For the determination of MBC, the agar dilution method was used. The MBC of the extracts was determined by removing a loop full of each culture medium from the wells that no bacterial growths were streaked on different sterile nutrient agar plates. The agar plates were incubated at 37 °C for 12 hours. The lowest concentrations of the *S. cordatum* fruit pulp and seed extracts that exhibited the complete killing of test microorganisms were considered as the MBC (Qaralleh *et al.*, 2012).

### **3.5.4 Total activity**

Total activity (TA) is the volume at which the test extract can be diluted with the ability to exert antibacterial activity. It is calculated by dividing the amount of extract from 1 g plant material by the MIC of the same extract and is expressed (ml) (Singariya *et al.*, 2012).

TA = extract per gram dried plant part / MIC of extract

## **3.6. Cytotoxicity assay**

### **3.6.1 Cell culture**

Human colorectal adenocarcinoma cells (Caco-2) were cultured on minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS), glutamine (2 mM) and 1% penicillin-streptomycin in static flask. The cells were incubated in a humidified atmosphere at 37 °C in 5% CO<sub>2</sub> for 24 hours.

### 3.6.2 MTT assay

The methanolic *S. cordatum* fruit pulp extract were tested for *in vitro* cytotoxicity using Caco-2 cells according to Akhir *et al*, (2011), with some modifications. The cells were plated in a 96-well-plate with cell suspensions of  $1 \times 10^5$  cells/ml concentrations. The cells were allowed to attach for 48 hours before being seeded with different concentrations from the crude extracts (150 mg/ml) using serial dilutions, administered in media containing 1% of FBS and returned to the incubator for 48 hours. After 48 hours incubation, the cell viability was determined removing the old medium and adding the (Merck) tetrazolium salt as a cytotoxicity indicator. 100  $\mu$ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) (5 mg/ml) in phosphate buffered saline (PBS) and 100  $\mu$ l medium was added to each well and incubated at 37 °C for 4 hours. The media with MTT were then aspirated from the wells and the formed formazan crystals were solubilized in 100  $\mu$ l of dimethyl sulfoxide (DMSO). The optical density of the solutions was measured at 570 nm using a Mindray-96A micro plate reader. The median inhibitory concentration (IC<sub>50</sub>) was determined.

### 3.6.3 Therapeutic Index (TI)

Therapeutic Index (TI) is the ratio of the highest cytotoxic concentration of the therapeutic extract to the inhibitory concentration of the extract that causes the desired efficacy (Edith *et al.*, 2013). TI was calculated according to Eloff *et al*, (2014), using the formula below;

TI = median inhibitory concentration / minimum inhibitory concentration

### 3.6.4 Statistical analysis

The mean and standard error mean of three experiments were determined. Statistical analysis of the differences between mean values obtained for experimental groups were calculated using Microsoft Excel Program, 2010 and Origin 6.0 for IC<sub>50</sub>. Data were subjected to one way analysis of variance (ANOVA). P values  $\leq 0.05$  were regarded as significant and P values  $\leq 0.01$  as very significant.

## **3.7 *In vivo* antidiarrhoeal and antimotility activities**

### **3.7.1 Animals**

Ethical clearance for the use of animals was collected from the Research Animal Ethics Committee (RAEC) of the UZ and the twelve white *Sprague-Dawley* rats (150–260 g) were collected from the animal house in the Department of Biochemistry and Microbiology at the same institution. Prior to the determination of the antidiarrhoeal and antimotility activities, rats were fed with standard food and given free access to water for one week to adapt to the laboratory conditions (temperature  $23 \pm 2$  °C and 12 hour light dark cycle). The rats were then fasted for 18 hours before the start of the experiment, but allowed free access to water (Orhan *et al.*, 2013).

### **3.7.2 Antidiarrhoeal activity**

The method used for determination of antidiarrhoeal activity was adopted from Teke *et al.* (2007), with some modifications. The experimental rats were divided into four groups of four rats each namely: Group A, Group B, Group C and Group D. Group A served as a negative control. It received vehicle distilled water (2 ml/kg) orally. Group D served as a positive control. It received atropine at the dose of 5 mg/kg orally by gavage. Group B and Group C received the seed and fruit-pulp extracts (400 mg/kg), respectively. Each rat was put in its own cage. Diarrhoea was introduced to each rat by orally administering 0.2 ml of castor oil. After 30 minutes of administration of castor-oil, observation of the defecation was done for 5 hours. The onset time of faeces and number of normal and wet faeces were the determined parameters. A score based on stool consistency was assigned as follows; normal-stool = A and wet-stools = B. The presence of normal stools was recorded as a positive result, indicating protection offered by the controls and the extracts from diarrhoea while the presence of watery stools was recorded as negative results.

### 3.7.3 Antimotility activity

The method used in the antimotility test was adopted from Teke *et al*, (2007), with some modifications. The animals were divided into four groups of four rats each namely: Group A, Group B, Group C and Group D. Diarrhea was introduced to each rat in all groups by orally administering 0.2 ml of castor oil. After 30 minutes of administration of castor-oil, all rats received different treatments. Group A served as a negative control and received distilled water (2 ml/kg) orally. Group D served as a positive control. It received atropine at the dose of 5 mg/kg orally by gavage. Group B and C received the seed extract and fruit pulp extract of 400 mg/kg, respectively. Thereafter, each rat was put in its own cage after the administration of 2 ml of charcoal meal (3 % deactivated charcoal in distilled water) orally. The rats were sacrificed 30 minutes thereafter for determination of gastrointestinal motility. The intestinal distance moved by the charcoal meal from pylorus to caecum was measured and expressed as a percentage of distance travelled from pylorus to caecum. The mean movement of charcoal meal in ratio to the intestinal length and percentage of inhibition were arithmetically measured. The following formulas were used:

$$\% \text{ travelled} = \text{length travelled by charcoal} / \text{total length of small intestine} \times 100$$

$$\% \text{ inhibition} = \text{mean length of duodenum} - \text{length of travelled charcoal} / \text{mean length of duodenum} \times 100$$

## CHAPTER FOUR

### 4. Results:

#### 4.1 Physiochemical qualities

The physiochemical qualities of *S. cordatum* fruit pulp and seed extracts are presented in Table 1 below.

