The potential of tropane alkaloids and other phytochemical compounds of the South African *Erythroxylum* species to target pathogenic factors in rheumatoid arthritis

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Abstract

Rheumatoid arthritis is the third most common type of arthritis worldwide; and it is also one of the most common autoimmune diseases found today. Studies have shown that the life expectancy of a patient can be reduced by as much as 10 to 15 years due to the co-morbidities associated with chronic inflammation, a hallmark of the disease. Inflammation is characterized by pain, swelling and oxidative stress leading to tissue damage. Extract of three of the southern African erythroxylums were investigated as a potential modifying drug for chronic inflammation. All three extracts exhibited very good antioxidant activity, especially *E. emarginatum* with an EC_{50} of 3µg/ml. This is a very good indication that the extract will prevent further tissue damage and inhibit inflammatory proliferation. *E. emarginatum* also showed very good COX inhibitory results with selectivity to the COX-2 enzyme. An EC_{20} of 1.38µg/ml was obtained for COX-2 inhibition. This is a key step in preventing inflammatory pain and stopping the inflammatory process early on. Moderate antibacterial activity was also observed indicating elimination of the initiating factor of the disease. Furthermore these extracts proved to be non-toxic in cell culture studies, and were found to be chemically rich and diverse, containing compounds from all classes including 1.35 ± 0.063 (SD) mg of methylecgonine per gram of leaf material in *E. emarginatum* and trace amounts of atropine, hyoscyamine, tropacocaine, transcinnamoylcocaine and anhydrous methylecgonine. Interestingly, *E. emarginatum* contained high levels of methylecgonine, the precursors to cocaine, compared to *E. coca*. This could indicate a blockage in the cocaine synthesis pathway. Gene sequencing results showed that a version of the cocaine synthase gene is present in *E. emarginatum*. Point mutations observed in the gene sequence could explain the inability of the *E. emarginatum* to produce cocaine. Overall the results indicate very good potential for these plants to be used medicinally as chronic inflammation modifying drugs.

Keywords: Inflammation, COX, *Erythroxylum,* Methylecgonine, Inflammation modifying drugs

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Contents

Chapter 4 Cytotoxicity

Chapter 5 Antibacterial screening assay

List of figures

List of Tables

List of abbreviations

bp	Base pairs
BLAST	Basic local alignment search tool
COX	Cyclooxygenase (enzyme)
DNA	Deoxyribonucleic acid
DMARD	Disease modifying antirheumatic drugs
ELISA	Enzyme linked immunosorbent assay
IC_{50}	Concentration where inhibition occurs in 50% of cases
MIC	Minimum inhibitory concentration
min	Minutes
NSAIDS	Nonsteroidal anti-inflammatory drugs
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
TD_{50}	Dose where toxicity occurs in 50% of cases
T-cell	T-lymphocyte
UTI	Urinary tract infection

Chapter 1

Introduction

1. 1 Introduction and Literature review

1.2 Aim and Objectives

1.3 References

1.1 Introduction and Literature Review

Rheumatoid arthritis is the third most common type of arthritis worldwide; and it is also one of the most common autoimmune diseases found today. Affecting as much as 1% of the world population, people from all ethnic backgrounds are at risk of developing this disease. Rheumatoid arthritis is a complex, multifactorial condition with an unclear aetiology and no cure (Goh and Midwood, 2011). Studies have shown that the life expectancy of a patient can be reduced by as much as 10 to 15 years due to the co-morbidities associated with chronic inflammation, a hallmark of the disease (Rheumatoid arthritis support network, 2016). Co-morbidities include accelerated atherosclerosis, as well as an increased risk of stroke and heart attacks (Gullo *et al*., 2014). It is estimated by Hootman *et al*., 2017, that by 2040, 26% of adults in the United States of America will be diagnosed with a form of arthritis. Currently 1 in 4 people suffering from arthritis have reported work limitations due to the arthritis (Hootman *et al*., 2017). These disorders represent an important medical and economical problem because they have a devastating impact on quality of life and require long-standing medical care (Tlaskalova´-Hogenova *et al*., 2011). Preserving an active lifestyle and preventing joint damage by early detection and treatment aimed at reducing inflammation is currently one of the only options available to medical practitioners as treatment (Hodkinson *et al*., 2012: Oxenham, 2010).

Rheumatoid arthritis symptoms are triggered when a person's antibodies attack the synovial joint fluid, causing chronic inflammation and accumulation of reactive oxygen species (Gullo *et al*., 2014; Rheumatoid arthritis support network, 2016). This leads to erosion of bone, joint destruction, pain and disability (Cock and Van Vuuren, 2014). There is increasing evidence that reactive oxygen species produced at sites of chronic inflammation not only contribute to the destruction of tissue but also have genotoxic effects. Raised levels of DNA oxidation products have been associated with rheumatoid arthritis, systemic lupus and inflammatory bowel syndrome with a direct correlation found between oxidative stress and the increased incidence of cancer in ulcerative colitis and Crohn's disease (Tak *et al*., 2000)

Inflammation consists of an array of responses to tissue injury and is characterized by heat, redness, swelling and pain. Prostaglandins are key inflammatory mediator molecules during the initiation and maintenance of inflammation and therefore are significant therapeutic targets (Whicher and Evans,

1992). Prostaglandins are synthesized through the cyclooxygenase (COX) enzyme with arachidonic acid forming the skeletal structure for these molecules. The inhibition of the COX enzyme is therefore at the centre of current anti-inflammatory research therapies (Murphy, 2012).

Two isozymes of the COX enzyme exist. COX-1 is a constitutively expressed enzyme found in almost all tissue, and is responsible for regulating renal blood flow, gastric mucosal integrity and platelet dependant homeostasis. COX-2 on the other hand is highly inducible and is expressed in response to hormones, growth factors, pro-inflammatory cytokines and bacterial endotoxins. Experimental evidence has shown that the inhibition of the COX-1 enzyme by non-steroidal anti-inflammatory drugs (NSAID's) is associated with the side effects of these drugs, while inhibition of the COX-2 enzyme is associated with the therapeutic activity (Laufer *et al*., 2003). Current medicine cannot be used chronically because of these side effects, which may lead to kidney, liver and sometimes cardiac failure (Serhan *et al*., 2010). Thus, direct inhibition of the COX-2 enzyme is seen as an efficient pharmacological approach to treat chronic inflammation, as exemplified by the active development of COX-2 inhibitors such as celecoxib (Buchanan and DuBois, 2006; Deeks *et al*., 2002; Fujimura *et al*., 2007).

Studies have shown that COX-2 inhibitors isolated from plants may have an advantage to chemically synthesized COX-2 inhibitors that has thus far reached the market. Selectivity for the COX-2 enzyme seems to be greater, accompanied with less risk of long term side effects (Bruno, 2009). Numerous double-blind clinical trials conducted since 1998 have shown ginger (*Zingiber officinale*) to be effective in relieving inflammation, pain and stiffness in patients suffering from rheumatoid arthritis, osteoarthritis and muscular discomfort. Patients that consumed ginger or ginger extracts daily for two and a half years reported no side effects (Srivastava and Mustafa, 1992). Purified 10-gingerol, 8-shogaol and 10-shogaol isolated from ginger, showed no COX-1 inhibition, but inhibited COX-2 with IC_{50} values of 32 µM, 17.5 µM and 7.5 µM, respectively, helping to explain gingers anti-inflammatory activity (Breemen *et al*., 2011).

Numerous *Erythroxylum* species have been reported to have been traditionally used as medicine by local tribes, with many being used as a pain remedy. These include both species that contain the tropane alkaloid cocaine and those that do not. Some examples include the *E. coca*, found in Southern America. The leaves of the *E. coca* is used to relieve hunger, fatigue, stimulate stomach function, cause sedation, treat asthma, colds, and other ailments, treat altitude sickness and to numb eye, nose, and throat pain; and to narrow blood vessels (Biondich and Joslin, 2016). *E. catuaba* is local to Brazil and is traditionally used as a central nervous stimulant, aphrodisiac, for the treatment of agitation, nervousness, nerve pain and weakness, as well as

forgetfulness (Wilmer, 2012). A 1992 study showed the antibacterial activity of the species against *Escherichia coli* and *Staphlococcus aureus*, where a 1997 study indicated significant pain relieving activity *in vivo*. *E. catuaba* does not contain the alkaloid cocaine (Taylor, 2012). Another Brazilian species that doesn't contain cocaine, *E. suberosum*, is used as an antidiarrheal, astringent, antirheumatic, and anaesthetic agent (De Oliveira *et al*., 2015)

Locally *E. delagoense* and *E. pictum* have been reported to be used for the treatment of dysentery and diarrhoea. The fruits of the *E. delagoense* are used for throat and respiratory ailments in infants (Corrigan *et al*., 2011), while *E. emarginatum* has traditionally been used to treat asthma, kidney problems, arthritis, child bearing problems and influenza (De Wet, 2011).

Very few studies have been done to confirm the biological activity and identify the chemical constituents of these southern African species. *E. delagoense*, *E. pictum* and *E. emarginatum* were tested for antimicrobial properties and found to have good activity against *Bacillus subtillus*, *Klebsiella pneumoniae* and *Staphylococcus aureus* (De Wet, 2011). A study conducted in 2005 showed that the alkaloid calystegine A3 was present in the bark of *E. pictum*, but no study was done on the leaf alkaloid content (Brock *et al*., 2005). An unpublished study in 2014 by Daneel, demonstrated good preliminary anti-inflammatory activity from these plants as well as the presence of numerous tropane alkaloids present in the leaves of these plants. Some of the alkaloids include the cocaine related methylecgonine, tropacocaine and benzoylecgonine. It is suspected that these alkaloids may contribute to the anti-inflammatory activity observed.

Tropane alkaloids are widely used for their medicinal activity and almost all of these are isolated from plants to be used in either their natural or a semi-synthetic form (Grynkiewicz and Gadzikowska, 2002). The tropane moiety is part of over 20 commercially produced pharmaceutically active ingredients used as anti-emetics, anaesthetics, bronchodilators as well as mydriatic agents (Grynkiewicz and Gadzikowska, 2008). With the increased demand for these products various studies are looking at the tropane alkaloid content of plants and methods to genetically or biologically manipulate the plant to increase tropane yields. The closer the structure to the basic tropane moiety, the easier it is to use in semisynthetic reactions to create valuable medicinally active compounds. For instance tropinone can chemically be used as the basis of any tropane alkaloid (Grynkiewicz and Gadzikowska, 2008).

Erythroxylum delagoense Schinz, is commonly known as the small- leaved coca-tree. It is found in the north-eastern parts of South Africa and some in Swaziland. It is a perennial tree growing 1-6m in height and found in dry sandy or stony soils (Figure 1.1) (Balkwill *et al*., 2004; Robson, 1963). The leaves are petiolate and rounded, pale green on top and slightly paler at the bottom. Flowers are small, white to greenish white, in bloom from September to January, with small drupe red fruits occurring from October to April (Robson, 1963; Schmidt *et al*., 2002).

Figure 1.1: *Erythroxylum delagoense* (Daneel, 2014)

Figure 1.2: *Erythroxylum pictum* (Daneel, 2014)

Erythroxylum pictum E. Mey ex Sond, also known as the forest coca tree or blue-leaved coca-tree can also be found along the eastern coast line of South Africa, but extends more south than *E. delagoense* (Balkwill *et al.,* 2004). The tree is often found near streams and as the name suggests in forest areas. *E. pictum* is a small to medium sized tree, often with some yellow leaves at the canopy. The leaves are oval shaped and generally bluish green on top and slightly paler on the bottom (Figure 1.2). Flowers are small and white, with small bright red drupe fruits (Van Wyk and Van Wyk, 2007).

Erythroxylum emarginatum Thonn. is much wider spread than the previous two species and can be found extending the region from the Eastern Cape into tropical Africa. The species is commonly known as the African coca-tree, and can occur in a variety of habitats including sandveld, bushveld and forests (Schmidt *et al.,* 2002).

E. emargiantum is a woody tree or shrub that can grow up to 5 m in height (Figure 1.3)

Figure 1.3: *Erythroxylum emarginatum* (Daneel, 2014)

(Balkwill *et al*., 2004). The leaves are oval with the apex rounded, dark green above and paler below. The flowers are small, white and occur between September and January. The fruits are red, drupe and edible and occur from November to February (Schmidt *et al.,* 2002).

1.2 Aim and Objectives

The aim of this study was to holistically evaluate the southern African *Erythroxylum* species as potential medicinal agents in the fight against debilitating inflammatory disorders such as rheumatoid arthritis. This was done by:

- Evaluating the broad chemical profile of the species to enable future studies to directly link activity to compounds.
- Evaluating the potential for cocaine production in these species due to the precursor molecules present in them through isolating the cocaine synthase gene.
- Establishing the cytotoxicity of the species to evaluate the viability of using the extracts medicinally.
- Measure the antibacterial activity of the species as a method of eliminating the environmental cause of rheumatoid and other forms of arthritis during early infection.
- Investigating the antioxidant activity of the species to combat destruction of tissue through oxidative species.
- Investigate the COX inhibitory activity of the species to determine if the species have a direct effect on blocking inflammatory mediators.

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Chapter 2

Phytochemical Composition and Alkaloid Quantification

2.1. Introduction

In 2014 it was estimated that 350 000 known plant species existed of which only about 30% have been phytochemically defined in detail (Jirschitzka, 2014). A report by The Royal Botanical Gardens has estimated that 390 900 vascular plants are currently known with 2034 newly described plant species in 2015 alone (RBG Kew, 2016). When considering the vast amount of plant species that have yet to be phytochemically described, the estimated 200 000 chemical compounds known to be produced by plants is possibly only a fraction of what is still to be discovered. Because of the intricate structures of some of the plant produced products some compounds take up to 19 years to be synthesised in laboratories. Not all natural products can be fully synthesized and many natural products are too difficult and expensive to synthesize on an industrial scale. These include drugs such as penicillin, morphine, and formerly paclitaxel (Lahlou, 2013). Therefore compound isolation from natural sources is still important in discovering new medicinal compounds. The potential is even bigger when considered that, of the identified plant compounds, only a limited amount have been subjected to biological or pharmacological screening, emphasizing the necessity of such studies (Wang, 2016).

It is often difficult to classify secondary metabolites because of overlap in structures and biosynthetic pathways. Commonly used classes to describe plant compounds include the alkaloids, referring to nitrogen containing molecules, terpenes that are made from the basic isoprene unit and phenols where hydroxyl groups are attached to an aromatic ring (Taiz and Zeiger, 2006). Natural compounds however often have numerous of these groups incorporated in a single compound. Each of these classes have varying biological activity that can be utilized medicinally. Table 2.1 demonstrates some of the classes of secondary metabolites based on their structure and important examples of medicinal compounds that falls within these specific structural classes.

Specific secondary compounds are often restricted to a narrow set of species within a phylogenetic group (Wink, 2003; Chizzali and Beerhues, 2012). Considering this, it is often expected that species from the same genus may produce similar medicinal effects.

The *Erythroxylum* species are well known for their alkaloid content and are especially famous for their cocaine producing members (Chin *et al.* 2006). Despite this very little is published on the overall chemical profile of most of these species (Evans, 1981). The genus *Erythroxylum* consists of 200 species found in tropical regions of South America, Africa and Madagascar. Besides tropane and

other alkaloid derivatives, there have been reports of diterpenoids, flavonoids, tannins, and triterpenoids in some species of the genus (Chin *et al*., 2006).

Table 2. 1: Classes of phytochemicals used medicinally (Marker and Krueger, 1940; Stierle *et al*., 1993; Frachini *et al.*, 2002; Thevis, 2003; Chemocare, 2016).

Class	Trade name	Medicinal compound	Use
Tannins	Toposar	Etoposide	Anticancer drug
Alkaloids	Adcodol	Codeine	Analgesic
Saponins	Diosgenin	Diosgenin	Precursor of semisynthetic cortisone
Cardiac glycosides	Lanoxin	Digitoxin	Congestive heart failure and other heart conditions
Terpenes	Onxol	Taxol	Anticancer drug
Flavanoids	Hepatron	Silymarin	Hepatoprotective
Phenolics	Disprin	Salicylic acid	Analgesic, antipyretic

Research on the southern African *Erythroxylum* species has been focused on terpenes. Diterpenes have been reported in all three South African species with 29 different diterpenes reported in *E. pictum*, including labdane, kaurane, beyerane, rosane, dolabrane, pictane and manool (Ansell *et al*., 1993). Anhydroecgonine methyl ester (Figure 2.1 (a)), anhydroecgonine methyl ester N-oxide (Figure 2.1 (b)) and calestigine A3 (Figure 2.1 (c)) found in the twigs and bark of *E. emarginatum* are the only alkaloids reported in literature to be present in the South African *Erytroxylum* species (Brock *et al*., 2005; Nishiyama *et al*., 2007). The limited knowledge on the phytochemical profile of the southern African Erythroxylaceae species and the range of medicinal activity observed in other *Erythroxylum* species justifies an in depth look at the chemical makeup of these three species.

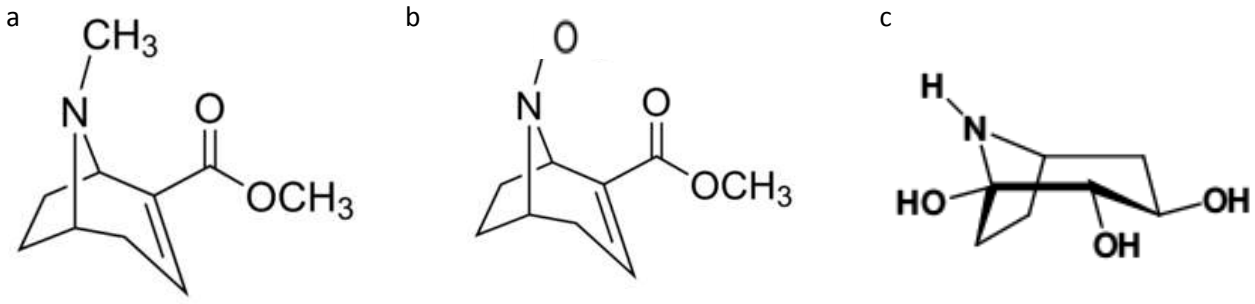

Figure 2.1 a) Anhydroecgonine methyl ester b) anhydroecgonine methyl ester N-oxide c) calestigine A3

2.2. Aim

The aim of this chapter was to elucidate the phytochemical composition of the southern African *Erythroxylum* species and to quantify some of the tropane alkaloids found in these species to establish potential medicinal value of the species. This was followed by fractionation of the *Erythroxylum emarginatum* extract with the aim of purifying some of the compounds for future studies.

2.3. Methodology

2.3.1. Plant collection and extraction

Leaf samples of the *E. delagoense, E. emerginatum,* and *E. pictum* were collected from the Lowveld National Botanical Gardens situated in Nelspruit (Mbombela), Mpumalanga in September 2015. Voucher specimens were authenticated and deposited at the H.G.W.J Schweikerdt herbarium at the University of Pretoria (Appendix 1). The leaves were freeze dried on a Virtis K-series benchtop freeze dryer (United Scientific, South Africa). The leaves were then extracted with methanol (Merck, South Africa) on a Buchi E-916 speed extractor (Labotec, South Africa). Extraction conditions consisted of 4 cycles of 1 min heating to 60°C, holding the sample and methanol for 9 min at a 100bar pressure and 60°C followed by discharge of 5 min. Five technical replicates were made during the extraction process. An alkaloid fraction was isolated from the crude extracts for further bioactivity testing. This was done by liquid-liquid extractions as described by Turner *et al.* (1981); and demonstrated in Figure 2.2.

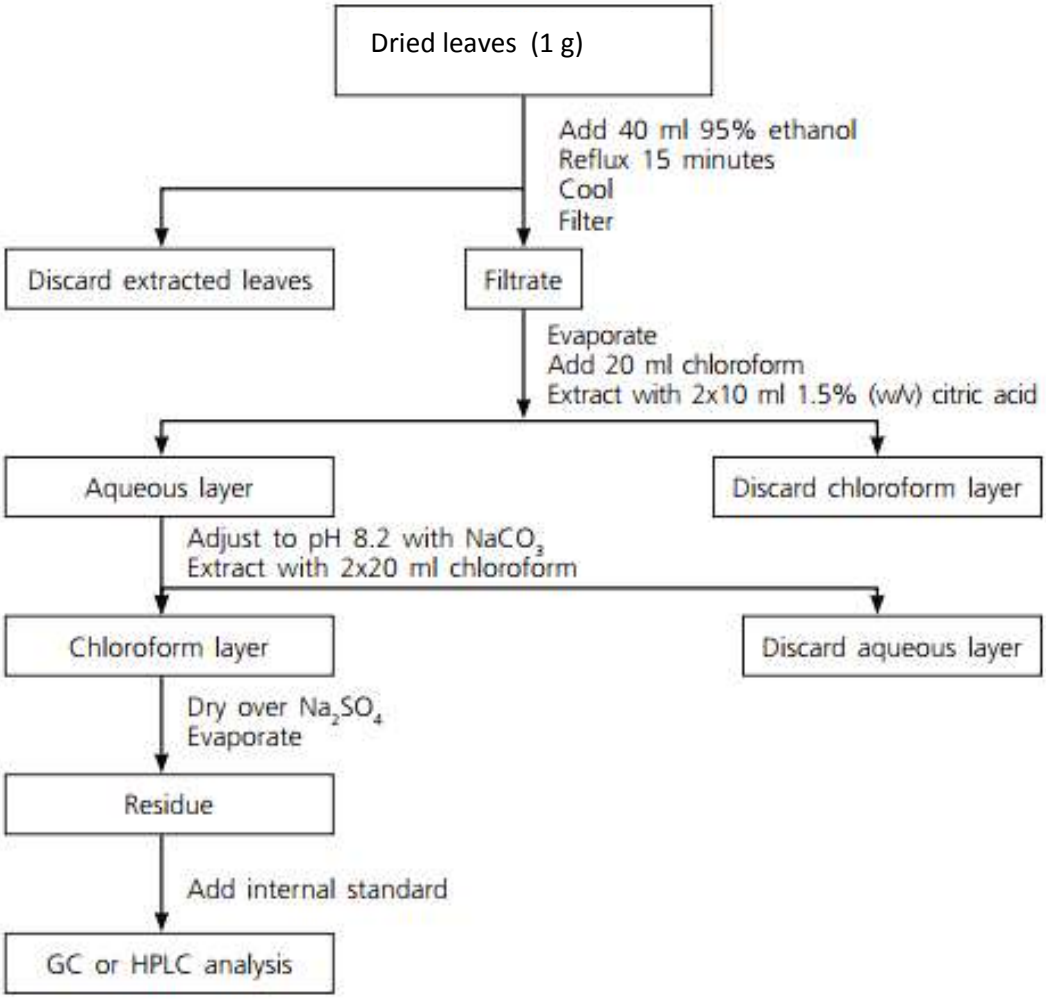

Figure 2.2: Flow chart for extraction of alkaloids from coca crude extracts.

