

CHAPTER 1

1. INTRODUCTION

Throughout the ages, humans have relied on nature for their basic needs such as food-stuffs, shelter, clothing, transport, fertilizers, flavours, fragrances and medicines. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies. Although some of the therapeutic properties attributed to plants have proven to be erroneous, medicinal plant therapy is based on the empirical findings of hundreds of years (Rios and Recio, 2005).

There is continuing interest in the evaluation of natural products as potential chemotherapeutic agents. This is encouraged by the previous isolation of natural products which became important drugs in modern medicine. Natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world. During the last 40 years, at least a dozen potent drugs have been derived from flowering plants, e.g. diosgenin, which was derived from *Dioscorea* species and used to synthesise contraceptive agents (Sala *et al.*, 2002). Other examples include reserpine from *Rauwolfia* species. Two powerful anticancer agents were derived from *Catharanthus roseus* as well as laxative agents from *Cassia (Senna)* species (Gurib-Fakim, 2006). These examples are just a few of many plant derived drugs that are important in the treatment of various medical conditions. About one quarter of all medical prescriptions are formulations based on substances derived from plants or plant-derived synthetic analogues.

Although the isolated natural products are utilised in modern medical practice, the vast majority of people still rely on their traditional *materia medica* (medicinal plants and other materials) for their everyday healthcare needs. One quarter of all medical prescriptions are formulations based on substances derived from plants or plant-derived synthetic analogues. According to the World Health Organization (WHO), 80% of the world's population, primarily those of developing countries, rely on plant-derived medicines for their healthcare (Gurib-Fakim, 2006). In most of developing countries, South Africa included, traditional herbal medicine remains the core of healthcare, especially in rural settlements. Although not well regulated, the majority of people consult traditional healers especially those who cannot access Western medicines due to high costs and unavailability.

In the developing countries, large numbers of the World's population are unable to afford pharmaceutical drugs and they continue to use their own systems of indigenous medicine that are mainly plant based. Thus, there is a great need to harness scientific and clinical research in order to investigate the quality, safety and efficacy of these herbal therapies (Phillipson, 2001).

The current advancement in science has made it possible for the isolation of compounds of medical importance from traditional medicinal plants. Plant parts such as roots, leaves, stems and rhizomes are extracted with different solvents to yield extracts that are biologically active against various disease conditions or active in various *in vivo* and/or *in vitro* testing systems (van Wyk and Wink, 2004). Some of these compounds are effective in combination with others (i.e., as crude extracts); while others are active as pure compounds (Balunas and Kinghorn, 2005). The

isolation of such compounds remains a challenging and a mammoth task. Scientists have succeeded to isolate and characterize bioactive compounds from medicinal plants. The isolation of bioactive compounds is preceded by the determination of the presence of such compounds within plant extracts through a number of bioassays. The presence of bioactive compounds, such as antifungal compounds, in plant extracts can be determined by bioautographic methods as reported by Masoko and Eloff (2005). Martini and Eloff (1998) reported on the isolation of some flavonoids with antibacterial activity from *Combretum erythrophyllum*.

Senna species belongs to the family Fabaceae and is well known for its therapeutic properties in the folk medicine of some countries. *Senna* species is being widely used traditionally to treat sexually transmitted diseases (Tshikalange *et al.*, 2005). Some *Senna species* from Venda in South Africa have shown antibacterial activities and as treatment of sexually transmitted diseases (Tshikalange *et al.*, 2005). A literature survey on the chemical constituents of the genus *Senna* revealed the presence of alkaloids, quinines and anthraquinones. These types of compounds have been isolated from heartwood, seeds, root bark, roots and leaves of the genus *Senna* (Barbosa *et al.*, 2004). Thus, the biological activity of *Senna* species is very important to study based on its wide usage in traditional medicine practice.

The current study evaluates the effect of the acetone extract of *Senna* species on the proliferation of Jurkat T cells and on the growth of selected bacterial strains. We also determine the free radical scavenging power of the extract of *Senna* species on 2, 2-diphenyl-1-picrylhydrazyl, DPPH. The current study also reports on the isolation and identification of a compound with free radical scavenging properties.

1.1. MEDICINAL PLANTS: A SOURCE FOR DRUG DISCOVERY

Plants have been utilized as medicines for thousands of years (Gurib-Fakim, 2006; Phillipson, 2001). These medicines initially took the form of crude drugs such as tinctures, powders and other herbal formulations. The specific plants to be used and the methods of application for particular ailments were passed down through oral history (van Wyk and Wink, 2004). Eventually, information regarding medicinal plants was recorded in herbals. It was not until the 19th century that man began to isolate the active principles (constituents) of medicinal plants. In more recent history, the use of plants as medicines has involved the isolation of active compounds.

Drug discovery from medicinal plants led to the isolation of early drugs such as cocaine, codeine, etc. (Butler, 2004). The isolation and use of natural products such as morphine and quinine has resulted in replacing the plant extracts used with single chemical entities. There is a basic supposition that any plant possessing clinical effectiveness must contain an active ingredient which can completely replace the plant extract. However, Phillipson (1995) argues that this may not necessarily be true. Such argument can hold since the synergistic activity of the constituents of plant extracts cannot be ruled out. Isolation and characterization of pharmacologically active compounds from medicinal plants continue to be much relevant today. More recently, drug discovery techniques have been applied to the standardisation of herbal medicines to elucidate analytical markers (Newman *et al.*, 2000).

Despite these discoveries, the impact of phytochemistry on new drug development declined and inevitably the innovative pharmaceutical industry turned to synthetic chemicals. Successful clinical agents emerged from multidisciplinary research teams

in which pharmacologists and synthetic chemists collaborated. In recent years, the attention of the pharmaceutical industry has switched once more to the natural world and this may be illustrated by reference to three clinical drugs: taxol, etoposide and artemisin. The isolation and structure determination of taxol followed on from experiments that showed that a crude extract of *Taxus brevifolia* was active against cancer cells in laboratory tests. Although this activity was discovered in the early 1960s, it was not until 1971 that the structure elucidation of this complex diterpene was determined (Pieters and Vlietinck, 2005).

Drug discovery from medicinal plants has evolved to include fields of inquiry and various methods of analyses. Collection may involve species with known biological activity for which active compound(s) have not been isolated (e.g., traditionally used herbal remedies) or may involve taxa collected randomly for a large screening program. Phytochemists (natural product chemists) prepare extracts from the plant materials, subject these extracts to biological screening in pharmacologically relevant assays and commence the process of isolation and characterization of the active compound(s) through bioassay-guided fractionation. Molecular biology has become essential to medicinal plant discovery through the determination and implementation of appropriate screening assays directed towards physiologically relevant molecular targets (Balunas and Kinghorn, 2005).

1.1.1. *Importance of medicinal plants in drug discovery*

Numerous methods have been utilized to acquire compounds for drug discovery which include isolation from medicinal plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modelling. Despite the interest in

combinatorial chemistry and other synthetic techniques by pharmaceutical companies and funding organizations, medicinal plants remain an important source of new drugs. These drugs could either be new drug leads or new chemical entities (NCEs) (Balunas and Kinghorn, 2005).

Drugs derived from medicinal plants can serve not only as new drugs themselves but also as drug leads suitable for optimisation by medicinal and synthetic chemists. Even when new chemical structures are not found from medicinal plants during drug discovery, known compounds with new biological activity can provide important drug leads (Kramer and Cohen, 2004). Natural products have played an important role as new chemical entities (NCEs). Approximately 28% of NCEs between 1981 and 2002 were natural products or natural product-derived. Another 20% of NCEs during this time period were considered natural products mimics, meaning that the synthetic compound was derived from the study of natural products. Thus, combining these categories, research on natural products accounts for approximately 48% of the NCEs reported from 1981 to 2002 (Newman *et al.*, 2003).

The stereochemistry of these natural products plays a vital role towards their biological activity. Many structural features common to natural products (e.g., chiral centers, aromatic rings, complex ring systems, degree of molecule saturation and number and ratio of heteroatoms) have been shown to be highly relevant to drug discovery efforts. Natural products provide a starting point for new synthetic compounds, with diverse structures and often with multiple stereocenters that can be challenging to synthesize (Koehn and Carter, 2005).

The new chemical entities isolated from medicinal plants need to be screened for biological activity using relevant bioassay models. Relevant molecular targets can be used to determine the biological activity of natural products. Since the sequencing of the human genome, many new molecular targets have been identified as important in various diseases. With the advent of high-throughput screening assays directed towards these targets, known compounds from medicinal plants may show promising and possibly selective activity. Several known compounds isolated from traditionally used medicinal plants have already been shown to act on newly validated molecular targets (Xiao *et al.*, 2002).

Other known compounds have also been shown to act on novel molecular targets, thus reviving interest in members of these frequently isolated plant compound classes. Three examples are cucurbin I, which was found to be highly selective in inhibiting pathways in tumours; β -lapachone, which selectively kills cancer cells over normal cells through direct checkpoint activation during the cell cycle; and betulinic acid, with selective melanoma cytotoxicity through the activation of p38.

1.1.2. *Anti-cancer drug discovery*

Cancer is one of the most dreaded diseases of the 20th century and spreading further with continuance into the 21st century. It is considered as an adversary of modernization and advanced pattern of socio-cultural life dominated by western medicine. Multidisciplinary scientific investigations are making best efforts to combat this disease, but the sure-shot, perfect cure is yet to be brought into the world of medicine. Recently, a greater emphasis has been given towards the search on complimentary and alternative medicine that deals with cancer management. Several

studies have been conducted on herbs under a multitude of ethnobotanical grounds. Data have been collected on about 3 000 plants, those of which possess anticancer properties and have subsequently been used as potent anticancer drugs (Balachandran and Govindarajan, 2004).

Despite many therapeutic advances in the understanding of the processes in carcinogenesis, overall mortality statistics are unlikely to change until there is a reorientation of the concepts for the use of natural products as new chemopreventive agents. Cancer chemoprevention was first defined as “a strategy of cancer control by administration of synthetic or natural compounds to reverse or suppress the process of carcinogenesis” (Kingham *et al.*, 2003). Natural or semi-synthetic compounds may be used to block, reverse or prevent the development of invasive cancers. Cellular carcinogenesis forms the biological basis for the identification of preventive products, the assessment of their activity, and ultimately the success or failure of a therapy (Reddy *et al.*, 2003).

Since 1990 there has been an increase in cancer incidence and mortality with the four most frequent cancers being lung, breast, colorectal and stomach. Drug discovery from medicinal plants has played an important role in the treatment of cancer. Indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied towards combating cancer. The majority of available anticancer drugs are natural products or derived from natural products (Balunas and Kinghorn, 2005).

Many natural products are available as chemopreventive agents against commonly occurring cancers occurring worldwide. A major group of these products are the powerful antioxidants (others are phenolic in nature) and the remainder includes reactive groups that confer protective properties. These natural products are found in vegetables, fruits, plant extracts and herbs. A host of plant constituents could be responsible for the protective effects, and it is likely that several of them play a role under some circumstances. Most of the non-nutrients in these foods are phenolic or polyphenolic compounds such as isoflavones (Galati and O'Brien, 2004).

Numerous types of bioactive compounds have been isolated which includes alkaloids, diarylheptanoids, fatty acids, flavonoids, phenanthrene derivatives, polyacetylenes, triterpenoids, phenols, etc. Gurib-Fakim (2006) highlighted that perhaps one of the significant breakthrough in the field of anticancer drugs came from the Madagascan Periwinkle (*Catharanthus roseus*) in the 1970s. This plant had been in use and patent medicines such as vinculin in England and covinca in South Africa have been marketed for diabetes. The Periwinkle has a long history of treating a wide variety of diseases. Its use as a source of anticancer alkaloids arose from its reputation as a cure for diabetes. This breakthrough led to isolation of medically important compounds from medicinal plants.

Since then more than 150 alkaloids have been isolated and characterised, a number of which have been found to be indole alkaloids including dimeric and *bis*-indole alkaloids. Thus, bioassay-guided isolation of the plant extracts led to the characterisation of the active complex alkaloidal compounds: vincristine and vinblastine. These compounds have been proven to be effective agents against

childhood leukaemia, breast cancer and Hodgskin's disease (a cancer of the lymph nodes) and choriocarcinoma (Voss *et al.*, 2006).

Another example of plant-derived anticancer drug is paclitaxel, more commonly known by its trademark name, Taxol. Taxol, a complete terpene-based molecule is derived from the Pacific yew (*Taxus brevifolia*). Extracts of Pacific yew were found to stop the growth of several mouse tumours. The plant was traditional used for lung ailments and not specifically for cancers or tumours. Should the traditional usage been strictly followed this could have overshadowed the usage of paclitaxel for treatment of tumours. Paclitaxol is now being used to treat lung cancers that do not respond to other therapies. Thus, although a particular plant species is ethnobotanically used for a particular condition that does not rule out other usage towards different conditions. Ethnobotanical usage can only serve as a lead to screen the plant species for biological activity.

One more other example is the roots of the African bush willow (*Combretum caffrum*) from southern Africa region which is commonly used in traditional medicine against body pain. Screening of the plant extracts have led to the isolation of the Combrestatins. Among these, combrestatin A-4 (CA-4), isolated from a South African tree *Combretum caffrum*, is one of the most potent antimetabolic agents. Combrestatins are a family of stilbenes, which act as antiangiogenic agents causing vascular shutdown in tumours and resulting in tumour necrosis when tested against solid tumours.

CA-4 has shown strong cytotoxicity against a variety of cancer cells, including multidrug resistant cancer cell lines (Dziba *et al.*, 2002). It has also been demonstrated

to exert highly selective effects in proliferating endothelial cells. CA-4 disodium phosphate (CA4DP), a water-soluble pro-drug of CA-4, has also shown potent antivasular and antitumour effects in a wide variety of preclinical tumour models. Combrestatin A-4 phosphate has undergone successful Phase I clinical trials and has also exhibited the absence of cumulative toxicity. This has led to a significant number of compounds based upon the combrestatins skeleton to have been synthesized in the search for more effective anticancer agents (Dziba *et al.*, 2002).

Cancer is formed through a process known as carcinogenesis. Carcinogenesis is a multistage process by which a normal cell is transformed into a cancerous cell. Transformation involves initiation, typically from DNA damaging agents; promotion, during which cell proliferation is increased; and progression, involving additional genetic alterations. Chemoprevention strategies target each of these steps including anti-initiation strategies (e.g., DNA repair, detoxification, free radical scavenging and carcinogen metabolism) and anti-promotion/anti-progression strategies (e.g., free radical scavenging, proliferation suppression, differentiation induction, immunity enhancement, inflammation reduction, increase in apoptosis, altered gene expression and decrease in angiogenesis).

Because cancer chemotherapy is designed to occur from the onset of cancer diagnosis, little to no toxicity can be tolerated (chemoprevention can also apply to preventing cancer recurrence in which case a slightly higher toxicity levels are acceptable). As such, herbal medicines, botanicals, dietary supplements and edible plants have all been suggested as potentially important in cancer chemoprevention due to their long history of human consumption (Balunas and Kinghorn, 2005).

1.2. SECONDARY PLANT METABOLITES IN DRUG DISCOVERY

Secondary metabolites or “natural products” are low-molecular weight compounds that do not play a role in primary plant metabolism. They constitute the active ingredients of medicinal plants (Kinghorn *et al.*, 2003). In most cases secondary metabolites are unique to individual plant species and they usually occur in low concentrations. It is assumed that their function or importance is mainly related to ecological aspects as they are used for defense against predators, parasites and diseases, for interspecies competition, and to facilitate the reproductive processes (e.g., colouring agents, attractive smells, etc) (Schulz and Baranska, 2007).