**Table 1: Physiochemical qualities of *S. cordatum* PE and SE extract.**

Physical parameters		Solvents and Observations
1. Extracting solvent:	PE	Methanol
	SE	Methanol
2. Yield (%):	PE	10 %
	SE	6 %
3. Colour:	PE	Black-brown
	SE	Black-green
4. Odour (smell):	PE	Fruity
	SE	Fruity
5. Texture:	PE	Jelly like
	SE	Waxy
6. pH:	PE	2.94
	SE	3.48

Key: PE denotes pulp extract and SE denotes seed extract

#### 4.2 Proximate analysis

The pulp of fleshy fruits and seeds are nutritive tissues for major and micro nutrients. The body gets its energy from the catabolic actions of these nutrients to support the metabolic reactions. The results obtained from the proximate analysis of *S. cordatum* fruit pulp and seed extracts are provided in Table 2, Table 3 and Table 4 below.

**Table 2: Preliminary nutritional composition of *S. cordatum* PE and SE extracts**

<b>Nutrients</b>	<b>Tests</b>	<b>Samples</b>	<b>Results</b>
Total carbohydrates	Molisch	PE SE Glucose Distilled water	+ + + -
Soluble sugars	Benedict	PE SE Glucose Distilled water	+ + + -
Starch	Iodine	PE SE Starch Distilled water	- + + -
Fats/ Lipids	Emulsion	PE SE Cooking oil Distilled water	+ - + -
Vitamin C	Indophenol	PE SE Ascorbic acid Distilled water	+ + + -
Proteins	Biuret	PE SE Milk Distilled water	- + + -
Free Amino acids	Ninhydrin	PE SE Alanine Distilled water	+ + + -

PE or SE mean pulp and seed extract, + or - show presence or absence, respectively.

**Table 3: Proximate analysis of *S. cordatum* PE and SE extracts.**

Sample	% Moisture	% Fat	% Fibre	% Ash	% Protein	% Carbohydrates	Nutritive Energy (KJ)
PE	23.8	7.7	1.7	2.3	0.6	63.9	327.3
SE	18	6	1.3	1.3	12.9	60.9	347

Key: PE denotes fruit pulp extract and SE denotes seed extract

**Table 4: Microelements detected from *S. cordatum* PE and SE extracts in mg/L.**

Elements	K <sup>+</sup>	Ca <sup>2+</sup>	Na <sup>+</sup>	Zn <sup>2+</sup>	Fe <sup>3+</sup>	Ag <sup>+</sup>	B <sup>3+</sup>	Al <sup>3+</sup>
PE	38	121	0	0.334	0.25	0.25	0.050	0
SE	61	151	0	0.53	0.15	0	0	0

Key: PE denotes fruit-pulp extract and SE denotes seed extract

### 4.3 Phytochemical screening

Phytochemicals are non-nutritional bioactive chemicals from plants that help plants to survive biotic and abiotic environmental changes. The pharmacological activity of the extracts against GIT infections depends on the presence and concentrations of phytochemicals. *S. cordatum* fruit pulp and seed extracts had the total phenolic contents of 16.4±1.8 and 21.4±1.4µg/mg, respectively. The results obtained from qualitative and quantitative analysis of some phytochemicals are presented in Table 5 and 6.

**Table 5: Preliminary phytochemical screening of *S. cordatum* PE and SE samples and extracts**

Phytochemicals	Tests	Samples	Results
Alkaloid:	Dragendorff's	PE	+
		SE	++
	Mayer's	PE	+
		SE	++
Flavonoids	Alkaline Reagent	PE	+
		SE	-
Saponins	Frothing	PE	++
		SE	+
Cardiac glycosides	Sodium nitroprusside	PE	+
		SE	+
Tannins	Ferric chloride	PE	+
		SE	+
Phenols	Ferric Chloride	PE	+
		SE	++
Terpenoids:	Salkowski	PE	+
		SE	+
Betulinic acid (BA)	TLC	PE	+
		SE	+

Key: - denotes absence, + denotes low concentration, ++ denotes moderate concentration, +++ denotes high concentration, TLC denotes Thin layer chromatography, PE denotes fruit pulp extract and SE denotes seed extract



**Table 6: Total Phenolic Content in 500 µg/ml of crude methanolic *S. cordatum* PE and SE extracts.**

Assay	Expression of results	Concentration (µg/mg) original sample) ± SER
Total Phenolic	TAE: Fruit-pulp	16.4±1.8
	Seed	21.4±1.4

Note: (n = 2, X ± SEM). The results were expressed as µg/mg – where TAE denotes tannic acid equivalent. PE denotes fruit pulp extract and SE denotes seed extract.

#### 4.4 Antibacterial activity

The results of the MIC, MBC and total activity are as presented in Table 7. The *S. cordatum* fruit pulp showed the lowest MIC value of 3.13 mg/ml against *S. aureus* (ATCC 25925), *B. cereus* (ATCC 10102), *E. hirae* (ATCC 8043) and *P. aeruginosa* (ATCC 7700) isolates and the seed extract had the MIC value of 6.25 against *S. aureus* (ATCC 25925) and *E. hirae* (ATCC 8043). The fruit pulp extract had the highest MIC value of 6.25 mg/ml against the other four isolates. The seed extract showed the highest MIC value of 25 mg/ml on *V. vulnificus* (AL 042). The pulp extract had the lowest MBC value of 5 mg/ml against *E. hirae* (ATCC 8043) while the seed extract had the lowest MBC value of 12.5 mg/ml against the isolates *S. aureus* (ATCC 25925), *E. coli* (ATCC 25922), *B. cereus* (ATCC 10102), *E. hirae* (ATCC 8043) and *P. aeruginosa* (ATCC 7700).