2.3.2. Isolation via column chromatography

A column was packed with 200g of C_{18} reverse phase silica gel (Sigma-Aldrich, South Africa) and conditioned with 50:50 water to methanol solvent system. Methanol dissolved crude *E. emarginatum* extract (8 g) was mixed with C_{18} reverse phase silica gel, after which the mixture was left until complete dryness. The dry silica-plant extract mixture was added on top of the column, 10 ml fractions were collected throughout the duration of fraction collection. The solvent elution system can be seen in Appendix 1, Table 2.2.

2.3.3. Phytochemical analysis

All chemicals used were supplied by Merck, South Africa, unless otherwise stated.

I. Tannins

2 ml of 5% $FeCl_3$ was added to 2ml of aqueous extract. The formation of yellow to brown precipitate was observed indicating the presence of tannins (Harborne, 1998).

II. Alkaloids

1% HCl was added to 2ml of MeOH extract. After heating of the solution in a warm bath, 6 drops of Dragendorff reagent were added (Harborne, 1998). Formation of orange to brown precipitate was observed indicating the presence of alkaloids.

III. Saponins

Aqueous extracts of 2g dried plant material was prepared and shaken vigorously. If a persistent froth was observed the presence of saponins were confirmed (Harborne, 1998).

IV. Cardiac glycosides

According to the Keller-kiliani test, 1ml glacial acetic acid, 1-2 drops $FeCl_3$ and 1ml concentrated H_2SO_4 were slowly added to 2ml of methanol extract to form layers. A brown ring at the interface of the solvent layers indicated the presence of a deoxy sugar; characteristic of cardenolides (Kiliani, 1895).

V. Terpenes

5 ml $CHCl_3$, 1 ml glacial acetic acid and concentrated H_2SO_4 were slowly added to 2 ml of an aqueous extract to form layers. The red-brown discoloration of the interface indicated the presence of terpenes (Harborne, 1998).

VI. Flavonoids

The Shinoda test was carried out by the addition of a few drops of concentrated hydrochloric acid to 2ml of methanol extract and 0.5g of magnesium turnings. A red-pink discoloration after 3 min indicated the presence of flavanoids (Harborne, 1998).

I. Phenolics

1ml of 1% ferric chloride solution was added to 2ml of methanol extract. A blue or green colouration indicated the presence of phenols (Harborne, 1998).

2.3.4. Quantification

Quantification was done on an Agilent Gas chromatograph (GC) combined mass spectrometer (MS) in electron impact ionization mode (70eV). The GC was fitted with a DB-5ms column (30m, 250µm, 0.25µm). The oven temperature was initially at 50°C and held for 1 min, after which the temperature was increased by 60°C/min up to a temperature of 320°C and held for 4.5 min. The injection port was set at 300°C, operated in splitless mode with 1µl of sample injected and a total flow of 54.6ml/min. The transfer line was set to 280°C. The mass detector was operated in selected ion monitoring (SIM) mode monitoring ions 82m/z, 85m/z, 96m/z, 139m/z for tropinone and 82m/z, 85m/z, 96m/z, 199m/z for methylecgonine. Tropinone standard was obtained from Sigma Aldrich and methylecgonine standards (Sigma Aldrich) were generously provided by the South African Police Service's forensics unit, from which standard curves were established. Quantification was done on the 82 ion in both cases.

2.4. Results and Discussion

Table 2.2 represents the chemical composition of the three South African *Erythroxylum* species and the fractions collected from the reverse phase column chromatography on *E. emarginatum*.

The Table supports the hypothesis that the Erythroxylaceae is a family rich in chemical diversity. All the tested classes of compounds were observed in *E. emarginatum*, while only one or two classes were not observed in *E. delagoense* and *E. pictum* respectively. It is important to take note here that it was possible to identify alkaloids in the fractions of *E. emarginatum*, but not in the crude plant extract. This may be because the concentration in the crude sample was too low to observe in comparison to the matrix effect. This observation must be taken into account when considering the

phytochemical profile of the other two species. None the less, the major compounds present in the plant species can be reported.

Table 2.2: The secondary compound classes found in each plant species' crude extract and fractions from *E. emarginatum's* column chromatography

Sample	Tannins	Alkaloids	Saponins	Cardiac glycosides	Terpenes	Flavonoids	Phenolic compounds
E. pictum	●			●	●	●	●
E. delagoense	●	●		●	●	●	●
E. emarginatum	●		●	●	●	●	●
Fraction 1				●			
Fraction 2	●		●	●		●	●
Fraction 3	●		●	●	●	●	●
Fraction 4	●		●	●	●	●	●
Fraction 5	●		●	●	●	●	●
Fraction 6	●		●	●	●	●	●
Fraction 7	●		●	●	●	●	
Fraction 8	●		●	●	●	●	●
Fraction 9	●		●	●	●		●
Fraction 10			●	●	●		●
Fraction 11			●	●	●		●
Fraction 12		●		●	●		
Fraction 13				●			
Fraction 14				●			
Fraction 15				●	●		
Fraction 16				●	●		
Fraction 17				●	●		
Fraction 18				●	●		
Fraction 19				●	●		
Fraction 20				●			
Fraction 21				●			

Fraction 22				•			
Fraction 23				•	•		
Fraction 24			•	•	•		
Fraction 25				•	•		
Fraction 26				•	•		
Fraction 27				•			
Fraction 28				•	•		
Fraction 29					•		
Fraction 30					•		
Fraction 31							
Fraction 32				•			

Interestingly, cardiac glycosides were present in nearly all the fractions of *E. emarginatum*, indicative not only of a large amount of these compounds present, but also the diversity in structure and polarity. Terpene compounds are also a major constituent found throughout the column fractions. This supports the literature findings by Ansell *et al*., 1993 on the southern African *Erythroxylum* species that found diterpenes in all three species with 29 different diterpenes in *E. pictum*. Amongst others labdane was found that is a precursor molecule for the labdane terpenes which have been proven to have antibacterial, antifungal and anti-inflammatory properties (Atta-Ur-Rahman, 2003). Dolbrane diterpenes, also found in *E. pictum,* has been shown to have potent anti-inflammatory activity, inhibiting the migration of neutrophils to the site of infection (Rauter *et al.,* 2002). The number of fractions containing terpenes in the *E. emarginatum* may indicate potential for further research on these compounds in the plant as well as investigating the medicinal potential of these compounds.

The most famous group of compounds for the *Erythroxylum* species are the alkaloids. Tropane alkaloids are an important group of compounds due to their vast range of biological actions. Tropane alkaloids are used in medicine as analgesic, anaesthetics, anticholinergic, antiemetic and antihypertensive agents, with atropine and scopolamine being especially medicinally valuable (Chin *et al*., 2006). Surprisingly, alkaloids were the least detected of the compound classes in the three South African species. Quantification, however, indicated relatively high concentrations of methylecgonine relative to other species. This is of significance since higher yields will make isolation easier and commercial isolation more profitable than in other species.

1.35 ± 0.063 (SD) mg (R^2 value of 0.996) of methylecgonine was found per gram of leaf material in the leaves of *E. emarginatum.* No methylecgonine was found in the *E. delagoense* or *E. pictum*. Trace amounts of tropinone were detected in the leaves of the *E. delagoense* but not in the other two species. Other alkaloids observed and identified with the NIST 11 library based on their mass fractionation include atropine, hyoscyamine, tropacocaine, transcinnamoylcocaine and anhydrous methylecgonine in *E. emarginatum.* Anhydrous methylecgonine was found in *E. delagoense* while no tropane alkaloids could be identified in *E. pictum.*

Even though the presence of anhydrous methylecgonine in *E. emarginatum* has been reported by Nishiyama *et al*. (2007), they only reported the compound to be present in the twigs and bark of the plant. This study confirms the presence of the compound in the species and for the first time reports the presence in the leaves as well as the presence of methylecgonine. Tropinone is reported for the first time to be present in the leaves of the *E. delagoense.* The other tropane alkaloids identified are also reported for the first time in the leaves of the southern African *Erythroxylum* species.

Coca leaves is reported to be used for toothache, stomach ulcers, rheumatism, asthma, and even malaria (Grinspoon and Bakalar, 1981). Other *Erythroxylum* species such as *E. cuneatum* do not contain the alkaloid cocaine, but have also been reported to be active against pain (Saleh *et al.,* 2012). This may be an indication that the Erythroxylaceae family has a variety of compounds with analgesic and other medically relevant activity. Considering the differences in chemical profile of the three species tested, there is major potential for further investigations into biological activity of these three species and the family in general. It is also important to conduct these studies as these plants are already being used as traditional medicine. It will be important for physicians to take note of the alkaloids contained in these plants such as atropine when considering drug-drug interactions, as atropine is a very potent systemic drug.

2.5. Conclusion

This study evaluated the chemical profile of the southern African *Erythroxylum* species. A preliminary screen showed large chemical diversity in these plants with compounds in the classes of cardiac glycosides, alkaloids, phenolics, flavonoids, saponins, tannins and terpenes found. A wide range of tropane alkaloids have, for the first time, been identified in the leaves of these species. 1.35 ± 0.063 SD mg of methylecgonine was found in the leaves of the *E. emargiantum* and trace amount of tropinone was found in the *E. delagoense*. Other medicinally valuable compounds such as

atropine and hyoscyamine were also reported. This confirms that the Erythroxylaceae is a chemically rich family which may hold enormous medicinal value.

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Chapter 3

Isolation of cocaine synthase gene from *Erythroxylum emarginatum*

3.1 Introduction

Tropane alkaloids are medicinally active secondary metabolites that have been reported in the *Solanaceae, Euphorbiaceae, Proteaceae, Convolvulaceae, Brassicaceae, Rhizophoraceae* and *Erythroxylaceae* (Griffin and Lin, 2000). All tropane alkaloids are derived from L-ornithine or L-arginine (Dewick, 2002) and share an 8-methyl-8-azabicyclo[3.2.1]octane core structure, also referred to as a tropane ring. More than 150 different tropane alkaloids have been identified including the medicinally used agents atropine, hyoscyamine and cocaine (Docimo *et al.*, 2012).

Despite the overwhelming volume of publications available on *Erythroxylum coca* and cocaine in particular, the complete biosynthetic pathway of cocaine, including enzymes involved, was only elucidated as recently as 2015 by Schmidt *et al*. This is in stark contrast to the depth of knowledge available on the biosynthetic pathway of medicinally active tropane alkaloids in the Solanaceae family (Docimo *et al.*, 2012).

The first step in the biosynthesis of cocaine has been shown to be the conversion of ornithine through ornithine decarboxylase (ODC) or arginine through arginine decarboxylase (ADC) to putrescine (Figure 3.1) (Docimo *et al.*, 2012). Putrescine is methylated and then oxidised to 4-methyl-aminobutanal, which undergoes spontaneous cyclization to an N-methyl-D1-pyrrolinium cation. Oxobutanoic acid forms as an intermediate after the addition of two acetyl units, which in turn forms methylecgonone (Jirschitzka *et al*., 2013). Methylecgonone reductase then reduces methylecgonone to methylecgonine. The final biosynthetic step to produce cocaine in *E. coca* is the esterification of methylecgonine with a benzoyl moiety derived from cinnamic acid. In 2015, Schmidt *et al*. elucidated the role of the BAHD super family in this reaction. Two genes in particular, *EcBAHD7* and *EcBAHD8*, were isolated and after kinetic studies the *EcBAHD7* gene was designated the true cocaine synthase gene. This is because despite high (77%) sequence identity of the enzymes, cocaine synthase (EcBAHD7) has a 1000 times higher efficiency rate for the production of cocaine in comparison to EcBAHD8 (Schmidt *et al*., 2015). In order to get to these result Schmidt *et al.* (2015) identified eight BAHD acyl transferases expressed in *E. coca* from an available λZAPII cDNA library and transcriptome library. These genes were then heterologously expressed in *E. coli* for the purpose of enzymatic studies related to the production of cocaine.

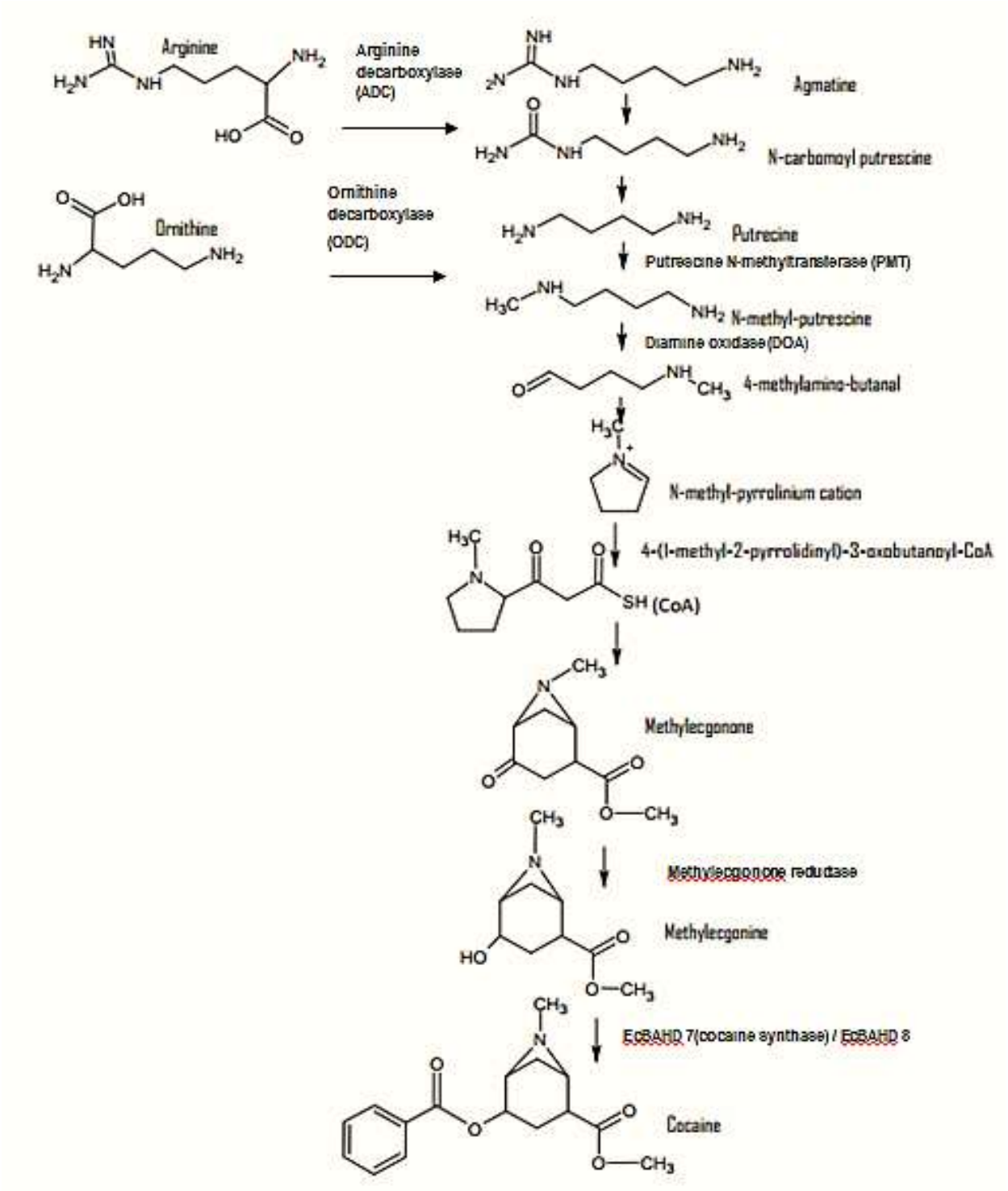

Figure 3.1: Cocaine synthase pathway in *E. coca*

The presence of cocaine has been reported in roughly 23 of the approximate 250 *Erythroxylum* species, however precursor compounds from the cocaine biosynthesis pathway are found abundantly in the genus (Oliveira *et al.*, 2010). It is believed that no old world species contain cocaine and thus far this has been the case. It was interesting however that the direct precursor to

cocaine, methylecgonine, was found in *E. emarginatum* (Alberts *et al*., 2018). This poses the question as to whether the biosynthesis pathway of cocaine was acquired or lost between species.

An unpublished study by Masike (2015) showed the presence of the *ODC* and *ADC* genes, in *E. emarginatum*. This, together with methylecgonone and methylecgonine found in the plant samples suggests that at least part of the cocaine synthase pathway may be present in the old world *Erythroxylum* species. The question this brings is whether the cocaine synthase gene is present in the old world species? To investigate, primers were designed to amplify the *cocaine synthase* and *BAHD8* genes within *E*. *emargiantum*.

3.2 Aim

The aim of this part of the study was to establish whether the *cocaine synthase* gene and *BAHD8* gene as described in the publication by Schmidt *et al*. (2015) are present in the southern African *Erythroxylum* species, indicating an evolutionary development in the cocaine biosynthesis pathway. Due to the presence of cocaine precursor compounds in *E. emarginatum*, we selected this species to screen for the presence of the *cocaine synthase* and related *BAHD8* gene.

3.3 Methodology

3.3.1 DNA extraction

All reagent and consumables were supplied by Merck (Johannesburg, South Africa) unless otherwise stated.

In order to extract genomic DNA from the leaves of *E. emarginatum* the CTAB protocol as described by Doyle and Doyle (1987) were used. Some modifications to the method included transfer of the ground leaf material into a 2ml tube before the addition of CTAB. The volume of CTAB added was doubled from 500µl to 1ml and the sample manually shaken. Furthermore the wash step with 500µl of 24:1 chloroform-isoamyl alcohol was repeated. These additional steps were performed to remove excess carbohydrates found in the leaves of this species. The concentration of the extracted DNA was determined using a nano-drop spectrophotometer (ThermoFischer Scientific, South Africa).

3.3.2 Primer design

Primers were designed based on *BAHD7* and *BAHD8* gene sequences available on NCBI, with accession numbers KC140149 and KC140150.1 respectively. The CLC genomics workbench 7 (Qiagen Aarhus A/S) software was used to design 4 primers for the whole gene amplification as well as internal fragments of the *BAHD7* and 5 primers for the amplification of the *BAHD8* gene (Table A2.1). Primers were designed over the start and stop codons of the genes, because more information on up and down stream sequences weren't available. The sequence available were derived from cDNA, therefore because the length of the amplicons from genomic DNA were unknown, internal fragments were designed to compensate for possible introns that might be part of the sequence. Primer sequences are available in Appendix 2. Primers for the *ODC* gene, used as control, were provided by Masike.

3.3.3 PCR amplification

The PCR reaction mix was prepared using the KAPA taq polymerase, according to the protocol by KapaBiosystems (2013). 12.5µl of KAPA Taq ready mix containing 1.5mM $MgCl_2$ and 0.2mM of each dNTP was added to 100ng template DNA and 10mM forward and reverse primer each. Reaction volumes of 25µL were prepared each time. Thin walled PCR tubes were used. PCR reactions were cycled as follows: an initial denaturing step of 95°C for 3 min followed by 25 cycles of 95°C for 30sec, 60°C for 30sec, 72°C for 60 sec. A final elongation step of 72°C for 10 min was included. After amplification, the PCR products were analysed using an agarose gel, one distinct PCR product band for each product was expected and no band for the non-template controls (Cullings, 1992). The *ornithine decarboxylase* (*ODC*) gene, part of the first step of cocaine synthesis and previously isolated by Masike in *E. emarginatum*, was isolated as positive control.

3.3.4 Agarose gel electrophoresis

Loading dye (Fermentas ready-to-use loading dye, ThermoFischer) was added to 5µl PCR amplification product and viewed on a 1% agarose gel (10µg/ml EtBr, Sigma-Aldrich) which was electrophoresed at 100V for 2h; in TAE buffer. For determination of the amplicon sizes the DNA was compared to the 100bp ladder. All gels were viewed using the BioDoc-It™ system (Bio-Rad, South Africa).