Although natural products, particularly secondary metabolites, have formed the basis of medicines, the presence of these compounds in the biochemistry of the plant is very often difficult to justify (Gurib-Fakim, 2006). It has been suggested that these compounds may have been synthesized by the plant as part of its defense system, e.g., plants are known to produce phytoalexins in response to attack by bacteria and fungi. Whatever the reason for the presence of these compounds in nature, they provide an invaluable resource that has been used to find new drug molecules. The figure below shows some of the drug molecules derived from plant isolated natural compounds:

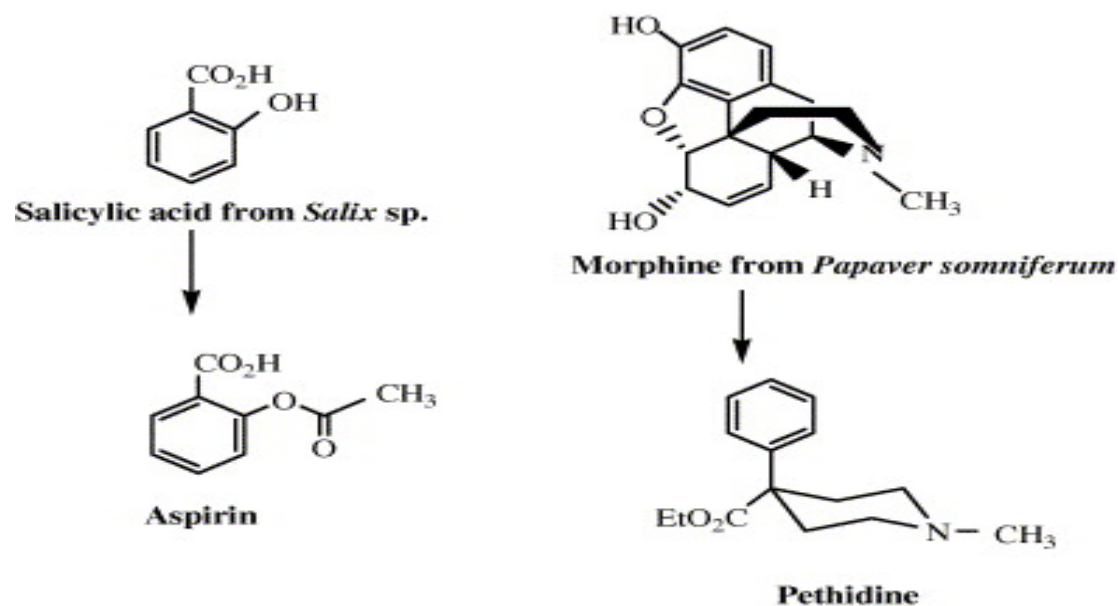


Figure 1.1: Medicinal agents derived from nature's lead molecules (Gurib-Fakim, 2006).

Approximately 20% of higher plants have been investigated in some depth so far, several ten thousands of secondary metabolites have already been isolated and their structures determined by mass spectrometry (MS), nuclear magnetic resonance (NMR) or X-ray diffraction. Three major groups of secondary metabolites can be recognised: nitrogen-containing substances, terpenes and phenolics (van Wyk and Wink, 2004). Numerous types of bioactive compounds have been isolated which include alkaloids, diarylheptanoids, flavonoids, phenanthrene derivatives, polyacetylenes, triterpenoids, phenols, etc (Kinghorn *et al.*, 2003).

1.3. STRATEGIES USED IN THE DETERMINATION OF BIOACTIVE COMPOUNDS IN MEDICINAL PLANTS

Epidemiological studies over the last decades have consistently linked certain diets and specific foods to disease conditions. At the same time, the number of bioactive compounds has increased dramatically. The terms “phytochemical”, “nutraceutical”

and “functional food” have been introduced to describe various aspects of this development. Bioactive compounds include a range of compounds with diverse chemical structures such as plant sterols, carotenoids, ω -fatty acids and phenols (Luthria *et al.*, 2006).

There is a lot of interest in plant polyphenols as witnessed by the numerous publications devoted to various aspects of these compounds. The use of plants and herbs as antioxidants in processed foods is becoming of increasing importance in the food industry as an alternative to synthetic antioxidants. Phenolics are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. These compounds also have metal chelating properties (Lee *et al.*, 2000). The antioxidant properties of these compounds are often claimed for the protective effects of plant-based beverages against cardiovascular disease, certain forms of cancer and photosensitivity reactions. It was also found that these antioxidant compounds inhibit ascorbate auto-oxidation, cytotoxic effects and tumour promotion (Zheng and Wang, 2001). These studies provide the basis for the current and rapidly increasing interest in the use of natural antioxidants as functional food ingredients and/or as food supplements.

Plant phenols embrace a wide range of secondary metabolites that are synthesized from carbohydrates *via* the Shikimate pathway. This is the biosynthetic route to the aromatic amino acids and is restricted to microorganisms and plants. Thus, phenolic compounds are ubiquitous in plant kingdom and are found in virtually all parts of the plant but with quantitative distributions that vary between different tissues of the plant and within different populations of the same plant species. The phenolic component

of plants constitutes a complex mixture, and only a small number of plants have been examined systematically for their phenolic content (Vinson *et al.*, 2001).

The determination of phenols encompasses a number of distinct aspects and the analytical strategy depends on the sample, analyte and nature of the problem (Luthria and Mukhopadhyay, 2006). Given the diversity of analytes and sample types and the number of permutations of the three, there is no global strategy that will suffice for a given phenol in all situations although a number of generalisations have been made. Thus, the general analytical strategy involves recovery of the phenol from the sample matrix followed by separation and identification (Robards, 2003). For most phenols, the recovery step typically involves solvent extraction using a range of solvents. However, special considerations apply to some phenols such as the anthocyanins and oligomeric species.

Considering the nature of the analytical problem, several roles can be identified although there is no rigid distinction and an investigation may encompass aspects of each other. In the first role, screening of bioextracts for biologically active natural products played a strategic role in the phytochemical investigation of crude plant extracts. The primary strategy for the discovery of bioactive natural products through the 19th century and into the 20th century was the structure elucidation of the active ingredients of plants with reported biological properties (Robards, 2003).

Methods of characterisation and identification of plant bioactive compounds (e.g., phenols) follow those in general use for natural products. Hence, preparation of an extract, biological screening, bio-guided fractionation, isolation and structure

elucidation is the usual approach where complete characterization is required. However, the number of biological assays that are available in a given laboratory is often limited and the range of activities screened is thus restricted. This is the approach that we pursued in the present study. A particular plant can be utilised for treatment of more than one disease condition in traditional medical practice. This observation encourages the screening of a plant extract over a number of biological assays. The numbers of biological assays screened in this study were limited by the availability of bio-assays material in our laboratory. Hence, we do not rule out the possibility that the medicinal plant we used in the present study could possess some other biological properties apart from the ones evaluated here.

An alternative approach employs “chemical screening” using coupled techniques at the earliest stage of separation of crude extracts. With this approach, extract preparation is followed by isolation of the phenol(s) and structure elucidation. This efficient and targeted isolation of compounds permits an optimization of an investigation and avoids the time consuming and costly isolation of “trivial” natural products.

1.3.1. *Bioextracts screening*

Bioassay is a very crucial stage in assessing the pharmacological actions of plant extracts and their ethnomedical uses. In the initial stages, *in vitro* testing has priority over *in vivo* studies involving laboratory animal models. *In vivo* studies may be preferable at the later stages of the research project but still depends on the amount and the nature of evidence of bioactivity already collected by means of *in vitro* studies, and the quest for additional information under life conditions. Bioactive

components that are candidates for therapeutic application will have to undergo extensive clinical and toxicological screening programmes before they can be registered as medicines (Pieters and Vlietinck, 2005).

Current strategies for choosing candidate plant species or tissues for isolation of bioactive components are based on ethnobotany, chemical ecology and plant anatomy. Phytochemicals such as flavonoids have been used historically to identify plants in chemotaxonomy. This role is being reversed and chemotaxonomy is generating bioactive compounds of the same or related molecular structures. The isolation of new bioactive compounds from plants can be directed by bioassays.

Alternatively, new uses of compounds can be identified when known compounds are tested in new bioassays. The availability of specific *in vitro* bioassays has facilitated the screening of numerous bioactivities of natural products. Screening has uncovered new pharmaceuticals and structure-activity relationships which has provided leads for designs of new drugs (Baker *et al.*, 1995).

There are many types of pharmacological screens that are specific for bacteria, fungi, protozoa, intestinal worms, viruses, etc. The efficacy of compounds against health problems such as cancer and inflammation is often probed while the effect on physiological and anatomical systems such as reproduction, digestion, etc., can be judged. Among the commonly used assays are the brine shrimp and antimicrobial (bacterial and fungi) screens:

- Brine shrimps are small aquatic animals that can be grown in solutions similar to seawater. In order to test the potential toxicity of the plant, and thus its

probability of containing anticancer agents, measured amounts of plant extracts are added to containers holding known numbers of brine shrimps. The surviving shrimps are counted after 6 and 24 hours.

- Bacteria are grown on agar medium in Petri dishes. Then, measured amounts of a plant extract are placed on paper disks or in wells set on the surface of the bacteria-inoculated agar under sterile conditions. After 18 to 24 hours, bacteria-free circles can be seen around the disk or wells, thus indicating that the extract has inhibited the microbes (Gurib-Fakim, 2006). Alternatively, antibacterial activity can be determined through the bioautography method using TLC plates as reported by Begue and Klein (1972), and modified by Masoko and Eloff (2005) for antifungal studies. The bioautographical antibacterial activity of the extracts is coupled with the determination of the minimum inhibitory concentrations (MICs) using the microtitreplate method (Eloff, 1998a).

Bioassays can range from molecular assays to whole-organism assays. Each has its advantages depending on the objectives. Many factors can complicate results when using bioassays or bioassay-guided fractionation. The choice of a solvent is important as many bioextracts have limited solubility. The selection of solvent must be considered carefully in relation to the nature of the bioassay in order to avoid false results. Other factors include synergistic effects, chemical changes during extraction and cancellation of activity by certain concentrations of substances. For example, in the isolation of leurosine, the crude alkaloid fraction exhibited no *in vitro* activity whilst the pure alkaloid showed pronounced cytotoxicity in the same test (Robards, 2003).

The most studied bioactivity of the phenols is their antioxidative status. Many medicinal plants contain large amounts of antioxidants such as polyphenols, which can play an important role in adsorbing and neutralising free radicals (Djeridane *et al.*, 2006). Antioxidants are known to interrupt the free radical chain of oxidation by donating hydrogen from phenolic hydroxyl groups and to form stable products, which do not initiate or propagate further oxidation of lipids (Kouri *et al.*, 2007).

The method widely employed in the evaluation of antioxidant activity is the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. DPPH radical is used as an indicator in testing hydrogen-donating capacity and thus antioxidant activity (Dorman *et al.*, 2003). The problem with bioassays is that they provide no data on individual compounds. Moreover, they do not distinguish between members of a class of bioactive compounds and provide only semi-quantitative measurements of substances detected. Nevertheless, they continue in use because of their simplicity, low cost and the potential to generate activity data of direct relevance (Villaño *et al.*, 2007).

1.3.2. *Phytochemical studies*

1.3.2.1. *Extraction of phytochemicals*

Structural diversity of the phenols affects physicochemical behaviour such as solubility and partitioning making optimisation of the recovery system difficult in all but the simplest cases. Extraction method and solvent choice are generally critical as well as extraction time and temperature (Asima *et al.*, 2003; Caldwell *et al.*, 2005; Hinneburg and Neubert, 2005). The choice of the extraction solvent is influenced by the nature of study the chemist wants to undertake. No single solvent will provide

optimum recovery of all phenols or even a limited range of phenols. Plant phenols are ionisable with typical pKa values ranging from 8 to 12 and oil/water partitioning. Thus, they exhibit considerable diversity in terms of acidity as well as polarity ranging from hydrophobic and hydrophilic in character. The range of physicochemical behaviours should be considered when determining sample handling strategies (Bidlack *et al.*, 2000).

Ethyl acetate and dimethyl sulfoxide have been used as extractants but aqueous mixtures of methanol, ethanol or acetone are often the solvents of choice for recovery of a wide range of phenols from diverse sample types. There are some important distinctions between fresh and dried samples. Furthermore, with dried materials, low polarity solvents and ethyl acetate will simply leach the sample whereas alcoholic solvents presumably rupture cell membranes and enhance the extraction of endocellular materials (Robards, 2003).

Though many traditional healers use water extracts, this route is often not followed due to complications related to working with water extracts, although the prominent factor is the difficulty in removing water from the extracts. The other reason is that with water, the extracted compounds are mainly polar and this rule out the extraction of active non- polar compounds. Although methanol is also a good extractant in terms of quantity, it is difficult to remove from the extracts. In this study the quantity was not the driving force rather much attention was paid to the bioactivity of the extract. Acetone is preferably used as a solvent for extraction because it extracts polar and non- polar components from the plant material, is miscible with water, very

volatile, has low toxicity in antimicrobial bioassays and is easily removed from the plant material at low temperature (Eloff, 1998b).

1.3.2.2. *Quantification of the extract's phenolic content*

Traditional methods for the determination of the phenolic component rely on calorimetric measurement of total phenols using one of a number of reagents of varying selectivity. A classic reagent for determination of the plant extract's total phenolic content is the Folin-Ciocalteu reagent. The blue colour formed after 15-60 minutes is measured at 725-735 nm and results are expressed in terms of molar equivalents of a commonly occurring phenol, for example, gallic acid, tannic acid or quercetin (Abdille *et al.*, 2005).

A disadvantage is the interference of reducing substances such as ascorbic acid. All phenols absorb radiation in the ultraviolet region and this provides the basis for an alternative measurement of total phenols. However, all spectrophotometric measurements lack specificity and give an overestimation of 'phenolic' content. Estimation of total phenolics by the Folin-Ciocalteu procedure also lacks specificity and is influenced by interference from reducing sugars, sulphites and amino acids (Singleton *et al.*, 1974). However, even though not specific to the identification of the phenolic compound, the determination of the total phenolic of the plant extract is of utmost importance. This is so because in most instances the extract's biological activity could be linked to its phenolic composition (Robards, 2003).

1.4. PURIFICATION OF NATURAL PRODUCTS

The purification of natural products remains a challenging, lengthy and a tedious task (Mahler and Thomason, 2005). Spectroscopic methods coupled with good separation techniques like chromatography, have contributed to the phenomenal success of natural product chemistry over the past 50 years. Sound strategies have helped in the isolation and characterization of many bioactive molecules (Rios *et al.*, 1991). Nowadays, bioassay-guided fractionation of medicinal plants is a routine feature in the attempt to isolate bioactive components from natural sources.

In practice as soon as the material is collected, in the case of plants, it needs to be identified by a taxonomist so as to ascertain the correct identity of the material. Various parts of the plant are collected (leaves, flowers, stem, wood, bark, root, root bark, etc.) and dried quickly in drying cabinets. Good ventilation conditions or high speed fans can be used to speedily dry the plant material which avoids degradation of the components of the plant material by air or by microbes.

Once the material has been dried to constant weight, it is ground up to smaller particles and extracted usually using a gradient solvent extraction. Numerous extraction techniques are available which include:

(i) Cold extraction

In this instance, the plant material is extracted in solvents of differing polarity at room temperature; this allows for maximum extraction of most components.

(ii) Hot percolation

The plant is heated in the solvent usually under reflux. This extraction method allows for extraction of a large number of metabolites, from the most insoluble material like the waxes to the lipophilic natural products.

(iii) Soxhlet extraction

Perhaps the most widely and commonly used extraction technique for the extraction of natural products. The polarity gradient of the solvent is applied. Although some components may be destroyed in the process, it is still the best method of extraction used in natural product chemistry.

Once the extraction is complete, the extractant is usually concentrated under vacuum. The activity within the obtained extract can then be demonstrated by bioassay methods using both the crude and the fractionated or semi-purified extracts. Fractionation has the added advantage of getting to the biologically active material faster. One of the simplest separation methods is solvent partitioning which is a widely used method as an initial extract purification step. A combination of solvents, miscible and immiscible ones, is used to separate the phytochemicals or ingredients making up the extract. This method relies on the ability of the components to be either soluble in water or the organic phase (Gurib-Fakim, 2006).

