A phylogeny-based comparative study of the phytochemical and pharmacological characteristics of *Croton* species occurring in KwaZulu-Natal

By

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Submitted in fulfilment of the requirements of the degree of Master of Science

Centre for Functional Biodiversity

Research Centre for Plant Growth and Development

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Pietermaritzburg Campus

April 2021

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Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our abilities and approved the final document for submission to the Faculty of Science and Agriculture Higher Degrees Office for examination by the University appointed examiners.

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CONFERENCE CONTRIBUTIONS

1. T. Mathe, B. Bytebier, J. F. Finnie and J. Van Staden. A phylogeny-based comparative study of the phytochemical and pharmacological characteristics of *Croton* species occurring in KwaZulu-Natal. BIMF-FBIP FORUM 14th–17th August 2017, Salt Rock Hotel and Beach Resort, KwaZulu-Natal, South Africa.

2. T. Mathe, B. Bytebier, J.F. Finnie and J. van Staden. A phylogeny-based comparative study of the phytochemical and pharmacological characteristics of *Croton* species occurring in KwaZulu-Natal, South Africa. BIMF-FBIP FORUM
   13th–16th August 2018, Cape St Francis Resort, Eastern Cape, South Africa

ACKNOWLEDGEMENTS

My greatest gratitude goes to my Heavenly Father for giving me the opportunity, patience, and capacity to do this project. I am so grateful for the love and grace. He continuously blesses me with.

I would also like to thank:

- My supervisor Dr. B. Bytebier for his guidance, support, and encouragement throughout this study. I have gained so much knowledge in my time working with him.
- My co-supervisors Prof. J.F. Finnie and Prof J. Van Staden for their guidance and contributions they have made in fulfilment of my MSc. study.
- Dr. MacMaster Vambe for his patience, guidance and the time he dedicated to assist me in different aspects of my studies. I am truly indebted to him.
- Dean Phillips for his help in my molecular studies and for assisting me with data analysis.
- Alison Young for being so kind with her time, accompanying me and assisting me during field trips.
- The CREW team (Custodians of Rare and Endangered Wildflowers) for allowing me to join them on their field trips and their assistance in finding my study species.
- The staff and my friends from Bews Herbarium, Prudence Magwaza, Dr. Christina Curry, Sifiso Mnxati, Ayanda Mnikathi and Simon Chetty for all the support, motivation and always checking up on my progress.
- My colleagues at the Research Centre for Plant Growth and Development, Dr. Fikisiwe Gebashe, Thabiso Thabethe, Nokuthula Sithole, Nelson Ngoroyemoto and Dr. Priya Gupta, for the shared times in the lab and continuous support and assistance.

- My friends Zombuso Mbatha, Nontando Ntuli Kholofelo Rammala, Neo Mohai, Sachin Doarsamy, and, Roy Caister, for the emotional support and keeping me going.
- My loving family, My grandmother, Mrs. M.N Mathe, who suggested I study *Croton* species and has shown so much interest in my study. Thank you. My sisters, Ya-ya, Lisa and Ziningi for all your support and for always providing loving words of encouragement.
- Finally the Foundational Biodiversity Information Programme (FBIP) for funding my studies. I am truly grateful for the opportunity they gave me.

ABSTRACT

Ethnobotanical enquiries often lead to the discovery of phytocompounds with pharmacological activities. Against this background a comparative and quantitative evaluation of the phytochemical and antioxidant activity of extracts from six *Croton* species; *C. gratissimus* Burch., *C. sylvaticus* Hochst., *C. menyhartii* Pax, *C. pseudopulchellus* Pax, *C. steenkampianus* Gerstner and *C. rivularis* Müll.Arg., all collected from KwaZulu-Natal (KZN), South Africa, was conducted. The analysis included a comparison of the different plant organs to explore the possibility of using leaves rather than bark for medicinal purposes. The latter would result in less destructive harvesting and would contribute to sustainable use of these medicinal plant resources.

Extraction of the different plants and their organs were done in water and, in different organics solvents, including methanol (MeOH), dichloromethane (DCM), and petroleum ether (PE). The extracts were screened for antibacterial and antifungal activities using the microdilution technique. All of the tested plant samples showed some notable antibacterial activity in one or two of their organs, except for *C. rivularis*, which was the only species in the list that had no record of medicinal use. The most potent antibacterial activity was exhibited by the dichloromethane (DCM) extracts of *C. steenkampianus* leaves and the petroleum ether (PE) extracts of *C. pseudopulchellus* stem bark, both sampled from the Durban Botanic Gardens, which yielded a minimum inhibition concentration (MIC) value of 0.04 mg/ml against *Enterococcus faecalis* (*E. faecalis*). The DCM stem bark and leaf extracts, as well as the PE twig extracts of *C. pseudopulchellus*, (Durban Botanic Gardens) also exhibited noteworthy activities against *S. aureus* (MIC value of 0.08 mg/ml). A broad spectrum of activity was observed in the DCM and PE twig extracts of *C. sylvaticus* collected

from Umdoni Park, with a MIC ranging from 0.31-0.94 mg/ml. This activity was against *E. faecalis*, *Staphylococcus aureus* (*S. aureus*) and *Klebsiella pneumoniae* (*K. pneunomiae*).

Noteworthy antifungal activity against *Candida albicans* was only observed in one extract, the MeOH leaf extract of *C steenkampianus* collected at Kosi Bay. The MIC of this extract was 0.6 mg/ml. Water extracts did not show any antimicrobial activity. The results of the pharmacological study suggested that the aerial plant organs, such as the leaves and the twigs, could replace bark as they exhibited significant antimicrobial activity when compared to the preferred bark. The study also revealed that the same species collected from different regions may not necessarily exhibit similar biological activities, as pharmacological activities are as a result of the phytochemicals present in the plant, which are triggered by environmental stimuli.

The phenolic profiles of aqueous (50%) methanol extracts obtained from the plants were assessed using the Folin and Ciocalteu (Folin C), butanol-hydrochloric acid and aluminium chloride assays. Aqueous (50%) methanol extracts were also run on thin layer chromatography plates and the plates were later stained with the Dragendorff reagent to determine the possible presence of alkaloids. Antioxidant activity was determined with two different assays, β-carotene /Linoleic model system and 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging assay. The overall EC_{50} values of the different *Croton* species displayed by the DPPH assay ranged from 1.76 to 5.35 µg/ml. The β-carotene /Linoleic model system displayed antioxidant activity that ranged between 48.66 to 81.97%. In the phytochemical study; the leaf extracts of *C. pseudopulchellus* from Mkuze exhibiting the highest phenolic content at 23.8±1.1 mg GAE/g Dry Weight. The highest condensed tannin content was from the leaf extracts of *C. gratissimus* from Southport at a concentration of 31.3± 0.1 mg CCE/g Dry Weight. The highest flavonoid content observed in the leaf extracts of *C. gratissimus* from Southport at a concentration of 31.2±0.7 mg CE/g Dry Weight. Higher

phytochemical contents were also observed in the leaves and twigs. These phytochemicals are believed to be the reason for most of the notable antimicrobial activities exhibited by these plant organs. The mutagenic potential of the most biologically active *Croton* extracts was tested. An Ames with two *Salmonella* tester strains (TA98 and TA102) revealed that the species are not toxic as they did not produce His+ revertant colonies in *Salmonella* tester strains that were more than twice the number of His+ revertant produced by the positive control, Nitroquinoline-N-oxide (4-NQO).

Standard DNA barcodes of the *Croton* species occurring in KZN were generated, since these are useful in plant identification and authentication. These were used to run a phylogenetic analysis in order to assess whether the phytochemical profile is clade-specific. The results showed that the quantity and quality of phytochemicals closely related species may vary. DNA barcoding may be a useful tool in medicinal plant identification, especially where fragments of the plants are traded and morphological identification is not possible.

In this study, the biological activity of different parts of six *Croton* species occurring in KZN, was investigated with the aim of replacing bark as the source of local medicine with other plant parts (leave and twigs) that can be more sustainably harvested, as this will contribute to the conservation of these species.

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4-NQO	Nitroquinoline-N-oxide
AA	Antioxidant activity
AIDS	Acquired immune-deficiency syndrome
$AlCl_3$	Aluminium chloride
ANOVA	Analysis of variance
ANT	Antioxidant activity
ASC	Ascorbic acid
ATCC	American Type Culture Collection
BHA	Butylated hydroxyanilosole
BHT	Butylated hydroxytoluene
BI	Bayesian Inference
bp	Base pairs
CAF	Central Analytical Facility
CCE	Cyanidin chloride equivalent
CE	Catechin equivalent
CFU	Colony forming unit
DBG	Durban Botanic Gardens
DCM	Dichloromethane
DMRT	Duncan's multiple range test
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picryl hydrazyl
EC_{50}	Effective Concentration
DW	Dry weight
GAE	Gallic acid equivalent

HCl	Hydrochloric acid
His+	Histidine independent
HIV	Human immunodeficiency virus
INT	*p*-Iodonitrotetrazolium chloride
ITS	Internal transcribed spacer
KZN	KwaZulu-Natal
MDR	Multiple drug-resistant
MeOH	Methanol
MH	Mueller-Hinton
MHz	Megahertz
MIC	Minimum inhibition concentration
ML	Maximum likelihood
NaOH	Sodium hydroxide
ORR	Oxidation rate ratio
PCR	Polymerase chain reaction
PE	Petroleum ether
PMB	Pietermaritzburg
RCPGD	Research Centre for Plant Growth and Development
RNA	Ribonucleic acid
RSA	Radical Savaging Activity
ROS	Reactive oxygenic species
SAM	S-adenosylmethionine
SASSB	Southern African Society for Systematic Biology
STI	Sexually Transmitted Infection
TBE	Tris-borate-EDTA
TBHQ	Tert-butylhydroquinone
TLC	Thin Layer Chromatography

UKZN	University of KwaZulu-Natal
UTI	Urinary Tract Infection
YM	Yeast Malt

CHAPTER 1

General Introduction

1.1. Historical and current therapeutic use of plants

Plants have been a reliable and valuable source of medicines throughout humankind's history and continue to be used by people in rural and disadvantaged communities as one of the primary sources of food and medicine (**Chen et al.**, 2010, **Tshisikhawe et al.**, 2012). Existing physical evidence indicates that the therapeutic use of plants dates back up to 60 000 years (**Halberstein**, 2005). Due to the high prices and the lack of trust in synthetic drugs, medicinal plants remain the treatment of choice in various rural communities across the world (**Halberstein**, 2005, **Fatemeh et al.**, 2018). Many people trust medicinal plants for their cultural relevance and the long history of use by their ancestors (**Masevhe et al.**, 2015, **Fatemeh et al.**, 2018). They have had great success in maintaining and restoring their health. Herbal medicines are believed to have several advantages over Western medicine, such as fewer side effects and greater affordability (**Singh and Prakash**, 2011). Mastering and understanding the use of plants for therapeutic gain and other human needs has taken humans a great deal of trial and error (**Fatemeh et al.**, 2018). Some of their knowledge was obtained by observing monkeys and apes, as they are believed to use plants to treat their own ailments (**Halberstein**, 2005, **Mamedov**, 2012). Much of the knowledge of traditional plants has been passed down orally from generation to generation by traditional healers (**Mamedov**, 2012, **Fatemeh et al.**, 2018).