3.3.5 DNA Sequencing and Data Processing

The PCR products obtained from previous steps were sent for Sanger sequencing at the sequencing facility at the University of Pretoria. The *ODC* gene was not sequenced as it was only used as an amplification control. Chromatograms received were edited; end trimmed and mapped to the reference sequences KC 140149 and KC 140150, downloaded from the NCBI database, with CLC genomics workbench 11 (Qiagen Aarhus A/S) software. The mapping was done according to a minimum aligned read length of 50 bases excluding the reference sequence in the contig. This was followed by extracting a consensus sequence. The consensus sequence was entered in an NCBI BLASTn search query as well as an NCBI BLASTx search query. The Blastn (DNA sequence and DNA database) search parameters filtered out any low complexity areas and used a gap cost of existence 5, extension 2. Further the match / mismatch were set to match 2, mismatch -3. The BLASTx (translated DNA sequences and protein database) were searched against the non-redundant protein sequences database with a PAM70 matrix, filtering out low complexity sequences and a gap cost of existence 10, extension 1 (Altschul *et al*., 1997).

3.4 Results and Discussion

Isolation of the DNA proved to be difficult as the samples seemed to have very high concentrations of carbohydrates resulting in a DNA solution that was difficult to pipette. Adjustments to the method was made in order to lower the viscosity and improve purity of the sample. Firstly, the amount of CTAB buffer was doubled in order to allow better separation of the polysaccharides and phenols from the sample, while secondly, the chloroform-isoamyl alcohol wash step was repeated twice in order to further enhance the separation of the polysaccharides and other organic contaminants from the nucleic acids.

The focus of this study was to determine the presence of the *BAHD7* and *BAHD8* genes in *E. emarginatum*. This was based on the hypothesis that the relatively high concentration of methylecgonine found in *E. emarginatum*, and not in the other species, may indicate a blockage in the cocaine synthesis pathway. Difficulty was experienced in optimizing the DNA extraction for each of the three southern African species, with only *E. emarginatum* providing relatively pure DNA. Amplification of the *ODC* gene was included as a positive amplification control, since this gene has previously successfully been amplified from *E. emarginatum* genomic DNA.

The *EcBAHD7* and *EcBAHD8* genes isolated by Schmidt *et al.* in 2015 from *E. coca*, was done by making use of cDNA, therefore no introns would be present in these sequences deposited in the

NCBI. This complicated the predictions of expected gene length. Considering this, primers binding to internal fragments of these genes were created to compensate for long gene sequences that might be present due to introns. It was very interesting later to note that considering the alignment of the *BAHD 7* and *8* genes from *E. emarginatum* and *E. coca*, it seems that there are no introns present in these genes. An uncommon feature in Eukaryote genes.

Each of the amplified segments was compared to the *EcBAHD* gene sequences in terms of estimated number of base pairs, visually depicted in Figure 3.2. The band sizes obtained from the amplified segments correlated well with the expected band sizes predicted from *EcBAHD7* and *EcBAHD8* (Figure 3.3). The segment amplified from *E. emarginatum* with the BAHD8 FWD and BAHD8 REV primers gave the expected approximate 100 bp for the two internal primers, 600 bp for the forward primer and internal reverse primer, but only approximately 600 bp instead of an expected 1000 bp for the internal forward and reverse primer. The *BAHD7* gene fragments amplified from *E. emarginatum* as expected and yielded band sizes of approximately 600 bp, 200 bp and 700 bp. No results were obtained for the BAHD7 FWD and BAHD7 REV primers; therefore the complete gene was not amplified in a single reaction. The lengths of the amplified fragments, suggests that both of these genes are present in *E. emarginatum*.

Figure 3.2: Visual representation of fragments amplified in comparison to the reference transcript sequences from *E. coca*. Primer sequences in table A2.1.

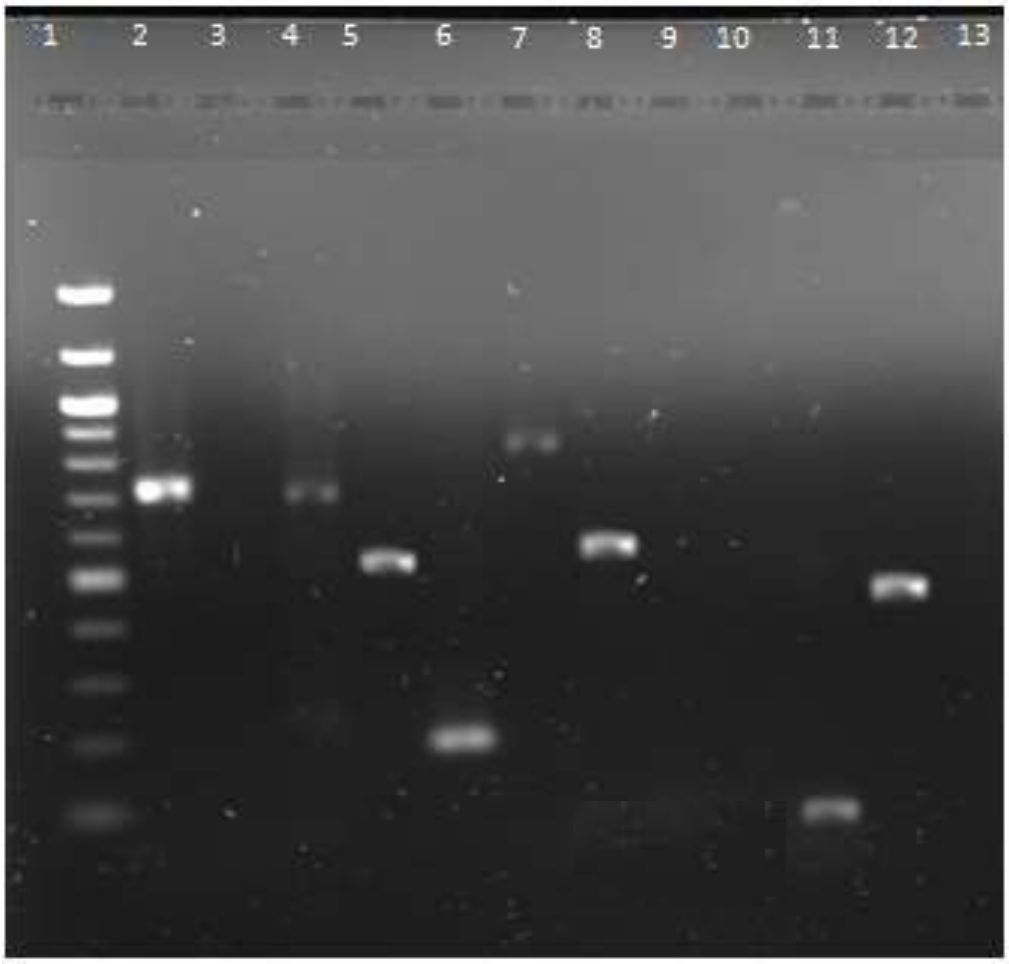

Figure 3.3: Agrose gel electrophoresis of *BAHD7* and *BAHD8* PCR amplification products from *E. emarginatum*. 1) 100bp ladder; 2) Positive control (ODC FWD : ODC REV) 3) BAHD 7 FWD – BAHD REV 7; 4) EcBAHD 7 FWD *I* – BAHD 7 REV; 5) BAHD 7 FWD – EcBAHD 7 REV *I*; 6) EcBAHD 7 FWD *I* – EcBAHD 7 REV *I*; 7) BAHD 8 FWD 1 – BAHD 8 REV; 8) BAHD 8 FWD 1 – EcBAHD 8 REV *I*; 9) BAHD 8 FWD 2 – BAHD 8 REV; 10) BAHD 8 FWD 2 – EcBAHD 8 REV *I*; 11) EcBAHD 8 FWD *I* – EcBAHD 8 REV *I*; 12) EcBAHD 8 FWD *I* – BAHD 8 REV; 13)Negative (non-template) Control

To confirm this observation, the PCR fragments were sent for further clean-up and Sanger sequencing. The segments of amplified DNA showed remarkable similarity to the original *EcBAHD7* and *EcBAHD8* genes isolated from *E. coca*. Mapping the sequences to the *EcBAHD8* gene and performing a BLASTn search query on the consensus sequence produced a pairwise identity of 97%. Similarly when the sequenced fragments were mapped to the *EcBAHD7* (cocaine synthase) gene and a BLASTn search query were performed on the consensus sequence, an 87% pairwise identity was obtained (Appendix 2). In both cases the E value was 0. For each of the consensus sequences a BLASTx search query was also performed. This showed that the amplified segments from the *BAHD8* consensus sequence showed a 94% identity (E-value of 0) with the EcBAHD8 protein sequence

(AGT56098.1), where the amplified segments from the *BAHD7* gene consensus sequence showed 75% identity (E-value of $5e^{-94}$) with the cocaine synthase (AGT56097.1) enzyme. This shows a high degree of conservation in both the gene sequences and amino acid sequences. Despite the degree of conservation, 20 amino acid replacements are seen in the BAHD8 translated amino acid sequence, while 24 amino acid replacements are seen in the BAHD7 translated amino acid sequence. Both of these sequences have more than one radical amino acid change, likely influencing the structure and function of the protein. This should further be investigated with protein folding studies.

There are enough differences between the translated nucleotide and the amino acid sequences to explain the lack of cocaine in *E. emarginatum*. Islam (2011) compiled a phylogenetic tree based on information from three loci, one based in the chloroplast and the other two in the nuclear genome of the *Erythroxylum* genus. The phylogenetic tree indicates a split between the African clade and the clade containing the *E. coca* early in the genus history. Interestingly despite the distance in ancestry, there are still high levels of conservation in these genes. Isolation studies of these genes in the other members of the Erythroxylaceae family and determining if the coded proteins would be functional might indicate whether the genes are conserved within the family and might be serving a role other than cocaine synthesis. The *EcBAHD* genes are part of the super-family of BAHD acetyl transferase enzymes. These enzymes are often associated in plants with secondary phenolic compounds used in plant defences (Chedgy *et al*., 2015). It would therefore be very possible that cocaine synthase and *EcBAHD8* are derived from functioning *BAHD* genes found in the other Erythroxylum family members.

3.5 Conclusion

To establish whether the genes encoding enzyme responsible for cocaine synthesis, namely *BAHD7* as well *BAHD8* are present in the *E. emarginatum,* total genomic DNA was extracted from young leaves, according to the CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle, 1987). Due to high concentrations of carbohydrates in the samples the method had to be optimised.

The results obtained indicate that both genes are present in the plant species. Even though the complete *BAHD7* gene was not amplified, all the fractions to build up the gene were amplified. To verify these results and to establish why the *E. emarginatum* lacks in the production of cocaine, these gene fragments were sequenced and compared to the Ec*BAHD7* and Ec*BAHD8* gene sequences from the *E. coca*.

This data shows that versions of the *BAHD7* and *BAHD8* genes are present in *E. emarginatum*. There are however differences in the *BAHD7* and *BAHD8* gene sequences between *E. coca* and *E. emarginatum*. Future studies should firstly establish if the *BAHD7* and *BAHD8* genes present in *E. emarginatum* codes for functional proteins. Given that these two species are well separated on a phylogenetic tree, the genes are well conserved between species, suggesting a conserved functional role as a BAHD acetyl transferase.

The presence of the *BAHD7* and *BAHD8* genes in other members of the Erythroxylaceae family should also be established. The level of similarity between the genes from each species can contribute to our understanding of the evolution of these genes and ultimately cocaine synthesis. It would also be interesting to compare the protein structures of the cocaine producing enzymes *versus* the non-cocaine producing enzymes to establish the key point mutation.

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Chapter 4

Cytotoxicity

4.1 Introduction

Drug safety continues to be one of the most challenging aspects of drug discovery especially in terms of financial expenses. Hepatic, cardiac and nephrotoxicity in both early and late stages of clinical trials are one of the main reasons that drug trials fail. This has caused more emphasis to be placed on early detection of substance toxicity through *in silico* and cytotoxic models, before major financial investment in research and development takes place (Lin and Will, 2012).

Cultured immortal cell lines are an *in vitro* model system that is used for the study of xenobiotic toxicity, the detection of cytotoxic and genotoxic agents as well as carcinogens (Mersch-Sundermann *et al*., 2004). Models based on human hepatoma cell lines such as HepG2 cells are widely used for these studies as they have the advantage of avoiding the inter-species differences to hepatoxins. HepG2 cells retain many morphological and biochemical characteristics of normal hepatocytes, although it is accepted that no transformed cell-line can be an exact substitute for *in-vivo* studies (Thabrew *et al*., 1997).

A study by Lin and Will (2012), found that approximately 95% of compounds had no significantly different cytotoxic effect in three different cell lines representing the major toxicity routes namely the HepG2 cell line for hepatotoxicity, H9c2 cell line for cardiotoxicity and NRK-52E cell line for nephrotoxicity. Therefore testing the toxicity of a compound on one of these cell lines with the XTT viability assay can give an indication of the general toxicity of the compound. Organ specific toxicity cannot accurately be measured with such a simple approach. Aspects such as cardiotoxicity due to ion channel inhibition, liver toxicity due to bile salt efflux transporter inhibition and allergic responses cannot be predicted in this type of model (Lin and Will, 2012).

The XTT (sodium 30-[1-(phynyl-aminocarbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitrobenzene] sulfonic acid hydrate)) viability assay is a colorimetric assay based on the ability of viable cells to reduce the yellow tetrazolium salt to an orange formazan dye in the mitochondrion of the cell. This is a commonly used assay to determine the compound dose toxic to 50% of the cells (TD_{50}) and a good first step in determining the safety profile of a drug (Xu and Urban, 2011).

Most traditional medicinal plants have never exhaustively been investigated for their toxicological effects, as they are assumed to be safe because of their extended traditional use. Even though traditional use is a good indication of safety, research has shown that numerous commonly used

plant species can cause *in vitro* mutagenic, toxic or carcinogenic effects. Therefore it is important to test all new and known compounds for toxicity early in research and development studies.

Some cytotoxicity studies for *Erytroxylum* species using the HepG2 immortal cell line have been done. *E. cuteanum* as an example showed cell growth inhibition over a 72h period with IC_{50} values of 125 ± 12µg/ml, and lactate dehydrogenase leakage detected at 251 ± 19µg/ml. This indicates that the *E. cuteanum* plant extract causes cell death via necrosis at a concentration of 251 µg/ml (Wesam *et al.*, 2014). *E. minutifolium* and *E. confusum* on the other hand both showed cytotoxic effects on rat hepatocytes at 200µg/ml but hepato-protective effects at lower concentrations. These protective effects are speculated to be due to their antioxidant capacity (Rodeiro *et al*., 2008). These results stress the importance of testing the southern African *Erythroxylum* species for cytotoxicity.

4.2 Aim

The aim of this part of the study was to establish the general toxicity and therefore basic safety profile of the southern African *Erythroxylum* species on the HepG2 immortalized hepatic cell line.

4.3 Methodology

HepG2 cells were cultured in complete Minimal Essential Eagle's Medium (EMEM) supplemented with 10% foetal bovine serum (Separations, RSA) and 1% penicillin, streptomycin and fungizone (Highveld biological, RSA). The cells were grown at 37°C in a humidified incubator set to 5% CO_2. The cells were detached by treating them with Trypsin-EDTA (0.25% trypsin containing 0.01% EDTA) for 10 min at 37°C and then adding complete medium to inhibit the reaction.

The HepG2 cells were added to the 96-well plate with approximately 10 000 cells per well. The plated cells were incubated overnight in a humidified atmosphere at 37°C with 5% CO_2. After incubation the methanol-water extracts (Chapter 2), as well as Actinomycin-D as positive control were plated in triplicate at a concentration range between 400µg/ml to 3.125µg/ml for the extracts and a final concentration of 0.001µg/ml for the Actinomycin-D control. The plates were incubated under the same conditions for a further 72h. After incubation cytotoxicity was determined by adding 50µg/ml XTT (sodium 30-[1-(phynyl amino carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitrobenzene] sulfonic acid hydrate) reagent (1mg/ml XTT with 0.383mg/ml PMS (N-methylphenazonium methyl sulphate)) to all wells and incubating under the same conditions for a

further 3h. The absorption of the wells were measured at 450nm with a reference wavelength of 690nm (BIOTEK Power-wave XS multi-well reader). The experiments were done in triplicate. Graph Pad Prism 4 (Version 4 Graph Pad Prism Software, USA) was used to analyse the results. A 95% confidence interval was used with a sigmoidal dose response curve fit.

4.4 Results and Discussion

The IC_{50} (µg/ml) values obtained for the cytotoxicity test on the HepG2 cell line is indicated in Table 4.1. An IC_{50} value lower than 50µg/ml indicates that the tested sample is toxic while a value between 50µg/ml and 100µg/ml indicates a moderately toxic sample. A sample with an IC_{50} value of more than 400µg/ml is generally seen as non-toxic. This is in regards to the effective dose (ED_{50}) of the compounds. The larger the difference between the ED_{50} and the $IC_{50,}$ the safer the drug is to consume (Xu and Urban, 2011). Therefore as a general rule a higher IC_{50} indicates a better chance of the compound to be able to be used medicinally.

Plant extract	**IC_{50} values (µg/ml) ± CE**
E. delagoense	> 400.0
E. emarginatum	> 400.0
E. pictum	> 400.0
Actinomycin D	0.011

Table 4.1: IC_{50} (µg/ml) values for HepG2 cytotoxicity

Actinomycin D was used as the positive control to indicate the sensitivity of the cells, as it is a known toxicant. A very low IC_{50} value was obtained for Actinomycin D, 0.011 g/ml, as expected for the positive control. The results of the present study are similar to the results obtained for the actinomycin D control used by Lall *et al*. (2013). In their experiments they obtained an IC_{50} value of 0.005 g/ml, indicating comparability of results.

4.5 Conclusion

The results show that all three *Erythroxylum* species had an IC_{50} value greater than 400µg/ml, therefore no toxicity towards the HepG2 cell line. According to the experimental data from Lin and Will (2012), no significant difference in toxicity between extracts is seen for the three major toxicity routes, namely hepatotoxicity, cardiotoxicity and nephrotoxicity. Therefore the methanol/water extracts from *E. delagoense, E. emarginatum* and *E. pictum* can generally be regarded as safe. It is however very important to take into account that this method cannot accurately measure organ specific toxicity and therefore cannot replace clinical trials. This should especially be kept in mind when taking into account the cardio glycosides observed in the plants (Chapter 2). Current results justify further studies on these plants in the pursuit of finding new medicinally active chemicals.

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Chapter 5

Antibacterial screening assay

5.1 Introduction

Molecular mimicry (Figure 5.1) is a concept that has been linked to the development of autoimmunity since 1985. Since then numerous publications have supported the hypotheses making it the favoured mechanism for causing autoimmune diseases (Direct MS, 2016). An autoimmune disease occurs when an immune response is initiated towards healthy host tissue, mistaking it for harmful pathogens or irritants (Nordqvist, 2015). Molecular mimicry is an intricate concept entailing a deep understanding of the functioning immune system. The model is based on the observation that a string of amino acids from a specific protein can closely resemble a string of amino acids from another protein molecule (Cunningham, 2009).

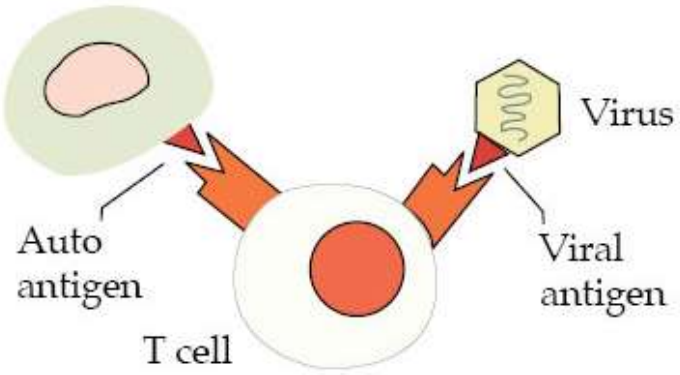

Figure 5.1: A representation of molecular mimicry where a great degree of similarity between an antigen and an endogenous structure can mistakenly trigger autoimmunity; causing antibodies to attack the host tissue (Acharya *et al*., 2010).

During an immune response inflammation increases the blood circulation around the affected area, allowing more immune cells to reach the area and consequently strengthen the immune response (The Chronie, 2015). During this process macrophage molecules will engulf an invading molecule and break it down into fragments. The macrophage will then present protein fragments of the invader on its surface. A T-cell that has a receptor that can recognise this specific amino acid sequence will bind to the fragment and so be activated. The activated T-cell then initiates other parts of the immune system to attack all proteins that has a similar amino acid sequence to the initiating sequence within them (Lawrence *et al.*, 2008). The process of inflammation should only last as long as the infection exists. If the foreign antigen is not eradicated, the helper T cells do not recognise the fact or if the immune cells receive a signal to stay-alive from an alternative source, such as with molecular

mimicry, then chronic inflammation may develop (The Chronie, 2015). It has been established that a variety of similar, yet slightly different amino acids sequences can be recognised by the same T- cell. If a T-cell recognises a foreign protein that has a similar sequence to a self-protein found naturally in the human body, cross reactivity may occur which can keep the T-cells active and trigger auto immunity (Cunningham, 2009).

An individual's genetic make-up is what predisposes some entities to developing autoimmune diseases, while leaving others resistant. A specific T-cell gene repertoire will allow the individuals immune system to recognise a part of a foreign protein sequences of which a similar sequence occur in self-proteins. Non-susceptible individuals will have a different set of T-cell genes that recognise a different part of the foreign protein of which a similar sequence does not occur in self-proteins and therefore cross-reactivity will not occur (Direct MS, 2016).