For the separation of compounds within the extract, chromatographic techniques are employed. Chromatographic techniques have been instrumental in the separation of natural products. Chromatography is a process whereby a mixture of solutes may be resolved into components by exploiting differences in affinity of the solutes for

particles of an insoluble matrix over which a solution of the components is passing. The insoluble matrix is called the stationary phase, while the solution which passes through it is called the mobile phase.

One of the fastest and most widely used chromatographic techniques instrumental in the separation of natural products is thin layer chromatography (TLC). TLC method employs glass or aluminium plates pre-coated with the sorbent (e.g., silica gel) to varying thickness depending on the amount of the sample to be loaded. The compound mixture is loaded both in preparative or analytical plates at around 1-2 cm from the bottom of the plate and lowered in a tank containing the solvent. The latter migrates up the plates and separates the compound mixture according to the polarity of the components. Several reagents are available for visualization of the separated materials. TLC has the advantage of being a highly cost-effective qualitative technique since a large number of samples can be analysed or separated simultaneously. The few drawbacks include poor detection and control compared to high performance liquid chromatography, HPLC (Ferenczi-Foder *et al.*, 2006).

HPLC is a very popular method and widely used for the analysis and isolation of bioactive natural products. The analytical sensitivity is further enhanced depending on the detector that is being used. The detectors can be UV detection such as a photodiode array (PDA), which enables the acquisition of UV spectra of eluting peaks between 190 nm to 800 nm. PDA ultraviolet detection has the advantage of detecting even compounds with poor UV characteristics and this is particularly useful in the analysis of natural products such as terpenoids or polyketides, which may not necessarily have chromophores that will rise to a characteristic UV signature.

Coupled with electronic library searching of compounds along with the “fingerprinting” of biologically active extracts, HPLC becomes a very powerful quality control technique of herbal medicines. It has now become a tool of choice for the analysis of a majority of natural products in the pharmaceutical industry. It suffers from one drawback in the sense that it is expensive both from the machine and consumable view-points (Gurib-Fakim, 2006).

1.5. STRUCTURAL CHARACTERISATION OF PURIFIED NATURAL PRODUCTS

Once the biological evaluation has been performed and the separation of the natural product has been achieved, the chemist will attempt the structural characterization of the compounds (Pieters and Vlietinck, 2005). Structural elucidation is crucial in assessing the biological activity of the molecule as it is a well-known fact that biological activity depends to a large extent on the 3-D arrangement of functional groups on the molecule. Structure elucidation depends on classical spectroscopic techniques such as: Nuclear Magnetic Resonance (NMR), Infra Red (IR) and UV-Visible, Mass Spectrometry (MS) and X-Ray analyses (Balunas and Kinghorn, 2005; Lindsey *et al.*, 2006).

Since the development of the high resolution NMR spectrometer in the 1950s, NMR spectra have been a major tool for the study of both newly synthesised and natural products isolated from plants, bacteria, etc. In the 1980s a second revolution occurred. The introduction of reliable superconducting magnets combined with newly developed, highly sophisticated pulse techniques and the associated Fourier transformation provided the chemist with a method suitable to determine the 3-

dimensional structure of very large molecules. Since drugs in clinical use are mostly synthetic or natural products, NMR spectroscopy has been mainly used for the elucidation and confirmation of structures (Lindsey *et al.*, 2006). In the present study, NMR techniques were used to characterise the structure of the isolated biologically active compound. This report systematically follows the isolation and identification of a free radical scavenging chalcone (flavone) from the roots of *Senna* species.

2. RATIONALE OF THE STUDY

Medicinal plants have provided mankind with valuable natural products for many decades and remain the core of healthcare for many in the developing countries. These plants are administered as traditional medicine. Medicinal plant parts such as roots, leaves, stems and rhizomes possess biologically active compounds. The possession of biologically active compounds by medicinal plants has not been fully exploited.

It has been reported that antioxidative compounds are important in the prevention and treatment of various human diseases. Antioxidant compounds could be isolated and used for the prevention and treatment of free radical-related disorders. Therefore, research to isolate and identify antioxidative compounds is of high importance. Although it remains unclear which of the compounds of medicinal importance are active, polyphenols have recently received increasing attention because of some interesting new findings regarding their biological activities (Djeridane *et al.*, 2006). Although a variety of herbs are known to be sources of phenolic compounds, studies dealing with the isolation of polyphenols have rarely been carried out.

The current study has chosen to investigate *Senna* species, based on two criteria: first, it is its frequent ethnopharmacological usage amongst many communities in the Limpopo province of South Africa. The plant is used as a remedy for various disease conditions (such as sexually transmitted diseases and some forms of intestine complications) which suggest its diverse biological properties. The second criterion was based on its abundance and availability. This second criterion is important because extensive scientific studies about the plant can be carried out without endangering the species.

3. HYPOTHESIS

The roots of *Senna* species possess bioactive compounds with multiple biological activities. The presence of these bioactive compounds may account for the diverse biological activities and wide usage of this species in traditional medicinal practice.

4.1. AIM:

The purpose of the study was to evaluate the biological activity of the extracts of the roots of *Senna* species and to isolate and identify the bioactive compound(s) responsible for this activity.

4.2. OBJECTIVES:

The specific objectives of the study were:-

- To obtain acetone extract from the roots of *Senna* species.
- To obtain spectral profile of the acetone extract of *Senna* species.
- To evaluate the total phenolic content of the acetone extract using the Folin-Ciocalteu method.

- To assess the antiproliferative activity of the acetone extract on Jurkat T cancer cell line.
- To determine the free radical scavenging activity of the acetone extract using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay.
- To evaluate the antibacterial activity of the acetone extract using bioautography.
- To isolate and purify the bioactive compound(s) from the acetone extract.
- To identify the isolated bioactive compound(s) using spectroscopic and other related techniques (e.g., chromatography).

CHAPTER 2

MATERIALS AND METHODS

2.1. PLANT MATERIAL

2.1.1. *Extraction*

The roots of *Senna* species were collected from Bolahlakgomo village (Zebediela region, Limpopo province, R.S.A). After collection, the roots were dried at room temperature and ground to a fine powder using a grinder (ML 90L4, Monitoring and Control Laboratories (Pty) Ltd, R.S.A). The ground powder (150 g) was extracted with absolute acetone using cold extraction method (Masoko, 2006). The acetone extract was then filtered and concentrated using a rotary evaporator (Büchi Labotec rotavapor Model R-205, Switzerland) at 40°C. The concentrated extract was then transferred into pre-weighed beakers, dried under a stream of air and weighed.

2.2. PHYTOCHEMICAL STUDIES

2.2.1. *Total phenolic content*

Total phenolic content of the acetone extract of the roots of *Senna* species was determined using Folin-Ciocalteu method as described by Abdille *et al.* (2005). Two hundred microliter (200 μ l) of 1:10 diluted sample was added to 1 ml of 1:10 diluted Folin-Ciocalteu reagent. After 4 min of incubation, 800 μ l of sodium carbonate (75 g/l) was added and incubated for 2 h at room temperature. The absorbance was measured at 765 nm using a microtitre plate reader (Multimode detector DTX 880, Beckman-Coulter, Austria). Tannic acid (0 – 10 mg/ml) was used for plotting a standard curve. Results were expressed as tannic acid equivalents (TAE)/g dry weight of plant material.

2.2.2. *Thin Layer Chromatography fingerprinting and spectral profiles*

Thin Layer Chromatography (TLC) spectral profile of the extract was obtained. Small aliquots (10 μ l of 10 mg/ml stock) of the acetone extract were loaded onto the baseline of the silica TLC plate, silica gel 60 (Macherey-Nagel, 0.20 mm-UV₂₅₄, Germany) and separated with three mobile phases, viz: chloroform: ethyl acetate: formic acid, CEF (5:4:1, v/v/v), intermediate polarity/acidic; benzene: ethanol: ammonia hydroxide, BEA (90:10:1, v/v/v), non-polar/basic and ethyl acetate: methanol: water, EMW (40:5.4:5, v/v/v), polar/neutral (Kotze and Eloff, 2002). Once developed, the separated compounds were observed under UV light at 360 nm and 254 nm for visualization of the fluorescing (360 nm) or quenching (254 nm) compounds. To visualise the separated compounds, vanillin-sulphuric acid and *p*-anisaldehyde were sprayed on the chromatograms and heated at 110°C for optimal colour development. The UV-Visible spectral profile of the extract (0.1 mg/ml) was obtained using a spectrophotometer (UV- Visible, CARY, Model 1E, Australia).

2.3. **BIOASSAYS**

2.3.1. *Antiproliferative activity*

The antiproliferative activity of the extract was evaluated on Jurkat T cells. Jurkat T cells (American Type Culture Collection, ATCC, Manassas, USA) were grown in RPMI-1640 supplemented with 10% foetal bovine serum (FBS) and 1% penicillin, streptomycin, neomycin (PSN) at 37°C in a 5% CO₂, 95% humidified atmosphere. For cytotoxicity effects, Jurkat T cells were seeded at 2×10^4 cells/ml in a 12-well culture flask and then treated with different concentrations (0; 100; 200, 400, 800 μ g/ml) of the extract. The extract was filtered through a 0,22 μ m filter (Millex, USA) to sterility before treating the experimental cultures. Cell viability and number were

assayed at 24 h interval for 72 h using trypan blue dye exclusion method. The extract was suspended in Dimethylsulfoxide (DMSO), hence DMSO at 0.01% was used as a control in addition to untreated control cells.

2.3.2. Free radical scavenging activity

The free radical scavenging activity of the extract was evaluated using the 2, 2-diphenyl-1-picrylhydrazil (DPPH) assay (Abdille *et al.*, 2005). Briefly, aliquots of 10 μ l of the acetone extract of *Senna* species were loaded onto the baseline of the TLC plate. The compounds were then separated with three mobile phase systems (CEF, BEA and EMW). The developed TLC plates were dried and stained with 20% DPPH in methanol solution to visualize specific bands that possess free radical scavenging power. The plates were observed for 10 min after spraying with DPPH.

The radical scavenging activity of the acetone extract was compared to that of a known standard, vitamin C, as described by Abdille *et al.* (2005). Different concentrations (0 to 0.3 mg/ml) of acetone extract of *Senna* species were prepared into a microtiter plate. A 0.1 mM methanolic solution of DPPH was added into the extract solutions and allowed to stand at room temperature for 30 min. The control was prepared as above without the inclusion of the extract. The changes in the absorbance of the samples were measured at 517 nm using microtitre plate reader (Multimode detector DTX 880, Beckman-Coulter, Austria). The decrease in absorbance indicated the increase in radical scavenging activity.

2.3.3. Antibacterial activity

2.3.3.1. Bioautographic method

The TLC plates were developed with mobile phases (CEF, BEA and EMW). The plates were dried for a week using a fan to remove the solvents and sprayed with actively growing bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*) and incubated in a humidified atmosphere. After 24 h the plates were sprayed with 0.2 mg/ml of *p*-iodonitrotetrazolium violet (INT) (Sigma-Aldrich, USA) to visualize antibacterial activity on the plate (Begue and Kline, 1972).

2.3.3.2. Minimum inhibitory concentrations (MICs)

The minimum inhibition concentrations (MICs) of the extract were determined. A 50 μ l sample of a 1:10 diluted acetone extract of *Senna* species was serially diluted with 100 μ l of distilled water in a microtiter plate (Eloff, 1998a). Then 100 μ l of test bacterial strains were added to the serially diluted extract and incubated for 24 h in a humidified environment. After overnight incubation, a 100 μ l of 0.2 mg/ml INT was added. The lowest concentration that inhibited the growth of the bacterial strain was taken as the MIC value of the extract with regard to that strain. Gentamycin (0.1%) was used as positive control.

2.4. ISOLATION OF BIOACTIVE COMPOUND(S)

2.4.1. Extraction of plant material

Serial exhaustive extraction (SEE) method was used to extract the ground root powder (700 g) of *Senna* species with n-hexane, dichloromethane, acetone and methanol. The ground roots were extracted three times with 2 l of each solvent. The extracts were

filtered and concentrated using a rotary evaporator (Büchi Labotec rotavapor model R-205, Switzerland). The concentrated extracts were transferred into pre-weighed beakers, dried under a stream of air and weighed.

2.4.2. Column chromatography

After serial exhaustive extraction of the roots of *Senna* species, the antioxidant activity of the extracts was evaluated as described in section 2.3.2. Acetone extract, which demonstrated a convincing antioxidant activity, was further fractionated with the solutions of CHCl_3 : MeOH of different ratios, starting with 100% CHCl_3 increasing the concentration of MeOH until 100% MeOH was reached. A 500 mg sample of acetone extract was fractionated into seven fractions of chloroform: methanol in the following ratios: 1:0; 9:1; 8:2; 7:3; 1:1; 3:7; 0:1 (v/v) using column chromatography packed with 50 g Silica gel 60 (63-200 μm). The yields of the fractions were recorded and their antioxidant activity was evaluated using TLC-DPPH method. Fraction 9:1 (v/v) was found to possess antioxidant activity and thus chosen for compound isolation (further purification). Accordingly, 2.1 g of the remaining acetone extract was eluted with 2 l of CHCl_3 : EtoAc 9:1 (v/v) on a silica gel column.

2.4.3. Determining a suitable mobile phase for elution of the bioactive compound

After column fractionation of the acetone extract with the solutions of different ratios of CHCl_3 : MeOH, the antioxidant activity of the resultant fractions was evaluated. A suitable separation mobile phase for isolating an active compound was determined through TLC chromatography. Various ratios of the solvent combinations were used (i.e., CHCl_3 : EtoAc 3:1; 3:2; 1:1; 1:3, v/v). The developed TLC plates were visualised with vanillin-sulphuric acid and assayed for free radical scavenging active bands

using DPPH. The ratio of the mobile phase that gave a better resolution of the antioxidant active compound on the plate was found to be CHCl_3 : EtoAc 9:1 (v/v) and was used for the elution of the CHCl_3 : MeOH (9:1, v/v) fraction from the Silica gel column.

2.4.4. *Elution and structural characterisation of the antioxidant compound*

A 450 mg of fraction CHCl_3 : MeOH (9:1, v/v) was dissolved in acetone and packed on a column with silica-gel 60 (45 g) and eluted with chloroform: ethyl acetate (1:1, v/v). Tubes with similar TLC profiles (tube 13-18) were pooled together and re-eluted with chloroform: ethyl acetate (1:1, v/v) to obtain a pure compound (tube 13-31) with a yield of 13 mg. The free radical scavenging activity of the isolated compound was assayed using DPPH on TLC plate prepared using chloroform: ethyl acetate (1:1, v/v) as mobile phase. The ^1H -NMR and ^{13}C -NMR spectra of the isolated compound were analysed at the University of Botswana using an NMR spectrophotometer (Bruker, USA). For NMR analysis the compound was dissolved in absolute acetone. The structure of the compound was elucidated at the University of Botswana.

CHAPTER 3

RESULTS

3.1. Acetone extract and its phenolic content

The roots of *Senna* species were extracted with acetone and gave a yield of 1.87% (Table 3.1). The total phenolic content of the acetone extract was 0.86%. This phenolic content is not absolute but just a measure of phenolics with reducing activity.

Table 3.1: The yield and the total phenolic content of the acetone extract of the roots of *Senna* species.

| Yield (g) | Yield (%) | Phenolic content TAE/ g dry weight (mg/ml) | % Phenolic content (w/w) |
|----------------------|----------------------|---|-------------------------------------|
| 2.8 | 1.87 | 24.08 | 0.86 |

3.2. PHYTOCHEMICAL ANALYSIS

3.2.1. TLC-fingerprinting of the acetone extract

TLC fingerprinting of the extract showed the presence of many compounds with different polarities when visualized with anisaldehyde (Figure 3.1a) and vanillin in sulphuric acid reagent (Figure 3.1b).