Medicinal plant trade and demand in South Africa are rapidly growing and estimated to be worth about R270 million per annum, with over 700 plant species traded for medicinal purposes (**Dold and Cocks**, 2002). This increase in trade and demand for traditional medicinal plants in this country is threatening plant biodiversity. This is mostly due to the immense surge in unsustainable bark harvesting practices (**Tshisikhawe et al.**, 2012). The depletion of natural resources such as medicinal plants causes concerns about the negative impact this will have on people and communities that rely on the plants for their livelihood, health care and financial income (**Hamilton**, 2004).

Plant overexploitation was not a problem for the early humans as they had vast knowledge about medicinal plants; they valued and strived to maintain healthy ecosystems through sustainable practices (**Tshisikhawe et al.**, 2012). Only skilled traditional healers were responsible for harvesting. They followed strict collection practices, which observed collection times and quantities (**Williams et al.**, 2000). Nowadays, the increased demand for medicinal plants has led to the rise of many untrained harvesters who do not consider that plants may go extinct due to their unsustainable harvesting practices (**Williams et al.**, 2000, **Tshisikhawe et al.**, 2012).

However, not all is lost. The conservation status of medicinal plants can be improved by developing sustainable use and harvesting practices. These include acquiring adequate information about the medicinal plants such as their distribution and the genetic diversity of wild populations and relatives. Furthermore, studies should be conducted that determine the annual yield of plants that can be harvested without damaging populations. This information is essential in developing sustainable use strategies (**Schippmann et al.**, 2002).

1.2. Sustainable use of plants as medicine

A rapid decrease in biodiversity has been observed in ecosystems globally. This decline not only limits the availability of medicinal plants, but also causes an imbalance in the ecosystem where many ecosystem services have been disturbed because certain species have been lost or have gone extinct (**Shinwari and Qaiser**, 2011). Indiscriminate harvesting has significantly contributed to the decline of species in ecosystems (**Aremu**, 2009). Unlike aerial parts harvesting, such as that of fruits and leaves, the harvesting of the roots, bulbs, or bark damages the plant. Bark harvesting is especially harmful when the tree is ring-barked, which disturb the tree's long term growth and reproductive activity (**Williams et al.**, 2000). An observation made in South Africa is that the bark is the most harvested and sought after product in the traditional medicine market. The effects that bark harvesting has on medicinal plants in South Africa require attention from all research fields to preserve the country's rich floral heritage and traditional health care (**Grace et al.**, 2002). The threat to biodiversity harms the livelihoods of many people that depend on and trade natural resources. This has led to a realization that there is an urgent need for sustainable use and conservation of all plants, all of which will only be successful when local communities are involved (**Van Wilgen et al.**, 2013).

Various agencies suggested the cultivation of medicinal plants to conserve the plants in the wild and minimize the pressure on wild resources. However, this method is not without its disadvantages and may not be the perfect solution to solve plant exploitation. Disadvantages include a decrease in genetic diversity and a lessened interest from locals to conserve wild populations (**Schippmann et al.**, 2002). Some strategies listed as significant in promoting and achieving conservation include conducting studies on the habitat, distribution, and abundance of medicinal plants. Conducting studies on the spread of medicinal plant knowledge and use in various societies; studying medicinal properties, quality and the

interaction of the plants with their environment are also possible strategies of promoting and achieving plant conservation. These will be followed by the establishment of methods to monitor the sustainability of current harvesting processes of medicinal plants. Finally the implementation of sustainable harvesting methods (**Hamilton**, 2004). Several of the strategies mentioned above are covered in the current *Croton* study and, therefore, the results may be an example of the efforts to improve the conservation strategies of other medicinal plants. **Schipmann et al.** (2003) noted sustainable harvesting as an essential conservation strategy for wild-harvested plants, notwithstanding their significance to the economies of local communities. **Schipmann et al.** (2003) further explained that the main idea of sustainable harvesting involved conducting non-destructive harvests, thus maintaining the populations and ecosystem diversity. Hence this study focused on comparing various phytochemical and pharmacological properties of the plant parts of the studied *Croton* species.

1.3. Role of plants in drug discovery

The process of drug discovery from plants is multifaceted, with biological, botanical, ethnobotanical, and phytochemical expertise collectively integrated (**Jachak and Saklani**, 2007). This process is often initiated by the efforts of ethnobotanists who research relationships between people and plants. Here, knowledge about indigenous use of plants as remedies by local herbalists gives clues to plants with pharmacologically active compounds (**Cox and Balick**, 1994). Pharmacological and phytochemical studies validate the selected plants' use and safety (**Light et al.**, 2005). These plants may be potential cures for a range of ailments, including HIV/AIDS, malaria, Alzheimer's, pain, and cancer (**Cox and Balick**, 1994, **Jachak and Saklani**, 2007).

The selection of plants as potential sources of novel drugs is considered advantageous because of their long history of use by humans. There is an expectation that bioactive compounds extracted from these plants would be less toxic to humans (**Fabricant and Farnsworth**, 2001). Some of the most prominent early plant-derived drugs include the most potent analgesics for chronic pain, such as codeine and morphine, which are obtained from opium poppy *Papaver somniferum* L.; the cardiac glycoside digoxin obtained from *Digitalis* species; aspirin derived from the bark of the willow tree (*Salix* sp.), atropine, a muscaric antagonist, obtained from *Atropa belladonna* L. and many more (**Houghton**, 1995, **Brower**, 2008, **Dutra et al.**, 2016). Medicinal plants have played a significant role in oncology therapeutics as well. About 60% of the drugs that have been used to fight cancer over the past century are plant-derived (**Balunas and Kinghorn**, 2005, **Dutra et al.**, 2016). These plant-derived chemotherapeutic agents such as taxol, camptothecin, vincristine, and vinblastine, have had a major impact in improving the treatment of various kinds of cancer (**Raskin et al.**, 2002, **Shoeb**, 2006)

Studies have proposed traditional based medicine as a reliable alternative treatment for fever, cold, cough, chest pain, asthma, throat and ear infection worldwide. Thus, medicinal plants' significance in treating respiratory diseases is supported (**Dutra et al.**, 2016). The proven efficacy of various medicinal plants tested against a wide range of respiratory diseases globally has led to hopes that medicinal plants could be used as potent medicine against the current pandemic; COVID-19 (**Khan et al.**, 2020). Plants continue to be a relevant source of leading pharmaceuticals (**Raskin et al.**, 2002). This is attributed to the ethnobotanical approach, which has proven to be highly productive (**Cox and Balick**, 1994). Together with ethnopharmacology, this expertise could play a significant role in the global HIV/AIDS pandemic (**Light et al.**, 2005). A more recent drug discovery approach involves using various

techniques to standardize herbal medicine and interpret analytical marker compounds (**Jachak and Saklani**, 2007).

1.4. Medicinal plant misidentification and use of adulterants

The increased use and international trade of medicinal plants have given rise to more challenges, such as the inability to instantly and accurately identify traded plants, mainly because only part of the plant is being sold (**Chen et al.**, 2010). Accurate identification and authentication procedures for medicinal plants are crucial for people's safety and health. These procedures will help prevent problems, especially when plants are traded (**Techen et al.**, 2004). The traditional medicine industry is known to be plagued with fraudulent activities, whereby medicinal plant adulterants and substitutions of certain medicinal plants sold to people in the absence of the required plants. The substitutions are usually closely related species. This causes great concern because the adulterant may not be effective or could possibly be toxic (**Techen et al.**, 2004, **Techen et al.**, 2014).

Consumption and use of misidentified herbs have been the leading cause of death in the traditional medicine industry (**Rates**, 2001, **Ndhlala et al.**, 2011). In order to use medicinal plants and the indigenous knowledge associated with them more effectively with little to no identification problems, there is a need to develop DNA barcode-based identification tools, chemical profiles and the use of phylogenetic analysis to identify clades of species that contain high levels of the desired phytochemicals (**Singh and Prakash**, 2011). Analysis of plant DNA barcodes is a reliable and effective pharmacognostic method of solving plant identification problems caused by identification uncertainty (**Selvaraj et al.**, 2012). A medicinal plant DNA barcode reference library could be created and used to support plant identification (**Ganie et al.**, 2015).

1.5. The genus *Croton*

The genus *Croton* belongs to the family Euphorbiaceae and consists of about 1300 species (**Salatino et al.**, 2007). Many of them have a long history of use in traditional medicine in South America, Africa, and Asia (**Salatino et al.**, 2007, **Adelekan et al.**, 2008). Ailments that *Croton* species are commonly used for include fever, digestive problems, cancer, constipation, hypertension, external wounds, malaria, inflammation, diabetes, chest complaints, rheumatism, bleeding gums, cough, pain, and ulcers (**Salatino et al.**, 2007). Despite their extensive use, there have been only a few scientific studies to validate the healing properties and efficacy of local *Croton* species in South Africa (**Van Wyk et al.**, 1997). *Croton* is known for its chemical complexity; species within this genus contain a wide variety of chemical compounds (**Van Wyk et al.**, 1997). In KwaZulu-Natal (KZN) and nearby Eswatini/southern Mozambique, five *Croton* species occur (**Table 1.1**) and all are used for medicinal purposes. The two most commonly used species are *Croton gratissimus* Burch. and *Croton sylvaticus* Hochst. These two *Croton* species share a common Zulu name 'umahlabekufeni,' and their bark is mostly used in treating the same ailments (**Van Wyk and Gericke**, 2000).