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by chronic synovitis, inflammation in surrounding tissue and sometimes other organs, bone destruction and eventually functional damage of the joints (Nordqvist, 2015). The etiopathogenesis of the disease is still not clearly understood although it has been established that the disease have both a genetic and environmental factor contributing to disease initiation. Among the known genetic factors, strong associations have been observed between RA and the presence of HLA-DR1 and HLA-DR4 haplotypes containing the sequence Gln-Arg-Arg-Ala-Ala (EQRRAA) at position 70 to 74 in the DRB1 chain (Albani *et al.*, 1995; Ebringer *et al.*, 2010). The frequency of these genes in the general population is about 35% while over 90% of individuals suffering from RA possess these genes (Ebringer *et al.*, 2010).

Evidence suggests an infectious aetiology where opportunistic infections by *Proteus mirabilis* may be responsible (Gomez *et al.*, 2012). Studies have found significantly (p 0.001) increased levels of antibodies directed to *Proteus* species in RA patients which was absent in other autoimmune diseases (Ebringer *et al.*, 2010; Tlaskalova *et al*., 2011). These findings are further supported by studies where susceptible mice strains reared in 'germ free' conditions fails to develop RA and had decreased clinical and autoimmune markers of the disease. Other supporting evidence is the close relationship observed between urinary tract infections (UTI) and RA. Rheumatoid arthritis is found 3-4 times more frequently in woman than in men, it is seen more frequent in smokers and onset is often after pregnancy, each of these cases correlate to the incident frequencies of UTI's (Ebringer, 2010; Rashid and Ebringer, 2008). Correspondingly *P. mirabilis* cultures have consistently been isolated in urine from RA patients (Cock and Van Vuuren, 2014).

P. mirabilis is a facultative anaerobic, rod shaped, Gram-negative bacterium. It is part of the normal gut microbiota and is estimated to be the cause of 90% of all opportunistic *Proteus* infections. The bacterium produces high level of urease, which converts urea to ammonia, making the urine more alkaline. If left untreated, the increased alkalinity can lead to the formation of crystals which may lead to kidney stones. The bacteria can be found throughout the kidney stones and can therefore reinitiate infection after antibiotic treatment (O'Hara *et al*., 2000). It is further reported that *P. mirabilis* contain an endotoxin which is responsible for the induction of the inflammatory response system.

It was established through computer analysis that there is a sequence similarity between *Proteus* haemolysin from amino acid position 32-36 (ESRRAL) which is almost identical in shape to the EQR(K)RAA sequence found in HLA-DR1/4. Antibodies against *Proteus* haemolysin will then cause cross reactivity towards the HLA-DR1/4 epitope and following immune activation cause inflammation with consequent tissue damage as seen in RA (Ebringer *et al.,* 2010). It has also been established that the IRRET sequence found in the *Proteus* urease molecule from amino acid position 337-341 shows molecular mimicry towards alpha-2 type XI collagen sequence LRREI from amino acid position 421-425. Therefore cross-reactive anti-bodies between *Proteus* urease and alpha-2 collagen will occur in predisposed individuals. This type of collagen is mainly found in hyaline cartilage which is found in the joints of hands and feet, cross reactive antibodies attacking this cartilage can explain the clinical features of phalangeal inflammation seen in RA (Ebringer *et al.*, 2010).

Ankylosing Spondylitis (AS) is another autoimmune disease where inflammation affects the vertebrae, muscles, ligaments and sacroiliac joints, eventually breaking down the cartilage (Nordqvist, 2015). The disease typically has an early onset affecting more men than women and has a strong genetic association with the HLA-B27 gene. A microbial agent, as cause of this disease, is supported by the study where none of the genetically identical mice from a susceptible inbred line develop AS. It was also established that mice reared under microbe-free conditions do not develop AS, but as soon as they are exposed to faeces from mice reared under normal conditions AS started to develop in the susceptible mice (Rehakova *et al*., 2000). A study by King's College in London has found molecular mimicry between the HLA-B27 gene and two surface molecules of *Klebsiella* microbes, implicating *Klebsiella pneumonia* as the microbe responsible for AS (Rashid and Ebringer, 2007). This is further supported by increased numbers of anti-*Klebsiella* antibodies isolated from patients suffering from AS (Tlaskalova *et al.*, 2011).

K. pneumonia is a Gram-negative, facultative anaerobic rod shaped bacterium that makes up a part of the normal gut microflora. Opportunistic diseases linked with this bacterium include pneumonia,

urinary tract infections, lower biliary tract infections and wound infections (Podschun and Ullmann, 1998).

It is proposed that using anti-bacterial compounds to eliminate the *P. mirabilis* infection could greatly reduce the symptoms of RA, the same is true in the case of AS and *K. pneumonia*. Getting rid of the bacteria is expected to reduce the inflammation, reduce the production of cross-reactive antibodies and so decrease disease progression (Cock and Van Vuuren, 2014). In fact, therapeutic regimens that theoretically target the intestinal flora have been used since the 1940's to treat inflammatory diseases. Several of them even became disease-modifying antirheumatic drugs (DMARDs), such as sulfasalazine and minocycline (Brusca *et al.*, 2014). Such as the case with many other bacteria however, resistance to commonly used antibiotics have been reported for both *K. pneumonia* and *P. mirabilis* (Groopman, 2008; Gonzalez, 2015).

Various researchers have investigated the ability of plants to produce compounds that have antibacterial activity. Kock *et al.* (2014) published an article indicating 23 extracts made from 13 South African plant species to have antibacterial activity against *Proteus* species. An article by De Wet, 2011 showed the potential of South African *Erythroxylaceae* species as anti-bacterial agents against various bacterial species including *K. pneumonia*, which is suspected to be the cause of AS. This not only highlights the potential of South African plant species as a source of anti-bacterial agents but the *Erythroxylum* species in particular as a promising source of anti-bacterial compounds.

In order to investigate the antibacterial capability of a plant extract INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium), which is a tetrazolium salt, can be used. The salt is able to diffuse through the bacterial membrane and change colour when it is reduced by the intracellular environment, forming a formazan dye. However if the bacterium is dead no respiratory functions will take place and therefore the salt cannot be reduced. Therefore a change to pink or red indicates living cells whereas no colour change indicates the inhibition of bacteria (Marshall *et al*., 1995).

5.2 Aim

The aim of this section was to establish whether the South African *Erythroxylum* species exhibit anti-bacterial activity towards the bacteria *Proteus mirabilis* and *Klebsiella pneumonia*; believed to be the causative agents for the two autoimmune diseases, rheumatoid arthritis and ankylosing spondylitis respectively.

5.3 Methodology

5.3.1 Minimum inhibitory concentration

The method used to determine the minimum inhibitory concentration of the plant extracts was based on the method described by Eloff (1998). Both *P. mirabilis* (TTC® 43071) and *K. pneumanoia* (ATTC® 13883) were grown on nutrient agar from quick sticks (Microbiologics, USA). Nutrient broth (Sigma Aldrich) were inoculated with each bacterium and incubated at 37°C for 24 h. Sterile 96 well plates were prepared by adding 100µl of nutrient broth to each well. This was followed by the addition of 100µl of 15mg/ml plant extract (extracted as described in chapter 2) dissolved in 5% methanol. Serial dilutions were performed. The positive control used was Tetracycline (2mg/ml), a 5% methanol solvent control and negative control was also added. The bacteria were then diluted to 3 x 108cfu/ml according to McFarland standards, before plating 100µl into each well. After 24 h incubation of the plates at 37°C p-iodonitrotetrazolium (INT) (Sigma, Austria) (2mg/ml) was added as a visualization agent. A pink/red colour indicated bacterial growth whereas no colour changes or colourless is an indication of bacterial inhibition. The experiment was performed in duplicate.

5.3.2 Active fraction identification

Methanol extracts of *E. emarginatum*, *E. pictum* and *E. delagoense* were concentrated and spotted on four identical C_{18} thin layer chromatography (TLC) plates (TLC Silica gel 60 F254, Merk). The TLC's were developed using a 7 : 3 acetonitrile : methanol eluent system (Merck). Once the eluent system was well evaporated, two of the plates were sprayed either with *P. mirabilis* (TTC® 43071) or *K. pneumonia* (ATTC® 13883) and incubated at 37°C for 24h in a moist environment. After this incubation period the plates were sprayed with INT (1mg/ml) and incubated at room temperature for 2h. White zones on the TLC plates indicated inhibition of bacteria and were isolated from the duplicate plates by using UV comparison and R_f values. The R_f values were calculated by dividing the distance travelled by the compound trough the distance travelled by the solvent front. The silica on relevant areas of the TLC plate was scratched off with a spatula and collected in an Eppendorf tube. Methanol (Merck) was added to dissolve the active compound and the mixture was filtered with a 0.2µm syringe filter. The isolated fraction was analysed using gas chromatography – mass spectroscopy. The fractions were analysed on a Shimadzu QP2010 ultra GC-MS in electron impact ionization mode (70eV). The GC was fitted with an Rxi -5ms column (30m, 0.25mm id, 0.25µm film thickness). The injector port was set at 250°C and operated in splitless mode with helium used as a carrier gas. The oven temperature was held at an initial temperature of 50°C for 2 min, then increased by 50°C/min to 225°C, held for 0.25 min,

increased by 25°C/min to 300°C and held for 5.25 min. The mass detector was operated in full scan mode, acquiring ions of m/z from 50 to 500. Compounds were identified using GC-MS solutions (Shimadzu) and the NIST 11 library with a library hit of 80% or more and comparison of mass spectral fragmentation data to library data.

5.4 Results and Discussion

5.4.1. Minimal Inhibitory concentration

The minimal inhibitory concentration (MIC) values were interpreted as follows: Below 5mg/ml was seen as having some antibacterial activity, below 2mg/ml are considered as good bacterial inhibitory agents, while MIC values below 1mg/ml are considered to be noteworthy (De Wet, 2011; Kock *et al*., 2014). Table 5.1 shows the inhibitory concentrations of the crude plant extracts as well as the alkaloid fractions of the plant extract as described in Chapter 2.

Table 5.1: MIC values (mg/ml) of South African *Erythroxylum* species extracts and alkaloid fractions on *P. mirabilis* and *K. pneumonia*

Plant	***Proteus mirabilis***	***Klebsiella pneumonia***
E. pictum	2.50	2.50
***E. pictum* alkaloids**	0.63	0.63
E. delagoense	5.00	2.50
***E. delagoense* alkaloids**	5.00	5.00
E. emarginatum	2.50	2.50
***E. emarginatum* alkaloids**	1.25	1.25
Tetracycline (positive control)	< 0.04	< 0.04
Negative control	No inhibition	No inhibition

The Tetracycline positive control was effective at killing the bacteria at all tested concentrations. The solvent control containing 5% methanol showed no bacterial inhibition as well as the negative control containing only growth media.

The alkaloid fraction of *E. pictum* was the only extract of note with inhibition of both bacteria tested at an MIC value of 0.625mg/ml. It is interesting to note that the alkaloid fraction of both *E. pictum* and *E. emarginatum* show better inhibitory activity than the crude plant fraction, but this is not the case for *E. delagoense*. The overall inhibition of *E. delagoense* is also somewhat weaker than seen in the *E. emarginatum* and *E. pictum*. This may indicate a similar compound in the alkaloid fractions of

E. emarginatum and *E. pictum*, not found in the *E. delagoense*, that could explain the inhibitory results observed. The concentration of active compounds could also play a role in the observed activity.

When comparing the MIC values of the methanol extracts of *E. emarginatum*, *E. delagoense* and *E. pictum* for the inhibition of *K. pneumonia* it is less remarkable than found by De Wet, 2011. De Wet found MIC values with the methanol extracted leaves of 0.5mg/ml, 0.5mg/ml and 0.25mg/ml respectively. This could indicate that the compound may only be present under stressed conditions or be linked to seasonal or other variations. It is important to take note of this variation in results as it may play a critical role in further development of a commercially viable product.

5.4.2. Active fraction identification

The active fractions were clearly visible on the TLC plates and easily isolated on the duplicate plates. Through the analysis of the inhibitory fractions *via* GC-MS a number of compounds; likely responsible for the bactericidal or bacteriostatic effects were identified. Each compound's reported antibacterial activity was investigated after analytical artefacts were eliminated. Thirteen constituents were detected from *E. delagoense* (Table 5.2), 43 from *E. emarginatum* (Table 5.3) and 27 from *E. pictum* (Table 5.4), all with a hit percentage above 90% identity to the NIST 11 library. The fact that only 13 compounds were identified in the *E. delagoense* fraction and only 6 have been reported to show antibacterial activity could to some extent explain the less potent activity seen by *E. delagoense*. *E. emarginatum* and *E. pictum* in comparison have more constituents reported in their active fractions correlating to their smaller MIC values.

Table 5.2: GC-MS identified constituents in the antibacterial fraction of *E. delagoense*

Compound	Retention time	% Peak area	Previous antibacterial activity reported	Reference to previously reported antibacterial activity
Undecane	5.105	0.24	No	
3-Hexadecene, (Z)-	6.515	0.99	No	
1-Hexadecanol	6.741	1.22	Yes	Togashi *et al.*, 2007
Myristic acid	6.947	1.31	Yes	Agormoorthy *et al.*, 2007
Tetratetracontane	7.046	2.09	No	
Decane, 2,3,5,8-tetramethyl-	7.078	1.58	No	
Phytol, acetate	7.173	5.39	Yes	Rajab *et al*., 1998
Hexadecanoic acid, methyl ester	7.418	5.92	Yes	Shettima *et al*., 2013
Oleic acid methyl ester	7.977	3.58	No	
Methyl stearate	8.040	3.04	Not active	Kabara *et al*., 1972
Benzoic acid, pentadecyl ester	8.684	2.70	No	
4-Methoxy cinnamic acid	8.750	9.05	Yes	Rahman and Moon, 2007
Benzoic acid, tetradecyl ester	9.022	3.85	No	

Table 5.3: GC-MS identified constituents in the antibacterial fraction of *E. emarginatum*

Compound	Retention time	% Peak area	Previous antibacterial activity reported	Reference to previously reported antibacterial activity
Undecane	5.183	0.89	No	
+ Camphor	5.461	1.05	Yes	Gupta and Saxena, 2010
1-Undecanol	6.319	0.48	Yes	Togashi *et al.*, 2007
Lauric acid	6.503	0.62	Yes	Agormoorthy *et al.*, 2007
1-Pentadecene	6.510	0.53	No	
E-14-Hexadecenal	6.550	0.70	No	
1-Hexadecene	6.564	0.64	Yes	Belakhdar *et al.*, 2015
9-Octadecene, (E)-	6.570	0.48	No	
1-Hexadecanol	6.766	1.08	Yes	Togashi *et al.*, 2007
α-Pinene, 10-(dimethylaminomethyl)-	6.826	6.15	No*	
Myristic acid	6.975	1.09	Yes	Agormoorthy *et al.*, 2007
Farnesane	7.078	1.21	Yes	Vuddhakul *et al.*, 2007
Tetratetracontane	7.096	0.87	No	
i-Propyl 12-methyl-tridecanoate	7.131	1.03	No	
Tetradecanoic acid, dodecyl ester	7.139	1.02	No	
Phytol, acetate	7.189	9.48	Yes	Rajab *et al.*, 1998
Phytol	7.188	6.37	Yes	Natural Agricultural Library, 2016
Pentadecanoic acid	7.244	0.66	Yes	Natural Agricultural Library, 2016
Citronellyl propionate	7.256	1.79	Yes	Sestras *et al.*, 2012
1-Eicosanol	7.297	7.47	Yes	Karthi *et al.*, 2015
Methyl palmitate	7.415	3.27	Not active	Roller, 2003
Pentadecanoic acid, 14-methyl-, methyl ester	7.426	2.55	Yes	Akpuaka *et al.*, 2013
Oleic acid	7.491	0.69	Yes	Kabara *et al.*, 1972

Palmitic acid	7.536	6.77	Yes	Agormoorthy *et al.*, 2007
Isopropyl palmitate	7.717	1.52	Not active	Cardoso *et al.*, 2006
Hexadecen-1-ol, trans-9-	7.925	26.11	No	
Oleic acid, methyl ester	7.981	2.24	No	
Methyl stearate	8.040	2.55	Not active	Kabara *et al.*, 1972
Heneicosyl acetate	8.298	0.85	No	
N-Methoxydiacetamide	8.327	2.43	No	
Benzoic acid, tridecyl ester	8.354	2.11	No	
Benzoic acid, pentadecyl ester	8.685	2.48	No	
4-Methoxy cinnamic acid 2-ethylhexyl ester	8.745	12.74	No	
Benzoic acid, tetradecyl ester	9.022	3.58	No	
Ethanol, 2-(octadecyloxy)-	9.235	5.14	No	
1-Decanol, 2-octyl-	9.237	6.01	No	
Oxalic acid, 2-ethylhexyl pentadecyl ester	9.266	2.39	No	
Benzene, (2-nitropropyl)-	9.298	20.02	No	
Benzene, (5-iodopentyl)-	9.300	11.74	No	
Pentacosane	9.601	3.95	Yes	Konovalova *et al.*, 2013
Octocrylene	10.099	2.56	No	
Supraene	10.713	18.91	Yes	Tjan, 2011
Cholest-5-en-3-ol (3β)-, carbonochloridate	11.519	2.20	Yes	Natural Agricultural Library, 2016

*non derivatised form active

Table 5.4: GC-MS identified constituents in the antibacterial fraction of *E. pictum*

Compound	Retention time	Peak area	Previous antibacterial activity reported	Reference to previously reported antibacterial activity
E-14-Hexadecenal	6.555	0.57	No	
Pentadecanoic acid	6.968	0.92	No	
Farnesane	7.098	1.09	Yes	Vuddhakul *et al.*, 2007
i-Propyl 12-methyl-tridecanoate	7.133	1.31	No	
Phytol, acetate	7.200	14.99	Yes	Rajab *et al.*, 1998
Phytol	7.256	2.36	Yes	Natural Agricultural Library, 2016
Cyclododecanol	7.258	2.14	No	
Pentadecanal-	7.266	3.94	Yes	Selvin *et al.*, 2009
1-Hexadecanol	7.312	8.01	Yes	Togashi *et al.*, 2007
Oxirane, tetradecyl-	7.324	9.53	No	
Pentadecanoic acid, 14-methyl-, methyl ester	7.428	2.39	Yes	Akpuaka *et al.*, 2013
Methyl palmitate	7.438	5.26	Not active	Roller, 2003
Palmitic acid	7.532	4.46	Yes	Agormoorthy *et al.*, 2007
Isopropyl palmitate	7.715	1.64	Not active	Cardoso *et al.*, 2006
Hexadecen-1-ol, trans-9-	7.923	11.57	No	
N-Methoxydiacetamide	8.331	4.07	No	
Benzoic acid, tridecyl ester	8.351	1.63	No	
Benzoic acid, tetradecyl ester	8.354	0.85	No	
4-Methoxy cinnamic acid 2-ethylhexyl ester	8.746	12.41	No	
1-Decanol, 2-hexyl-	9.236	5.34	No	
Tritetracontane	9.236	2.18	No	
Ethanol, 2-(octadecyloxy)-	9.239	6.43	No	
Benzoic acid, pentadecyl ester	9.383	2.56	No	
Supraene	10.709	20.12	Yes	Tjan, 2011

5. Conclusion

Some antibacterial activity was observed for all three *Erythroxylum* species' alkaloid extracts. The *E. pictum* alkaloid fraction showed the best antibacterial activity with an MIC value of 0.625mg/ml for both bacterial species tested. With the use of GC-MS some of the compounds possibly responsible for the anti-bacterial activity observed could be identified. 4-Methoxy-cinnamic acid, hexadecen-1-ol, trans-9- and benzene(5-iodopentyl) were the compounds that made up the biggest relative percentage of the antibacterial fraction for *E. delagoense, E. emarginatum* and *E. pictum* respectively. Only 4-methoxy-cinnamic acid out of the three compounds, have been reported in literature to exhibit antibacterial activity (Rahman, 2007). Numerous other compounds identified with GC-MS present in smaller quantities in the plant extracts have been found, according to literature, to be active as antibacterial agents. However a number of the identified compounds have no literature report of antibacterial activity tested on the compound.

Antibacterial activity of the three methanol plant extracts against *K. pneumonia* was found to be less effective than the methanol extracts reported by De Wet, 2011. This could be due to a number of environmental factors or stresses. It may be interesting to evaluate the antibacterial activity of the plants under different stress conditions. Fluctuation in the chemical profile and biological activity may indicate which compounds play a bigger role in the antibacterial activity observed. This can lead to the isolation of the most active compound or compounds that may exhibit synergistic interactions.

According to Cock and Van Vuuren, 2014, it is expected that the prognosis of autoimmune diseases can be improved by the elimination of the bacterial agents that is proposed to initiate these diseases. The *Erythroxylum* species have been found to show good antibacterial action against two opportunistic pathogens linked to autoimmune diseases. The findings by De Wet, 2013, also indicate good antibacterial activity by these species. Therefore *E. delagoense*, *E. emarginatum* and *E. pictum* show promise in being utilised in the prevention and early treatment of autoimmune diseases such as rheumatoid Arthritis and Ankylosing Spondylitis.

Future studies can focus on the non-volatile fraction of antibacterial agents found in the plants, as well as considering the influence that stresses or environmental factors can have on the chemical profile and biological activities of the plant extracts. Various compounds identified via GC-MS have no literature evidence as being tested for antibacterial activity; this is another possible focus point.

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Chapter 6

Antioxidant and anti-inflammatory activity

6.1 Introduction

Inflammation is a local response to cellular injury that is marked by blood vessel dilatation, redness, heat, and pain and that serves as a mechanism initiating the elimination of harmful agents and of damaged tissue. The process is regulated by cytokines, which is defined as any of a class of immunoregulatory proteins that are secreted by cells especially of the immune system (Longmore *et al.*, 2014).