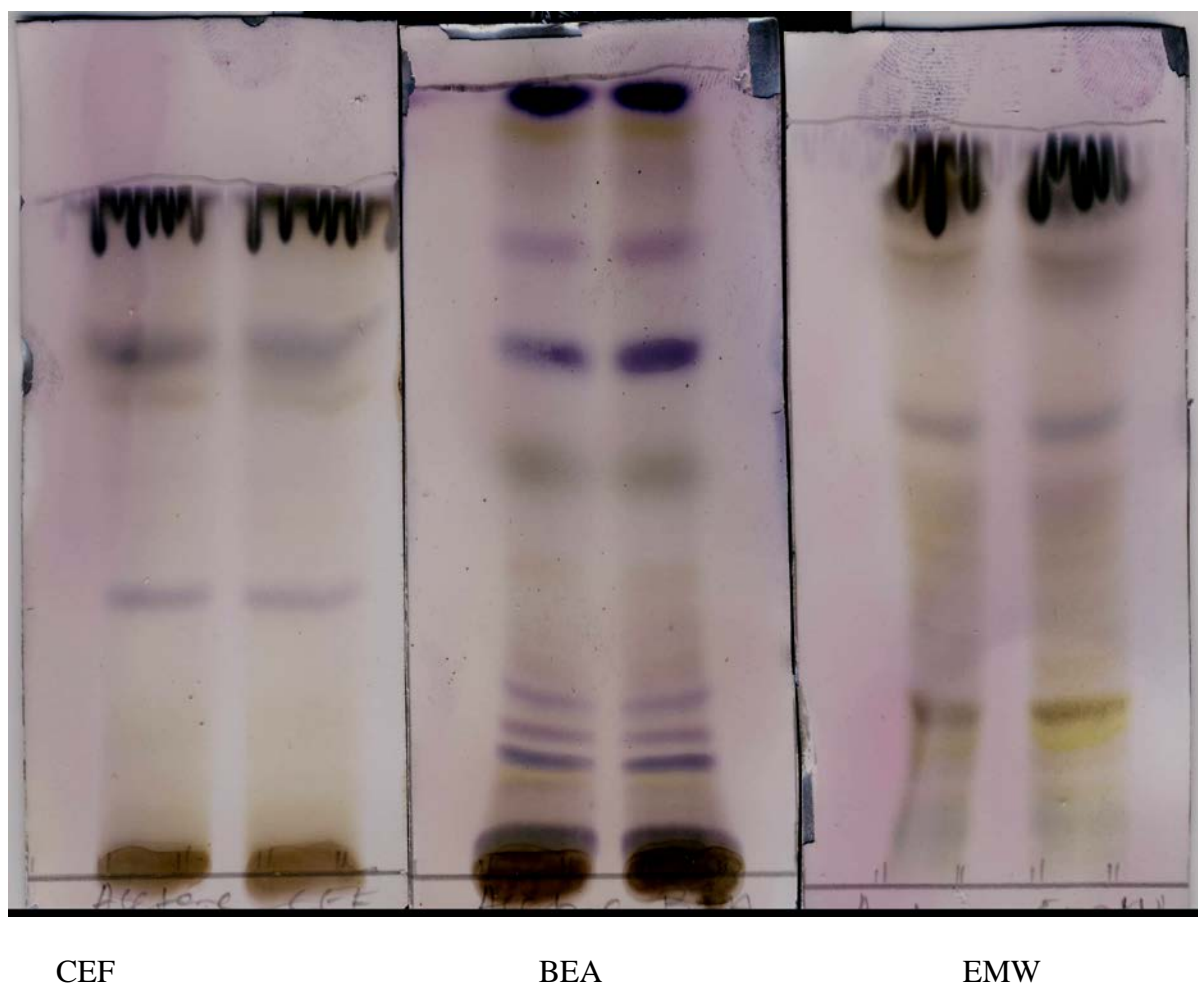


Figure 3.1a: The TLC profile of crude acetone root extract of *Senna* species separated with BEA (non-polar), CEF (intermediate polarity) and EMW (polar). The individual compounds were visualized with anisaldehyde.



BEA

CEF

EMW

Figure 3.1b: The TLC profile of crude acetone root extract of *Senna* species separated with BEA, CEF and EMW. The individual compounds were visualized with vanillin-sulphuric acid reagent.

3.2.2. The spectral profile of the acetone extract

The UV-Visible spectrum of the extract indicates major absorbance at a wavelength range of 280 nm to 450nm (Figure 3.2).

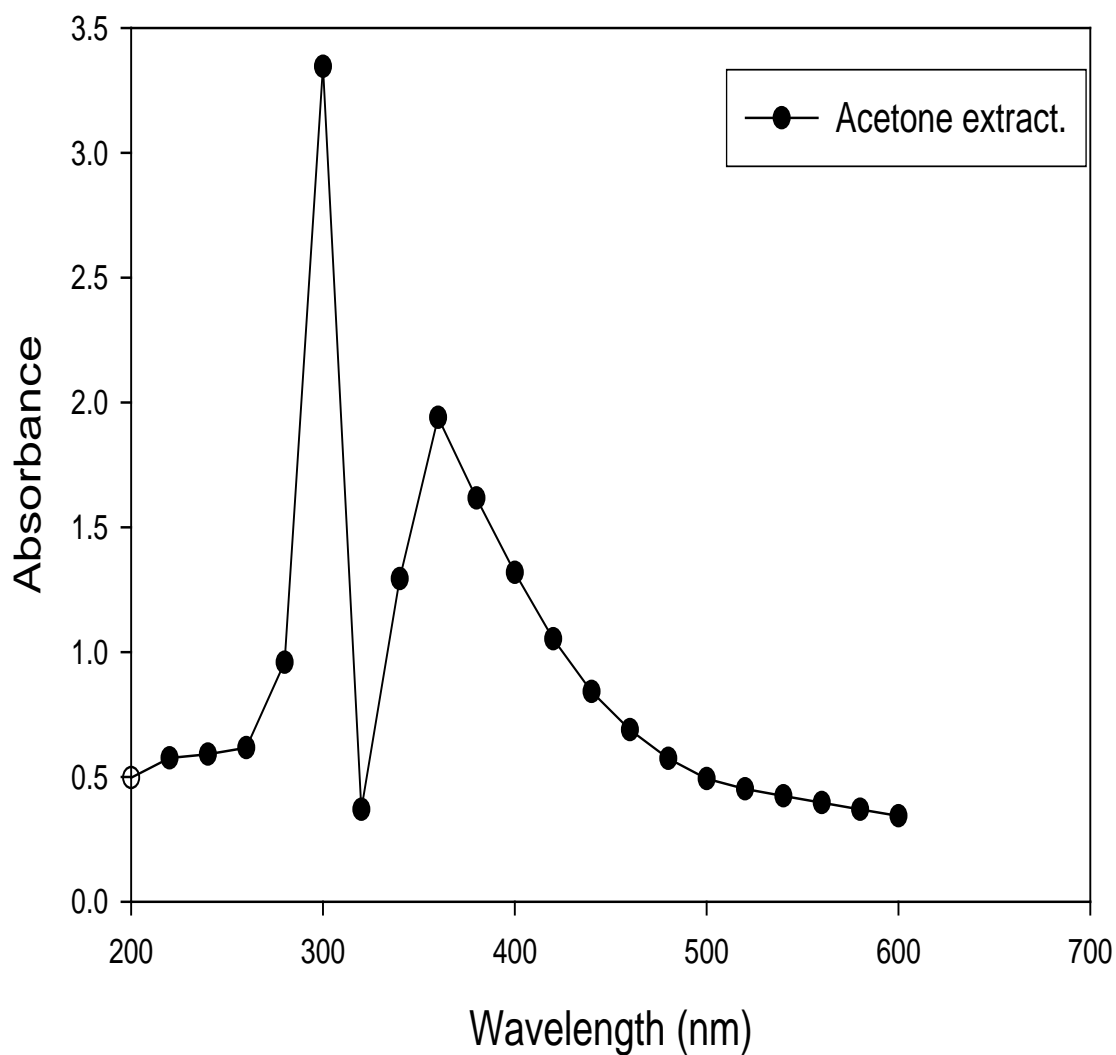


Figure 3.2: The UV-Visible spectral profile of the acetone extract of the roots of *Senna* species. Major absorption of light was shown within a range of 240 nm to 450 nm which suggests the presence of flavonoids, prosalins and caretenoids.

3.3. BIOASSAYS

3.3.1. Antiproliferative activity of the acetone extract

The acetone extract of *Senna* species demonstrated some inhibitory effects on the growth and viability of the Jurkat T cells. The inhibitory effects of the extract on the growth (Figure 3.3a) and viability of Jurkat T cells (Figure 3.3b) showed a time- and concentration-dependent effect.

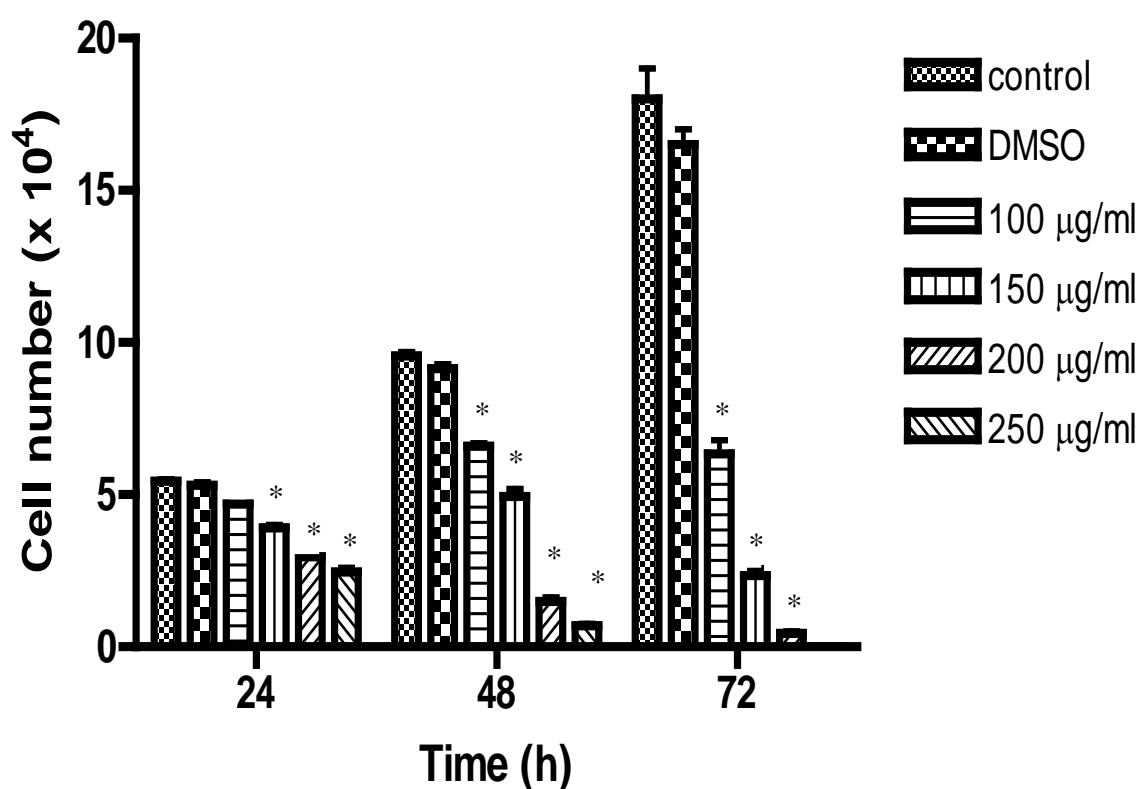


Figure 3.3a: The effect of the crude acetone extracts of the roots of *Senna* species on the proliferation of Jurkat T cells treated with increasing concentrations at 24 h intervals. Cell growth was inhibited in a dose- and time-dependent manner. (* $p < 0.01$).

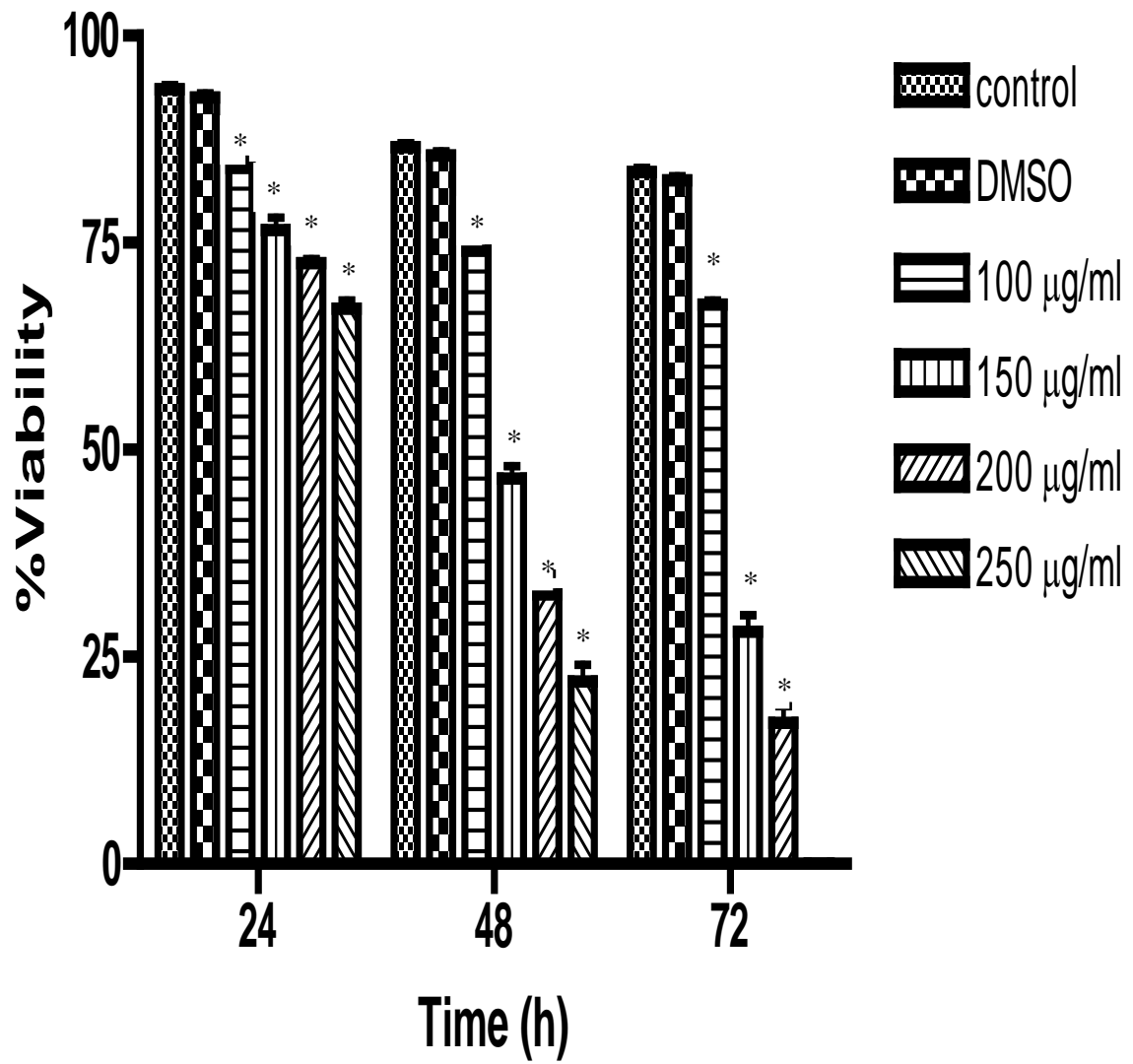


Figure 3.3b: The effect of the acetone extract of the roots of *Senna* species on viability of Jurkat T cells treated with increasing concentrations of crude acetone extract at 24 h intervals. Cell viability was reduced in a dose- and time-dependent manner. (* $p < 0.01$)

3.3.2. Free radical scavenging activity of the acetone extract

The extract of *Senna* species possessed free radical scavenging properties on DPPH (Figure 3.4a) and compared against vitamin C (Figure 3.4b).