1.6. Morphology, geographical distribution and ethnomedicinal uses of *Croton* species found in KZN

Croton gratissimus Burch. (Lavender Croton) is a shrub or tree that grows to a height of 10 meters. The tree is slender with drooping leaves and a crown with hanging terminal branches that extend in a V-shape. The leaves have a dark-green colour on the upper surface and the lower surface is silver to grey with brown spots (**Coates Palgrave**, 2000). It is mostly distributed in Africa's tropics, over various forest types, and a wide range of altitudes,

frequently associated with rocky outcrops. The tree occurs in warm and dry regions on stony and sandy hillsides. When crushed, the leaves produce a pleasant smell that can be used in perfumes (**Boon**, 2010).

This is one of the many plants used by the Venda people to treat candidiasis and other fungal infections (**Masevhe et al.**, 2015). All of its parts are known to have medicinal value (**Van Vuuren and Viljoen**, 2008). The leaves are used to make a decoction to treat coughs (**Mulholland et al.**, 2010) and sores associated with sexually transmitted infections (**Van Vuuren and Naidoo**, 2010). The bark is used to treat respiratory illnesses such as intercostal neuralgia (**Mulholland et al.**, 2010), stomach disorders, and bleeding gums (**Mthethwa et al.**, 2014). It also alleviates skin inflammation, wounds, and chest pain (**Hutchings**, 1996). A study done by (**Mthethwa et al.**, 2014) confirmed that extracts from *C. gratissimus* exhibit antimicrobial and anti-HIV properties. Thus it may have potential as both an antiviral and an antimicrobial agent. Even with its extensive use, there is still a need for further scientific studies to validate the therapeutic properties that *C. gratissimus* claimed to have by users (**Van Vuuren and Viljoen**, 2008).

Croton sylvaticus Hochst. (Forest Croton) is a semi-deciduous shrub or large tree (**Boon**, 2010). The bark is grey and becomes rough with age. It has large, smooth, alternating leaves, which are ovate with a pointed apex. It is commonly found in forests or dense woodlands (**Boon**, 2010).

Studies have shown that *Croton sylvaticus* is traditionally used to treat about 24 human and animal diseases (**Maroyi**, 2017). It is used to treat uterine and abdominal disorders, fever, and internal inflammation (**Hutchings**, 1996, **Selowa et al.**, 2010). It is also used to treat respiratory ailments, cough, tuberculosis (**Sawhney et al.**, 1978), rheumatism, and pleurisy,

(**Netshiluvhi**, 1996) and is used as a purgative (**Elgorashi et al.**, 2002). Extracts from leaves are used to treat ear infections, malaria and cancer (**Maroyi**, 2017). The leaves are also used to prepare a poultice for treating pleurisy (**Palgrave**, 2002). The bark is highly sought after and stocked in the South African muthi markets (**Maroyi**, 2017).

Croton steenkampianus Gerstner (Maputaland Croton) is a shrub or tree. It grows on edges of sand forests and thickets in Maputaland (**Boon**, 2010). It has smooth, basal much-branched stems. The leaves are large (12 × 7 cm) and mostly ovate-heart shaped with a pointed apex, dark green at the top, and grey on the underside (**Boon**, 2010). This plant is endemic to specific areas in central Africa and the eastern part of Southern Africa (**Coates Palgrave**, 2000).

C. steenkampianus is used to treat back pain, joints problems, and rheumatism (**Hutchings**, 1996, **Selowa et al.**, 2010). Steam from a leaf decoction is inhaled for pain relief (**Pooley**, 1993, **Adelekan et al.**, 2008).

Croton pseudopulchellus Pax (Small Lavender Croton) is a shrub or small tree that grows up to 3-5 m tall (**Boon**, 2010). It looks similar to *C. gratissimus* but is shorter, and the leaves are 6 × 2 cm in size, with red to brown scale dots on the surface (**Hargreaves**, 1991). It is multi-stemmed with a rough brown-grey bark when matured (**Boon**, 2010)

The leaves are used as an ointment by Tanzanians to relieve chest infirmities. *C. pseudopulchellus* is known to have antiviral properties due to its action against tussive conditions (**Langat et al.**, 2012). The species is also used to treat asthma (**Hargreaves**, 1991) and malaria (**Salatino et al.**, 2007).

Croton menyharthii Pax (Rough-leaf Croton) is a small shrub that grows up to 4 m tall (**Boon**, 2010). It is similar to *C. gratissimus;* however, its leaves are smaller (up to 7 x 3 cm) with yellow scale speckles scattered on the surface (**Hargreaves**, 1991). The plant has elliptic to ovate-lanceolate, dull-green rough hairy leaves and hairy twigs. Its distribution is scattered across South Africa, usually forming dense stands in bushveld and thickets (**Boon**, 2010).

The leaves, roots, and root bark of *C. menyhartii* have been used in folk medicine to manage and treat intestinal obstruction, ascites, hepatitis, and dysmenorrhoea (**Aderogba et al.**, 2013).

Croton rivularis Müll.Arg. is a small shrub growing up to 2 m tall, commonly found in bushveld along rivers (**Boon**, 2010). Stems and twigs are hairy. Leaves are dark green and ovate-lanceolate in shape (up to 12 ×7 cm). They have unevenly serrated margins and are covered in star-shaped hairs (**Boon**, 2010).

To the best of our knowledge, there is no record of the medicinal use of *C. rivularis*. However, it occurs in KZN and will be tested in this study for potential pharmacological properties.

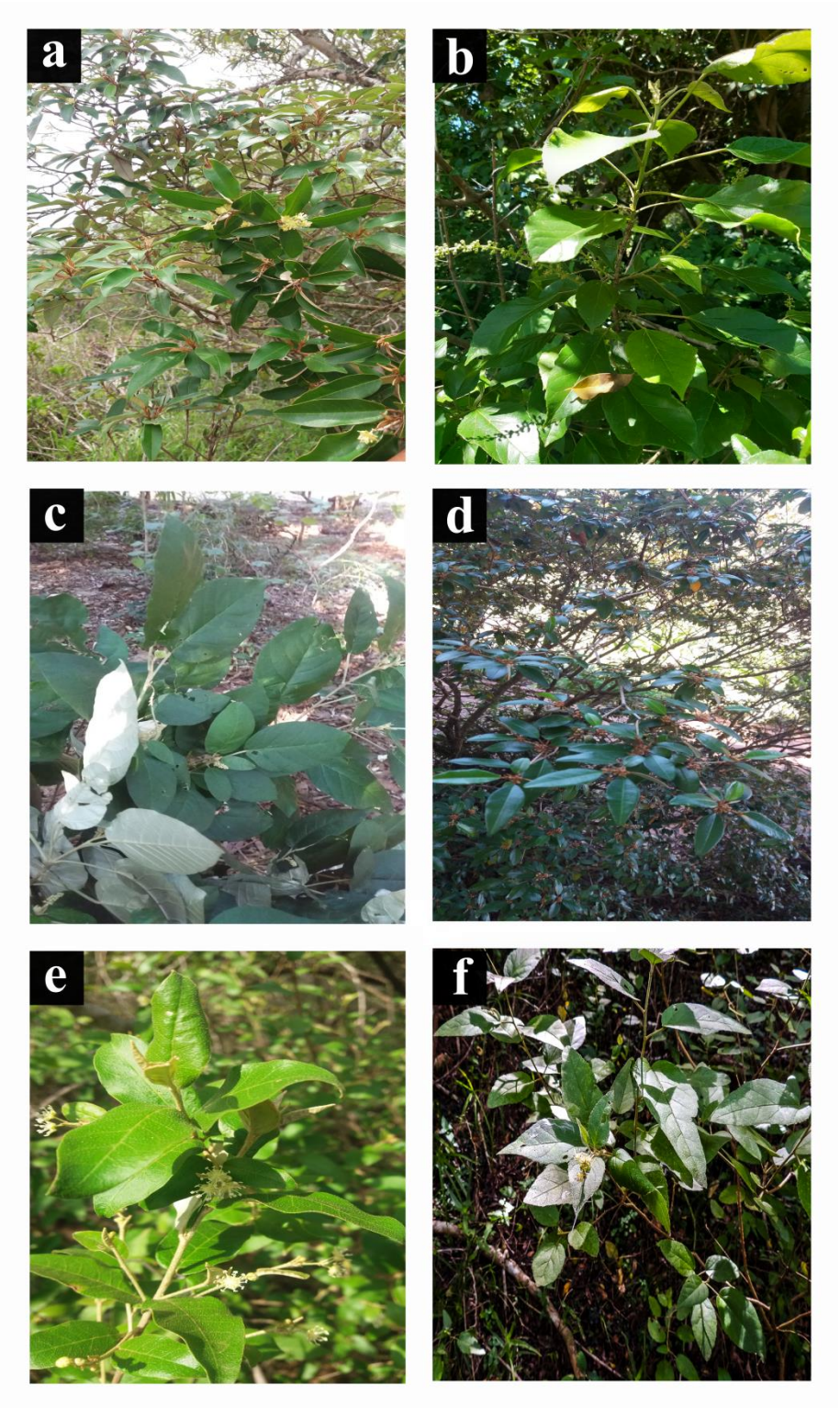


Figure 1.1: Images of *Croton* species that occurring in KZN, South Africa. a=*C. gratissimus*, b=*C. sylvaticus*, c=*C. steenkampianus*, d=*C. pseudopulchellus*, e=*C. menyhartii*, and f=*C. rivularis*. Images a-e by Tanya Mathe and image f by Graham Grieve.

1.7. Disease-causing bacteria

1.7.1. *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, facultative aerobic bacterium from the family Staphylococcaceae that can also grow in the absence of oxygen by using alternative terminal electron accepters or fermentation (**Masalha et al.**, 2001). This bacterium can adapt to different environments (**Balasubramanian et al.**, 2017). Humans are natural carriers of non-pathogenic *S. aureus;* these are more common than pathogenic strains (**Chambers**, 2001). However, *S. aureus* has proven to be a human pathogen responsible for a wide range of nosocomial infections (**Chan and Foster**, 1998). It is also known to occur on the skin (**Feng et al.**, 2000). *S. aureus* infection is mostly responsible for soft tissue, skin, joint, bone endovascular, and respiratory disorders (**Lowy**, 1998). Even though it is rare *S. aureus* does cause urinary tract infections (UTI), with symptoms such as fever, dysuria, altered mental status, hematuria, and suprapubic pain (**Tong et al.**, 2015). Skin infections caused by the presence of *S. aureus* include cutaneous abscesses, impetigo, pyomyositis, and folliculitis (**Balasubramanian et al.**, 2017).