**Table 7: MIC, MBC in mg/ml and total activity (T.A) (ml) of the *S. cordatum* PE and SE extracts on the selected bacterial strains known to cause GIT infections**

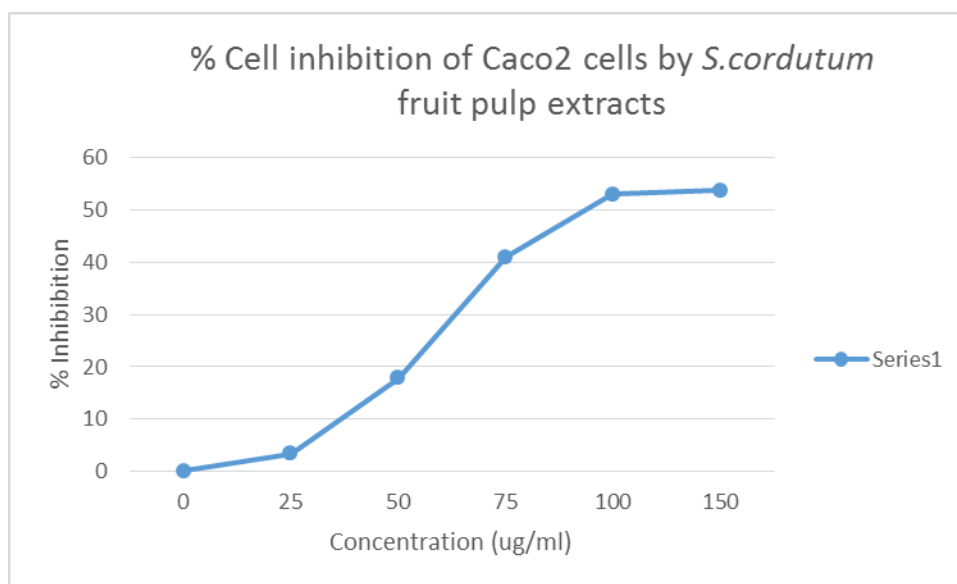
Bacterial Strains	Pulp extract			Seed extract			Ciprofloxacin	
	MIC	MBC	T.A	MIC	MBC	T.A	MIC	MBC
<i>S. aureus</i> (ATCC 25925)	3.13	6.25	31	6.25	12.5	16	3.13	6.25
<i>E.coli</i> (ATCC 25922)	6.25	6.25	16	12.5	12.5	8	3.13	6.25
<i>V. vulnificus</i> (AL 042)	6.25	12.5	16	25	50	4	1.56	12.5
<i>B. cereus</i> (ATCC 10102)	3.13	6.25	31	12.5	12.5	8	3.13	3.13
<i>S.typhimurium</i> (ATCC70003)	6.25	6.25	16	12.5	25	4	1.56	6.25
<i>E. hirae</i> (ATCC 8043)	3.13	3.13	31	6.25	12.5	8	1.56	3.13
<i>P. aeruginosa</i> (ATCC 7700)	3.13	6.25	31	12.5	12.5	4	3.13	12.5
<i>V. fluvialis</i> (AL 019)	6.25	12.5	16	25	50	4	1.56	12.5

Key: MIC denotes minimum inhibitory concentration (mg/ml), MBC denotes minimum bactericidal concentration (mg/ml), TA denotes total activity (ml), PE denotes fruit pulp extract and SE denotes seed extract.

#### 4.5 Cytotoxicity

The fruit pulp extract exhibited a concentration dependent cytotoxic effect on Caco-2 cells. The inhibitory concentration required for 50% cytotoxicity ( $IC_{50}$ ) was 92  $\mu\text{g/ml}$ . The results are presented (Figure 4) below.

**Figure 4:** % cell inhibition of the Caco-2 cells by *S. cordatum* fruit pulp extract.



#### 4.6 Therapeutic Index (TI)

Therapeutic Index (TI) is the ratio of the highest cytotoxic concentration of the therapeutic extract to the inhibitory concentration of the extract that causes the desired efficacy. Therapeutic index (0.1 - 0.3) of the fruit pulp extract was obtained on the selected bacterial species (Table 8).

**Table 8: Therapeutic index of *S. cordatum* fruit pulp extract at 25 µg/ml**

<b>Bacterial Strains</b>	<b>Therapeutic index of the extract</b>
<i>S. aureus</i> (ATCC 25925)	0.3
<i>E. coli</i> (ATCC 25922)	0.1
<i>V. vulnificus</i> (AL 042)	0.1
<i>B. cereus</i> (ATCC 10102)	0.3
<i>S. typhimurium</i> (ATCC70003)	0.1
<i>E. hirae</i> (ATCC 8043)	0.3
<i>P. aeruginosa</i> (ATCC 7700)	0.3
<i>V. fluvialis</i> (AL 019)	0.1

#### **4.7 Antidiarrhoeal and antimotility activities**

*S. cordatum* fruit pulp and seed extracts exhibited different percentages (49 and 41, respectively) of inhibition against the diarrhoeal activity in castor oil induced-rats (see Table 10). *S. cordatum* fruit pulp and seed extracts both reduced the number of wet stools, total number of stools and onset time generally in comparison to the negative control (distilled water). *S. cordatum* fruit pulp and seed extracts, in a dose-related manner (400 mg/kg of rat), exerted the antidiarrhoeal property by reducing intestinal motility. The results are tabled in Table 9 and 10 below.

**Table 9: Effects of the crude methanolic *S. cordatum* PE and SE extracts on castor oil-induced rats**

Groups	Treatments	Doses	Onset times (minutes)	Stools	
				normal	Wet
A	Distilled water plus Co	2 ml/kg	51	13±0.15	10.3±0.21
B	SE plus Co	400 mg/kg	68	8.25±0.17	2.75±0.31
C	PE plus Co	400 mg/kg	98	5.25±0.20	1.5±0.41
D	Atropine plus Co	5 mg/kg	127	1.25±0.19	0

Key: (n = 4, X ± SEM). PE denotes fruit pulp extract, SE denotes seed extract and Co denotes Castor oil.

**Table 10: Antimotility activity of crude methanolic *S. cordatum* PE and SE extracts on castor oil-induced rats.**

Groups	Treatments	Doses (ml/kg or mg/kg)	Mean total length of small intestines	Mean distance travelled by charcoal	Percentage Inhibition
A	Distilled water	2 ml/kg	130.8±3.97	106.8 ±6.54	0
B	SE plus Co	400 mg/kg	125.5±4.22	74.3 ±3.45	41
C	PE plus Co	400 mg/kg	114.8 ±3.68	59±3.34	49
D	Atropine	5 mg/kg	115.5±6.28	41.3±3.97	64

Key: (n = 4, X ± SEM). PE denotes fruit pulp extract, SE denotes seed extract and Co denotes Castor oil.

## CHAPTER 5

### 5. Discussion and conclusion

#### 5.1 Discussion

All the results obtained during the conducted experiments are discussed here.