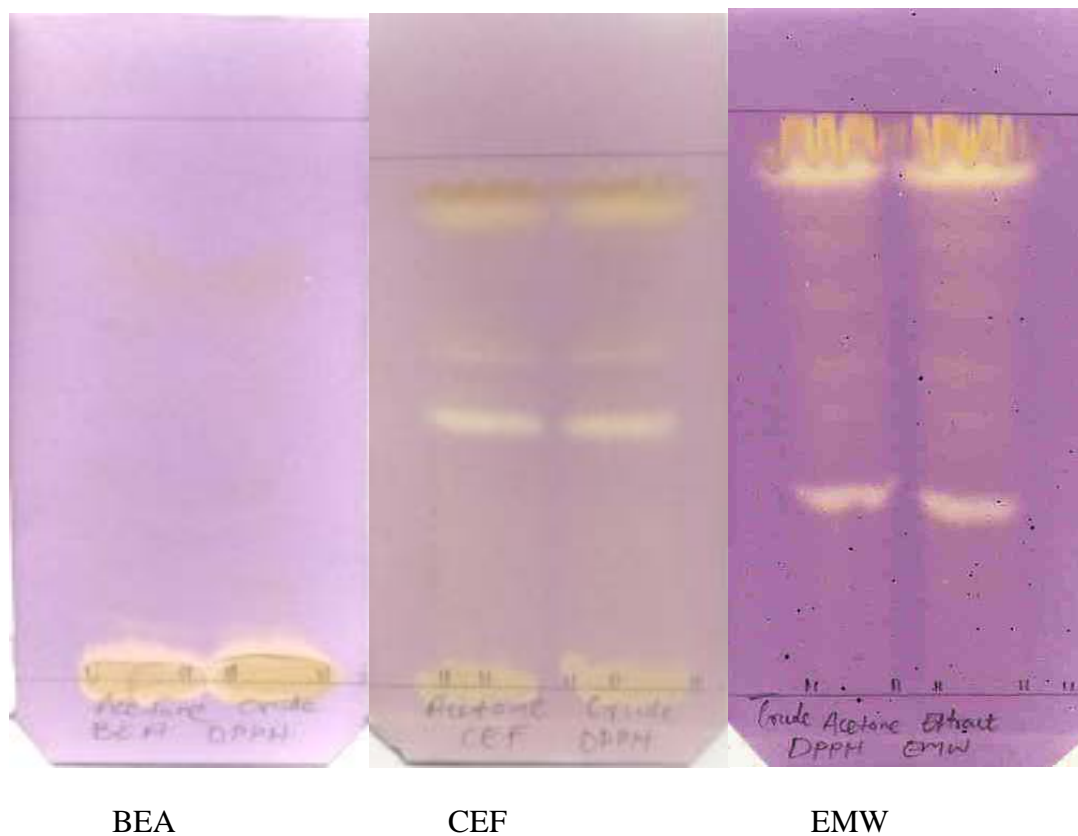


Figure 3.4a: The free radical scavenging power of the acetone extract of the roots of *Senna* species as evaluated by the DPPH assay. The yellow zones on the plates indicate areas containing compounds with free radical scavenging activity.

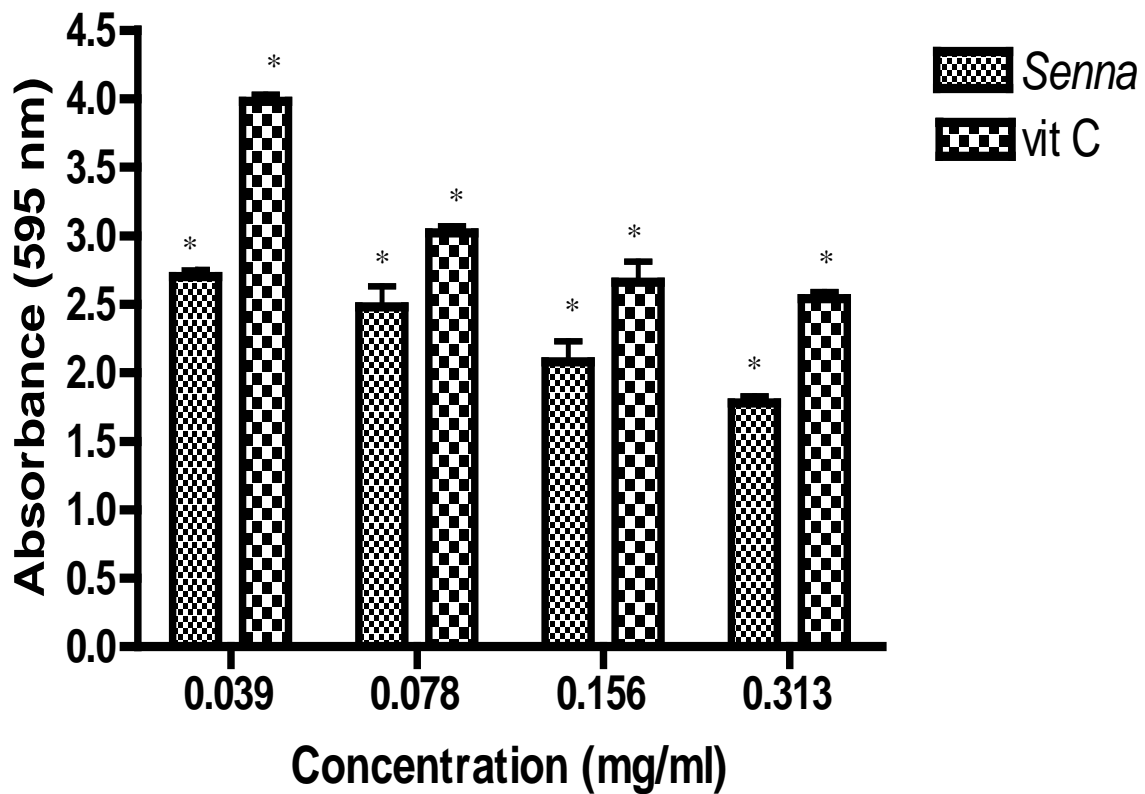


Figure 3.4b: The comparison of the free radical scavenging power of the acetone extract of the roots *Senna* species with vitamin C. Low absorbance values indicate high free radical scavenging power. The acetone extract displayed more radical scavenging power compared to vitamin C. (* $p < 0.01$).

3.3.3. Antibacterial activity of the acetone extract

The roots of *Senna* species showed antibacterial activity on test organisms (Figures 3.5a and 3.5b) with minimum inhibition concentrations ranging from 0.078 to 0.16 mg/ml (Table 3.2). The results show the inhibition of bacterial growth by the acetone extract.

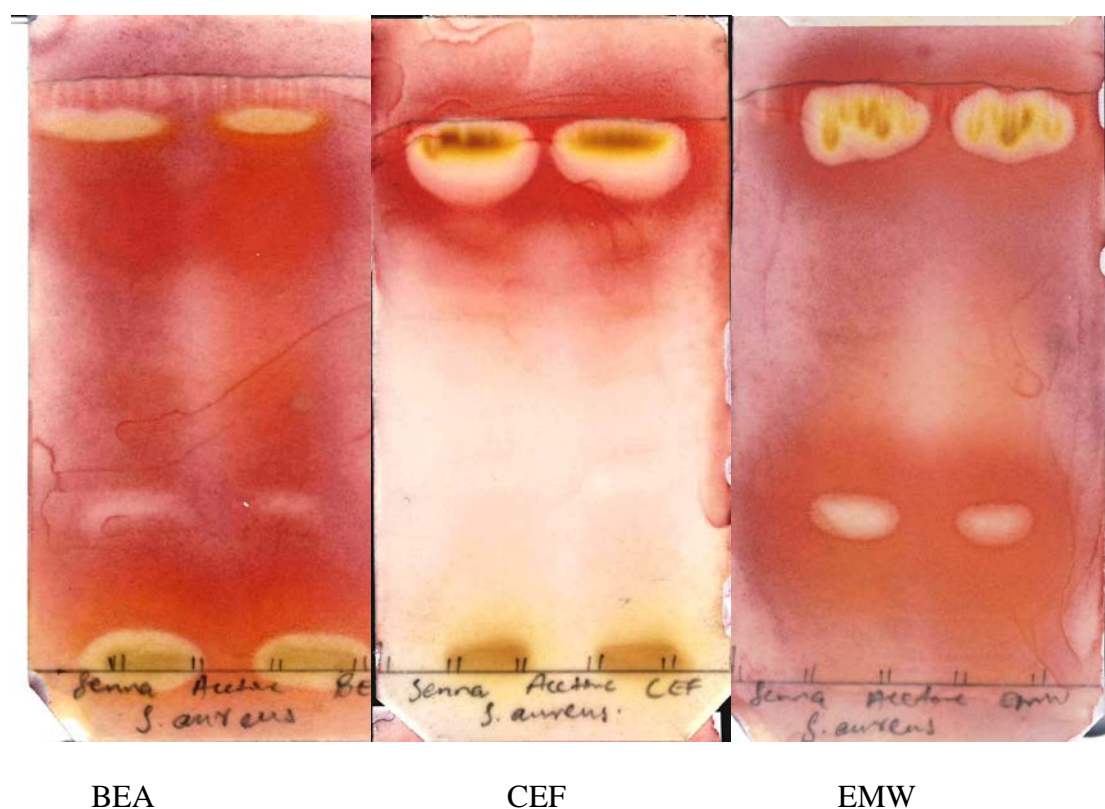
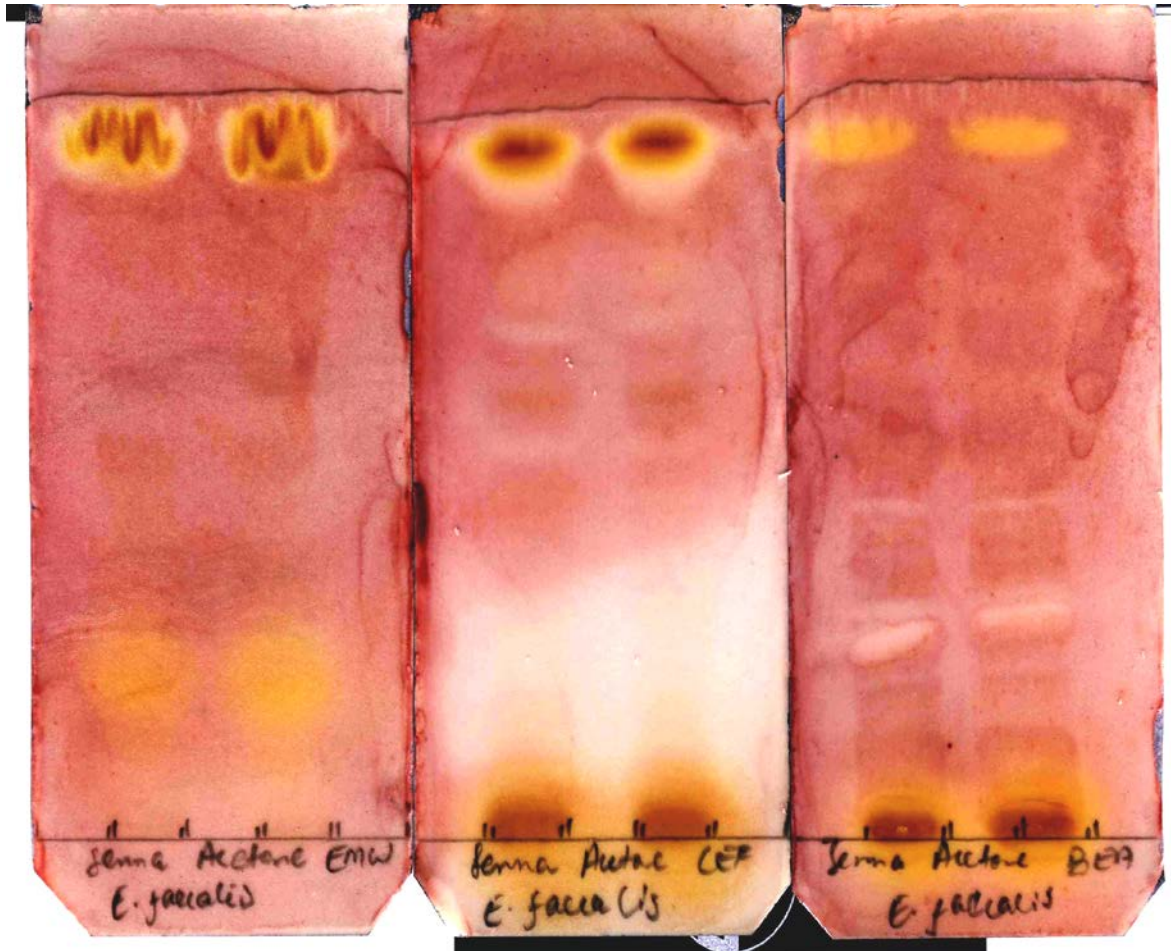


Figure 3.5a: The antibacterial activity of the acetone extract of the roots of *Senna* species evaluated on *E. faecalis*. The clear zones on the plates indicate areas where inhibition of bacterial growth occurred.



EMW

CEF

BEA

Figure 3.5b: The antibacterial activity of the acetone extract of the roots of *Senna* species evaluated on *S. aureus*. The clear zones on the plates indicate areas where inhibition of bacterial growth had occurred.

Table 3.2: Minimum inhibitory concentrations (MICs) values of the acetone extract tested on various microorganisms. Gentamycin was used as positive control. The results suggest that test organisms, *E. faecalis* and *E. coli*, are the most sensitive to the acetone extract in terms of MIC values.

| Microorganism | MIC value (mg/ml) | Gentamycin MIC (mg/ml) |
|----------------------|--------------------------|-------------------------------|
| <i>P. aeruginosa</i> | 0.16 | 0.039 |
| <i>E. faecalis</i> | 0.078 | 0.039 |
| <i>E. coli</i> | 0.078 | 0.039 |
| <i>S. aureus</i> | 0.16 | 0.039 |
| Average | 0.12 | 0.039 |

The R_f values of compounds of the acetone extract were determined from TLC fingerprinting plates. A comparison of R_f values of compounds and analysis of bioactivity plates indicated that some compounds possess both antibacterial and antioxidant properties (Table 3.3).

Table 3.3: Summary of the biological activity of the acetone extract of the roots of *Senna* species compiled from TLC fingerprinting.

| SOLVENT SYSTEM | R_f VALUES | ANISALDEHYDE | VANILLIN | BIOLOGICAL ACTIVITY |
|----------------|--------------|--------------|----------|---------------------------|
| BEA | 0.98 | + | + | Antibacterial |
| | 0.45 | + | + | No activity observed |
| | 0.36 | - | + | Antibacterial |
| | 0.11 | + | + | No activity observed |
| | 0.21 | + | - | No activity observed |
| | 0.17 | + | - | No activity observed |
| | 0.053 | + | - | Antibacterial/Antioxidant |
| CEF | 0.76 | + | - | No activity observed |
| | 0.69 | + | - | No activity observed |
| | 0.40 | + | + | Antioxidant |
| | 0.91 | - | + | Antibacterial |
| | 0.86 | - | + | Antioxidant |
| | 0.61 | - | + | Antioxidant |
| | 0.14 | - | + | No activity observed |
| EMW | 0.88 | + | + | Antibacterial/Antioxidant |
| | 0.60 | + | + | No activity observed |
| | 0.53 | + | + | No activity observed |
| | 0.24 | + | + | Antibacterial/Antioxidant |
| | 0.12 | - | + | No activity observed |

(+: for presence; -: for absence).