1.7.2. *Escherichia coli*

Escherichia coli is a Gram-negative, rod-shaped bacterium from the family Enterobacteriaceae. The bacterium grows in both aerobic and anaerobic conditions (**Croxen et al.**, 2013). *E. coli* is common in humans' gastrointestinal tract (**Feng et al.**, 2000). Although it is not pathogenic, there are some pathogenic strains, causing extra-intestinal diseases and diarrhea. Different variants of *E. coli* (pathotypes or pathovars) have been studied and attributed to globally cause disease and fatalities (**Croxen et al.**, 2013). Conditions related to the different *E. coli* pathotypes include enteric diseases with symptoms

ranging from diarrhea to severe dysentery. When colonizing the urinary tract, some *E. coli* pathotypes may result in cystitis or pyelonephritis or cause extra-intestinal infections, in addition it can cause septicemia and meningitis (**Donnenberg and Whittam**, 2001).

1.7.3. *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative, non-motile bacterium of the family Enterobacteriaceae. It is found widely spread in the environment and is found to inhabit the human gastrointestinal tract (**Lawlor et al.**, 2005). *K. pneumonia* is also present as a saprophyte in the intestinal tract and the nasopharynx (**Podschun and Ullmann**, 1998). Various *K. pneumoniae* infections have been shown to occur after gastrointestinal colonization, and the gastrointestinal tract is known to transmit bacteria (**Struve and Krogfelt**, 2004). Numerous diseases can result from *K. pneumonia* infection; these include bacteremias, urinary tract infections, liver abscesses, and pneumonia. Hyper-virulent and antibiotic-resistant strains of *K. pneumoniae* have emerged and spread with time; this has resulted in a wide range of people being susceptible to infections. This has made it hard to treat (**Paczosa and Mecsas**, 2016).

1.7.4. *Enterococcus faecalis*

E. faecalis is a commensal Gram-positive bacterium from the family Enterococcaceae (**Shioya et al.**, 2011). It is a fermentative, non-spore forming, facultative anaerobe, with the majority of its strains being non-motile and non-hemolytic (**Rôças et al.**, 2004). *E. faecalis* was listed as one of the primary causative agents of urinary tract infections (**Shankar et al.**, 2001). *E. faecalis* causes wound infections such as intra-abdominal abscesses, peritonitis, and infected surgical wounds. Entry of *E. faecalis* in the genital or gastrointestinal tract leads to

bacteremia, followed by *E. faecalis* colonizing the heart tissue and producing acute or sub-acute bacterial endocarditis (**Guzman et al.**, 1989). It has also been found that *E. faecalis* occupies the oral cavity where it causes endodontic infections (**Rôças et al.**, 2004). In a study conducted by **Rôças et al.** (2004) it was revealed that the prevalence of *E. faecalis* is commonly associated with persistent endodontic infection or failed endodontic therapy than with primary infection.

1.8. Fungi as a human pathogen

Research done on fungi has estimated the number of existing fungal species to be approximately 611 000, with 600 species being human pathogens (**Mayer et al.**, 2013). The infections caused by fungi in humans range from mild skin infections to severe dermal and lethal systematic infections (**Mayer et al.**, 2013). A high percentage of fungal infections clinically diagnosed are related to pathogens from the genera *Aspergillus*, *Candida*, and *Cryptococcus.* These fungi are ubiquitous and have been the cause of high mortality rates (**Karkowska-Kuleta et al.**, 2009). Fungal species were rated the fourth common cause of nosocomial infections in the United States of America and caused 50% death of infected individuals (**Mayer et al.**, 2013). In review based on scattered data **Brown et al.** (2012) estimated that the mortality rate associated with fungal infections has been higher than 50%. This has been a cause of great concern in the medical sector (**Brown et al.**, 2012).

Various factors are responsible for this alarming rise in mortality, even after intensive antifungal drugs have been administered to the patients. These include the development of antifungal drug resistance by the pathogens, late diagnosis of the cause of disease in the patient, and immunodeficiency in the patient (**Karkowska-Kuleta et al.**, 2009). Ninety percent of HIV infected individuals contract at least one fungal infection. These fungal

infections are responsible for the mortality of about 10-20% of these HIV infected individuals (**Samie et al.**, 2010). It is interesting to note that most healthy individuals rarely contract fungal infections, and if contracted, their immune system can fight them off without medication assistance (**Brown et al.**, 2012). Just as in the case of bacteria, many fungal species have developed resistance against a wide range of commercial antifungal agents. However, most useful drugs are expensive and inaccessible to most people (**Samie et al.**, 2010). One of the most common human pathogens is *Candida albicans.*

Candida has been proven to be a highly opportunistic pathogen mostly prevalent in immune-compromised patients like those infected with HIV (**Motsei et al.**, 2003, **Amoo et al.**, 2011, **Mukanganyama et al.**, 2011).

With the alarming increase in HIV/AIDS infections in South Africa, Candida's opportunistic infections become unmanageable, thus requiring an intense search for alternative control methods or sources of medicine (**Samie et al.**, 2010). Some of the most common infections caused by *Candida* are oral or vaginal candidiasis and lethal systematic infections (**Motsei et al.**, 2003). While *C. albicans* is notorious for causing pathogenic infections in humans, it may also form part of the normal micro-flora in most individuals, residing commensally in most healthy individuals (**Naglik et al.**, 2003, **Mayer et al.**, 2013). In the case of an imbalance in the normal micro-flora or if the immune system is compromised, *Candida* species become pathogenic (**Naglik et al.**, 2003).

1.9. Aims and objectives

The aims and objectives of this study were:

- to investigate and compare the antibacterial and antifungal properties of different *Croton* species found in KZN and parts of the plants;
- to analyse and compare the phytochemical composition of different *Croton* species and various plant parts:
- to investigate the genotoxicity of the various species and their parts to determine their safety as medicines;
- to provide DNA a barcodes for locally sourced specimens of all the *Croton* species occurring in KZN;
- to perform a basic phylogenetic analysis in order to confirm that sequences of KZN specimens group with other existing of the same taxon collected elsewhere and establish the phylogenetic relationships of taxa previously not included in any study; and
- to investigate if phylogenetic relatedness influences phytochemical properties.

CHAPTER 2

Antimicrobial susceptibility testing

2.1. Introduction

Waksman (1947) and **Willey** et al. (2008) defined antibiotics as chemical substances of microbial origin that possess the ability to control and inhibit the growth and/or metabolic activities of bacteria. Millions of lives have been spared over the past decades through the use of antibiotic therapy (**Abdallah**, 2011, **Yadav and Agarwala**, 2011). However, the extensive use and misuse of antibiotics have resulted in the emergence of different kinds of antibiotic-resistant bacterial strains (**Hart and Kariuki**, 1998, **Selowa et al.**, 2010). Some pathogenic strains have developed resistance to more than one antimicrobial agent (multiple drug-resistance), whereas others resist the effects of nearly all known antibiotics (**Levy and Marshall**, 2004, **Levy**, 2005). For instance; nearly all *Staphylococcus aureus* strains worldwide have developed resistance to penicillin (**Gibbons**, 2005, **Sibanda and Okoh**, 2007, **Hemaiswarya et al.**, 2008, **Chambers and DeLeo**, 2009). Similarly, *Escherichia coli*, *Klebsiella pneumoniae* and *Enterococcus faecalis* have all developed some kind of resistance to some of the currently available antimicrobial agents (**Tenover**, 2006, **Abdallah**, 2011). Drug-resistant pathogens cause treatment failures (**Sibanda and Okoh**, 2007, **Abdallah**, 2011) and hence cause spikes in morbidity and mortality rates especially in the wake of the HIV/AIDS pandemic (**Levy and Marshall**, 2004, **Samie et al.**, 2010). The unprecedented evolution and global spread of anti-infection resistant pathogens necessitates an urgent need to develop novel and effective therapeutic drugs (**Ahmad and Beg**, 2001, **Gibbons**, 2005, **Brown et al.**, 2014).

Plants can be a reliable source of therapeutic compounds as they contain bioactive secondary metabolites which they produce as part of their natural defence system (**Bennett and Wallsgrove**, 1994, **Jachak and Saklani**, 2007). The process of drug discovery from plants is multifaceted, with biological, ethnobotanical and phytochemical expertise collectively integrated (**Jachak and Saklani**, 2007). This process is often initiated by the efforts of ethnobotanists to research relationships between people and plants. Here knowledge about indigenous use of plants as remedies by locals gives clues of plants with possible pharmacological compounds (**Cox and Balick**, 1994). Pharmacological and phytochemical bioassays often validate the traditional use of medicinal plants and also ascertain their safety (**Light et al.**, 2005). The first medicines produced from these plants are usually in the form of crude drugs such as tinctures, teas, poultices, powders and other herbal formulations (**Balunas and Kinghorn**, 2005, **Jachak and Saklani**, 2007). About 109 antibacterial drugs were approved between the years 1981 and 2006; from these 69% were derived from natural products, and, 21% of the antifungal drugs originated from natural products or compounds that resemble natural products (**Newman**, 2008, **Savoia**, 2012).

Croton species are used widely in South African traditional medicine (**Hutchings**, 1996). Detailed investigations aimed at validating the traditional use of various *Croton* species were previously conducted by **Van Vuuren and Viljoen** (2008) and **Langat et al.** (2011). However, none of the studies focused on comparing the pharmacological properties of the species growing in different regions. The current study was designed to compare the antibacterial and antifungal efficacy of different *Croton* species occurring within different parts of the KwaZulu-Natal province, South Africa. This study further compares the antibacterial and antifungal activities of the different plant organs of the various *Croton* species occurring in KwaZulu-Natal province, South Africa. The results from this

comparative study will be beneficial in the efforts of addressing the problem of over harvesting and exploitation of medicinal plant.

2.2. Materials and methods

2.2.1. Plant material harvest and preparation of samples

Information on the location of different *Croton* species in the KwaZulu-Natal (KZN) province, South Africa was obtained from the Bews Herbarium (NU), University of KwaZulu-Natal (UKZN), Pietermaritzburg (PMB), South Africa. Different organs of the plants (leaves, twigs and stem bark) were collected from different geographical locations within KZN. The identity of the species was confirmed by Alison Young and Benny Bytebier, Curator, Bews Herbarium, UKZN-PMB and voucher specimens (**Table 2.1**) were prepared and deposited at the same herbarium. Once harvested, different plant parts were oven-dried (50 C), ground in a milling machine to fine powders and stored at room temperature in brown paper bags for further use.