##### 5.1.1 Percentage yields obtained from *S. cordatum* fruit pulp and seed extract

The percentage yields obtained from *S. cordatum* fruit pulp and seed extracts were 10 and 6, respectively, after extracted with methanol. The ability of methanol solvent to obtain good percentage yields is owed to its polarity. The percentage yields as showed by both extracts are important when the extracts are investigated for their biological activities.

##### 5.1.2 Proximate analysis

Ninety percent of ascorbic acid (Vitamin C) is provided by fruits in the human diet (Velisek, 2014). Traces of ascorbic acid (Vitamin C) were detected qualitatively in both fruit pulp and seed extracts. Vitamin C is an antiscorbutic substance which prevents and cures scurvy. Vitamin C is a co-factor in the synthesis of collagen, noradrenalin, carnitine and also acts as a co-factor for enzymes involved in translational hydroxylation of amino acids (proline). Vitamin C reduces the liability to infections due to its antimicrobial activity (Webb, 2011). The absorption of non-haem iron is increased by dietary ascorbic acid when the two nutrients are ingested simultaneously. Thus, consumption of *S. cordatum* fruit pulp and seeds has potential to increase the nutritional status of humans and their protection against GIT infections due to the presence of ascorbic acid. The major part of the edible portion of most fresh fruits consists of water content of approximately 75-95% (Alasalvar and Shahidi, 2013; Velisek, 2014). *S. cordatum* fruit pulp and seed extracts demonstrated the moisture content of 25% and 12% respectively, which was relatively lower than of most fruits. Moisture affects the keeping quality of fruits (Velisek, 2014). Fruits with less water have longer shelf lives since microbial growth is disturbed (Belitz *et al.*, 2009). *S. cordatum* fruit pulps and seeds stand a good chance to survive microbial decay due to the low water content and thus have long shelf lives.

The functions of proteins to humans are to provide energy and essential nutrients for body maintenance. Fruit pulps normally have a concentration of 0.2-1.3% of protein content while the seeds have 8-20% (Bewley *et al.*, 2013). *S. cordatum* fruit pulp extract showed 0.6% of total protein content while the seed extract showed 12.9%. According to the study conducted by Downs and Wilson, (2012), *S. cordatum* fruits possessed approximately 5.91% of protein. Eisenman *et al.*, (2013), stated that the amount of metabolites production in tissues significantly depend on the season of the year, climate, habitat, altitude and soil composition. Thus, the difference in results obtained in this study with the other study by Downs and Wilson, (2012), might have been due to some of these factors. In summary, *S. cordatum* fruits have enough protein content to provide energy for the maintenance of tissues and cells in the human body.

Most fruits have a low fat content of less than 0.5 g/100 g of the edible portion. *S. cordatum* fruit pulp and seed extracts had 7.7% and 6% respectively. The high fat content implied that the *S. cordatum* fruits have potential to yield high amounts of energy. According to the study conducted by Downs and Wilson, (2012), *S. cordatum* fruits possessed approximately 0.72% lipid contents. The percentage was little lower than those obtained in this study. Eisenman *et al.*, (2013), stated that the amount of metabolite production in tissues significantly depends on the season of the year, climate, habitat, altitude and soil composition. Thus, the difference in results obtained in this study with the other studies might be owed to some of these parameters.

Ash refers to the inorganic residue remaining after either ignition or complete oxidation of organic matter in food stuff. The ash content of most fresh fruits is rarely greater than 0.2-0.8% (Belitz *et al.*, 2009). *S. cordatum* fruit pulp extract had 2.3% of ash content while the seed extract had 1.3%. The high levels of ash content implied that *S. cordatum* fruit pulp and seeds have high levels of minerals. Hence, some of the trace elements that were qualitatively and quantitatively detected in mg/L in both extracts (K (fruit pulp 38, seed 61), Ca (fruit-pulp 121, seed 151), Zn (fruit pulp 0.334, seed 0.53), Fe (fruit pulp 0.25, seed 0.15), Ag (fruit pulp 0.25, seed 0), B (fruit pulp 0.050, seed 0) were high in concentrations.

Carbohydrates are important in food as a major source of energy (Alasalvar and Shahidi, 2013; Velisek, 2014; McWilliams, 2014). *S. cordatum* fruit pulp and seed extracts had 63.9% and 60.5% respectively. This implicates that *S. cordatum* fruit pulps and seeds are good energy sources for man. *S. cordatum*

fruit pulp and seed extracts had 1.7% and 1.3% of dietary fibre composition respectively. Dietary fibre lowers the risk of constipation and incidence of GIT infections (De la Rosa *et al.*, 2010; Velisek, 2014). Dietary fibre holds water; soften stools and decreases stool transit time in the intestines (Chu, 2014). Thus the daily intake of *S. cordatum* fruits can be of great significance to man in the prevention of GIT infections.

The pH values of *S. cordatum* fruit pulp and seed extracts were 2.67 and 3.36 respectively. The difference was believed to be owed to the concentration of ascorbic acid in the pulp extract as compared to the seed extract. The pH value of 2.5-5.5 tends to prolong fruit shelf lives by inhibiting microbial growth and thus fruit spoilage (Belitz *et al.*, 2009). The mean pH value (3.02) of *S. cordatum* fruit pulp and seed extracts prolongs the potential of the shelf life of *S. cordatum* fruits. The energy value for most fruits has a range of 84-2500 KJ 100 g<sup>-1</sup> sample (Mbosso *et al.*, 2012). *S. cordatum* fruit pulp and seed extracts had the energy values of 327.3 KJ/g and 347 KJ/g respectively. The energy values fell within the required energy range of most fruits. However, the energy values were far below the energy requirement of 12552 KJ per day for adults (Umar *et al.*, 2013). The high energy level was mostly contributed by the carbohydrates constituent while the minor difference of energy values of the extracts was due to higher protein level yielded in seed extract (12.9%) in comparison to that of fruit pulp extract (0.6%). In general, *S. cordatum* fruit pulps and seeds can supply sufficient energy to support the metabolic actions within the human system.