3.4. ISOLATION OF A COMPOUND WITH ANTIOXIDANT ACTIVITY

3.4.1. Extraction of the plant material

The roots of *Senna* species (700 g) were extracted with hexane, DCM, acetone and methanol in a serial exhaustive extraction (SEE) pattern. Methanol was the best extractant in terms of yield with acetone being the lowest yield extractant (Table 3.4).

Table 3.4: Different mass yields from the serial exhaustive extraction of roots of *Senna* species with hexane, DCM, acetone and MeOH.

| EXTRACT SOLVENT | YIELD (g) | TOTAL YIELD (g) | YIELD (%) |
|----------------------------|----------------------|----------------------------|----------------------|
| Hexane 1 | 2.8 | 4.8 | 0.69 |
| Hexane 2 | 1.3 | | |
| Hexane 3 | 0.7 | | |
| DCM 1 | 3.5 | 6.2 | 0.89 |
| DCM 2 | 1.5 | | |
| DCM 3 | 1.2 | | |
| Acetone 1 | 2.3 | 4.6 | 0.66 |
| Acetone 2 | 1.4 | | |
| Acetone 3 | 0.9 | | |
| MeOH 1 | 17.9 | 32.3 | 4.6 |
| MeOH 2 | 8.5 | | |
| MeOH 3 | 5.9 | | |

3.4.2. TLC-fingerprints of the extracts of *Senna species*

The compounds within the resultant serial exhaustive extraction extracts of the roots of *Senna species* were visualized with anisaldehyde (Figure 3.6). Compounds were visualized across the polarities with n-hexane composed of mostly non-polar material.

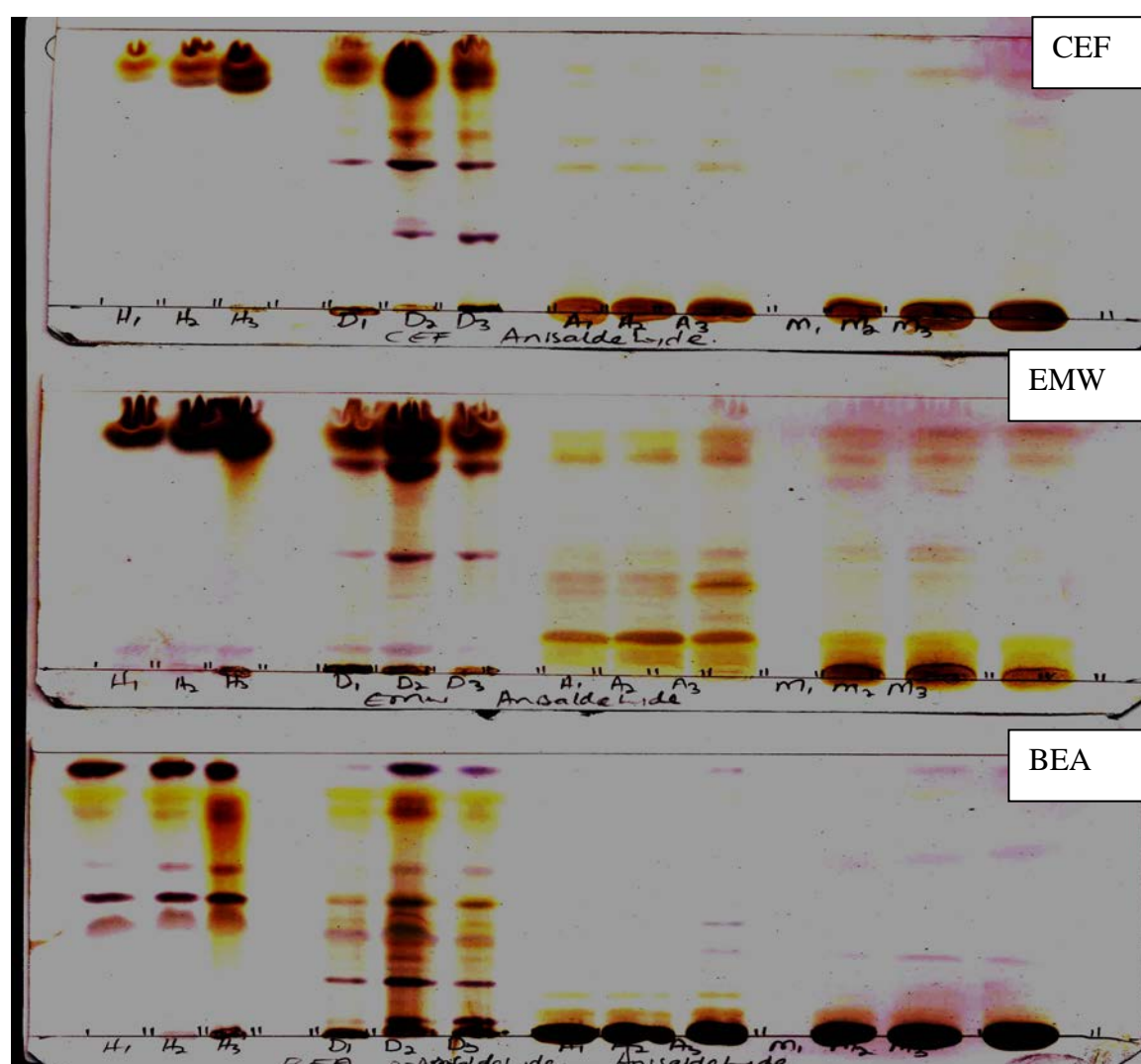


Figure 3.6: The visualisation of individual compounds of the extracts obtained from the serial exhaustive extraction of the roots of *Senna species* with anisaldehyde. Compounds visualized range from non-polar to very polar.

3.4.3. Free radical scavenging activity of the extracts of *Senna species*

The resultant SEE extracts possess free radical scavenging power (Figure 3.7).

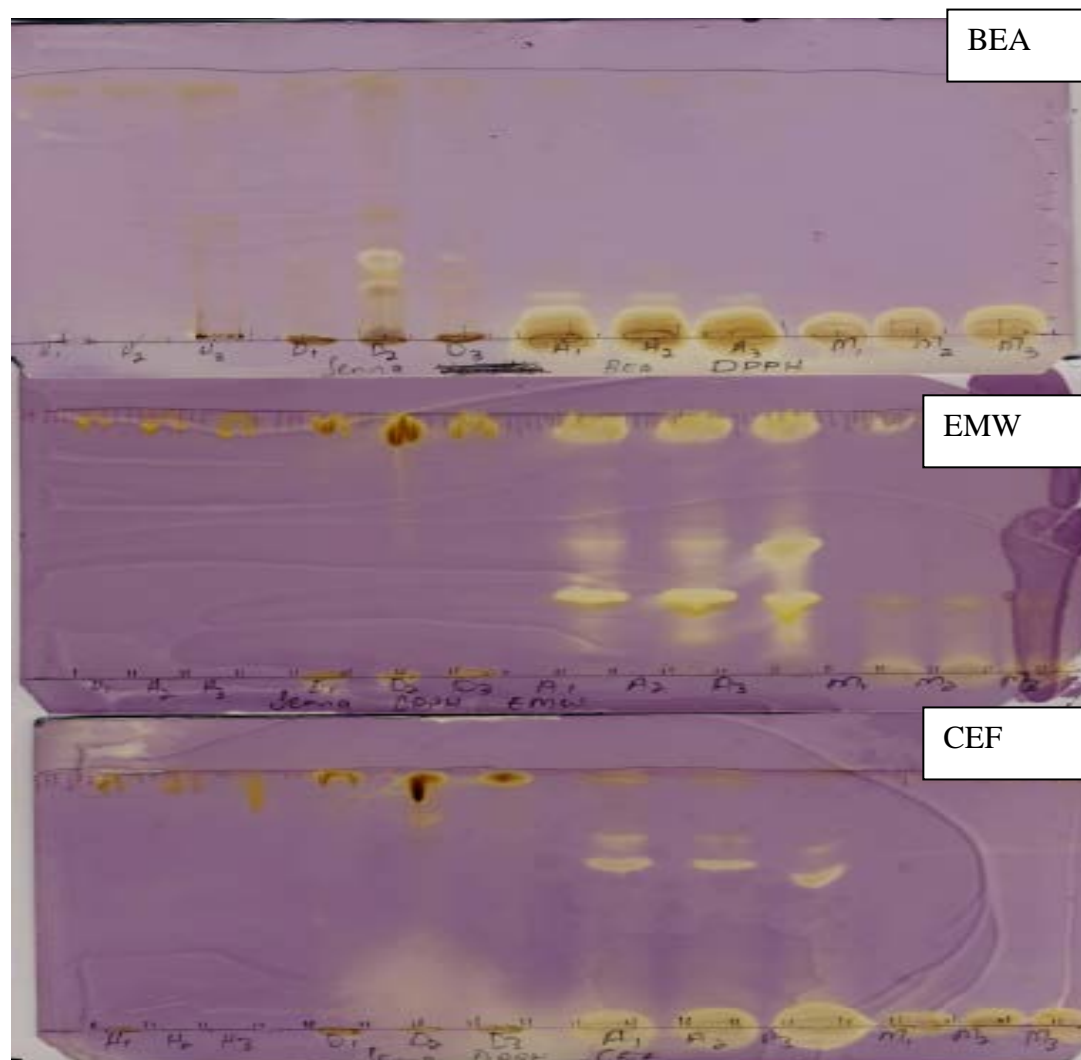


Figure 3.7: The evaluation of free radical scavenging activity of the extracts of the roots of *Senna species*. The plates were developed with BEA, EMW and CEF. Acetone extract displayed the highest antioxidant activity.

3.4.4. Fractionation of the acetone extract

The acetone extract of the roots of *Senna* species from SEE was fractionated with various ratios of chloroform: methanol (Table 3.5) as the first step on the isolation of the bioactive compound. Fraction 8:2 gave the highest yield (237 mg) followed by fraction 9:1 (125 mg), with fraction 0:1 giving the lowest yield (14 mg).

Table 3.5.: Fractionation of the acetone root extract (500 mg) with CHCl₃: MeOH different ratios on silica gel chromatography (elution was pursued to the end, hence different volumes).

| SYSTEM | CHCl ₃ (%) | MeOH (%) | SOLVENT QUANTITY(ml) | YIELD (mg) |
|--------|--------------------------|-------------|-------------------------|---------------|
| A | 100 | - | 500 | 58 |
| B | 90 | 10 | 700 | 125 |
| C | 80 | 20 | 1200 | 237 |
| D | 70 | 30 | 500 | 30 |
| E | 50 | 50 | 600 | 25 |
| F | 30 | 70 | 500 | 16 |
| G | - | 100 | 600 | 14 |

3.4.5. *TLC-fingerprinting of the fractions of acetone extract and their free radical scavenging activity.*

Compounds within the fractions of the acetone extract were visualized with vanillin.

Fraction 9:1 and 8:2 showed prominent antioxidant activity (Figure 3.8).

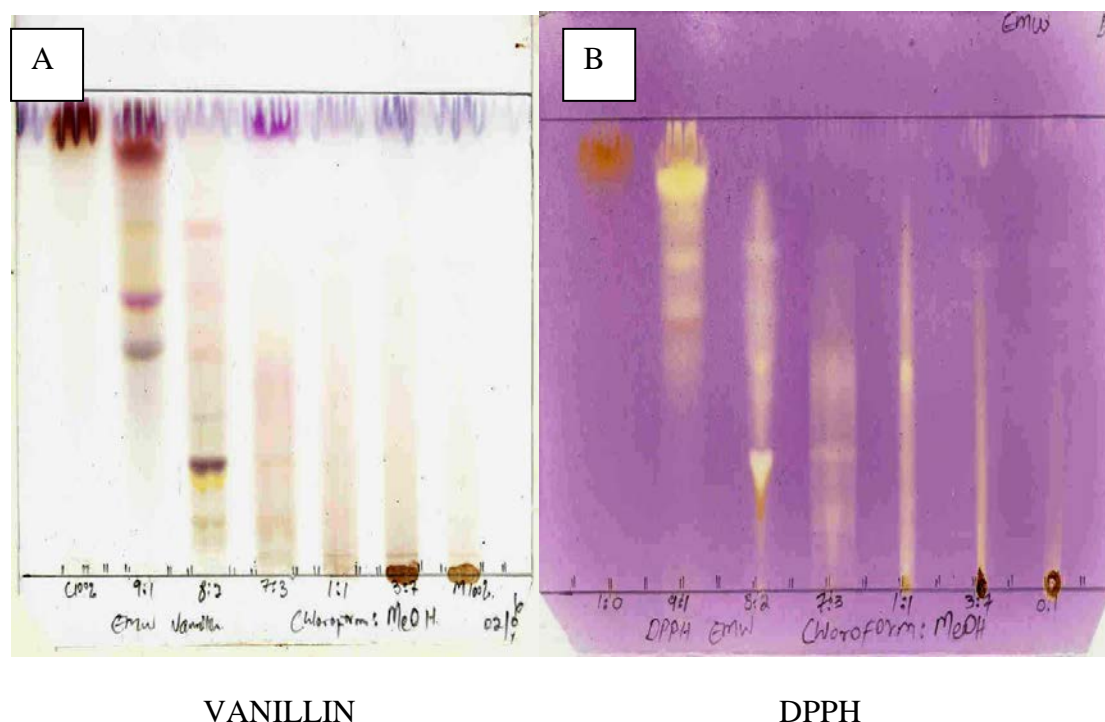


Figure 3.8: TLC chromatograms of the CHCl₃: MeOH fractions of the acetone extract species developed in EMW and sprayed with vanillin (A) and DPPH (B). Yellow zones on B indicate antioxidant activity.

3.4.6. Suitable mobile phase for elution of bioactive compounds

The chloroform: ethyl acetate ratio of 1:1 (v/v) was found to give better resolution of the active compound within the CHCl_3 : MeOH (9:1, v/v) fraction (Figure 3.9). The mobile phase was used to elute the bioactive compound through the silica gel column chromatography.

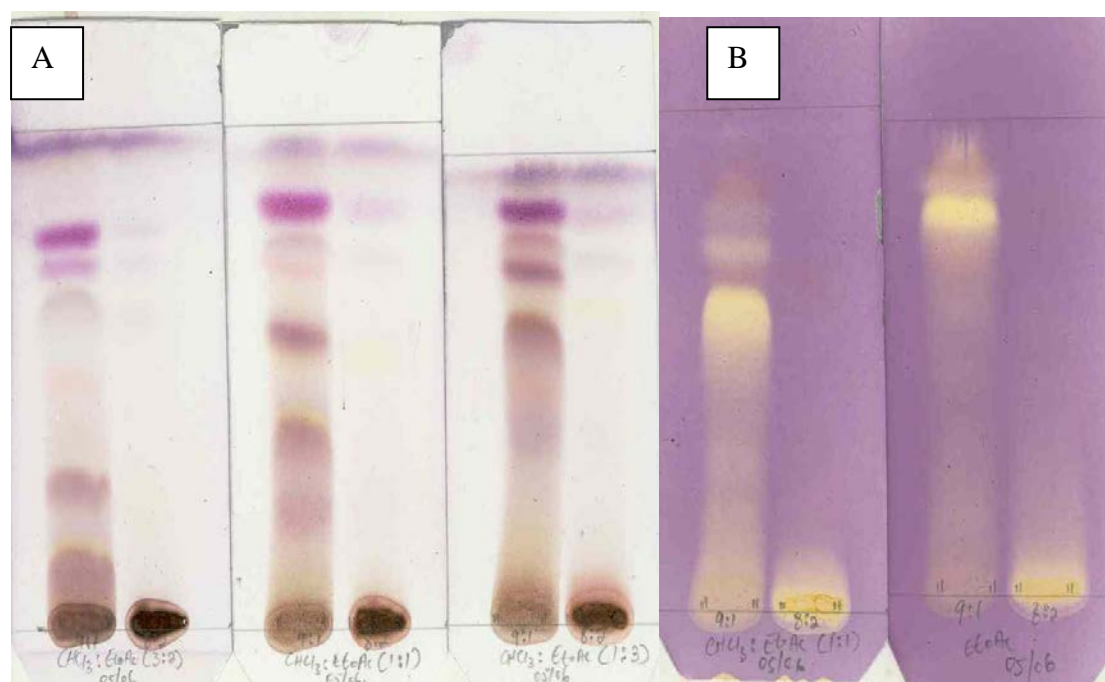


Figure 3.9: Determination of the mobile phase for the elution and separation of the active compound on a silica gel column prepared from the CHCl_3 : MeOH (9:1, v/v) fraction. CHCl_3 : EtOAc (1:1, v/v) gave better resolution of the bioactive compound (A). Position of the bioactive compound on the TLC plate (B) indicated by a yellow band.