Table 2.1: *Croton* species collected in KwaZulu-Natal, south Africa, with their voucher and Bews Herbarium (NU) barcode numbers

Plant species	**Locality and date of collection**	**Voucher and barcode number**
Croton gratissimus Burch.	Southport–20 April 2017	T2-NU0090740
	Durban Botanic Gardens-24 May 2017	T6-NU0090736
	Muden5–26 January 2018	T19-NU0087082
Croton sylvaticus Hochst.	Southport–20 April 2017	T3-NU0090725
	Umdoni Park-20 September 2017	T11-NU0087085
	Vernon Crook-21 September 2017	T14-NU0090728
	Ntunjambili–9 May 2017	T5-NU0090731
Croton steenkampianus Gerstner	Durban Botanic Gardens-24 May 2017	T7-NU0090730
	Kosi Bay-16 November 2017	T18-NU0087087
Croton pseudopulchellus Pax	Durban Botanic Gardens-24 May 2017	T8-NU0090737
	Mkuze Game Reserve–15 November 2017	T17-NU0087084
	Southport-20 September 2017	T12-NU0090738
Croton menyhartii Pax	Kranskop-9 May 2017	T4-NU0090732
	Mkuze Game Reserve–14 November 2017	T16-NU0087086
Croton rivularis Müll.Arg	Umzimkhulu Valley-16 February 2018	T20-NU0087083

2.2.2. Extraction of plant material

Dry and powdered plant materials were mixed with different solvents (distilled water, petroleum ether (PE), 80% methanol (MeOH) and dichloromethane (DCM) at a ratio of 10:1 (10 ml/g). Various phytochemicals are extracted in solvents of differing polarity based on their chemical composition, since no one solvent can accurately extract all of the phytochemical and antioxidant compounds found in plant material (**Lin and Giusti**, 2005, **Nawaz et al.**, 2020). Different solvents were used to facilitate extraction of all biologically active compounds in the plant material. The resultant solutions were stirred at room temperature for 12 h at 150 rpm in a rotary shaker (Edmund Bühler, Tübingen, German), after which they were sonicated on ice for 1 h (Julabo GMBH, Germany). The plant extracts (only solvent extracts) were then filtered under vacuum using Whatman No 1 filter paper in Büchner funnels and later concentrated using a rotary evaporator (Heldolph vv2000, Germany) at 35 C. The concentrated extracts were transferred to glass pill vials and dried under a fan. All water extracts were freeze-dried (Virtis freeze-drier, Benchtop Pro). Both aqueous and organic solvent extracts were stored in the dark at 10 C for further use. Dry plant material were weighed and resuspended in 20% dimethyl sulfoxide (DMSO) to make a final concentration of 10 mg/ml.

2.3. Antibacterial assay

2.3.1. Bacterial stock preparation

A colony of each bacterial strain (*Escherichia coli* ATCC 11775, *Klebsiella pneumoniae* ATCC 13883, *Staphylococcus aureus* ATCC 12600 and *Enterococcus faecalis* ATCC 19433) was inoculated in 5 ml sterile Muller-Hinton (MH) broth and incubated over night at 37 C in a water bath with an orbital shaker (40 rpm). Agar plates were prepared by pouring 20 ml of

hot sterile MH agar into Petri dishes and were allowed to cool and set. The bacterial test strains were each then streaked on different agar plates and incubated at 37 C for 24 h. The resulting plates were stored in a refrigerator (4 C) and the bacterial strains were sub-cultured on a monthly basis to maintain cell viability.

2.3.2 Microdilution bioassay

The microdilution antibacterial assay (**Eloff**, 1998) was used to determine the antibacterial activity of the plant extracts. Single colonies from the test bacterial strains (*E. coli*, *K. pneumoniae*, *S. aureus* and *E. faecalis*) were inoculated in 5 ml MH broth and incubated overnight at 37 C in a water bath with an orbital shaker. The absorbance of overnight bacterial cultures were read using a UV-visible spectrophotometer (Varian Cary 50, Australia) at 600 nm and the cultures were diluted with MH broth to a final inoculum of approximately of 10^6 cfu/ml (colony forming units). One hundred microliters of the resuspended plant extracts (10 mg/ml) were two-fold serially diluted with distilled water to make up concentrations ranging from 2.5-0.02 mg/ml, in triplicates, down a 96-well micro-plate (Greiner Bio-one, Germany). A two-fold serial dilution of neomycin (0.1 mg/ml) was used as a positive control against each test bacterial strain. Sterile MH broth, distilled water and 20% DMSO were used as the negative controls. The plates were then covered with parafilm and incubated overnight at 37 C. Growth of bacteria was detected by adding 50 µl of 0.2 mg/ml p-iodonitrotetrazolium chloride (INT) to each well and the plates were further incubated for 1 h at 37 C. Bacterial growth was indicated by a red-pink colour, whereas clear wells indicated inhibition of bacterial growth by the test plant extract. Minimum inhibitory concentration (MIC) values were recorded as the lowest concentration of the extract showing a clear well. The assay was done twice; also in triplicates to confirm results. Plant extracts

with MIC values lower than 1 mg/mg were considered to have noteworthy antibacterial activity (**Eloff**, 1998).

2.4. Antifungal assay

2.4.1. Fungal stock preparation

Candida albicans (ATCC 10231) bought from the African Bureau of Standards was used in the present study. One hundred microlitres of the fungal stock kept in a -70°C freezer was inoculated in 5 ml Yeast Malt broth (YM) and incubated overnight at 37°C. The overnight culture was streaked onto a Petri dish with solidified yeast malt agar and incubated further overnight at 37°C. The resulting agar culture plates were stored in the refrigerator at 4°C.

2.4.2. Microdilution bioassay

The antifungal activity of the assessed *Croton* species was determined by following the micro-dilution assay as previously described (**Eloff** (1998)) and modified for fungi by **Masoko** et al. (2007). The fungal cultures growing on YM agar plates (20 ml) was inoculated in 5 ml of YM broth and incubated in a shaker at 37°C overnight. Four hundred microliters of the overnight *C. albicans* culture was added to 4 ml of sterile saline (0.85% NaCl). The absorbance was read at 530 nm. The McFarland standard culture solution of 0.5 M (ranging from 0.2500-0.2800) was obtained and adjusted by adding sterile saline. The standardised fungal solution was further diluted with sterile YM broth making a 1:1000 dilution. The plant extracts were resuspended in 20% DMSO at a concentration of 50 mg/ml. In a 96-well micro-plate (Greiner Labortechnik) each resuspended extract was two-fold serially diluted with sterile distilled water to make up concentrations ranging from 12.5-0.098 mg/ml in the wells.

A similar two-fold dilution was done with Amphotericin B (0.25 mg/ml) as a positive control. Water, DMSO (20%), and sterile YM were used as the negative controls. One hundred microliters of the diluted fungal culture were added to each well. The plates were covered with parafilm and incubated at 37°C for 24 h. To obtain MIC values, 50 µl of INT (0.2 mg/ml) were added to each well and further incubated for 24 h. Wells that remained clear indicated that there was inhibition of fungal growth, while a red-pink colour in the wells indicated the growth of *C. albicans.* The MIC was recorded as the lowest concentration where there was no fungal growth. Here MIC<1 mg/ml were considered noteworthy. The experiment was repeated twice and was done in triplicates.

2.5. Results and discussion

Antimicrobial activity results of 160 extracts from six *Croton* species is presented in **Table 2.2**. Here not all plant organs were collected for all plants, thus only 160 extracts. The efficacy of the extracts was measured as the MIC. This is the lowest concentration at which microbial growth is inhibited by the extract. Minimum inhibitory concentrations less than 1 mg/ml were considered as noteworthy for both antibacterial and antifungal activity (**Eloff**, 1998, **Katerere and Eloff**, 2008).

There has been previous scientific research conducted to validate the use of *C. gratissimus* in traditional medicine. In a study conducted by **Van Vuuren and Naidoo** (2010), essential oils extracted from *C. gratissimus* leaves displayed noteworthy inhibitory activity against *Neisseria gonorrhoeae* and *Gardnerella vaginalis*, two bacterial species associated with Sexually Transmitted Infections (STI's). These findings are significant as *C. gratissimus* is traditionally used to treat STI's and wounds associated with them (**Van Wyk and Gericke**, 2000, **Koenen**, 2001, **Van Vuuren and Viljoen**, 2008). Therefore its use has been validated.

In the current study all *C. gratissimus* organs displayed noteworthy antibacterial activities, except for twig and stem bark extracts (*C. gratissimus*) from plants collected at Muden. *C. gratissimus* from the Durban Botanic Gardens and Southport had more extracts with noteworthy inhibitory activity in their organs compared to other samples of the same species. The noteworthy activities were only for two of the tested bacterial species; these were *S. aureus* and *E. faecalis*. These leaf extracts displayed noteworthy antibacterial activities against two Gram-positive bacterial strains namely *S. aureus* and *E. faecalis*, and a Gram-negative strain (*K. pneumoniae*) with MIC values ranging from 0.16-0.63 mg/ml. They were the only leaf extracts to demonstrate noteworthy antibacterial activities against *S. aureus* (MIC 0.16-0.24 mg/ml) and *K. pneumoniae* (MIC = 0.63 mg/ml) within the species *C. gratissimus*. The most effective *C. gratissimus* extracts were the Durban Botanic Gardens DCM and PE leaf extracts with MIC values of 0.24 and 0.16 mg/ml, respectively. Generally, DCM and PE extracts of leaf samples collected in Durban Botanic Gardens, Southport and Muden demonstrated noteworthy antibacterial activities against *E. faecalis* (MIC range: 0.31-0.94 mg/ml).

Extracts from twigs collected from Southport yielded noteworthy antibacterial activities against three of the four evaluated bacterial strains, while those from the Durban Botanic Gardens displayed noteworthy activities only against *E. faecalis* and *S. aureus*. Twigs from Muden yielded MIC ≥1.25 mg/ml. Some of the extracts of *C. gratissimus* stem bark collected at the Durban Botanic Gardens and Southport yielded noteworthy antibacterial properties (MIC range: 0.31-0.94 mg/ml), while those collected in Muden did not show noteworthy antibacterial properties (MIC ≥ 1.25 mg/ml).