### 5.1.3 Phytochemical screening

The presence of phenolics, alkaloid, saponins, cardiac glycosides, tannins, phenols, terpenoids, betulinic acid was obtained during the phytochemical screening and the absence of the flavonoids was found in the seed extract (Table 2). The total phenolic contents of the fruit and seed samples were also measured (Table 3). Phytochemicals have been reported to possess strong antimicrobial, antidiarrhoeal and gastroprotective properties (Neethirajan *et al.*, 2012). The phytochemicals detected in varying proportions in both extracts were phenolic compounds, alkaloids, cardiac glycosides, phytosterols, flavonoids, saponins, terpenoids and betulinic acid. The quantitative analysis showed that the crude seed extract possessed a significantly higher content of total phenolic compounds ( $21.4 \pm 1.4$   $\mu\text{g}/\text{mg}$ ) in comparison to the fruit-pulp extract ( $16.4 \pm 1$   $\mu\text{g}/\text{mg}$ ). The detected phytoconstituents implied that *S. cordatum* fruit pulps and seeds are potential sources for novel lead substances with potential therapeutic and preventative applications against GIT infections.

### 5.1.4 Antibacterial activity

Ciprofloxacin is a broad-spectrum fluoroquinolone antibiotic that exerts antibacterial activity by inhibiting bacterial DNA gyrase of both Gram-negative and Gram-positive bacteria (WHO, 2001; Volans and Wiseman, 2010). Ciprofloxacin has a bactericidal effect against *E. coli*, *Salmonella spp.*, *Pseudomonas aeruginosa*, *Staphylococcus spp* and *Streptococcus* strains (Paw and Shulman, 2010). Ciprofloxacin is readily absorbed in GIT and is widely used to treat urinary and respiratory infections as well as gastroenteritis (WHO, 2001; Volans and Wiseman, 2010). Ciprofloxacin (20  $\mu\text{g}/\text{ml}$ ) was used as a positive control on the selected bacteria in this study. Ciprofloxacin had an inhibitory effect on all the selected bacteria with the lowest MIC values of (1.56 mg/ml) on *V. vulnificus* (AL 042), *V. fluvialis* (AL 019) and *S. typhimurium* (ATCC 700030). The highest MIC value (3.13 mg/ml) of ciprofloxacin was recorded on all other selected bacterial strains.

Many naturally occurring compounds found in fruit pulp and seed extracts have been reported to possess antimicrobial activities. *S. cordatum* fruit pulp extract showed broad-spectrum antibacterial action with the lowest MIC value of 3.13 mg/ml on *S. aureus* (ATCC 25925), *B. cereus* (ATCC 10102), *E. hirae* (ATCC 8043) and *P. aeruginosa* (ATCC 7700) while the seed extract had the lowest MIC value (6.25 mg/ml) on *S. aureus* (ATCC 25925), *B. cereus* (ATCC 10102) and *E. hirae* (ATCC 8043). Even though the antibacterial action of *S. cordatum* fruit pulp extract was more pronounced on all Gram-positive bacterial strains, the extract did also show remarkable antibacterial activity against Gram-negative bacterium (*P. aeruginosa* (ATCC 7700) as well as with the same MIC value of 3.13 mg/ml. Gram-negative bacteria, in addition to a thin peptidoglycan layer (2 to 7 nm), possess about 7 to 8 nm of the outer membrane. This outer membrane consists of an additional protective lipopolysaccharide layer that exhibits toxicity and antigenicity against antimicrobials or chemotherapeutic agents (Martinko and Madigan, 2006). It was concluded that the high resistance shown by some Gram-negative bacteria as compared to Gram-positive bacteria to both *S. cordatum* fruit pulp and seed extracts was due to the mechanism of action of this layer. Gram-positive bacteria do not possess this layer and therefore they were generally, highly sensitive to the action of the antibacterial phytochemicals detected in both extracts. Gram-positive bacteria allow the direct contact of the extract constituents with the phospholipid bilayer of the cell membrane, enabling the antimicrobials to inhibit microbial growth easily.

The MIC values shown by *S. cordatum* fruit pulp were found to be lower than those of *S. cordatum* seed extract. The low MIC values displayed by the fruit pulp extract indicated its higher efficacy against bacteria causing GIT infections than the seed extract. The difference in efficacy of *S. cordatum* fruit pulp and seed extracts may be due to the unique mechanism of action displayed by various phytochemicals (flavonoids) that were detected in the *S. cordatum* fruit pulp extract but not in the *S. cordatum* seed extract, although the total phenolic content of seed extract ( $21.4 \pm 1.4 \mu\text{g/ml}$ ) was much higher than that of the *S. cordatum* fruit pulp extract ( $16.4 \pm 1.8 \mu\text{g/ml}$ ). According to Jayashree *et al.* (2014), the good potency of methanolic fruit extract has an MIC value ranging from 3.125 to 12.5 mg/ml. This implied that *S. cordatum* fruit pulp and seed extracts have a potential to be used as sources of novel antibacterial agents since they both possessed MIC values ranging from 3.13 to 12.5 mg/ml against selected bacterial strains.

Antimicrobial substances are considered as bactericidal agents when the ratio is  $\text{MBC/MIC} \leq 4$  and bacteriostatic agents when the ratio is  $\text{MBC/MIC} > 4$  (Erhabor *et al.*, 2013). Both *S. cordatum* fruit pulp

and seed extracts exhibited bactericidal effect on all selected bacterial species. However, ciprofloxacin showed bactericidal effect on all selected bacterial species with the exception on *V. fluvialis* (AL 019) and *V. vulnificus* (AL 042) where it showed a bacteriostatic effect. *S. cordatum* fruit pulp had the highest total activity of 31 mg/ml on *S. aureus* (ATCC 25925), *B. cereus* (ATCC 10102), *E. hirae* (ATCC 8043) and *P. aeruginosa* (ATCC 7700) while the seed extract exhibited total activity (16 mg/ml) on *S. aureus* (ATCC 25925) (see Table 7). This implied that if a gram of extractable phytochemicals present in one gram of ground *S. cordatum* fruit pulp and seed samples were dissolved in 31 ml and 16 ml, respectively, they would still inhibit the bacterial growth of the selected strains (Bag *et al.*, 2013; Adamu *et al.*, 2014).