Oxygen derived free radicals are known to play an important role in the pathogenesis of chronic inflammatory disorders (Ratheesh *et al*., 2009). Oxidative stress is a condition characterised by an elevation in cellular reactive oxygen species, causing cellular damage via the process of oxidation, due to impairment in the balance between oxidants and antioxidants.

Free radicals are molecules that have unpaired electrons in their outer orbits; due to this configuration they are extremely reactive. The radicals will react with adjacent molecules, "stealing" an electron to achieve a paired electron configuration, resulting in cellular instability. This leaves the adjunct molecule with an unpaired electron, in turn creating a new free radical (Milam *et al*., 1998). In this fashion a destructive chain reaction is established stopping only when two radicals react.

Antioxidants are molecules that inhibit this chain reaction by either accepting or donating an electron, effectively neutralizing the free radical. Often the antioxidant will become a free radical itself, but are less reactive and therefore less dangerous than the neutralized free radical. This is because antioxidant molecules regularly contain an aromatic ring structure that can delocalise the unpaired electron, stabilizing the configuration (Lu *et al*., 2010).

Under normal physiological conditions, a fine balance exists between free radical production and free radical scavenging mechanisms; as it has been shown that free radicals act as secondary messenger molecules in the catabolic pathway of arachidonic acid and the turnover of extracellular matrix. Some gene regulation may also be subject to free radical control (Ratheesh *et al*., 2009). However if these free radical mediated reactions proceed unchecked, or accumulation of free radicals occur, it could be extremely damaging to tissues, especially if maintained over a long period of time (Reuter *et al*., 2010). Radicals are able to destroy membrane lipids, proteins, DNA, hyaluronic acid and cartilage resulting in a diseased state as result of membrane and cellular instability (Ratheesh *et al*., 2009; Milam *et al*., 1998).

Various studies have shown the contribution of oxidative stress in rheumatoid arthritis (RA) as both an inflammatory mediator, as it releases arachidonic acid, as well as having a role in the destruction of the joints through direct tissue damage (Jikimoto *et al*., 2001). A study by Jikimoto *et al*. (2002) showed that natural antioxidant levels correlated with disease activity and almost normalized when the disease state was well controlled by therapy. Another study found that the oxidative damage found in RA is much higher compared to normal or osteoarthritis patients (Babior, 2000). Furthermore it has been demonstrated that levels of reactive oxygen species, superoxides and reactive nitrogen species are increased in the synovial fluid (naturally occurring joint-space fluid) of RA patients (Tak *et al*., 2000). This supports the observation that RA patients suffer from high levels of oxidative stress, especially in the active stages of the disease, as a result of the underlying inflammatory process.

Several factors contribute to the development of oxidative stress in the joints of RA patients. The production of nitric oxide (NO) is a distinguishing factor in rheumatoid synovial tissue compared to non-immune related arthritis models. Nitric oxide levels are increased substantially by inducible NO synthase (iNOS) after stimulation by cytokines or bacterial products (Tak *et al*., 2000)

Reactive oxygen and reactive nitrogen species have been shown to inhibit type II collagen and proteoglycan synthesis and enhance the breakdown of hyaluronic acid and chondroitin sulphate (Hitchon & El-Gabalawy, 2004). All four are key components of cartilage synthesis and construction, explaining the RA symptoms of joint degeneration and loss of function over time. This can be viewed as a double edged sword in the pathology of RA, as it not only damages the normal tissue via oxidative stress, but prevents regeneration and remodelling of new tissue.

Lipid peroxidation, induced by oxygen free radicals, indirectly activates the inflammatory pathway through a protein kinase C-independent pathway. This deterioration of unsaturated fatty acids activates the release of cytokines and membrane bound arachidonic acid (Moreno, 2003). The free arachidonic acid is converted by the two isoforms of the cyclooxygenase (COX) enzymes into prostaglandins (Wieslander *et al*., 1998; Jaswal *et al*., 2003; Ratheesh *et al*., 2009). Prostaglandins are ubiquitous compounds that are involved in a variety of inflammatory and homeostatic processes throughout the body. COX-1 and COX-2, while similar in structure, have very different actions. COX-1 mediates physiological functions such as protection of the stomach lining (via mucus production) and is constitutively expressed within most tissues, while COX-2 is an inducible enzyme that is up-regulated in response to mitogenic or inflammatory stimuli (Awad *et al*., 2004).

Furthermore oxidative stress causes enhanced activation, signal transduction and gene expression of other pro-inflammatory cytokines, enhancing the inflammatory process and playing a role in inflammatory and neurological pain. Nociceptors, also referred to as pain receptors, sense tissue damage through these cytokines as well as other released chemicals, initiating inflammatory pain. If persistent it could become extremely debilitating (Murphy, 2012). In response to these inflammatory conditions an excess of reactive oxygen species and reactive nitrogen species are released (Tak *et al*., 2000). This sustained inflammatory/oxidative environment leads to a proliferative cycle, which damages healthy neighbouring cells (Reuter *et al*., 2010)

Currently RA is managed with steroidal and non-steroidal drugs to slow deterioration, by inhibiting the inflammatory response by targeting the COX enzyme and in so doing lessening pain and preserving the functionality of joints. Prostaglandin synthesis inhibition is at the centre of current anti-inflammatory therapies, and with the COX enzyme primarily responsible for prostaglandin synthesis it is the main inhibitory target. These drugs however have various long term side effects, mainly due to non-selective COX inhibition, emphasizing the importance to find alternatives for the management of the disease (Ratheesh *et al*., 2009). Thus, direct inhibition of the COX-2 enzyme is seen as an efficient pharmacological approach to treat inflammation, as exemplified by the active development of COX-2 inhibitors such as celecoxib (Deeks *et al*., 2002). Studies have shown that COX-2 inhibitors isolated from plants may have an advantage to chemically synthesized COX-2 inhibitors that has thus far reached the market. Selectivity for the COX-2 enzyme seems to be greater, accompanied with less risk of long term side effects (Bruno, 2009).

The central role that oxidative stress plays in the diseased state suggests the necessity for co-administration of antioxidants with the conventional treatments. This is supported by clinical trials that have reported the benefit of using antioxidant vitamins as adjunct therapy in RA (Jaswal *et al*., 2003) Early antioxidant intervention may help retard disease progression and significantly reduce damage while preserving joint function (Tak *et al*., 2000). Despite the evidentiary support prescribing antioxidants as a co-pharmacotherapy for RA is not common practice.

Numerous plants, such as *Anethum graveolens* seeds, have radical scavenging activity and are commonly used in traditional medicines for the management of RA (Kazemi, 2015). The polyphenolic fraction of green tea (*Camellia sinensis*), containing potent antioxidants, has been shown to prevent collagen-induced arthritis (Hitchon & El-Gabalawy, 2004). The beneficial effects seem to be due to epigallocatechin-3-gallate. Furthermore ROS-induced COX-2 expression is decreased by natural antioxidants such as resveratrol, while various plant phenols inhibit COX activity (Moreno, 2003). Wesam *et al*. (2003) found aqueous leaf extracts of *Erythroxylum cuneatum*

to have antioxidant properties, while it has been documented that the leaves and roots of *E. emarginatum* are traditionally used for pain relief (Nishiyama *et al*., 2007). This may be an indication that the South African *Erythroxylum* species possess radical scavenging activity that could potentially aid in the relief of oxidative stress in RA patients and possibly also possess a secondary mechanism of inflammatory pain relief through COX inhibition.

6.2 Aim

Because of the important facilitating role that oxidation plays in the advancement of inflammation and therefore inflammatory pain, the aim was to see if the South African *Erythroxylum* species have the potential to reduce oxidative stress; having a protective effect on the cells and thereby reducing inflammation and inflammatory pain. Secondly it was the aim to try and determine the compounds responsible for the antioxidant activity in the plants. Lastly the aim was to evaluate the potential for the species to directly inhibit the inflammatory process by inhibiting the COX enzyme, determining the selective COX-1 or COX-2 inhibition exhibited by the plant extracts.

6.3 Methodology

6.3.1. Antioxidant activity

6.3.1.1. Minimum Effective Concentration

E. emarginatum, E. pictum and the *E. delagoense* were extracted as decribed in chapter 2, and tested in triplicate for their antioxidant activity making use of a 1,1-diphenyl-2-picryl hedrazyl (DPPH) screening assay as described in Du Toit *et al.* (2001) with ascorbic acid (Merck) as the positive control. 20µl of extract and 200µl of distilled water were pipetted into a 96 well plate and serially diluted after which 90µl of a 0.04µg/ml DPPH solution was added to each well. Plant extract, serially diluted with the distilled water, but without the addition of DPPH were added as colour controls. The plates were incubated for 30 min in a dark room and read at 515nm.

6.3.1.2. Active Fraction Identification

Methanol extracts (see Chapter 2) of *E. emarginatum*, *E. pictum* and *E. delagoense* were concentrated and spotted on two identical C_{18} thin layer chromatography (TLC) plates (TLC Silica gel 60 F254, Merk). The TLC's were developed using methanol as the eluent system (Merck). Once the eluent system was evaporated, one of the plates was sprayed with 0.2% DPPH. White zones on the

TLC plates indicated antioxidant activity and were isolated from the duplicate plate by using UV comparison and RF values. The isolate was dissolved in methanol (Merck) and the silica gel filtered off using a 0.2µm filter. The isolated fraction was analysed using gas chromatography – mass spectroscopy. The fractions were analysed on the Shimadzu QP2010 ultra GC-MS in electron impact ionization mode (70eV). The GC was fitted with a SH-Rxi -5ms column (30m, 0.25mm id, 0.25µm film thickness). The injector port was set at 250°C and operated in splitless mode with helium used as a carrier gas. The oven temperature was held at an initial temperature of 50°C for 2 min, then increased by 50°C/min to 225°C, held for 0.25 min, increased by 25°C/min to 300°C and held for 5.25 min. The mass detector was operated in full scan mode, acquiring ions of m/z from 50 to 500. Compounds were identified using GCMS solutions (Shimadzu) and the NIST 11 library with a library hit of 80% or more.

6.3.2 Anti-inflammatory

6.3.2.1. COX inhibition screening assay

The EIA based COX inhibition screening assay (Kit item no. 560131) was conducted as described by the user manual (Cayman Chemical, 2017). Recombinant COX-1 and COX -2 (human) enzymes were pre-incubated with the plant extract samples for 10 min at 37°C. The reaction was started by the addition of 10µl arachidonic acid and allowed to proceed for 2 min. The reaction was terminated by the addition of 1M HCl solution containing $SnCl_2$. The reaction mixture were transferred to the antibody coated microtiter plate and incubated at 23˚C for 18 h to allow binding to the antibodies. After the plate was washed, Elman's reagent was added and the colour intensity measured at 210 nm by a Power wave XS (Bio-tek) spectrophotometer after 90 min. Acetylsalicylic acid (Sigma) was used as a standard. Plant samples were tested in duplicate at five concentrations. Plant samples were dissolved in methanol as recommended by the kit protocol.

6.4 Results and Discussion

6.4.1 Antioxidant activity

All three plant samples showed promising antioxidant activity when compared to ascorbic acid which is seen as the golden standard. In literature ascorbic acid has an EC_{50} of approximately 4µg/ml. In these experiments the EC_{50} of ascorbic acid was established at 9µg/ml. *E. emarginatum* and *E. pictum* showed the most promising results with an EC_{50} value of 3µg/ml and 5µg/ml respectively.

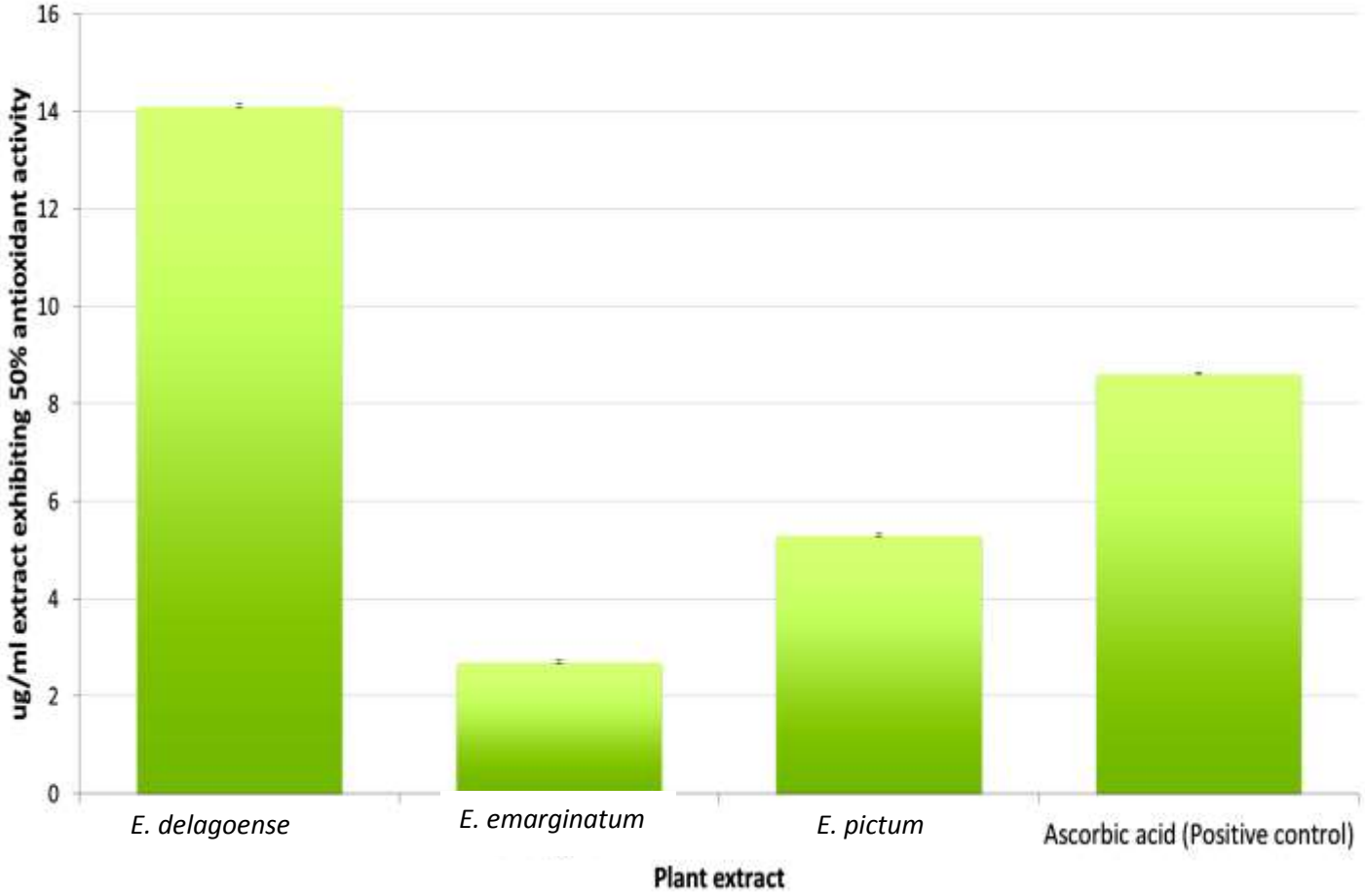

Figure 6.1: Anti-oxidant EC_{50} values for the South African *Erythroxylum* species

Each of the extracts was then subjected to TLC, from where the compounds in the active fraction were isolated and analysed via GC-MS. The tables below (Table 6.1, 6.2 and 6.3) indicate the compounds identified in each active fraction and any previously reported antioxidant activity of the compound.

Table 6.1: GC-MS results of *E. emarginatum* active antioxidant fraction isolation from TLC, with all hits above 85% identity

Compound	Retention time (min)	Peak area	Previous antioxidant activity reported	Source
1-Hexanol-2-ethyl	3.212	220380	N	
Undecane	3.434	526126	Y	Hong *et al.,* 2010
Benzoic acid methyl ester	3.46	108263	Y	Yoon *et al.*, 2006
2,4-Di-tert-butylphenol	4.649	1196776	Y	Abubakar and Majinda, 2015
Methyl palmitate	5.743	183549	Y	OECD SIDS, 2001
Benzenepropanoic acid, 3,5-bis(1,1-dimethyl)-4-hydroxy-, methyl ester	5.843	505434	N	
Cyclopropaneoctanal, 2-octyl	6.213	323227	Y	The chemical company, 2014
Methyl stearate	6.27	451075	Y	Chemicalland21, 2016
Phenol,2,2-methylenebis[6-(1,1-dimethylethyl)-4-methyl-	7.103	596556	N	

Table 6.2: GC-MS results of *E. pictum* active antioxidant fraction isolation from TLC, with all hits above 85% identity

Compound	Retention time (min)	Peak area	Previous antioxidant activity reported	Source
Ethane, 1,1,2,2-tetrachloro	2.824	1043752	N	
1-Hexanol, 2-ethyl	3.184	1196008	N	
Undecane	3.408	423179	Y	Hong *et al.*, 2010
Benzoic acid methyl ester	3.437	1066360	N	
1,2-Ethanediol, 1-phenyl-	4.075	370920	Y	Yehye *et al.*, 2015
Phenol,2-(1,1-dimethylethyl)-4-methyl-	4.181	500344	N	
Cyclodecane	4.276	237958	N	
Ethanol,2,2-dithiobis	4.426	522337	Y	Yoon *et al.*, 2006
2,4-Di-tert-butylphenol	4.471	5752940	Y	Yoon *et al.*, 2006
2,4-Di-tert-butylphenol	4.645	17279625	N	
E-14-hexadecenal	4.841	541781	Y	Sugihara *et al.*, 1993
Diphenylamine	5.022	1465662	Y	Kumari *et al.*, 2016
Methyl tetradecanoate	5.202	1401292	Y	Chandar and Ramasamy, 2016
1-Octadecene	5.387	958255	Y	OECID SIDS, 2001
Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy,	5.699	1909679	Y	Abubakar and Majinda, 2015

methyl ester				
Methyl palmitate	5.742	10508618	N*	
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	5.808	954437	Y	OECD SIDS, 2001
Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy, methyl ester	5.84	6999163	Y	Smaoui *et al.*, 2011
1-Nonadecene	6.166	2718146	Y	Abubakar and Majinda, 2015
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	6.207	21227049	Y	The chemical company, 2014
Methyl stearate	6.268	9590663	Y	Venkataramani and Sasikumar, 2012
E-15-heptadecenal	6.473	1095237	N	
N,N-dimethyldecanamide	6.624	2627888	N	
Benzenamine, 4-nitro-N-phenyl	6.735	2091695	N	
Eicosanoic acid, methyl ester	6.781	1005420	Y	National Agricultural Library, 2016
Gamma-sitosterol	10.396	2388996	Y	Kim *et al.*, 2013
Stigmasta-5,24(28)-dien-3-ol, (3beta,24Z)	10.492	1225963	N	Yehye *et al.*, 2015

* Breakdown product of antioxidant molecule

Table 6.3: GC-MS results of *E. delagoense* active antioxidant fraction isolation from TLC, with all hits above 85% identity

Compound	Retention time (min)	Peak area	Previous antioxidant activity reported	Source
1-Butanol, 3-methyl-	2.522	4088947	N	
2,3-Butanediol	2.573	5779729	N	
Ethanol, 2-butoxy-	2.806	3142464	Not active	Pomierny *et al.*, 2014
Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	4.33	12391334	N	
Benzoic acid, 4-ethoxy-, ethyl ester	4.843	4702228	N	
Hexadecanoic acid, methyl ester	6.363	5649575	Y	Abubakar and Majinda, 2015
D-Glucose, 2-O-methyl-, diethyl mercaptal	6.974	13518001	Y	National Agricultural Library
Alpha.-Tocopherol	7.478	2949354	N	
Benzamide, 2-hydroxy-N-[2-(tricyclo[3.3.1.1(3,7)]dec-1-yloxy)ethyl]-	7.85	409802	Y	National Agricultural Library
(2E,4E)-N-Isobutyltetradeca-2,4-dienamide	7.965	12664615	Not active	Amin and Hashem, 2012
(2E,4E)-N-Isobutyltetradeca-2,4-dienamide	8.44	6901206	N	Pomierny *et al.*, 2014
1-Dodecanone, 2-(imidazol-1-yl)-1-(4-methoxyphenyl)-	8.776	6566534	N	

Beta-sitosterol	9.302	2711000	Y	Paniagua-Pérez *et al.*, 2008
Deltamethrin	9.554	14984716	Not active	Abubakar and Majinda, 2015
Olean-12-en-3-ol, acetate	9.65	11677589	N	

These tables show a number of known antioxidants, but also numerous compounds that have never been tested as antioxidants. The compound making up the biggest portion of the *E. emarginatum* antioxidant active fraction, 2,4-di-tert-butylphenol, is used industrially as UV stabilizer and antioxidant for hydrocarbon-based products ranging from petrochemicals to plastics. The toxicity of this compound is very low with an LD_{50} of 9.2g/kg (Fiege *et al*., 2002). Sitosterol was identified in both the *E. delagoense* as well as the *E. pictum*. According to a Mexican study by Paniagua-Perez *et al*. (2008), beta-sitosterol dose-dependently reduced the level of free radicals in mice by up to 78%. Another study by Hitchon & El-Gabalawy, 2004 showed that prostaglandin-2 release was decreased 68% by beta-sitosterol. Therefore this compound shows dual potential of reducing inflammation and inhibiting the cytokine release responsible for pain and further inflammatory propagation. Interestingly according to the same study supplementation of the antioxidant vitamin E, present in the *E. delagoense*, has no effect on RA disease activity but did improve pain, suggesting a role in central analgesia mechanisms (Hitchon & El-Gabalawy, 2004).