Leaves of *C. sylvaticus* from Vernon Crookes Nature Reserve and Ntunjambili demonstrated extended-spectrum antibacterial activities with MIC ranging from 0.24-0.94 mg/ml. However, leaf extracts from Southport demonstrated noteworthy activities against only one of

the evaluated bacterial strains (*E. faecalis*, MIC=0.63 mg/ml). Both organic and aqueous solvent extracts of leaves collected from Umdoni Park yielded MIC values ≥ 1.25 mg/ ml.

C. sylvaticus twig extracts (Umdoni Park) demonstrated noteworthy antibacterial activities against three of the four tested bacterial strains (MIC range 0.31-0.94 mg/ml). However, extracts of twigs of the same plant species collected from Southport were effective only against *E. faecalis* and *S. aureus* (MIC 0.31-0.94 mg/ml). Twigs (PE extracts) collected from Ntunjambili displayed noteworthy bacteriostatic activities only against *E. faecalis* (MIC = 0.63 mg/ml).

Variations in antibacterial activities were also noticed in *C. sylvaticus* bark extracts from different locations within KZN. The organic solvent extracts of the stem bark from Umdoni Park yielded MIC values ranging from 0.31-0.94 mg/ml against two of the tested bacterial strains. However, both organic and aqueous solvent extracts from bark (*C. sylvaticus*) sample collected from Vernon Crookes Nature Reserve yielded MIC value ≥ 2.5 mg/ml.

Generally the most noteworthy inhibitory activity was observed in *C. sylvaticus* from Umdoni Park, which also showed a broad spectrum of activity in its DCM and PE twig extracts with MIC ranging between 0.31-0.94 mg/ml. A similarity was observed between this range and that of DCM and PE twig extracts of *C. sylvaticus* collected in Southport which was also 0.31-0.94 mg/ml, however this was only for two species of bacteria (*S. aureus* and *E. faecalis*). This may suggest that the principal biological active compounds responsible for inhibiting *S. aureus* and *E. faecalis* are similar for *C. sylvaticus* and are soluble in DCM and PE. Within this species the most effective extract was the DCM leaf extract collected in Vernon Crookes Nature Reserve 0.24 mg/ml against *E. faecalis*.

C. steenkampianus had two representative samples collected from the Durban Botanic Gardens and Kosi Bay. Only leaf and twig extracts of the plant species showed antibacterial

activity. Leaf extracts of *C. steenkampianus* collected at the Durban Botanic Gardens had the highest inhibitory activity with MIC ranging between 0.04-0.94 mg/ml. Leaf extracts of *C. steenkampianus* from Kosi Bay displayed noteworthy activities only against *S. aureus* (MIC range: 0.63-0.94 mg/ml).

Organic solvent extracts from the twigs of *C. steenkampianus* (Durban Botanic Gardens) yielded noteworthy activities against *S. aureus* and *E. faecalis*, while twigs of the same plant species collected in Kosi Bay yielded MIC values ranging from 0.47-0.63 mg/ml against *E. faecalis*.

The most effective *C. steenkampianus* sample was DCM leaf extract from the Durban Botanic Gardens (MIC=0.04 mg/ml) effective against *E. faecalis*. The most susceptible bacterial strain to *C. steenkampianus* was the Gram-positive *S. aureus* followed by Gram-positive *E. faecalis*. Only one DCM leaf extract was effective against Gram-negative *K. pneumoniae*. In a study conducted by **Selowa et al.** (2010) *C. steenkampianus* did not show any antimicrobial activity. This may be due to a number of factors such as the differences in the duration of extraction and the concentration of the solvent used.

C. pseudopulchellus leaf samples collected from Mkuze and Durban Botanic Gardens demonstrated noteworthy to potent antibacterial activities (MIC range: 0.08-0.94 mg/ml) against *S. aureus* and *E. faecalis*. Twig samples of the plant collected in Durban Botanic Gardens, Mkuze and Southport also demonstrated noteworthy antibacterial properties against the afore-mentioned bacterial strains (MIC range: 0.08-0.94 mg/ml). Extracts of the plant's stem bark collected from the Durban Botanic Gardens demonstrated better antibacterial properties (MIC range: 0.04-0.63 mg/ml) than bark samples of the same plant collected in Mkuze (MIC≥1.25 mg/ml).

C. pseudopulchellus showed the highest inhibitory activity in its stem bark extracts, compared to the stem bark extracts of the other species (MIC range: 0.04-0.94 mg/ml), this was followed by the stem bark extracts of *C. menyhartii* (MIC range:0.16 0.94 mg/ml). This validates the traditional use of these two species' bark in treating chest related infections, which may be caused by bacterial strains. Unlike other *Croton* species in addition to DCM and PE there were more methanol extracts that showed inhibitory activity from *C. pseudopulchellus*. This suggested that in addition to the DCM and PE soluble bioactive compounds possessed by *C. pseudopulchellus* there are other bioactive compounds that are soluble in methanol. This also suggests that *Croton* species may not all have the same chemical compounds and therefore may not all be used to treat the same ailments. A similarity in the inhibitory pattern of the leaf and twig MeOH, DCM and PE extracts against *S. aureus* and *E. faecalis* was observed.

C. menyhartii showed limited inhibitory activity in its extracts. The DCM stem bark of *C. menyhartii* collected at Mkuze showed the best MIC of 0.16 mg/ml. There was a clear difference in the inhibitory activity between the leaf extracts of this species collected in Mkuze and Kranskop. Leaves collected from Mkuze were active against *S. aureus* and *E. faecalis* (MIC: 0.63-0.94 mg/ml), while those collected from Kranskop were not active against any of the four evaluated bacterial strains. This is not what should be expected from specimens of the same species, however the difference in environmental stresses experienced by members of the same species in different regions may lead to the production or lack of certain biological active compounds. This may also explain why traditional healers are specific to the location where they collect their medicinal plants.

Twigs from Mkuze were active against both *S. aureus* and *E. faecalis* (MIC=0.63 mg/ml), while those from Kranskop demonstrated antibacterial activity only against *S. aureus.* (MIC=0.47 mg/ml).

There was only one collection of *C. rivularis*; which was from Umzimkhulu Valley. Extracts from this plant did not show any noteworthy activity. Conclusive information and a clear understanding to this could not be derived as there was one sample and no stem bark, which could have been the sample to show inhibitory activity.

Overall twig extracts generally demonstrated broad-spectrum antibacterial activities yielding MIC values ranging from 0.16-0.94 mg/ml. However, the highest inhibitory activity was observed in the DCM extract of *C. steenkampianus* leaf extract from the Durban Botanic Gardens against *E. faecalis* (0.04 mg/ml). PE extract of *C. pseudopulchellus* stem bark also from Durban Botanic Gardens showed similar inhibitory activity (0.04 mg/ml) against *E. faecalis*. The majority of the noteworthy antibacterial activities were observed in DCM extracts (**Table 2.2**). The results showed *E. faecalis* to be the most susceptible bacterial strain followed by *S. aureus.* This is no surprise as it has become a common occurrence to witness Gram-positive microorganisms showing more susceptibility to plants antibacterial extracts than Gram-negative microorganisms (**Fischbach and Walsh**, 2009, **Joshi et al.**, 2011). On the other hand, none of the plant extracts yielded any noteworthy inhibitory activity against *E. coli*. Only a few showed noteworthy inhibitory activity against *K. pneumoniae*, which are both Gram-negative bacteria. The resistance of gram-negative bacteria is attributed to the presence of an outer membrane which is made up of a lipopolysaccharide layer proteins and phospholipids. This membrane blocks the entry of substances into the bacteria. Therefore antibiotics cannot easily affect the organism (**Kumar et al.**, 2006, **Fischbach and Walsh**, 2009). The capability of *Croton* species to inhibit Gram-negative bacteria was demonstrated in a study by **Van Vuuren and Viljoen** (2008), where *C. gratissimus* leaf extracts exhibited the highest noteworthy inhibitory activity against three Gram-negative bacteria (*Pseudomonas aeruginosa, Escherichia coli,* and *Klebsiella pneumonia*) compared to the bark and roots of the same species. This highlighted the efficacy of leaf extracts.

There were a number of extracts that did not show noteworthy antibacterial activities. These included all the water extracts, the leaf and twig extracts of *C. rivularis* collected in Umzimkhulu Valley, which was the only sample of the species that was collected (due to the lack of known localities around KZN), and therefore there could not be compared with members of the same species collected in different regions. Water extracts have generally exhibited no inhibitory activity in antimicrobial studies of plants (**Lewu et al.**, 2006, **Fawole**, 2009). The water extracts in this study were not prepared in the exact way that traditional healers prepare their medicinal plants which involved boiling a medicinal plant for several hours or mixing and boiling two of more medicinal plants. This explains the inability to detect inhibitory activity of the aqueous extracts (**Clarkson et al.**, 2004).

Only one extract yielded noteworthy activity against *C. albicans*. This was from the MeoH leaf extracts of *C steenkampianus* collected at Kosi Bay, with the MIC value of 0.6 mg/ml.

2.6. Conclusions

Croton species are already known for their ethnomedicinal importance and some of the species have been subjected to phytochemical studies to validate their use (**Van Vuuren and Viljoen**, 2008, **Selowa et al.**, 2010, **Van Vuuren and Naidoo**, 2010), however the knowledge of the increased threat caused by large scale harvesting and trading of medicinal plants in South Africa (**Lewu et al.**, 2006, **Shai et al.**, 2009) prompted the necessity to conduct further comparative investigations. These were to gather information that would assist in improving sustainability of *Croton* species. This would be achieved through identifying and exploring possibilities of substitution across the species and plant organs.

There was no similar pattern in the inhibitory activity between samples of the same species collected from different regions. This may support the claim made by **Ndhlala et al.** (2013)

that geographic distribution has an influence in the chemical composition of plant species, thus the variations in antibacterial properties. Different environmental stresses are known to trigger the production of specific secondary metabolites in plants required for their survival, thus the variation in biochemical activities (**Mwine and Van Damme**, 2011). These environmental stresses may include altitude, climate, soil composition and harvesting time (**Atanasov et al.**, 2015).

A total of 107 extracts showed noteworthy inhibitory activities (MIC<1 mg/ml) against the test bacterial strains. **Figure 2.1** shows the percentages of the noteworthy activity from the different plant organs, of which twigs had the most noteworthy activity (44%) compared to the other organs. The stem bark had the lowest percent of noteworthy inhibitory activity (17%) and the leaves 39%. These results demonstrate that it is possible to substitute leaves and twigs for stem bark. Other studies by **Lewu et al.** (2006), **Van Vuuren and Viljoen** (2008), and **Van Vuuren and Naidoo** (2010) have shown results of aerial plant organs such as leaves and twigs exhibiting higher antibacterial activity compared to the bark or roots. This further suggests that the leaves and twigs are possibly more effective than the bark and that the leaves and twigs should rather be used in treatment of ailments.