### 5.1.5 Cytotoxicity

The higher the concentration of the *S. cordatum* fruit pulp extract, the more potential toxicity the extract revealed. The inhibitory concentration required for 50% cytotoxicity ( $IC_{50}$ ) was 92  $\mu\text{g/ml}$ . The toxicity threshold level for plant extracts is considered highly toxic ( $IC_{50} < 10 \mu\text{g/ml}$ ) and moderate ( $IC_{50} < 10-20 \mu\text{g/ml}$ ) (Boik, 2001). Thus, the fruit extract was confirmed to be partially non-toxic ( $IC_{50} = 92 \mu\text{g/ml}$ ). The results from crude aqueous *S. cordatum* stem bark obtained by Cordier, (2013) and Sibandze *et al.*, (2010) and the aqueous leaf extract (Amabeoku and Deliwe, 2013) both confirmed other parts of *S. cordatum* as non-toxic ( $IC_{50} > 10-20 \mu\text{g/ml}$ ). In addition to the cytotoxicity test, the therapeutic index (0.1 - 0.3) of the fruit-pulp extract was obtained for fruit-pulp extract safety-efficacy profile. A higher therapeutic index (TI >10) would have been more preferable as one would have to take much higher doses of the extract to actually reach its toxic threshold (More *et al.*, 2008). Thus, *S. cordatum* fruit pulp extract and presumably the seed extract are safe for consumption or usage at lower concentrations.

### 5.1.6 Antidiarrhoeal and antimotility activities

Castor oil is an effective emollient laxative agent. Castor oil causes a decrease in fluid and nutrient absorption, increase in the electrolyte secretion and water and produces alterations in intestinal motility (Priff and Harold, 2005). The diarrhoeal activity of castor oil is attributed to its active cathartic glyceride known as ricinoleic acid (Chamber *et al.*, 2015). Thus, castor oil-induced diarrhoea is as a result of the action of ricinoleic acid formed from the hydrolysis of its triglyceride in the duodenum by pancreatic lipase. The ricinoleic acid stimulates intestinal hypersecretion, hypermotility and decreases gastrointestinal transit time (Schellack, 2004). In this study, castor oil was used to induce diarrhoea in the test rats. Atropine was used in this study as a positive control in *in-vivo* antidiarrhoeal activity. Atropine is a tertiary amine belladonna alkaloid (Chamber *et al.*, 2015). It is a racemic hyoscyamine tropic acid ester of the base tropine and has high affinity for muscarinic receptors (Champe and Harvey, 2009; Hodgson and Kizior, 2014) and also exerts its pharmacodynamic effect by binding competitively at the muscarinic receptors to prevent acetylcholine to bind and thus reversing excessive secretions of fluids and electrolytes (Chamber *et al.*, 2015). Atropine actions reduce the intestinal hypertonicity and hypermotility of the GIT and thus act as an antidiarrhoeal agent (Lehne, 2004)

The experimental rats (Group C) were fed *S. cordatum* fruit pulp extract at a dose of 400 mg/kg of a rat weight. Group C had a stool onset time of 68 minutes, total normal stools of  $8.25 \pm 0.17$  and the total wet stools of  $2.75 \pm 0.3$  while the experimental rats that were fed seed extract (Group B) at the same dose as Group C, had a stool onset time of 98 minutes, total normal stool of  $5.25 \pm 0.20$  and the total wet stools of  $1.5 \pm 0.41$  in comparison with the experimental rats given distilled water (Group A), which had a stool onset time of 51 minutes, total normal stools of  $13 \pm 0.15$  and total wet stools of  $10.3 \pm 0.2$ . The experimental rats which were given atropine (Group D) had the longest onset time (127 minutes) and the least total number of normal stools ( $1.25 \pm 0.19$ ) with wet stools not being observed. *S. cordatum* fruit pulp and seed extracts exhibited the antidiarrhoeal activity by reducing the number of wet stools, total stools and onset time generally. Phytochemicals mediate antidiarrhoeal activity through antisecretory and antimotility mechanisms (Cowan, 2015). It was therefore esteemed that the antidiarrhoeal activity observed in Group B and C was due to the presence of these phytochemicals detected in *S. cordatum* fruit pulp and seed extracts. *S. cordatum* fruit pulp extract revealed higher antidiarrhoeal activity as compared to the *S. cordatum* seed extract as observed in the reductions of total number of normal and wet fecal stools and the stool onset time. The difference was assumed to be due to the relative difference in presence and concentrations of the phytochemicals in the fruit pulp and seed extracts. The extracts

mechanism of action in antidiarrhoeal activity was esteemed to mimic that of an atropine (positive control).

The results obtained during antimotility activity evaluation were presented in Table 10. The experimental rats in Group D were given an antimuscarinic drug (atropine) and had the highest inhibitory percentage of 64 followed by experimental rats that were fed fruit pulp extract (Group C), with 49. The experimental rats that were given seed extract (Group B) had the inhibitory percentage of 41 and there was zero inhibition in the experimental rats that were given distilled water (Group A). The reduction in the distance travelled by charcoal in the *S. cordatum* fruit pulp and seed extracts treated groups indicated that *S. cordatum* fruit pulp and seed extracts exerted antidiarrhoeal activity by decreasing the GIT motility. The reduction of GIT motility by extracts in comparison to the negative control (distilled water) was attributed to the presence of the detected phytochemicals (saponins, alkaloids, triterpenoids, flavonoids, tannins, betulinic acid). Phytochemicals exert a similar mode of action as antimotility agents like atropine (Ahmad *et al.*, 2006; Saleem *et al.*, 2010; Chollet and Gleason, 2012). Thus, *S. cordatum* fruit pulp and seed extracts might have exhibited the antimotility action through the same mechanism of action exerted by the drug-atropine. The results scientifically support *S. cordatum* fruit pulp and seed extracts as potential sources for effective, novel antidiarrhoeal agents.

## 5.2 Conclusion

Essential nutrients and phytochemicals do occur in fruit-pulps and seeds. The presence of phytochemicals and essential nutrients play a vital role in the therapeutic value of the fruit pulps and seeds against GIT infections and health promotion in man. *S. cordatum* fruit pulp and seed extracts did reveal a significant energy value with potential to support the metabolic reactions in the body. *S. cordatum* fruit pulp and seed extracts also demonstrated the therapeutic and biological efficacy (antibacterial, antidiarrhoeal and antimotility activities) with potential to prevent and treat GIT infections. Moreover, *S. cordatum* fruit pulp extract has a margin of safety as the level of safety efficacy threshold proved non-toxic. Due to the nutraceutical value and safety revealed by *S. cordatum* fruit pulp and seed extracts, *S. cordatum* fruit pulps and seeds can be regarded as satisfactorily, safe beneficial sources of nutraceutical compounds that are effective against GIT infections with health-promoting properties. Thus; *S. cordatum* fruit pulp and seeds can be harvested and developed as sources of novel nutraceuticals.