Numerous double-blind clinical trials conducted since 1998 have shown ginger (*Zingiber officinale*) to be effective in relieving inflammation, pain and stiffness in patients suffering from RA, osteoarthritis and muscular discomfort. Patients that consumed ginger or ginger extracts daily for two and a half years reported no side effects (Srivastava and Mustafa, 1992). Purified 10-gingerol, 8-shogaol and 10-shogaol isolated from ginger, showed no COX-1 inhibition, but inhibited COX-2 with IC_{50} values of 32µM, 17.5µM and 7.5µM, respectively (Breemen *et al*., 2011). Recent articles where South African plants have been screened for COX-2 inhibition activity has revealed potential for future anti-inflammatory drug development from these plants. Some of the best lead plants include *Agapanthus campanulatus* showing the highest COX-2 inhibition (83.7%) at 62.5µg/ml (Fawole *et al*., 2009). A low IC_{50} value of 12.66µg/ml was exhibited by the *Leucosidea sericea*. It is believed that it may be certain alkaloids present in the leaf of the *L. sericea* that is responsible for the COX-2 inhibiting properties (Aremu *et al*., 2010).

6.4.2. Anti-inflammatory

The COX inhibition results reported below in Table 6.4 are the IC_{20} results as an inhibition of 50% could not be obtained from the concentrations tested. The ratio of COX 2 vs COX 1 inhibitory activity is an indication of the selective inhibition of COX 2.

Table 6.4: 20% COX inhibition reported for each of the three crude plant extracts and their COX 2-COX 1 ratio

	COX 1 (ug/ml)	**COX2 (ug/ml)**	**COX2 / COX1 Ratio**
E. emarginatum	4.35 ± SD 1.88	1.38 ± SD 1.11	0.318
E. delagoense	Data out of range	Data out of range	
E. pictum	1.45 ± SD 1.33	10.007 ± SD 6.02	6.900

The extracts of *E. pictum* and *E. emarginatum* showed good COX inhibition, when comparing the results obtained to those published previously such as that of *Leucosidea sericea* and *Zingiber officinale. E. emarginatum* also showed preferential COX-2 inhibition to COX-1 inhibition, indicating that fewer side effects can be expected to traditional non-steroidal anti-inflammatory drugs (NSAIDS). The raw values obtained from *E. delagoense* were not in working range of the ELISA kit and could not be used. This is speculated to be due to interferences such as a large amount of phenolic compounds. When this sample is retested it is recommended to investigate the use of solid phase extraction to reduce the phenolic content of the sample. The next step would be to isolate compounds from the extracts, as pure compounds may exhibit better IC_{50} values and possibly even better COX-2/COX-1 ratios. Purified fractions may potentially also solve the interference seen in the *E. delagoense* sample. Literature has already shown one compound, beta-sitosterol a phytosterol widely found in vegetable oils, nuts and avocados to have activity as both an antioxidant as well as a

prostaglandin inhibitor. It would therefore be worth doing the isolation and testing of the compounds identified via GC-MS in the antioxidant active fraction.

6.5 Conclusion

Rheumatoid arthritis is characterized by increased generation of oxidants which leads to the failure of antioxidant defence mechanism to keep pace with oxidant generation (Jaswal and Metha, 2003). Since RA has no cure, antioxidant pharmacotherapy has emerged as a potential remedy (Wieslander *et al*., 1998).

All three extracts exhibited very good antioxidant activity, compared to the golden standard ascorbic acid which had an EC_{50} value of 9µg/ml. *E. emarginatum,* especially showed good results with an EC_{50} of 3µg/ml. *E. pictum* and *E. delagoense* showed EC_{50} values of 5µg/ml and 14µg/ml respectively. Various known and as of yet untested compounds have been identified as possible ani-oxidant molecules, responsible for these results. *E. emarginatum* also showed very good COX inhibitory results with selectivity to the COX-2 enzyme. An EC_{20} of 1.38µg/ml was obtained for COX-2 inhibition with a COX-2/COX-1 ratio of 0.318 indicating COX-2 selectivity.

These results are very promising, showing that the South African Erythroxylums, especially *E. emarginatum* should be further investigated and developed for the possible treatment of inflammatory diseases.

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Chapter 7

General Discussion and Conclusions

Now more than ever, in a society where inflammatory diseases are quickly becoming the number one health risk worldwide (Goh and Midwood, 2011) we are in dire need of remedies that do not pose a long term risk but are effective in reducing both the inflammation and debilitating pain that accompanies it. Plant extracts seem to be the way forward, as they have been shown to have better COX-2 selectivity and good oxidant scavenging abilities (Bruno 2009). The southern African *Erythroxylum* species, especially *E. emarginatum* seems to check all the boxes when it comes to a disease modifying agent for rheumatoid arthritis.

The extract displayed excellent anti-oxidant activity that will reduce inflammatory pain, long term damage to the cartilage as well as inhibit the proliferation of the inflammatory cascade. The extract exhibited fairly good COX inhibition when compared to other plant extracts that has been reported as having high potential, as well as exhibiting COX-2 selectivity (Breemen *et al*., 2011). This will reduce side-effects observed when using non-selective COX inhibiting agents such as the NSAIDS over an extended period of time. All three of the extracts showed relatively good antibacterial results towards the suspected causative agents in the initiation of inflammatory diseases such as rheumatoid arthritis as well as ankylosing spondylitis. By eliminating the causative agent the inflammatory process can be brought under control quicker. When tested on Hep-G2 cell lines none of the extracts exhibited any toxic activity towards the cells, showing preliminary safety of the extracts. Therefore these extracts seem to target the disease from multiple angles as shown in the results.

A very interesting discovery was the presence of mutated versions of the *E. coca BAHD7* and *BAHD8* genes in *E. emarginatum*. High levels of conservation were seen, despite the distance between the two phylogenetic branches. Point mutations in the genes however, explain the lack of cocaine in the *E. emarginatum.* Future studies should firstly establish if the *BAHD7* and *BAHD8* genes present in *E.*

emarginatum codes for functional proteins, explaining the high level of conservation observed. The presence of the *BAHD7* and *BAHD8* genes in other members of the Erythroxylum family should also be established. The level of similarity between the genes from each species can contribute to our understanding of the evolution of these genes and ultimately cocaine synthesis within certain members of the Erythroxylum genus.

Furthermore numerous active and untested compounds have been identified in this study for antibacterial activity as well as antioxidant activity. These compounds needs to be isolated and tested for activity in order to narrow down the effective compounds and their mechanism of action.

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Appendix 1

Phytochemical composition

Table A1.1 Herbarium specimen voucher numbers

Plant	**Voucher number**
Erythroxylum pictum	120711
Erythroxylum emarginatum	120712
Erythroxylum delagoense	120713

Table A1.2 Solvent systems used with the C_{18} reverse phase column

Solvent system	**Volume**
H_2O: MeOH 50:50	50 ml
H_2O: MeOH 25:75	50 ml
MeOH 100	750 ml
MeOH: THF 75:25	120 ml
MeOH: THF 50:50	250 ml
MeOH: THF 25:75	40 ml
Hexane 100	140 ml

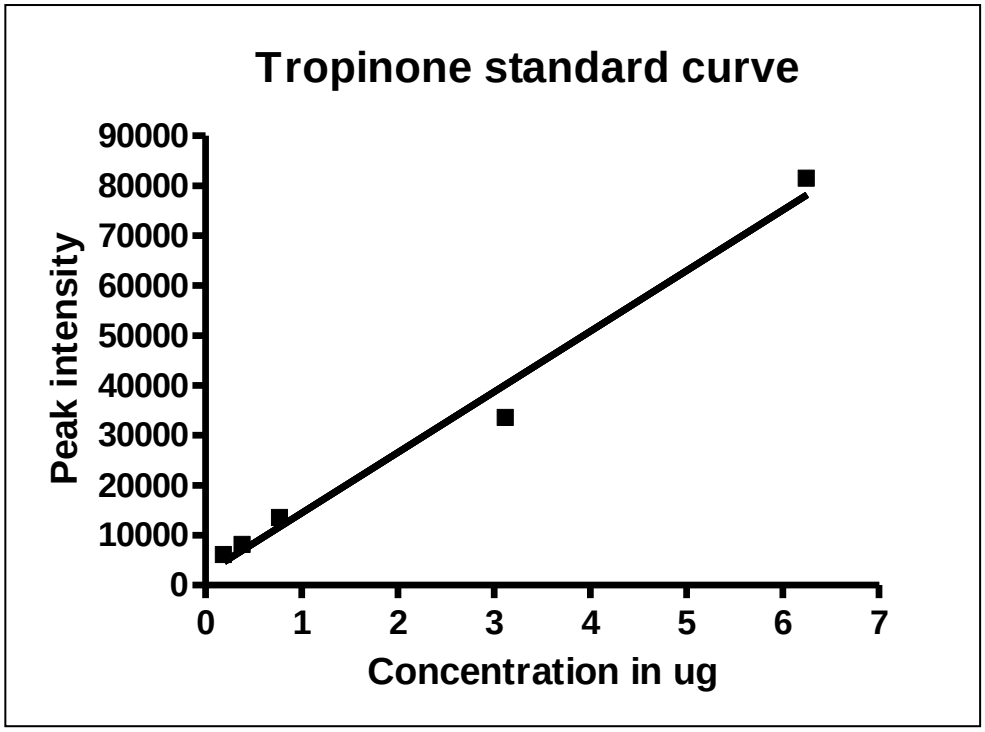

Figure A1.1 Tropinone Standard curve

Table A1.3 Goodness of fit values for tropinone standard curve

Descriptor	**Value**
r^2	0.9983
P-value	< 0.0001
Deviation from zero	significant
Number of values	6
Number of missing values	0

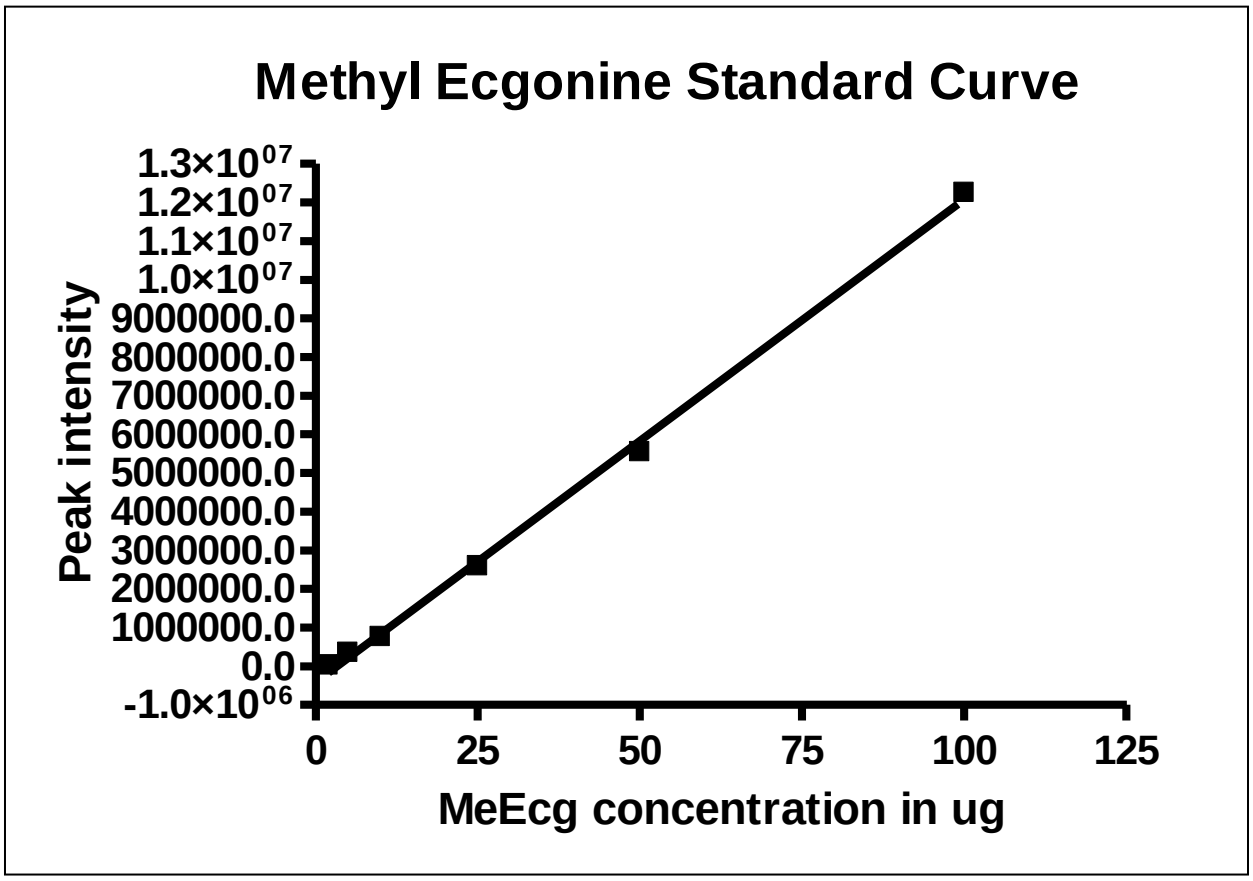

Figure A1.2 Methylecgonine standard curve

Table A1.4 Goodness of fit values for tropinone standard curve

Descriptor	**Value**
r^2	0.9845
P-value	0.0008
Deviation from zero	significant
Number of values	5
Number of missing values	0

Figure A1.3 Persistent foam layer showed a positive result for saponins.

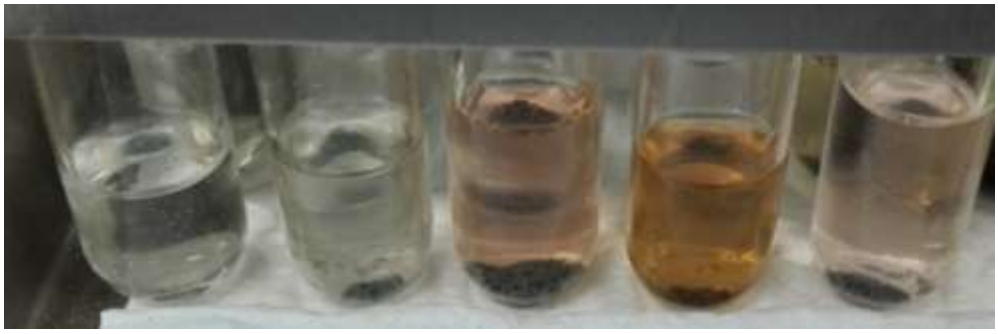

Figure A1.4 No flavanoids present in the two left samples but pink colouration indicates flavanoids in other 3 samples.

Figure A1.5 The brown ring at the interface of the two solvents is indicative of cardiac glycoside presence.

Figure A1.6 Brown precipitate is indicative of tannins

Appendix 2

Cocaine synthase gene isolation

Table A2.1 Primer sequences used for cocaine synthase and EcBAHD 8

Primer name	**Primer sequence (5' – 3')**
BAHD 7 FWD 1	ATGGAAATGTCTAAAAGAAGC
BAHD 7 REV 1	TCACATGCGAGAATATGCTGC
BAHD 8 FWD 1	ATGGAAATGCCTAACTTTCTG
BAHD 8 FWD 2	GCCTAACTTTCTGGAAATGCC
BAHD 8 REV 1	TCACATGCGAGAATATGCTGC
EcBAHD 7 FWD *i*	CTGGAACCATCATCCTCCCC
EcBAHD 7 REV *i*	CGCCATCACCAAATCAGC
EcBAHD 8 FWD *i*	GCCCTTCGCATAAACTCG
EcBAHD 8 REV *i*	CTGCCTTCCCGAGCTGTATT

```
Database: nr
Query= EcBAHD7 (Cocaine synthase gene) consensus
Length=683

                                                                      Score     E
Sequences producing significant alignments:                           (Bits)  Value

gi|531033427|gb|KC140149.1|  Erythroxylum coca cocaine synthas...   898     0E00
gi|531033429|gb|KC140150.1|  Erythroxylum coca BAHD8 mRNA, com...   822     0E00
gi|703113067|ref|XM_010101990.1|  PREDICTED: Morus notabilis B...   61     6E-05
gi|1039832135|ref|XM_008384054.2|  PREDICTED: Malus x domestic...   59     2E-04
gi|1477771522|gb|CP025649.1|  Ipomoea trifida cultivar NCNSP03...   55     3E-03
```

```
Query  241   TCGTTSAGKCNAGAGTTGGCTGCGAGATYGYAGAATTCNWTCAAATTTTTTCTGAWCTTA  300
             ||||| || | ||||||||| ||||||| | ||||||| |||||| ||||||||| ||||
Sbjct  1031  TCGTTGAGGCTAGAGTTGGCGGCGAGATTGCAGAATTCGTTCAAACTTTTTCTGAACTTA  972

Query  301   CCGGCTAACTCATTCAACTCAATCTGGTTTTCCTCTATTTTTGTTGTATWATACAACRNT  360
             |||||||||||||||||||||||||||||||||||||||||||| |||||||| |||||||  |
Sbjct  971   CCGGCTAACTCATTCAACTCAATCTGGTTTTCCTCTATTTGTGTTGTATAATACAACACT  912

Query  361   AAATTTCCAATGGTGTTCGCTGGCAATGGTGGTCTCACCCTTGTACGCATATTCACCAST  420
             | |||||||||||||| |||||||||||||||||||| ||| |||||| || ||||| |
Sbjct  911   ACATTTCCAATGGTGTTTGCTGGCAATGGTGGTCTCACTCTTTTACGCAAATCCACCACT  852

Query  421   TGAAACAACACATATGGACCTGAAGACGANTTGGACAACGACCTTGAAGCCAATATGGCA  480
             ||||| || |||||||||||||||||||| |||||||||| ||||||||||||||||||
Sbjct  851   TGAAATAATACATATGGACCTGAAGACGACTTGGACAACGATCTTGAAGCCAATATGGCA  792

Query  481   CNTTTCATATWTAACGCCNACACCAAATCAGMATCACTCAGATGTGGCTNTKCTGWGGAT  540
             | |||||||  |||||||  |||||||||||| |||||||| |||| ||| | ||| || |
Sbjct  791   CATTTCATAAATAACGCCATCACCAAATCAGCATCACTCATATGTTGCTTTCCTGTGGGT  732

Query  541   GATGAGSCTCCCCTTGTTTTTGTCCTGARTTGAGCTATTTTTGAGGCAGGGAAAACAAAC  600
             ||||||  |||  |||||||||||||| |||||||||||||||||||||||||||||||
Sbjct  731   GATGAGGGTCCGGTTGTTTTTGTCCTGAGTTGAGCTATTTTTGAGGCAGGGAAAACAAAC  672

Query  601   CTTCTCTTAACAAACTCTCCTGGTATTCCTGTTAGCATAGGCRYGTTACGTAAAGCGCTG  660
             |||||||||||  |||||||||||||||  ||||||||||||   ||||||||||  |||
Sbjct  671   CTTCTCTTAACGAACTCTCCTGGTATTTCTGTTAGCATAGGGGCGTTACGTAAAGACCTG  612

Query  661   GGTGGGGAGGA-GATGGTTCCAGA  683
             |||||||||||  ||||||||||||
Sbjct  611   GGTGGGGAGGATGATGGTTCCAGA  588
```

Figure A2.1 The nucleotide alignment of *E. emarginatum BAHD7* consensus sequence and the *E. coca EcBAHD7* gene using a BLASTn search query.

```
Database: nr
Query= KC140150 consensus
Length=1517

                                                                      Score     E
Sequences producing significant alignments:                           (Bits)  Value

gi|531033429|gb|KC140150.1|  Erythroxylum coca BAHD8 mRNA, com...   2142    0E00
gi|531033427|gb|KC140149.1|  Erythroxylum coca cocaine synthas...   1592    0E00
gi|658032282|ref|XM_008353422.1|  PREDICTED: Malus x domestica...   95    7E-15
gi|657999965|ref|XM_008394199.1|  PREDICTED: Malus x domestica...   95    7E-15
gi|1343991922|ref|XM_024022206.1|  PREDICTED: Quercus suber BA...   90    3E-13


ALIGNMENTS
>gb|KC140150.1| Erythroxylum coca BAHD8 mRNA, complete cds
          Length=1517

 Score = 2141.9 bits (2374),  Expect = 0E00
 Identities = 1256/1301 (96%), Gaps = 0/1301 (0%)
 Strand = Plus/Plus

Query  34    ATGCCAAAAAMGAAGCTTGAAATCATCTCAAGAAAAACAATCAAACCATCATCTTCTACA  93
             |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  34    ATGCCAAAAACGAAGCTTGAAATCATCTCAAGAAAAACAATCAAACCATCATCTTCTACA  93

Query  94    CCTCAACATCTTCAAACATTTCAGCTTTCTTTTTGGGACAAACCCATACCAGTTCCTCCT  153
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94    CCTCAACATCTTCAAACATTTCAGCTTTCTTTTTGGGACAAACCCATACCAGTTCCTCCT  153

Query  154   GAATATGGCACTATAATTTTTTTCTACCAGACCAATGACTGCCAAAATGATGATGATGAA  213
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  154   GAATATGGCACTATAATTTTTTTCTACCAGACCAATGACTGCCAAAATGATGATGATGAA  213
```