There was no significant antifungal activity demonstrated by all the extracts with the exception of the MeoH leaf extracts of *C. steenkampianus* collected from Kosi Bay. This was the only extract that showed noteworthy inhibitory against fungi with MIC value of 0.6 mg/ml.

The plants evaluated in the current study were all from the same genus (*Croton)* and are mostly used to treat similar ailments (**Table 1.1**), however they did not yield the same antimicrobial activity, and this could be due to variation in concentration of the bioactive chemical compounds contained by the different plants. Plants extracts that exhibited lower or

no inhibitory activity may not be suitable candidates for pharmaceutical production and use (**Rabe and Van Staden**, 1997). Previous studies have reported noteworthy activity of essential oil extracted from *Croton* species (**Martins et al.**, 2000, **Alviano et al.**, 2005, **Van Vuuren and Viljoen**, 2008). It may be worthwhile to explore this approach in future for the species in this study.

Table 2.2: Antibacterial and antifungal activity (Minimum inhibitory concentration, MIC) of different plant organs of *Croton* species collected within KwaZulu-Natal, South Africa.

Species and locality	Plant part	Solvent	Antibacterial MIC (mg/ml)				Antifungal MIC (mg/ml)
			Bacteria				Fungi
			E. c.	***S. a.***	***E. f.***	***K. p.***	**C.a.**
C. gratissimus Southport	Leaves	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	2.5	**0.31**	1.25	>12.5
		DCM	2.5	2.5	**0.31**	>2.5	6.3
		PE	2.5	2.5	1.25	>2.5	>12.5
	Twigs	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	2.5	2.5	2.5	>12.5
		DCM	>2.5	**0.63**	**0.63**	**0.47**	6.3
		PE	>2.5	**0.63**	**0.63**	**0.63**	>12.5
	Stem bark	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	2.5	>2.5	>2.5	>12.5
		DCM	2.5	**0.31**	**0.31**	2.5	>12.5
		PE	>2.5	**0.63**	**0.63**	>2.5	>12.5
C. gratissimus D.B.G.	Leaves	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	1.25	2.5	2.5	>12.5
		DCM	2.5	**0.24**	**0.31**	**0.63**	6.3
		PE	>2.5	**0.16**	**0.63**	>2.5	6.3
	Twigs	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	**0.47**	>2.5	>2.5	>12.5
		DCM	2.5	**0.24**	**0.63**	2.5	6.3
		PE	>2.5	**0.31**	1.25	>2.5	>12.5

Table 2.2 continued…

Species and locality	Plant part	Solvent	Antibacterial MIC (mg/ml)				Antifungal MIC (mg/ml)
			Bacteria				Fungi
			E. c.	***S. a.***	***E. f.***	***K. p.***	**C.a.**
	Stem bark	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	2.5	>2.5	>2.5	>12.5
		DCM	>2.5	**0.63**	**0.94**	1.9	>12.5
		PE	>2.5	1.25	2.5	>2.5	>12.5
C. gratissimus Muden	Leaves	Water	>2.5	>2.5	2.5	>2.5	>12.5
		MeOH	>2.5	>2.5	2.5	>2.5	1.6
		DCM	>2.5	1.25	**0.63**	>2.5	3.1
		PE	>2.5	2.5	**0.94**	>2.5	9.4
	Twigs	Water	>2.5	>2.5	2.5	>2.5	>12.5
		MeOH	>2.5	2.5	>2.5	>2.5	>12.5
		DCM	>2.5	1.25	1.25	>2.5	>12.5
		PE	>2.5	1.25	1.9	>2.5	>12.5
	Stem bark	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	1.25	2.5	2.5	>12.5
		DCM	>2.5	2.5	1.25	>2.5	>12.5
		PE	>2.5	2.5	>2.5	>2.5	>12.5

Table 2.2 continued…

Species and locality	**Plant part**	**Solvent**	**Antibacterial MIC (mg/ml)**				**Antifungal MIC (mg/m l)**
			Bacteria				**Fungi**
			E. c.	***S. a.***	***E. f.***	***K. p.***	**C.a.**
C. sylvaticus Southport	Leaves	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	>2.5	2.5	2.5	12.5
		DCM	2.5	2.5	**0.63**	>2.5	6.3
		PE	>2.5	2.5	1.9	>2.5	6.3
	Twigs	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	>2.5	>2.5	>2.5	>12.5
		DCM	2.5	**0.31**	**0.94**	2.5	6.3
		PE	>2.5	**0.31**	**0.63**	>2.5	>12.5
C. sylvaticus Umdoni Park	Leaves	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	2.5	2.5	>2.5	6.3
		DCM	>2.5	2.5	1.25	2.5	6.3
		PE	>2.5	1.25	1.25	2.5	12.5
	Twigs	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	1.25	>2.5	**0.63**	>12.5
		DCM	>2.5	**0.63**	**0.94**	**0.31**	>12.5
		PE	2.5	**0.63**	**0.63**	**0.63**	>12.5
	Stem bark	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	**0.94**	>2.5	**0.63**	>12.5
		DCM	>2.5	**0.31**	1.25	>2.5	>12.5
		PE	2.5	**0.63**	1.25	>2.5	6.3

Table 2.2 continued…

Species and locality	Plant part	Solvent	Antibacterial MIC (mg/ml)				Antifungal MIC (mg/ml)
			Bacteria				Fungi
			E. c.	***S. a.***	***E. f.***	***K. p.***	**C.a.**
C. sylvaticus Ntunjambili	Leaves	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	2.5	2.5	**0.63**	2.5	9.4
		DCM	2.5	**0.94**	**0.47**	**0.94**	4.7
		PE	2.5	1.25	**0.31**	2.5	>12.5
	Twigs	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	>2.5	>2.5	>2.5	>12.5
		DCM	>2.5	>2.5	>2.5	>2.5	6.3
		PE	>2.5	1.25	**0.63**	1.9	>12.5
C. sylvaticus Vernon Crookes N.R.	Leaves	Water	>2.5	>2.5	**>2.5**	>2.5	>12.5
		MeOH	>2.5	>2.5	1.25	>2.5	>12.5
		DCM	1.25	1.25	>**0.24**	**0.63**	>12.5
		PE	2.5	**0.63**	**0.63**	**0.63**	>3.1
	Twigs	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	2.5	>2.5	>2.5	>12.5
		DCM	>2.5	**0.63**	1.25	2.5	6.3
		PE	>2.5	**0.63**	**0.31**	>2.5	>12.5
	Stem Bark	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	>2.5	>2.5	>2.5	>12.5
		DCM	>2.5	2.5	2.5	2.5	>12.5
		PE	>2.5	>2.5	**>2.5**	>2.5	>12.5

Table 2.2 continued…

Species and locality	Plant part	Solvent	Antibacterial MIC (mg/ml)				Antifungal MIC (mg/ml)
			Bacteria				Fungi
			E. c.	***S. a.***	***E. f.***	***K. p.***	**C.a.**
C. steenkampianus D.B.G.	Leaves	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	2.5	**0.47**	**0.31**	1.25	3.1
		DCM	1.9	**0.31**	**0.04**	**0.94**	3.1
		PE	>2.5	1.25	**0.94**	>2.5	>12.5
	Twigs	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	**0.63**	>2.5	>2.5	>12.5
		DCM	2.5	**0.31**	**0.47**	1.9	>12.5
		PE	>2.5	1.25	**0.63**	>2.5	>12.5
	Stem bark	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	>2.5	>2.5	>2.5	>12.5
		DCM	2.5	1.9	2.5	2.5	>12.5
		PE	>2.5	>2.5	1.25	>2.5	>12.5
C. steenkampianus Kosi Bay	Leaves	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	2.5	>2.5	>2.5	**0.6**
		DCM	>2.5	**0.94**	>2.5	1.25	1.17
		PE	>2.5	**0.63**	2.5	1.9	3.1
	Twigs	Water	>2.5	>2.5	2.5	>2.5	>12.5
		MeOH	>2.5	**0.63**	>2.5	>2.5	>12.5
		DCM	2.5	**0.47**	1.25	1.9	>12.5
		PE	>2.5	1.25	1.25	>2.5	>12.5
	Stem bark	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	2.5	>2.5	>2.5	>12.5
		DCM	>2.5	1.25	1.25	>2.5	>12.5
		PE	>2.5	1.25	1.25	>2.5	3.1

Table 2.2 continued…

Species and locality	Plant part	Solvent	Antibacterial MIC (mg/ml)				Antifungal MIC (mg/ml)
			Bacteria				Fungi
			E. c.	***S. a.***	***E. f.***	***K. p.***	**C.a.**
C. pseudopulchellus D. B. G.	Leaves	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH		**0.31**	**0.94**	>2.5	>12.5
		DCM	2.5	**0.12**	**0.16**	2.5	3.1
		PE	>2.5	**0.12**	**0.16**	>2.5	>12.5
	Twigs	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	**0.31**	2.5	>2.5	>12.5
		DCM	>2.5	**0.31**	**0.63**	>2.5	>12.5
		PE	>2.5	**0.16**	**0.63**	>2.5	>12.5
	Stem Bark	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	**0.63**	>2.5	>2.5	>12.5
		DCM	>2.5	**0.08**	**0.63**	>2.5	>12.5
		PE	>2.5	**0.63**	**0.04**	>2.5	>12.5
C. pseudopulchellus Mkuze	Leaves	Water	>2.5	2.5	>2.5	>2.5	>12.5
		MeOH	2.5	**0.31**	1.9	2.5	>12.5
		DCM	2.5	**0.08**	**0.31**	2.5	6.3
		PE	>2.5	**0.31**	**0.94**	>2.5	12.5
	Twigs	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	**0.47**	1.25	>2.5	>12.5
		DCM	>2.5	**0.31**	**0.94**	>2.5	>12.5
		PE	>2.5	**0.47**	**0.94**	>2.5	>12.5
	Stem bark	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	1.25	>2.5	>2.5	>12.5
		DCM	>2.5	>2.5	>2.5	>2.5	>12.5