## 5.3 Suggestions for future studies:

Based on the results obtained, it is suggested that further studies should be conducted for:

- Isolation, characterisation and mechanism of action of the detected phytochemicals
- The activities of the extracts in combination with conventional antibiotics
- Nutraceutical development from the extracts

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## **Appendix**

### **1.0 Materials (reagents, equipment and media)**

Hexane, dichloromethane, ethyl acetate, methanol, ethanol, acetone, Sigma-Aldrich Iodonitrotetrazolium chloride (INT), refrigerator, vacuum filtration system (vacuum pump, Buchner funnel, Buchner flask and filter paper or glass wool), nutrient broth, Muller Hinton agar, 96 micro wells dilution plate, ruler, 10% Tween twenty, rotary evaporator, mechanical shaker, weighing balance, spectrophotometer, electric grinder, micropipette, micropipette tips, ciproflaxacin, refrigerator, autoclave, distilled water, cuvettes, incubator, common glassware, inoculation loop and Bunsen burner, anthrone reagent, biuret reagent, iodine solution, ninhydrin solution, ferrous sulphate solution, Dragendroff reagent, Mayer's reagent, sodium nitroprusside, gelatin solution, copper acetate solution, atropine, castor oil, syringe, tannic acid, fructose, glucose, sucrose, ascorbic acid, ferric chloride, alanine.

### **2.0 Preliminary testing**

#### **2.1 Serial exhaustive extraction**

The powdered *Syzygium cordatum* fruit pulp and seed (100 g each) were separately subjected to serial exhaustive extraction using 400 ml 100% hexane for defatting, ethyl acetate, water, dichloromethane and methanol respectively. Extractions were repeated three times per solvent. The third extractions were left for 24 hours on a mechanical shaker. The shaker ran at the speed of 200 rpm at 37 °C. The extracts obtained were filtered through Whatman filter and concentrated using a Büchi rotary evaporator at 45 °C. The yielded extracts were weighed. The yields were re-dissolved in 100ml of 10% tween 20 to the volume concentration of 100 mg/ml. The extracts were stored at 4 °C until use. The schematic diagram in figure 7 below represented the preliminary sequential extraction of powdered *S. cordatum* fruits (Bii *et al*, 2009).

## 2.2 Thin-layer chromatography for Betulinic acid

Betulinic acid (Fig 2) has been detected in the bark of *S.cordatum* in previous studies (Mativandlela, 2009). These triterpene has a high degree of antimicrobial activity (Moghaddam *et al.*, 2012). Thus, in the preliminary test, betulinic acid was firstly detected using thin-layer chromatography for its presence in *S.cordatum* fruits sample. Its presence in the extracted samples led to the choice of extraction solvent (methanol). Methanol demonstrated much better results as compared to other solvents (water, ethyl acetate, hexane and dichloromethane) regarding the presence of betulinic acid and thus methanolic extracts were used throughout the study.

## 3.0 Cytotoxicity results on human colon cells

<b>Samples</b>	<b>Control</b>	<b>25µg</b>	<b>50µg</b>	<b>75µg</b>	<b>100µg</b>	<b>150µg</b>
	0,034	0,034	0,021	0,018	0,013	0,012
	0,057	0,04	0,036	0,029	0,018	0,025
	0,026	0,039	0,039	0,022	0,024	0,017
<b>Average</b>	0,039	0,038	0,032	0,023	0,018	0,018
<b>Std Dev</b>	0,016	0,003	0,010	0,006	0,006	0,007
<b>%Cell Viability</b>	100	96,6	82,1	59,0	47,0	46,2

## **4.0 Preparation of reagents**

### **4.1 Preparation of DMSO**

10 ml of Tween twenty was added in 90 ml of distilled water.

### **4.2 Preparation of P-iodonitrotetrazodium violet (INT)**

The original concentration of INT is 0.2 mg/ml, to make 10 ml of INT solution 0.02 g of INT was added into 10 ml of distilled water.

### **4.3 Preparation of anthrone reagent**

1 g of anthrone was dissolved in 500 mL of 92% sulphuric acid. The mixture was shaken thoroughly, cooled and stored in 4 °C until used.

### **4.4 Preparation of Biuret reagent**

The Biuret reagent was prepared by adding 3 g of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and 9 g of sodium potassium citrate to 500 mL of 0.2 NaOH solution, followed by the addition of 5 g of KI. The resulting solution was then brought to a total volume of 1 L with 0.2 N NaOH

### **64.5 Preparation of Iodine Solution**

0.03 g  $\text{I}_2$  and 0.1 g KI were poured in 100 ml of water. The solution was shaken thoroughly until  $\text{I}_2$  and KI were dissolved (Acree *et al.*, 2005).

### **4.5 Preparation of ninhydrin solution**

0.2 g of ninhydrin powder was poured into 50 mL of acetone. The mixture was thoroughly shaken until all ninhydrin was dissolved

### **4.7 Preparation of ferrous sulphate solution**

4 g of clear crystals of *ferrous sulphate* was poured in about 50 ml of recently boiled and cooled water. The mixture was thoroughly shaken until ferrous sulphate was completely dissolved

## **5.0 Media preparation**

### **5.1 Biolab Nutrient agar**

31 grams of powdered Biolab nutrient agar was suspended into 500ml of distilled water in a 1L Schott's bottle. The bottle was carefully shaken and the mixture was adjusted to 1000ml by adding the distilled water. The bottle was sterilized in an autoclave at 121°C for 15 minutes. The sterilized media was cooled at room temperature and poured into petri plates for further use (Chan *et al.*, 1993).

### **5.2 Biolab nutrient broth**

16 grams of powdered Biolab nutrient broth was agar was suspended into 500ml of distilled water in a 1L Schott's bottle. The bottle was carefully shaken and the mixture was adjusted to 1000ml with the distilled water. The bottle was sterilized in an autoclave 121°C for 15 minutes. The sterilized media was cooled at room temperature and used in conducted experiments (Chan *et al.*, 1993).

### **5.3 Biolab Muller- Hinton agar**

Thirty eight grams of powdered Biolab Muller-Hinton agar was suspended into 500ml of distilled water in a 1L Schott's bottle. The bottle was carefully shaken and the mixture was adjusted to 1000ml by adding the distilled water. The bottle was sterilized in an autoclave at 15-lb pressure (121°C) for 15 minutes. The sterilized media was cooled at room temperature and poured into petri plates for further use (Chan *et al.*, 1993).