3.4.7. Elution of the bioactive compound

The 450 mg of CHCl_3 : MeOH (9:1, v/v) fraction of the acetone extract of *Senna* species was eluted with CHCl_3 : EtoAc (1:1, v/v) on the silica gel 60 column (Figure 3.10a) and tubes with the same chromatographic properties were pooled together for further purification (Figure 3.10b).

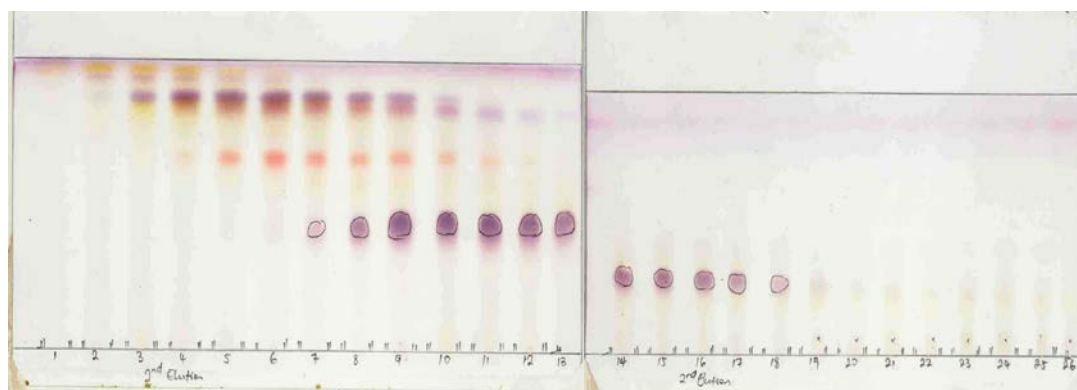


Figure 3.10a: TLC chromatogram obtained from the elution of CHCl_3 : MeOH (9:1, v/v) fraction with CHCl_3 : EtoAc (1:1, v/v) on a silica gel column.



Figure 3.10b: TLC chromatogram to check the purity profile of the fraction obtained from pooled tubes (13-18) with similar chromatographic properties.

3.4.8. Re-elution of the bioactive compound from a fraction of obtained from pooling different tubes together

Re-elution of the fraction obtained from pooling together a series of similar tubes with CHCl_3 : EtoAc (1:1, v/v) gave yield to compound 1 (Figure 3.11). The yield of the pure compound was obtained at 13 mg (which is 2.6 % of the 500 mg of the acetone extract fractionated).

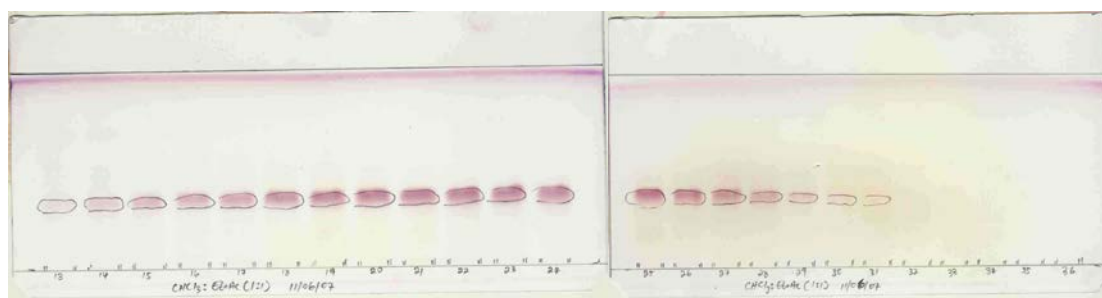


Figure 3.11: TLC chromatogram on fractions obtained from the re-elution of a fraction of pooled tubes with CHCl_3 : EtoAc (1:1, v/v) on Silica gel column to isolate compound 1.

3.4.9. Free radical scavenging activity of the isolated compound

Phytochemical profile was done to check the purity of the compound (Figure 3.12); further antioxidant activity was performed to check whether the isolated compound was the compound of interest (Figure 3.12).

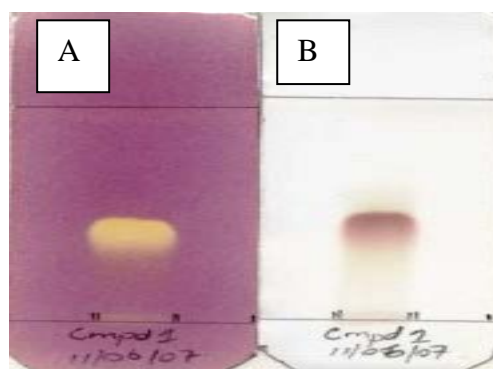


Figure 3.12: The vanillin visualization (B) and the free radical scavenging activity (A) of the isolated compound. The plates were developed with CHCl_3 : EtoAc (1:1, v/v).

3.4.10. Spectroscopic profile of the isolated compound

The ^1H - and ^{13}C -NMR spectra of the isolated compound were determined in Botswana and used to derive the chemical structure of the compound (Figures 3.13a and 3.13b).

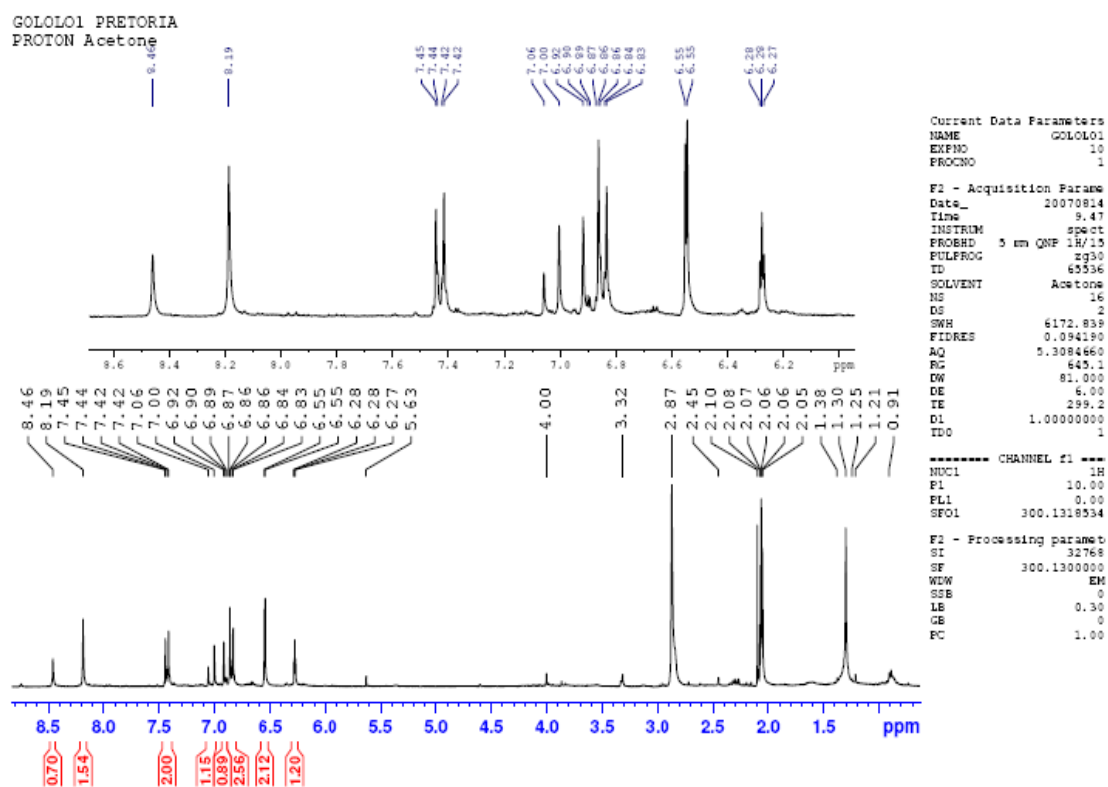


Figure 3.13a: The ^1H -NMR spectrum of Compound 1 isolated from the acetone extract of the roots of *Senna* species dissolved in acetone. Peaks around 6.5 to 7.5 ppm demonstrate the presence of aromatic hydrogens.

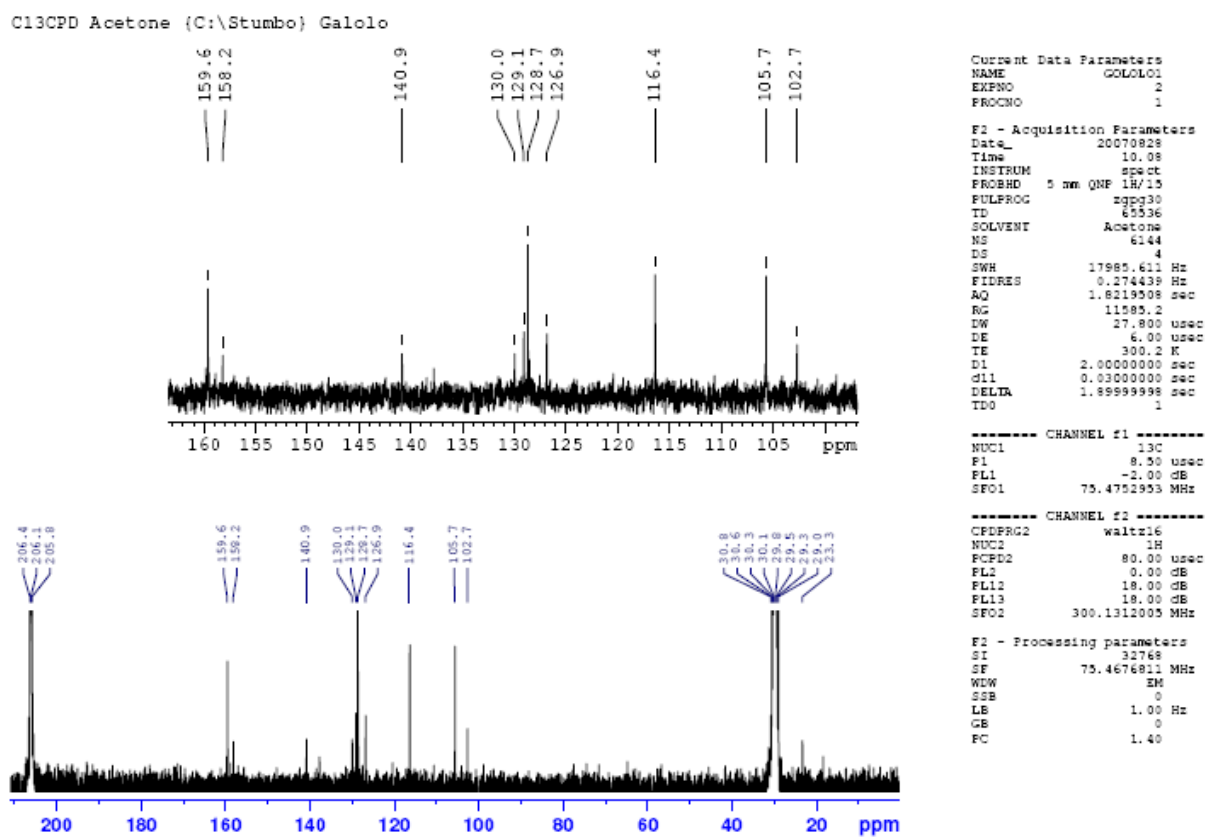


Figure 3.13b: The ^{13}C -NMR spectrum of Compound 1 isolated from the acetone extract of the roots of *Senna* species dissolved in acetone.

3.4.11. The structure of the isolated compound

The structure of the isolated compound was derived from the assignment of the peaks observed from the $^1\text{H-NMR}$ spectrum. The isolated compound possesses phenolic properties which could be responsible for the demonstrated antioxidant activity.

^1H NMR assignments

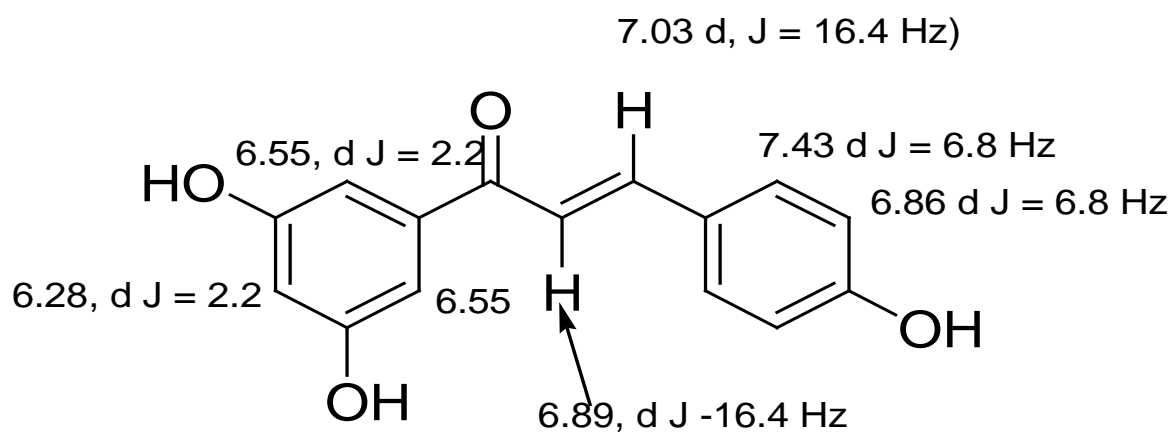


Figure 3.14: The structure of Compound 1 derived from ^1H - NMR spectrum peak assignment. The compound was identified as 1-(3, 5-dihydroxyphenyl)-3-(4-hydroxyphenyl) - 2-propen-1-one.