Query 214 ACTCTTTCAATGTTCTTGCAAAGGTCAAACACTCTACAGAAGTCACTCTCTCAAACACTC 273
Sbjct 214 ACTCTTTCAATGTTCTTGCAAAGGTCAAACACTCTACAGAAGTCACTCTCTCAAACACTC 273
Query 274 ACTCACTACTATCCATTAGCAGGGAGACTCAAAGATGATGCAACAGCAGTTGACTGCTTT 333
Sbjct 274 ACTCACTACTATCCATTAGCAGGGAGACTCAAAGATGATGCAACAGCAGTTGACTGCTTT 333
Query 334 GATGAAGGGGCTTATTTTGTGGTAGCTCGCATTGATTGCCAGCTTTCTACCTTACTTAAC 393
Sbjct 334 GATGAAGGGGCTTATTTTGTGGTAGCTCGCATTGATTGCCAGCTTTCTACCTTACTTAAC 393
Query 394 CATCCCGATGCCGATTTCTTGAGCCACTTCTGTCCTGCTTTGGATACAAACAACGTACCC 453
Sbjct 394 CATCCCGATGCCGATTTCTTGAGCCACTTCTGTCCTGCTTTGGATACAAACAACGTACCC 453
Query 454 TCAGGCTGTATGTTGGCCATTCAACTTACACTCTTCAACTGTGGAGGAATAGCCATTACT 513
Sbjct 454 TCAGGCTGTATGTTGGCCATTCAACTTACACTCTTCAACTGTGGAGGAATAGCCATTACT 513
Query 514 GTGAGCCCTTCGCATAAACTCGTAGATGCATCCTCATTGTGCACATTTGTCCAAAGTTGG 573
Sbjct 514 GTGAGCCCTTCGCATAAACTCGTAGATGCATCCTCATTGTGCACATTTGTCCAAAGTTGG 573
Query 574 RCATCAMTGAATACMRCTCAGGAAGGCAGCAGCAAAGTAGTAACACCTATACCTATATTT 633
Sbjct 574 ACATCACTGAATACAGCTCGGGAAGGCAGCAGCAAAGTAGTAACACCTATACCTATATTT 633
Query 634 CTGGAACCATACTCCTCGCCACCCAGTGTTYTGCTTAACATGCCTRAGCTAACCGGAATA 693
Sbjct 634 CTGGAACCACACTCCAAGCCACCCAGTGTTTTGCTTAACATGCCTAAGCTAACCGGAATA 693
Query 694 CCGGGAGACTTCGTTAGAAGAAGGTTTGTYGTCCYTGCYTCAAAAATAGCTCAACTCAGG 753
Sbjct 694 CCAGGAGACTTCRTTAGAAGAAGGTTTGTTGTCCCTGCCTCAAAAATAGCTCAACTCAGG 753
Query 754 ACAAAAACAACCCGATCCTCAACACCCCCAGCACAGCAATACCTCAGTGATTCTGATTTG 813
Sbjct 754 ACAAAAACAACCGGATCCTCAACACCCTCAGGCCAGCAATACCTCAGTGATTCTGATTTG 813
Query 814 GTGTTGGCGTTAATTATGAGAAGTGCCATATTGGCTTCAAGATCTTTGTTCAAGTCATCT 873
Sbjct 814 GTGTTGGCGTTAATTATGAAAAGTGCCATATTGGCTTCAAGATCTTTGTCCGAGTCATCT 873
Query 874 TCAGGTTCATATGTCCTGTTTCAAGTGGTGAATATGCGTAAAAGAGTGAGACCACCATTG 933
Sbjct 874 TCAGGTTCATAYGTGCTGTTTCAATTGGTGAATATGCGTAAAAGAGTGAGACCACCATTG 933
Query 934 CCAGGGAACACCATTGGAAATATAGCGTTGTATTATACAACAAAAATCGAAGAAAACCAG 993
Sbjct 934 CCAGCGAGCACCATTGGAAATATAGCGCTTTATTATACAACAAAAATCGAGGAAAACCAG 993
Query 994 ATTGAGTTGAATGAGTTAGCGGGTAAGTTCAGAAAAAGTTTGATTGAATTCTGCAATCTC 1053
Sbjct 994 ATTGAGTTGAATGAGTTAGCCGGTAAGTTCAGAAAAAGTTTGATTGAATTCTGCAATCTC 1053
Query 1054 CCGGACAACTCTAGCCTCAACGAAGAACCCAAGTTTAGCATCCAAGGTAGCCCTTATTGC 1113
Sbjct 1054 CCGGACAACTCTATCCCCAACGAAGAACCCAAGTTTAGCATCAAAGGTAGCCCTTATTGC 1113

```
Query  1114  TGCACTAACTTATGTGGCTTTCCGGTGTATGAGATAGATTTTGGTTGGGGTAAACCTTCA  1173
             ||||||||||||||||| |||||| ||||||||||||||||||| ||||||||||||||
Sbjct  1114  TGCACTAACTTATGTGGGTTTCCGTTGTATGAGATAGATTTTGGATGGGGTAAACCTTCG  1173

Query  1174  TGGGTCACTTCAACAGTTTTACGGTTCAGGAATATTATTGTGCTGCAGAAAACARAGGAA  1233
             |||||||||||||||||||||| ||||||||||||||||||||||||||||||| |||||
Sbjct  1174  TGGGTCACTTCAACAGTTTTACAGTTCAGGAATATTATTGTGCTGCAGAAAACAAAGGAA  1233

Query  1234  GGTGATGGTATAGAAGTTTGGATATCCTTGGACGAAAGAGAGATGGCCGTGTTTGAACAG  1293
             |||||| |||||||||||||||||| |||||||||||||||||||||| |||||||||
Sbjct  1234  GGTGATAGTATAGAAGTTTGGATATCCATGGACGAAAGAGAGATGGCCGTATTTGAACAG  1293

Query  1294  GATCATGATATTATTGCATATGCATCCAACAATCCTAGTGT  1334
             |||||||||||||||||||||||||||||| ||||||||||
Sbjct  1294  GATCATGATATTATTGCATATGCATCCAACGATCCTAGTGT  1334
```

Figure A2.2 The nucleotide alignment of *E. emarginatum BAHD8* consensus sequence and the *E. coca EcBAHD8* gene using a BLASTn search query

```
Database: nr
Query= EcBAHD7 (Cocaine synthase gene) consensus
Length=683

                                                                    Score     E
Sequences producing significant alignments:                         (Bits)  Value

gi|531033428|gb|AGT56097.1|  cocaine synthase [Erythroxylum coca]   327    5E-94
gi|531033430|gb|AGT56098.1|  BAHD8 [Erythroxylum coca]              306    1E-86

ALIGNMENTS
>gb|AGT56097.1| cocaine synthase [Erythroxylum coca]
          Length=437

 Score = 326.7 bits (770),  Expect = 5E-94
 Identities = 167/223 (74%), Positives = 179/223 (80%), Gaps = 0/223 (0%)
 Frame = -1

Query  671  SSPPSALRNXPMLTGIPGEFVKRRFVFPASKIAQXRTKTRGXSSSXXXPHLSDXDLVXAL  492
            SSPP +LRN PMLT IPGEFVKRRFVFPASKIAQ RTKT G SS     H+SD DLV AL
Sbjct  201  SSPPRSLRNAPMLTEIPGEFVKRRFVFPASKIAQLRTKTTGPSSPTGKQHMSDADLVMAL  260

Query  491  XMKXAILAXXXXXXXXSGPYVLFQXVNMRTRVRPPLPANTIGNLXLYXTTKIEENQIELN  312
             MK AILASRSLS SSSGPYVLFQ V++R RVRPPLPANTIGN  LY TT IEENQIELN
Sbjct  261  FMKCAILASRSLSKSSSGPYVLFQVVDLRKRVRPPLPANTIGNVVLYYTTQIEENQIELN  320

Query  311  ELAGKXRKNLXEFXBLAANSXLNEXPXXSIQGXPYCCTXXCGFPVYEXDFGWGKPSWXTS  132
            ELAGK RK+L EF +LAANS LNE P   IQG PYCCT  CGFP YE DFGWGKPSW T+
Sbjct  321  ELAGKFRKSLNEFCNLAANSSLNEEPEFIIQGSPYCCTNLCGFPFYEIDFGWGKPSWVTT  380

Query  131  EXLLFRNXIVLXKTKEGDGIEAWISMDXREMXLXEZDHDIJAY  3
            E L FRN IVL KTK+GDGIE W+SMD +EM L E+DHDI+AY
Sbjct  381  ELLWFRNIIVLQKTKDGDGIELWVSMDEKEMALFEQDHDIIAY  423
```

Figure A2.3 The amino acid alignment of translated *E. emarginatum BAHD7* consensus sequence and the *E. coca EcBAHD7* protein using a BLASTx search query

```
Database: nr
Query= KC140150 consensus
Length=1517

                                                                    Score     E
Sequences producing significant alignments:                         (Bits)  Value

gi|531033430|gb|AGT56098.1|  BAHD8 [Erythroxylum coca]               831     0E00
gi|531033428|gb|AGT56097.1|  cocaine synthase [Erythroxylum coca]    701     0E00

ALIGNMENTS
>gb|AGT56098.1| BAHD8 [Erythroxylum coca]
          Length=449

 Score = 831.4 bits (1972),  Expect = 0E00
 Identities = 406/434 (93%), Positives = 410/434 (94%), Gaps = 0/434 (0%)
 Frame = +1


 Query  34    MPKXKLEIISRKTIKPSSSTPQHLQTFQLSFWDKPIPVPPEYGTIIFFYQTNDCQNDDDE  213
              MPK KLEIISRKTIKPSSSTPQHLQTFQLSFWDKPIPVPPEYGTIIFFYQTNDCQNDDDE
 Sbjct  9     MPKTKLEIISRKTIKPSSSTPQHLQTFQLSFWDKPIPVPPEYGTIIFFYQTNDCQNDDDE  68

 Query  214   TLSMFLQRSNTLQKSLSQTLTHYYPLAGRLKDDATAVDCFDEGAYFVVARIDCQLSTLLN  393
              TLSMFLQRSNTLQKSLSQTLTHYYPLAGRLKDDATAVDCFDEGAYFVVARIDCQLSTLLN
 Sbjct  69    TLSMFLQRSNTLQKSLSQTLTHYYPLAGRLKDDATAVDCFDEGAYFVVARIDCQLSTLLN  128

 Query  394   HPDADFLSHFCPALDTNNVPSGCMLAIQLTLFNCGGIAITVSPSHKLVDASSLCTFVQSW  573
              HPDADFLSHFCPALDTNNVPSGCMLAIQLTLFNCGGIAITVSPSHKLVDASSLCTFVQSW
 Sbjct  129   HPDADFLSHFCPALDTNNVPSGCMLAIQLTLFNCGGIAITVSPSHKLVDASSLCTFVQSW  188

 Query  574   XSXNTXQEGSSKVVTPIPIFLEPYSSPPSVLLNMPXLTGIPGDFVRRRFVVXASKIAQLR  753
               S NT  EGSSKVVTPIPIFLEP S PPSVLLNMP LTGIPGDF RRRFVV ASKIAQLR
 Sbjct  189   TSLNTAREGSSKVVTPIPIFLEPHSKPPSVLLNMPKLTGIPGDFXRRRFVVPASKIAQLR  248

 Query  754   TKTTRSSTPPAQQYLSDSDLVLALIMRSAILASRSLFKSSSGSYVLFQVVNMRKRVRPPL  933
              TKTT SSTP  QQYLSDSDLVLALIM+SAILASRSL  SSSGSYVLFQ VNMRKRVRPPL
 Sbjct  249   TKTTGSSTPSGQQYLSDSDLVLALIMKSAILASRSLSESSSGSYVLFQLVNMRKRVRPPL  308

 Query  934   PGNTIGNIALYYTTKIEENQIELNELAGKFRKSLIEFCNLPDNSSLNEEPKFSIQGSPYC  1113
              P +TIGNIALYYTTKIEENQIELNELAGKFRKSLIEFCNLPDNS  NEEPKFSI GSPYC
 Sbjct  309   PASTIGNIALYYTTKIEENQIELNELAGKFRKSLIEFCNLPDNSIPNEEPKFSIKGSPYC  368

 Query  1114  CTNLCGFPVYEIDFGWGKPSWVTSTVLRFRNIIVLQKTXEGDGIEVWISLDEREMAVFEQ  1293
              CTNLCGFP YEIDFGWGKPSWVTSTVL FRNIIVLQKT EGD IEVWIS+DEREMAVFEQ
 Sbjct  369   CTNLCGFPLYEIDFGWGKPSWVTSTVLQFRNIIVLQKTKEGDSIEVWISMDEREMAVFEQ  428

 Query  1294  DHDIIAYASNNPSV  1335
              DHDIIAYASN+PSV
 Sbjct  429   DHDIIAYASNDPSV  442
```

Figure A2.4 The amino acid alignment of translated *E. emarginatum BAHD8* consensus sequence and the *E. coca EcBAHD8* protein using a BLASTx search query