### **5.4 Biolab Muller-Hinton broth**

Twenty one grams of powdered Biolab Muller-Hinton broth was suspended into 500ml of distilled water in a 1L Schott's bottle. The bottle was carefully shaken and the mixture was adjusted to 1000ml by adding the distilled water. The bottle was sterilized in an autoclave at 121°C for 15 minutes. The sterilized media was cooled at room temperature and poured into petri plates for further use (Chan *et al.*, 1993).



**ETHICAL CLEARANCE CERTIFICATE**

<b>Certificate Number</b>	<b>UZREC 171110-030-RA Level 02 PGM 2014/139</b>				
<b>Project Title</b>	<b>An evaluation of nutraceutical components of Syzygium cordatum fruits for the treatment of gastrointestinal tract disease</b>				
<b>Principal Researcher/ Investigator</b>	TS Maliehe				
<b>Supervisor and Co-supervisor</b>	Mr. JS Shandu	Prof AK Basson			
<b>Department</b>	Biochemistry and Microbiology				
<b>Nature of Project</b>	Honours/4 <sup>th</sup> Year	Master's	x	Doctoral	Departmental

The University of Zululand’s Research Ethics Committee (UZREC) hereby gives ethical approval in respect of the undertakings contained in the above-mentioned project proposal and the documents listed on page 2 of this Certificate.

- Special conditions:**
- (1) The Principal Researcher must report to the UZREC in the prescribed format, where applicable, annually and at the end of the project, in respect of ethical compliance.
  - (2) Documents marked “To be submitted” (see page 2) must be presented for ethical clearance before any data collection can commence.

The Researcher may therefore commence with the research as from the date of this Certificate, using the reference number indicated above, but may not conduct any data collection using research instruments that are yet to be approved.

Please note that the UZREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the documents that were presented to the UZREC
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research

Full Length Research Paper

**The antibacterial and antidiarrheal activities of the crude methanolic *Syzygium cordatum* [S.Ncik, 48 (UZ)] fruit pulp and seed extracts**

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Diarrheal infections are the major cause of high morbidity and mortality rates, especially in the developing countries. Different parts (roots, bark and leaves) of the species *Syzygium cordatum* have been used as antidiarrheal extracts with the exception of its fruit-pulps and seeds. This study aimed at evaluating the antibacterial and antidiarrheal activity of *S. cordatum* pulp and seed extracts so to find newer and more cost-effective means to prevent diarrhoea. The harvested fruits were separated into pulp and seeds, dried and extracted with methanol using Soxhlet extraction. The extracts were screened for phytochemicals. The antibacterial and *in-vivo* antidiarrheal activities were determined using the microdilution method and castor oil-induced rat model, respectively. The percentage yields of 10 for fruit-pulp extract and 6 for seed extract were obtained. The detected phytochemicals were phenolics, alkaloids, cardiac glycosides, phytosterols, flavonoids, saponins, terpenoids and betulinic acid with the total phenolic content of  $16.4 \pm 1.8$  and  $21.4 \pm 1.4$   $\mu\text{g}/\text{mg}$  for pulp and seed extracts, respectively. The pulp extract exhibited the lowest minimum inhibitory concentration (MIC) value of 3.13 mg/ml against some gram-positive and gram-negative bacteria while the seed extract had lowest MIC on. The *in vivo* antidiarrheal activity showed the percentage inhibition of 41 for the seed extract and 49 for pulp extract. The antibacterial and antidiarrheal activities were owed to the detected phytochemicals, and thus promoting *S. cordatum* fruit-pulps and seeds as potential sources of therapeutic compounds against diarrheal infections.

**Key words:** Antibacterial, antidiarrheal, antimotility, phytochemicals.

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## INTRODUCTION

Diarrheal infections are major causes of morbidity and mortality worldwide, especially in developing countries among infants and children. There are approximately 1.5 billion episodes of diarrheal infections per year. More than one in ten deaths of children under the age of 5

years are due to diarrhoeal infections (WHO and UNICEF, 2009). Diarrhoea is gastrointestinal disorder that is characterized by a decrease in the stool consistency and an increase in frequency, fluidity, or volume of the faeces during defecation for a period o

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Full Length Research Paper

## Evaluation of the antibacterial activity of *Syzygium cordatum* fruit-pulp and seed extracts against bacterial strains implicated in gastrointestinal tract infections

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Gastrointestinal tract (GIT) infections are the major cause of high morbidity and mortality rates, especially in the developing countries. Fruit and seed extracts possess phytochemicals that are active against bacterial strains implicated in GIT infections. Different parts of *Syzygium cordatum* trees have been investigated pharmacologically against GIT infections previously with the exception of the fruits and seeds. This study aimed at evaluating the antibacterial activity of *S. cordatum* fruits and seeds against bacteria causing GIT infections. The harvested fruits were separated into fruit-pulp and seeds, dried and extracted with methanol using Soxhlet extraction. The extracts were phytochemically screened and micro dilution assay was used to evaluate antibacterial activity of the fruit-pulp and seed extracts against the selected GIT infecting bacteria. The crude extracts of fruit-pulp and seed exhibited the percentage yield of 10 and 6, respectively. The extracts showed the presence of phytochemicals with the total phenolic content of  $21.4 \pm 1.4$   $\mu\text{g/ml}$  for seed extract and  $16.4 \pm 1.8$   $\mu\text{g/ml}$  for fruit-pulp extract. Antimicrobial activity of the pulp extract exhibited the lowest minimum inhibitory concentration (MIC) of 3.13 mg/ml against *Bacillus cereus* (ATCC 10102), *Staphylococcus aureus* (ATCC 25925), *Klebsiella pneumoniae* (ATCC 4352), *Pseudomonas aeruginosa* (ATCC 7700), *Enterococcus hirae* (ATCC 8043) while the seed extract had an equal MIC value against *Klebsiella pneumoniae* (ATCC 4352). The antimicrobial activity was due to the detected phytochemicals and thus promoting *S. cordatum* fruits and seeds as potential new and cost effective sources for prevention and treatment of GIT infections.

**Keywords:** Gastrointestinal, fruits, seeds, phytochemicals.