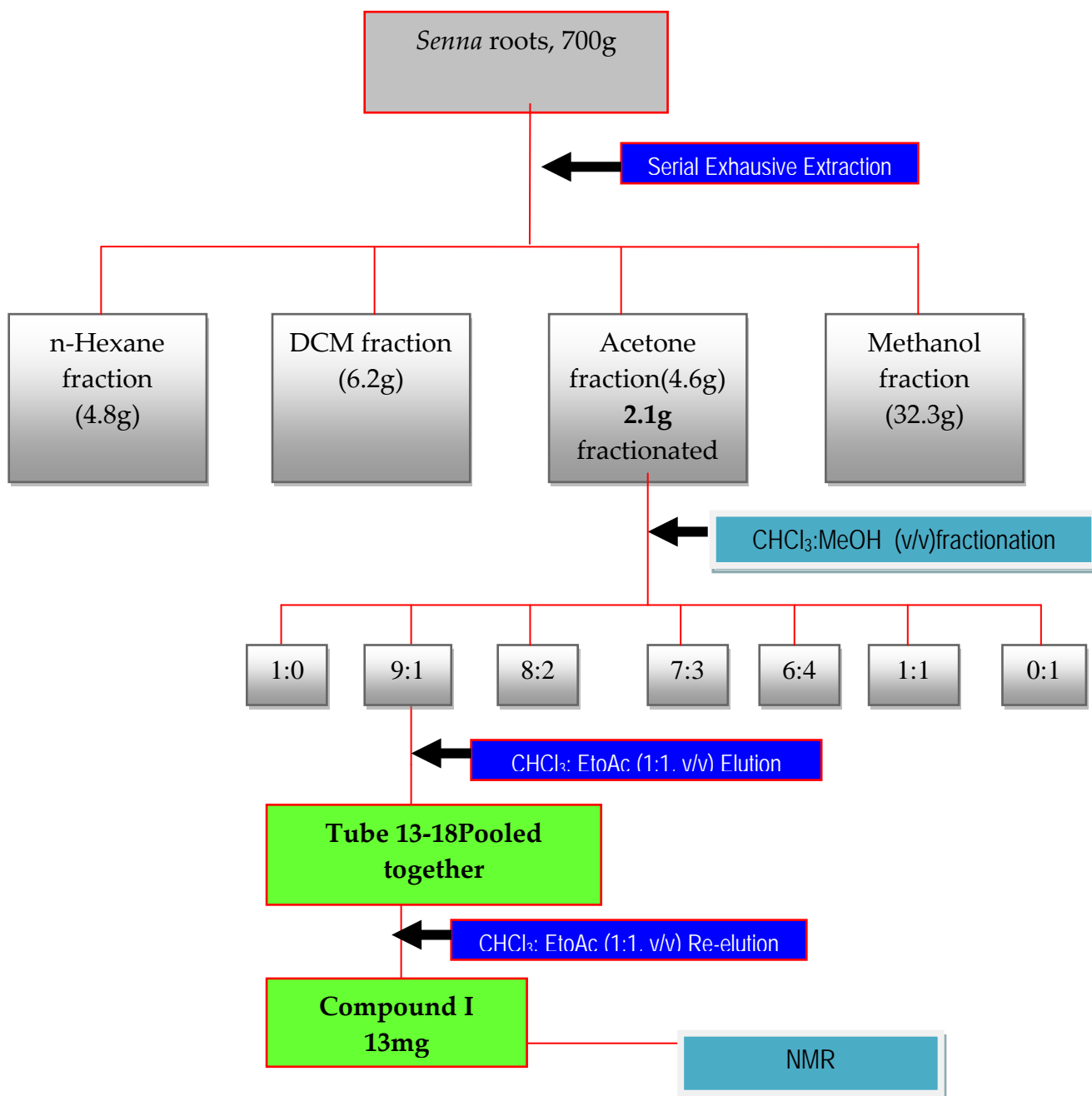


Figure 3.15: Flow diagram for the isolation of 1-(3,5-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one from the roots of *Senna* species.

CHAPTER 4

DISCUSSION

Our study used *Senna* species, a member of the Fabaceae family (subfamily Caesalpinaceae), for bioactivity screening and isolation of potential bioactive compounds. The selection of this species was based on its broad indigenous usage in traditional medicine for treatments of various diseases, such as sexually transmitted infections and some forms of intestinal complications (Gololo-personal communication; Tshikalange *et al.*, 2005). Traditionally, the roots of *Senna* are extracted with water by boiling and cooled before being orally taken.

The choice of the extraction solvent for biological activity screening is a very important step. This choice is influenced by the type of bioassays envisaged and the easiness of working with a particular solvent. Practitioners of indigenous medicine mainly use water (boiled water) to extract bioactive compounds. The use of water as the extractant is mainly based on its nontoxic nature towards human beings and, in particular, it is the only available extractant at the disposal of practitioners of indigenous medicine. In the present study, we chose acetone as our extractant for a number of reasons, *viz*: acetone was found to be a good extractant because of its nontoxicity towards test organisms during bioassays; its ability to extract compounds across different polarities and its easiness to remove from extracts (Eloff, 1998b).

The yield in grams and percentage of the extraction of the ground roots of *Senna* species as well as the total phenolic content of the extract are shown in Table 3.1. The determination of the total phenolic content of the plant's extract through the Folin-

Ciocalteu and other methods is not absolute. However, it is very significant to determine, as in most cases it is these phenolic compounds which contribute to the biological activities of the extracts. The acetone extract of *Senna* species showed reducing capacity relative to tannic acid, thus signifying the presence of phenolic compounds. We can therefore deduce that some of the biological activities of *Senna* species, as evaluated in the current study, could be due to the presence of these phenolic compounds. Indeed, the linkage between the biological activity of plant extracts and their phenolic content has been reported before (Frankel *et al.*, 1995).

The three mobile phase systems (BEA, CEF and EMW) used in this study separated compounds according to their polarities. The EMW mobile phase system separates polar and neutral compounds; the BEA system separates non-polar compounds, while the CEF system separates intermediate polarity and acidic compounds (Kotze and Eloff, 2002). Thus, the acetone extract of the roots of *Senna* species was found to possess polar, intermediate and non-polar compounds since all the three mobile phases used were able to separate the components inherent to the extract. This observation validates the findings by Eloff (1998b) that acetone is a good extractant in phytomedicine studies as it extracts both polar and non-polar compounds from plant materials; this is due to acetone's intermediate polarity.

Before spraying the TLC plates with anisaldehyde and vanillin the plates were visualized under UV light, which exposes fluorescent-quenching compounds in herbal extracts. UV light in most cases identifies fluorescing compounds that possess many double bonds while visible light detects coloured compounds, usually with conjugated bonds. Compounds containing aromatic rings absorb UV light at 254 nm and

therefore quench the fluorescence of the pigment present in the Silica gel (Masoko, 2006). The quenching of the fluorescence was observed with the acetone extract in the present study, which highlighted the presence of aromatic compounds.

The UV-Visible spectral profile of the acetone extract of the roots of *Senna* species is shown in Figure 3.2. As observed through the TLC fingerprinting, the spectral profile of the acetone extract of *Senna* species shows the presence of a number of compounds. These compounds absorbed light mostly within the range of 280 nm to 450 nm which could be due to, amongst others, the presence of flavonoids. It was shown that many fruit extracts, as in juices, contain ultraviolet absorbing phenolic substances (Kruger Food Laboratories, 2005). The UV-Visible absorption spectrum of the alcoholic clarified solutions of these fruit juices showed absorbance at the key wavelengths of 280, 325 and 443 nm, due to the presence of flavonoids, prosalins and carotenoids, respectively (Kruger Food Laboratories, 2005). Our current observations, as shown by Figure 3.2, suggest the presence of phenolic type of compounds which absorb light between 280 nm and 450 nm. This observation thus provides a qualitative correlation of the phenolic content of the extract which was determined by the Folin-Ciocalteu method (Table 3.1).

Previous studies have shown that most of the biological activities of plant extracts are contributed by the phenolic compounds present. The antiproliferative properties of the acetone extract of the roots of *Senna* species were evaluated on Jurkat T cells (Figures 3.3a and 3.3b). Figures 3.3a and 3.3b show that the acetone extract inhibit the proliferation and viability of Jurkat T cells in a dose- and time-dependent manner. The observation of cancerous cell-death is a bioassay model that indicates the potential of

the extract to inhibit the progression of cancer. The progression of cell proliferation is halted by the arrest of the cell division cycle at one of the checkpoints (either G₁/S or G₂/M interphases) in the cell division cycle. The arrest is mainly triggered by the irreparable or repairable damage in the cell's DNA.

In case of an irreparable DNA damage, the cell death pathways are triggered. The cell death could either be apoptotic or necrotic. The apoptotic cell death is the preferred mode of cellular demise than the necrotic pathway. To evaluate the mode of cell death triggered by the plant extract on cancerous cells, the expression levels of pro-apoptotic genes need to be evaluated. We can therefore deduce from the antiproliferation studies that the roots of *Senna* species possess some anticancerous properties. These anticancerous properties involve an effect on the cell division cycle which leads to cell death. The determination of the mode of cell death triggered by the extract of *Senna* species is not dealt with in this study and follow-up studies are in progress to explore this aspect.

The free radical scavenging activity of the acetone extract of the roots of *Senna* species were evaluated using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method as shown in Figure 3.4a. The radical scavenging activity was quantified using vitamin C as the standard (Figure 3.4b). The principle of the DPPH method is based on the reaction of the antioxidant compound with the stable free radical with decolouration (from purple colour to yellow). The degree of decolouration indicates the scavenging potential of the sample's antioxidative activity and depends on the hydrogen donating ability of the antioxidant (Es-Safi *et al.*, 2007). Because of the ease and convenience of this method, it now has widespread use during assessment of free radical

scavenging activity of extracts (Brand-Williams *et al.*, 1995; Sanchez-Moreno *et al.*, 1998).

The present study demonstrated that the roots of *Senna* species possess compounds with antioxidative activity against the free radical, DPPH. This is evidenced by yellow bands appearing on the TLC plate against the purple DPPH background (Figure 3.4a). The free radical scavenging activity of the plant extract contributes to the plant's antioxidant properties. Previous studies indicated that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (hydroquinone, pyrogallol, etc) and aromatic amines reduce and decolourise DPPH through their hydrogen donating ability (Abdille *et al.*, 2005). Thus, the acetone extract of the roots of *Senna* species may possess similar group of compounds with hydrogen donating ability that reduced DPPH (Figure 3.4a).

Free radicals are known to be a major factor in biological damages (Bondet *et al.*, 1997). Free radicals play a role in the aging process and in diseases such as atherosclerosis, diabetes, cancer and cirrhosis (Li and Thrush, 1994). The free radicals are continuously produced by our body's use of oxygen such as in respiration and some cell-mediated immune responses. Those free radicals are also generated through environmental pollutants, cigarette smoke, automobile exhaust, radiation, air pollution, pesticides, etc (Li and Thrush, 1994). The antioxidant defense systems in the body can only protect the body when the quantity of the free radicals is within the normal physiological level. But when this balance is shifted towards more free radicals, increasing their burden in the body either due to environmental condition or infections, it leads to oxidative stress, which may result in tissue injury and

subsequent diseases (Finkel and Holbrook, 2000). Thus, the search for new free radical scavenging compounds is very important.

Medicinal plants remain the reliable source of phenolic compounds with free radical scavenging power. The present study has shown that *Senna* species possesses impressive free radical scavenging power. The antioxidant activity of the plant extract could be attributed to its free radical scavenging power (Dizhbite *et al.*, 2004). We can thus deduce that it is this inherent antioxidant activity of the *Senna* species that contributes to the wide usage of this plant in traditional medicine. Therefore, the rationale behind the traditional usage of this plant against many disease conditions could be correlated to its antioxidant activity as demonstrated in this study. Ilavarasan *et al.* (2005) reported that plants which belong to Caesalpinaceae family are rich in flavonoids and that flavonoids are known for their anti-inflammatory and antioxidative activities.

Plants (fruits, vegetables, medicinal herbs, etc) contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g., phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids) and some other endogenous metabolites, which are rich in antioxidant activity (Cai *et al.*, 2003; Zheng and Wang, 2001). Previous studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antimutagenic, anticarcinogenic, antibacterial and, or antiviral activities to a greater or lesser extent (Owen *et al.*, 2000; Sala *et al.*, 2002). The current study also demonstrated that some of the antioxidant compounds of *Senna* species possess antibacterial activity (Table

3.3). This conclusion was arrived at by comparing the R_f values of compounds as shown in Figures 3.4a, 3.5a and 3.5b. However, more bioassay studies need to be carried out in order to confirm this assertion.

The antibacterial activity of the acetone extract of the roots of *Senna* species were determined using bioautography method (Begue and Kline, 1972) and quantified through the microtitre plate method (Eloff, 1998a). Figures 3.5a and 3.5b show the inhibition of bacterial growth on selected bacterial strains by some of the components of the roots of *Senna* species extracted using acetone. Table 3.2 shows the MIC values of *Senna* species extract against *P. aeruginosa*, *E. faecalis*, *E. coli* and *S. aureus* as 0.156, 0.078, 0.156 and 0.078 mg/ml, respectively. Although the MIC values of the extract are higher compared to that of the standard, Gentamycin (0.039 mg/ml), purification of these putative antibacterial compounds may enhance their activities and hence lower their MICs. Therefore, it could be argued that the acetone extract of the roots of *Senna* species possess potential antibacterial agents. Indeed, a number of antimicrobial compounds were observed (Figure 3.5a and 3.5b) with distribution across a range of polarities. All solvent systems that were used in bioautography assays (i.e., BEA, CEF and EMW) separated compounds with antibacterial activity.

The disparities in polarity characteristics of the solvent systems indicate the presence of compounds across the polarities which could be potential antibacterial agents. The observed wide array of antimicrobial components, and their somewhat broad spectrum, may explain why *Senna* species is so widely used for medicinal purposes in the Limpopo region of South Africa. Martini and Eloff (1998) indicated that the wide diversity in polarity of the antimicrobial components may provide clinically useful

leads. Therefore, the current results show that the roots of *Senna* species possess antimicrobial compounds which may also, in the future, provide useful clinical leads.

Once the biological evaluation has been performed, the separation or isolation of the active compound(s) is attempted for the comprehensive characterization of the medicinal plant's biological activity (Gurib-Fakim 2006; Phillipson, 2001; Pieters and Vlietinck, 2005). The present data clearly showed, to some extent, that the roots of *Senna* species possess antiproliferative, antioxidant and antibacterial properties. The data therefore afforded enough reason to attempt the isolation of potential bioactive compound(s) from the roots of *Senna* species. Accordingly, a bioassay-guided fraction was used to isolate these putative bioactive compounds pursuing the extract's inherent antioxidant activity.

Through the bioassay-guided fractionation a flavanoid (chalcone) compound with free radical scavenging activity was successfully isolated (Figure 3.14); the isolation procedure is summarised in Figure 3.15. The isolated compound was identified as 1,3-diphenol-2-propen-1-one which can also be referred to as 3',5',4'-trihydroxychalcone. The IUPAC systematic name of the isolated compound was assigned as 1-(3,5-dihydroxyphenyl)-3-(4-hydroxyphenyl)-2-propen-1-one. We report here for the first time the isolation of this compound from the roots of *Senna* species. There are reported synthesized trihydroxychalcones with hydroxyl group substitutions in different positions (i.e., 2',4',2(3)(4)-trihydroxychalcones). These synthesized chalcones are reported to be promising as new free radical scavengers as well as their antimicrobial activity (Ansari *et al.*, 2005). Therefore, the biological activities of *Senna* species investigated in this study could be partially accounted for by this

isolated chalcone compound. Yayli *et al.* (2007) have also reported on the antioxidant activity of the derivatives of the chalcones, azachalcones. The yield of the isolated compound was not enough to enable further biological evaluation and characterisation. This characterisation could have allowed an evaluation of whether this pure compound displays a more superior biological activity than is currently demonstrated by the crude extract.

Indeed, chalcones are a class of natural compounds classified as flavonoid type of phenolic compounds with a wide array of biological activities. Among the various biological activities of the chalcones are their insecticidal, antimicrobial, antiviral and bacteriostatic properties (Yayli *et al.*, 2007). Therefore, the presence of this isolated chalcone within the acetone extract of the roots of *Senna* species could explain the wide array of biological activities displayed by the crude extract. However, the presence of other compounds that may contribute to the observed biological activities observed in this study cannot be ruled out. The *Senna* species used in the current study could as well possess other biological activities apart from the ones evaluated.

CONCLUSION

The evaluation of the biological activities of the roots of *Senna* species demonstrated antiproliferative activity against Jurkat T cells, antimicrobial activity and antioxidant properties. These multiple biological activities could explain the diverse ethnobotanical usage of the species in traditional medicine. A flavonoid compound was successfully isolated and identified. The array of biological activities of *Senna* species that was demonstrated in this study could be explained by the presence of the isolated compound as well as other compounds present. The findings, indeed, validate

our hypothesis that the roots of *Senna* species possess compounds with multiple biological activities. Further work need to be done to evaluate the *in vivo* activity of both the crude acetone extract and the pure compound.

CHAPTER 5

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