Appendix 3

Cytotoxicity

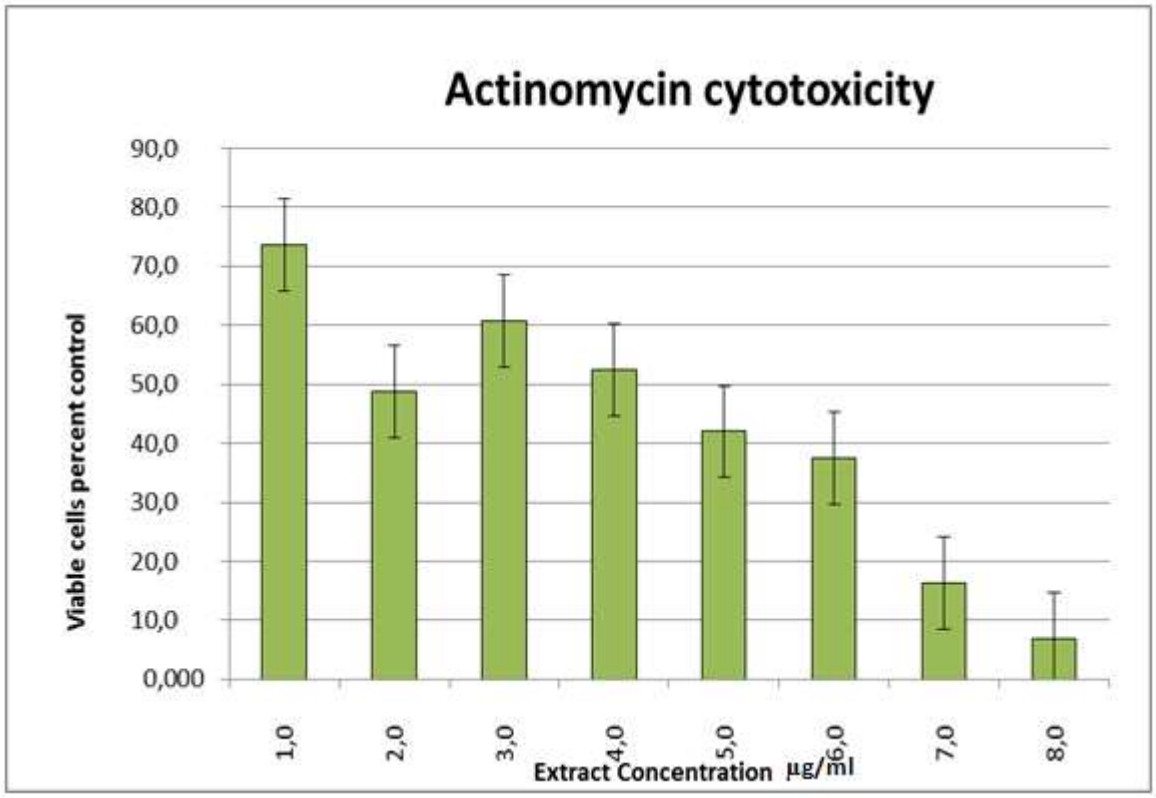

Figure A3.1 Cytotoxicity activity of positive control Actinomycin

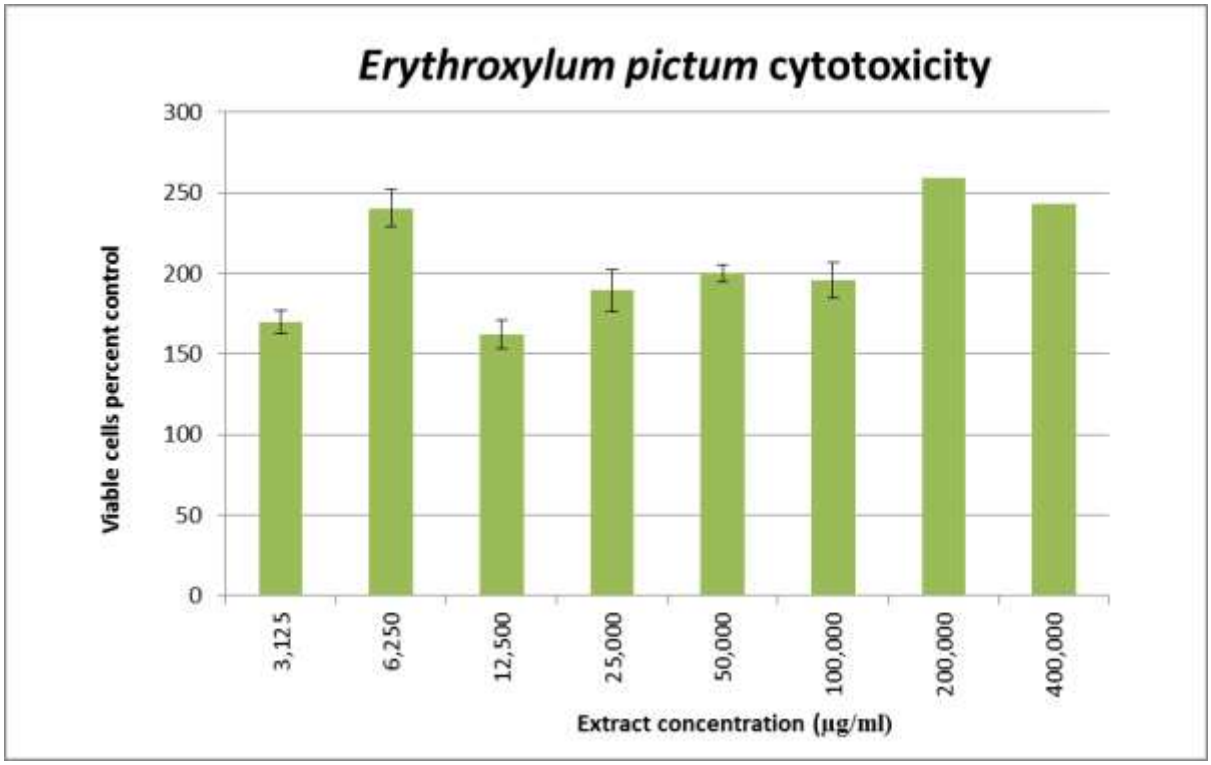

Figure A3.2 Cytotoxicity activity of *Erythroxylum pictum*

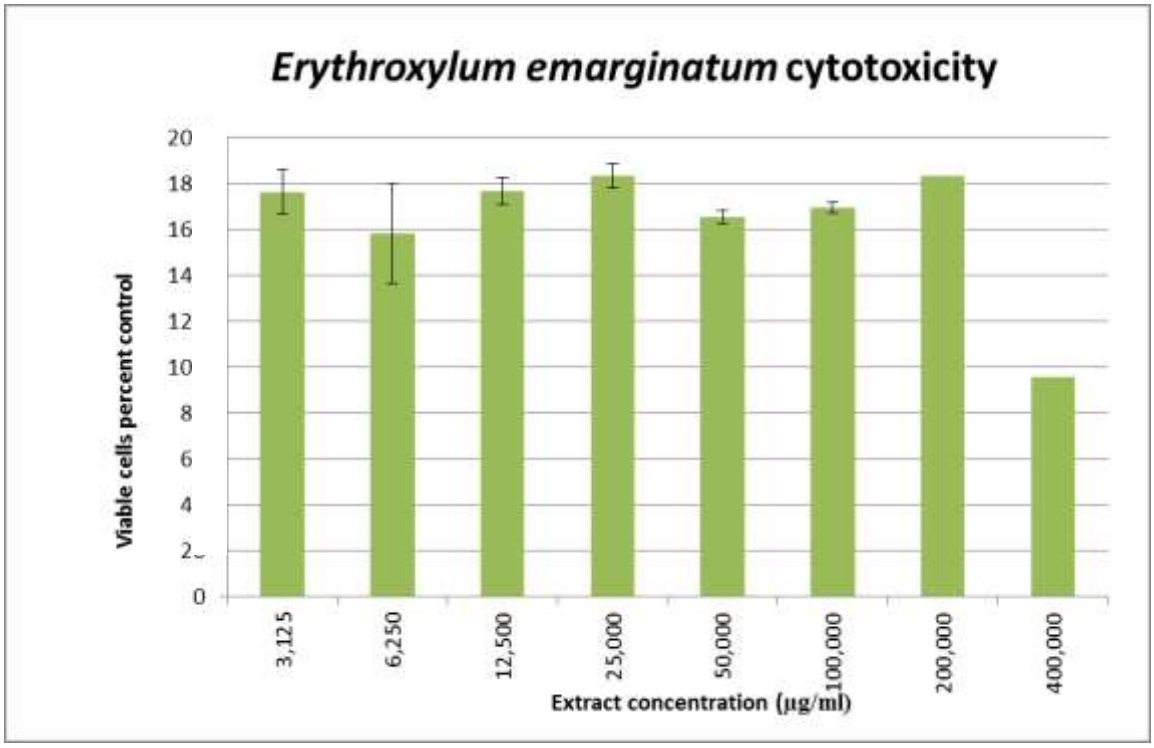

Figure A3.3 Cytotoxicity activity of *Erythroxylum emarginatum*

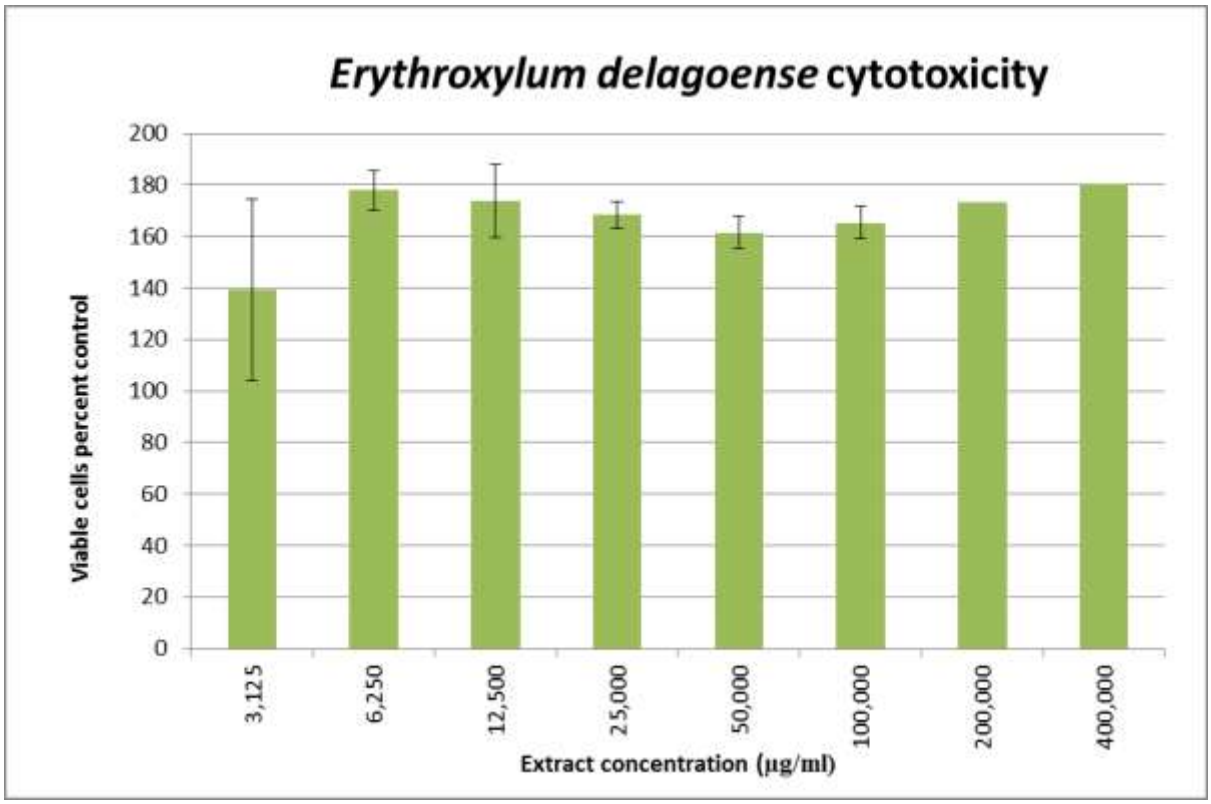

Figure A3.4 Cytotoxicity activity of *Erythroxylum delagoense*

Appendix 4

Antibacterial activity

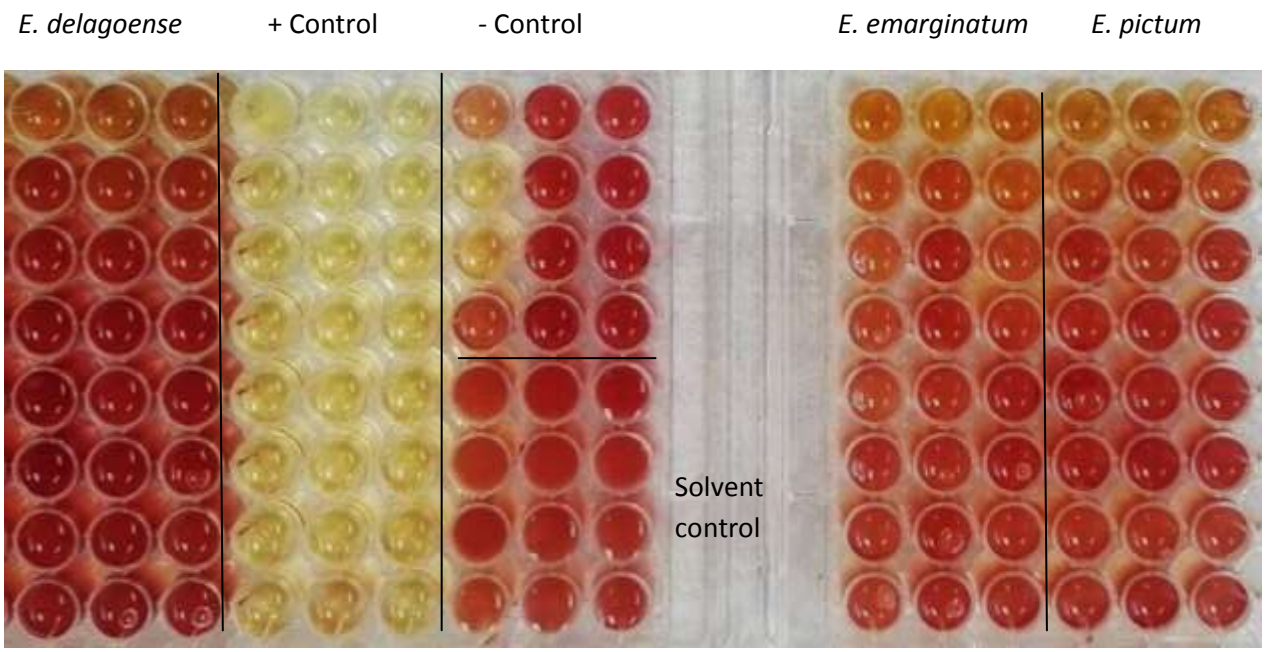

Figure A4.1 Microtiter plates with antibactrial activity results of plant extracts against *P. mirabilis*

Appendix 5

Antioxidant & Anti-inflammatory activity

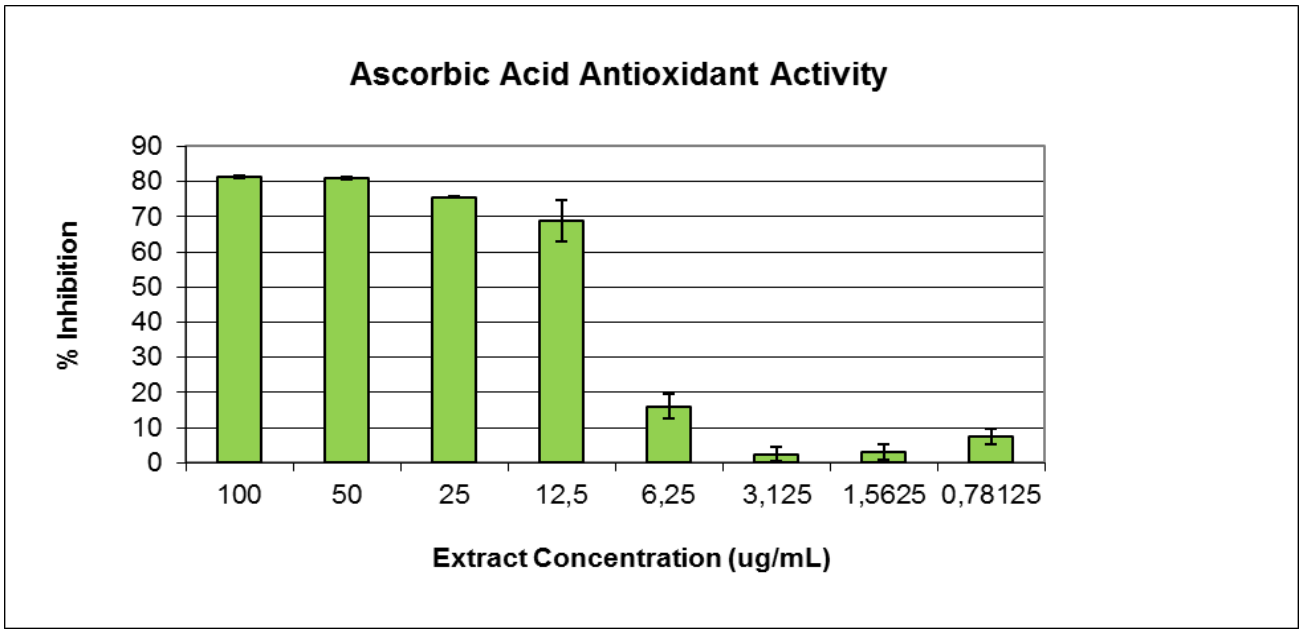

Figure A5.1 Antioxidant activity of the positive standard ascorbic acid

Table A5.1 Goodness of fit values for *E. pictum* antioxidant activity sigmoidal dose response curve

Descriptor	**Value**
r^2	0.9879
Log EC_{50}	0.01342
Log EC_{50} standard error	0.9631
Number of values	21
Number of missing values	6

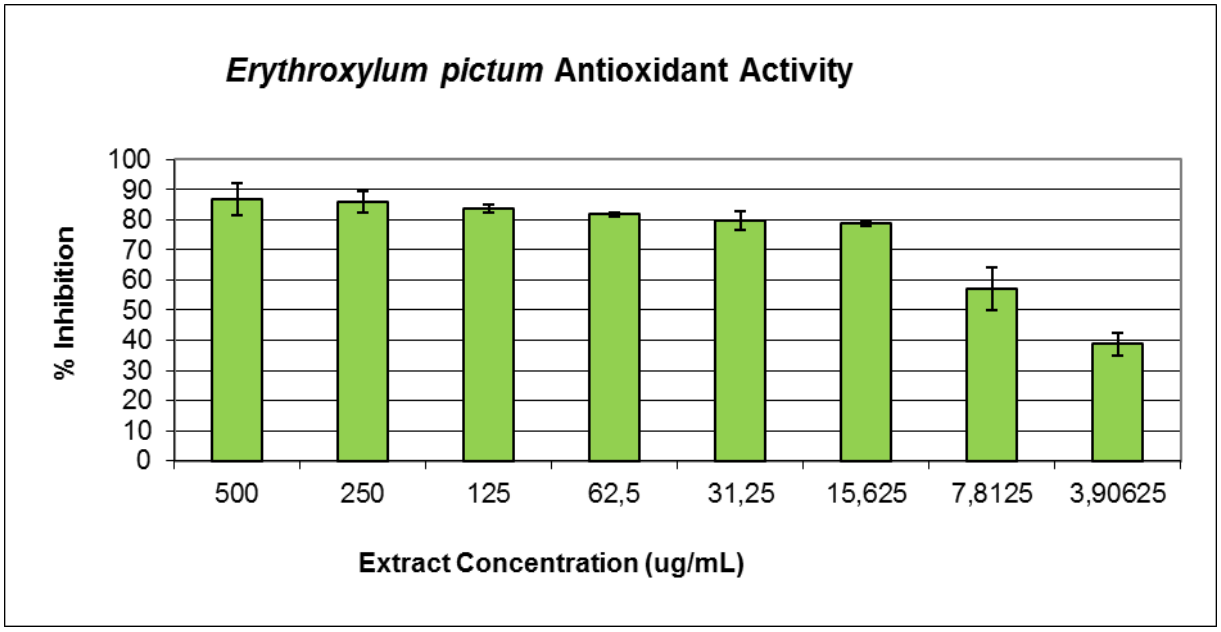

Figure A5.2 Antioxidant activity of the *E. pictum*

Table A5.2 Goodness of fit values for *E. pictum* antioxidant activity sigmoidal dose response curve

Descriptor	**Value**
r^2	0.9459
Log EC_{50}	4.608
Log EC_{50} standard error	0.02771
Number of values	23
Number of missing values	4

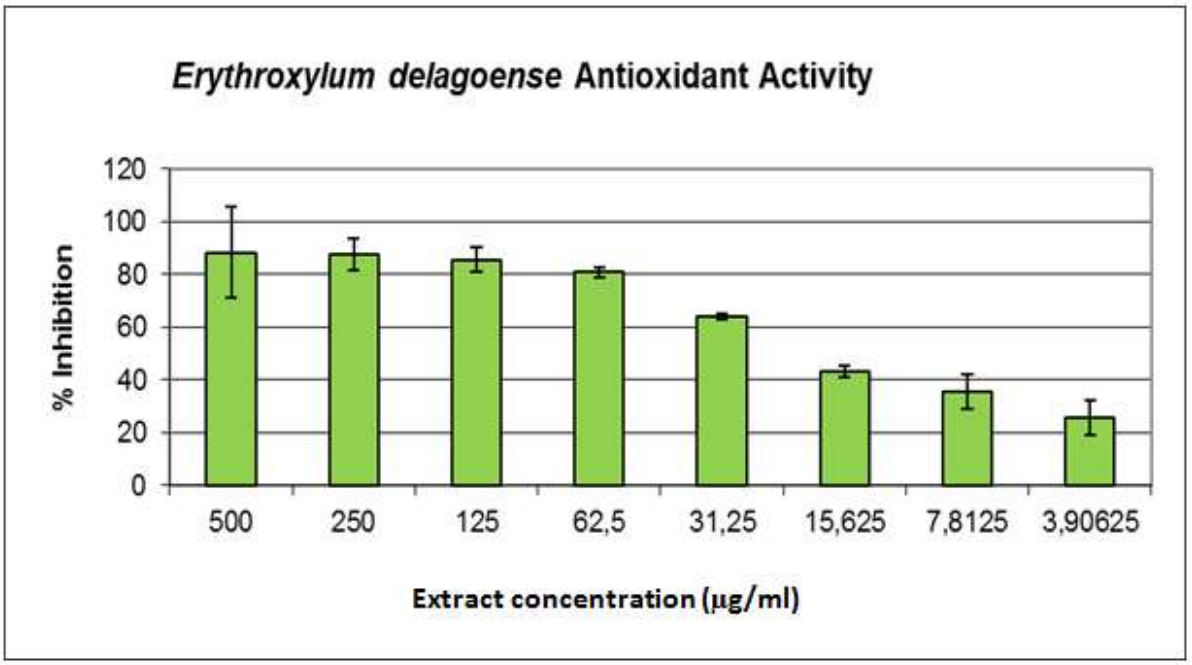

Figure A5.3 Antioxidant activity of the *E. delagoense*

Table A5.3 Goodness of fit values for *E. delagoense* antioxidant activity sigmoidal dose response curve

Descriptor	**Value**
r^2	0.9199
Log EC_{50}	15.47
Log EC_{50} standard error	0.04379
Number of values	22
Number of missing values	5

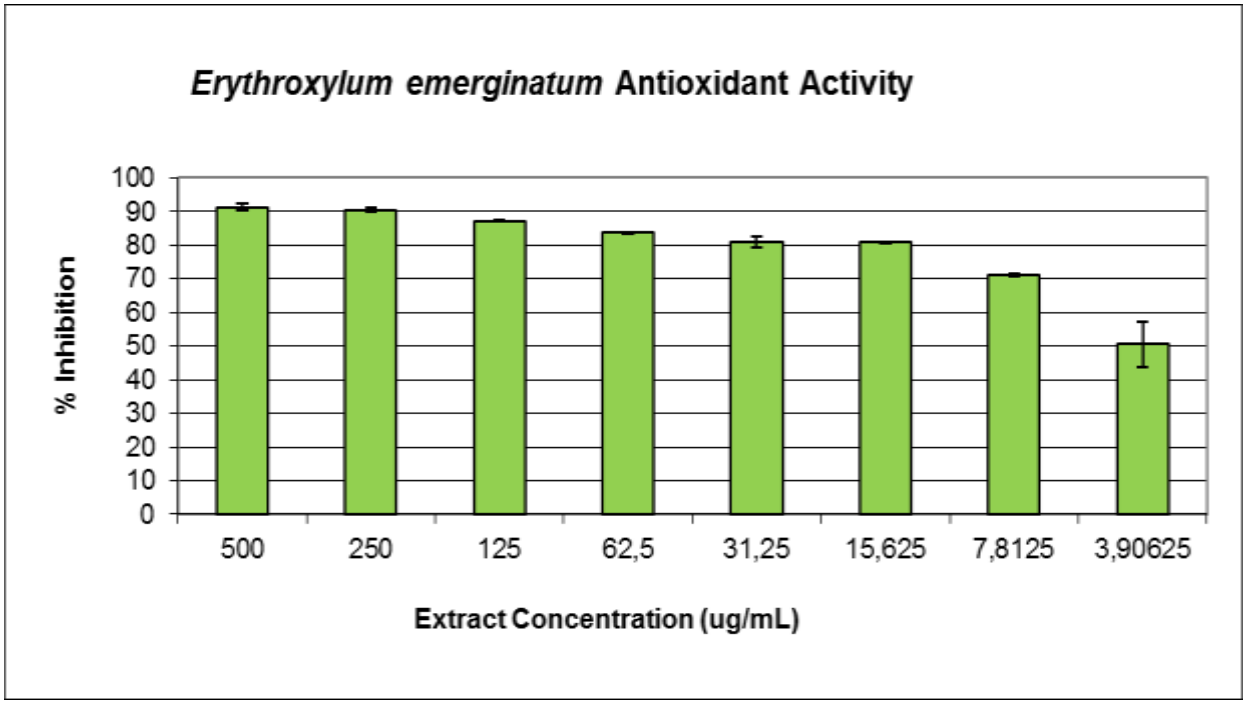

Figure A5.4 Antioxidant activity of the *E. emarginatum*

Table A5.4 Goodness of fit values for *E. emargiantum* antioxidant activity sigmoidal dose response curve

Descriptor	**Value**
r^2	0.959
Log EC_{50}	0.5482
Log EC_{50} standard error	0.03407
Number of values	24
Number of missing values	3

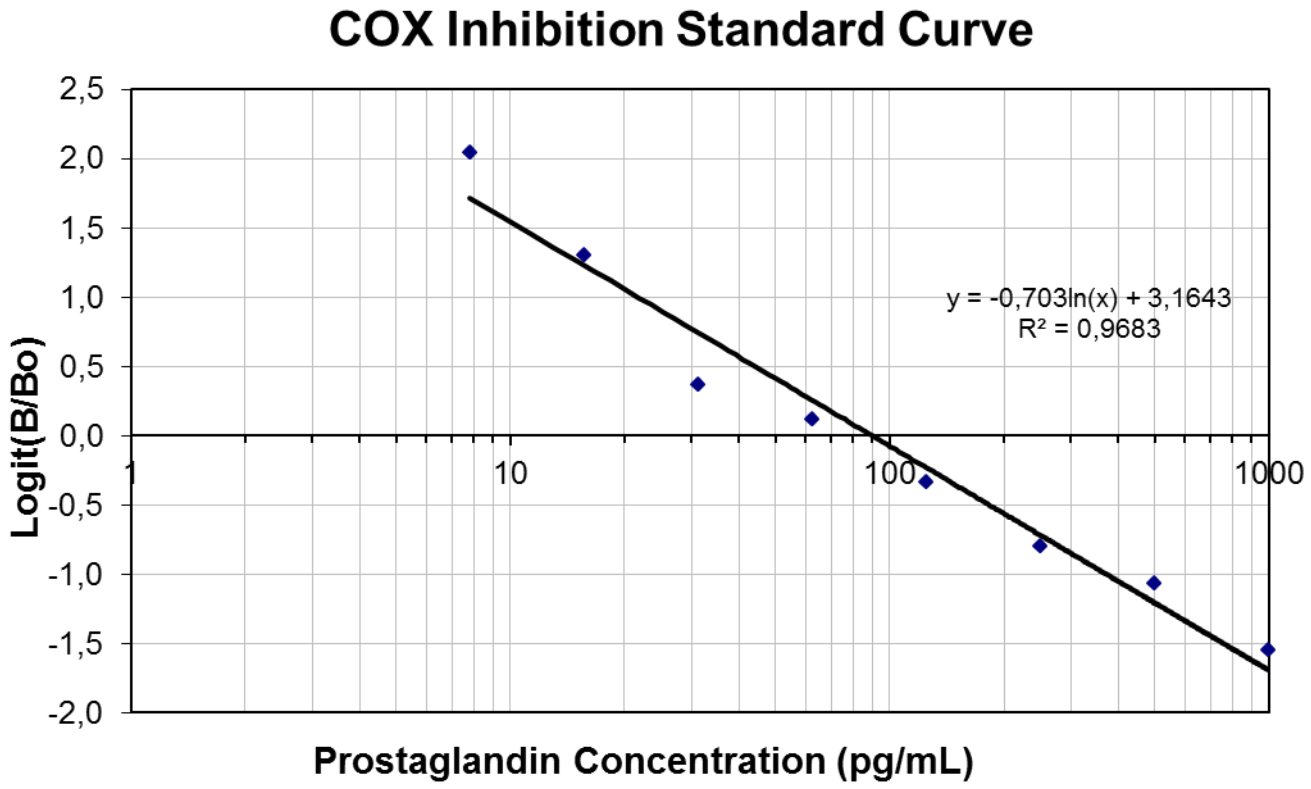

Figure A5.5 COX inhibitory standard curve with absorbance values versus prostaglandin concentration