Species and locality	Plant part	Solvent	Antibacterial MIC (mg/ml)				Antifungal MIC (mg/ml)
			Bacteria				Fungi
			E. c.	***S. a.***	***E. f.***	***K. p.***	**C.a.**
		PE	>2.5	>2.5	>2.5	>2.5	>12.5
C. pseudopulchellus Southport	Twigs	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	**0.31**	**0.63**	>2.5	>12.5
		DCM	>2.5	**0.12**	**0.31**	>2.5	>12.5
		PE	>2.5	**0.08**	**0.31**	>2.5	>12.5

Table 2.2 continued…

Species and locality	Plant part	Solvent	Antibacterial MIC (mg/ml)				Antifungal MIC (mg/ml)
			Bacteria				Fungi
			E. c.	***S. a.***	***E. f.***	***K. p.***	**C.a.**
C. menyhartii Mkuze	Leaves	Water	>2.5	>2.5	2.5	>2.5	>12.5
		MeOH	>2.5	2.5	>2.5	>2.5	6,3
		DCM	2.5	**0.94**	**0.94**	2.5	>12.5
		PE	>2.5	**0.63**	>2.5	>2.5	4,7
	Twigs	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	>2.5	>2.5	>2.5	>12.5
		DCM	>2.5	**0.63**	1.25	>2.5	>12.5
		PE	>2.5	**0.63**	**0.63**	>2.5	>12.5
	Stem bark	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	>2.5	>2.5	>2.5	>12.5
		DCM	>2.5	**0.16**	2.5	2.5	>12.5
		PE	>2.5	**0.63**	**0.63**	>2.5	>12.5
C. menyhartii Kranskop	Leaves	Water	>2.5	>2.5	2.5	>2.5	>12.5
		MeOH	>2.5	>2.5	>2.5	>2.5	>12.5
		DCM	>2.5	2.5	1.9	1.9	6,3
		PE	>2.5	>2.5	2.5	2.5	>12.5
	Twigs	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	2.5	>2.5	>2.5	>12.5
		DCM	>2.5	**0.47**	2.5	2.5	12,5
		PE	>2.5	**0.47**	2.5	2.5	12,5
	Stem Bark	Water	>2.5	>2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	2.5	>2.5	>2.5	>12.5
		DCM	>2.5	**0.94**	**0.63**	>2.5	>12.5
		PE	>2.5	**0.94**	1.9	>2.5	>12.5

Table 2.2 continued…

Species and locality	**Plant part**	**Solvent**	**Antibacterial MIC (mg/ml)**				**Antifungal MIC (mg/ml)**
			Bacteria				**Fungi**
			E. c.	***S. a.***	***E. f.***	***K. p.***	**C.a.**
C. rivularis Umzimkhulu Valley	Leaves	Water	>2.5	>2.5	2.5	>2.5	>12.5
		MeOH	>2.5	>2.5	2.5	>2.5	>12.5
		DCM	2.5	2.5	>2.5	2.5	9,4
		PE	>2.5	>2.5	>2.5	>2.5	>12.5
	Twigs	Water	>2.5	2.5	>2.5	>2.5	>12.5
		MeOH	>2.5	>2.5	>2.5	>2.5	>12.5
		DCM	>2.5	1.25	1.25	>2.5	>12.5
		PE	2.5	2.5	2.5	>2.5	6,3
Neomycin (µg/ml)			0.39	1.56	6.25	1.56	
DMSO 10 %			>2.5	>2.5	>2.5	>2.5	>12.5
Amphotericin (µg/ml)							0.15

MeOH=Methanol; DCM= Dichloromethane, PE= Petroleum ether, E.c=*Escherichia coli*, S.a= *Staphylococcus. aureus*, E.f= *Enterococcus. faecalis*, K.p= *Klebsiella. pneumoniae.* D.B.G. = Durban Botanic Gardens*Extracts with values in bold and red are considered active

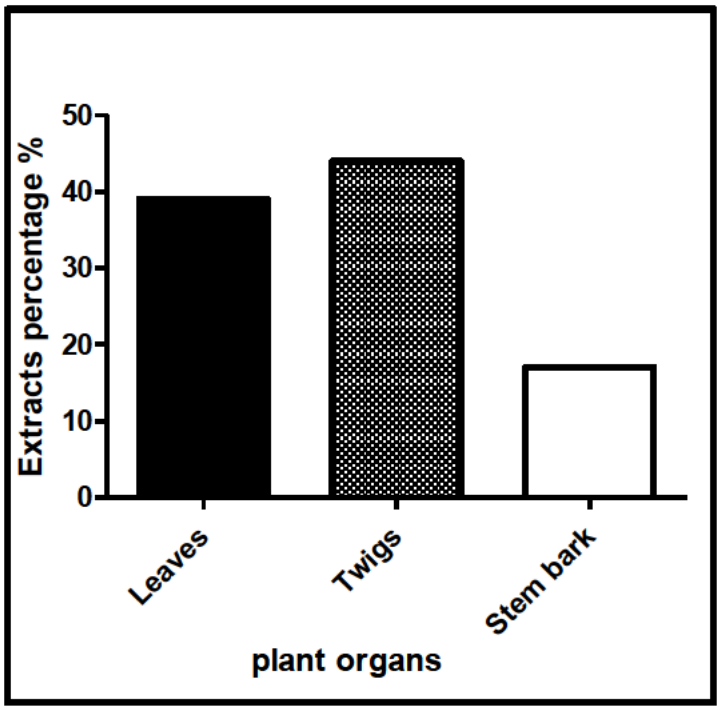


Figure 2.1: Comparison of the percentage of noteworthy antibacterial activity (MIC<1 mg/ml) between plant organs (leaves, twigs and stem bark) of six different *Croton* species collected at various locations in KZN, SA.

CHAPTER 3

Phytochemical analysis

3.1. Introduction

The World Health Organisation has identified plants as one of the best possible natural sources of new therapeutic drugs (**Nascimento et al.**, 2000). The use of phyto-medicines dates back to time immemorial. This is evident in the available successful drugs and medicines made from plant derivatives (**Savithramma et al.**, 2011). Recent reports suggest that plant-derived drugs contribute about 25% of the globally prescribed drugs (**Rates**, 2001, **Amoo et al.**, 2011). The concentration and availability of bioactive phyto-compounds often differ from one plant species to the other, as well as organs of the same plant species, (**Savithramma et al.**, 2011). This is evident in the therapeutic activity of different plant groups and species (**Briskin**, 2000). Similarly a single plant or plant organ may contain a great variety of bioactive compounds rendering it of considerable pharmacological importance, for this reason it is has been advised to screen plants for a variety of biological active compounds and activities (**Houghton et al.**, 2005, **Amoo et al.**, 2011). In this study methanolic extracts of six *Croton* species and their organs were evaluated for the presence and quantity of a series of phytochemicals. There was great variation observed in the antimicrobial activities of the different *Croton* species and their different organs. A study of the phytochemical composition of the species would give better understanding of the outcomes the antimicrobial tests done in the previous chapter.

3.2. Phytochemical profiling of selected medicinal plants

Plants defend themselves form herbivores, insects and microorganisms by producing a great variety of bioactive, secondary metabolites (**Savithramma et al.**, 2011).The ability of these compounds to either kill or inhibit the growth of pathogens, while inflicting little to no harm to humans or other hosts qualify phyto-compounds as perfect candidates for antimicrobial drug development (**Ahmad and Beg**, 2001).

Some of the most important bioactive phytochemicals include flavonoids, tannins, alkaloids and phenolics (**Edeoga et al.**, 2005). As medicinal plants continue to captivate the attention of scientists around the world, research efforts have been primarily targeted at elucidating the phytochemical and pharmacological properties of these valued natural resources. In a general, phytochemical studies involve chemical profiling of different parts of medicinal plants as well as the characterisation and identification of principal bioactive phyto-compounds. On the other hand, pharmacological research is also directed towards screening organic solvent, as well as aqueous medicinal plant extracts for pharmacological properties. Such studies may also entail elucidating the mode of action of bioactive phyto-compounds (**Briskin**, 2000, **Aremu et al.**, 2011). Phytochemical and pharmacological research is necessary in attaining information useful in developing novel therapeutic drugs (**Yadav and Agarwala**, 2011).

3.3. Secondary metabolites

Secondary metabolites are chemical compounds synthesized by plants and appear to have no designated roles in maintaining essential life processes such as plant growth and development. They have, however, been shown to have a significant role in the interaction of plants and their environment (**Bennett and Wallsgrove**, 1994, **Bourgaud et al.**, 2001, **Afrin et al.**, 2015). Thus, the evolution and production of secondary metabolites is a response to the

challenges exerted on plants by their natural environment (**Demain**, 1999). They are synthesized in small amounts, usually less than 1 % in dry weight (**Bourgaud et al.**, 2001, **Shakeri et al.**, 2012). This is also dependent on the growth stage, environmental conditions and physiology of the plant (**Bourgaud et al.**, 2001). Studies have shown plant secondary metabolites to be active antibacterial, antifungal, anti-carcinogenic, anti-mutagenic, anti-inflammatory, anti-oxidant, anti-diabetic and anti-tumour agents (**Moyo et al.**, 2010, **Somit et al.**, 2013). Many medicinally valued plants owe their antimicrobial characters to the various compounds produced during secondary metabolism in plants (**Briskin**, 2000, **Nascimento et al.**, 2000). These antimicrobial and therapeutic characters of plants may not often be ascribed to a single metabolite but may be a result of a combination of different secondary metabolites (**Ncube et al.**, 2008, **Joshi et al.**, 2011).

3.3.1. Tannins

Bate-Smith and **Swain** (1962) defined tannins as a group of water soluble phenolic compounds capable of turning animal hides into leather. Tannins are mostly found in vascular plants, occurring in different plant parts such as seeds, leaves and flowers but, they predominantly occur in the woody tissues of the plants (**Aremu**, 2009). In plants, these polyphenols occur mainly as hydrolysable or non-hydrolysable tannins (**Chung et al.**, 1998, **Santos-Buelga and Scalbert**, 2000). As shown in **Figure 3.1**, hydrolysable tannins are characterised by having a central carbohydrate molecule to which several hydroxyl groups and/or other functional groups are attached (**Mueller-Harvey**, 2001). Different types of tannins are formed when these functional groups react and join with other compounds. For instance, the esterification of gallic acid and polyol carbohydrates give rise to gallotannins, while ellagitannins are formed by the oxidative linkage of polyhydralic alcohols and ellagic acid (**Niemetz and Gross**, 2005).

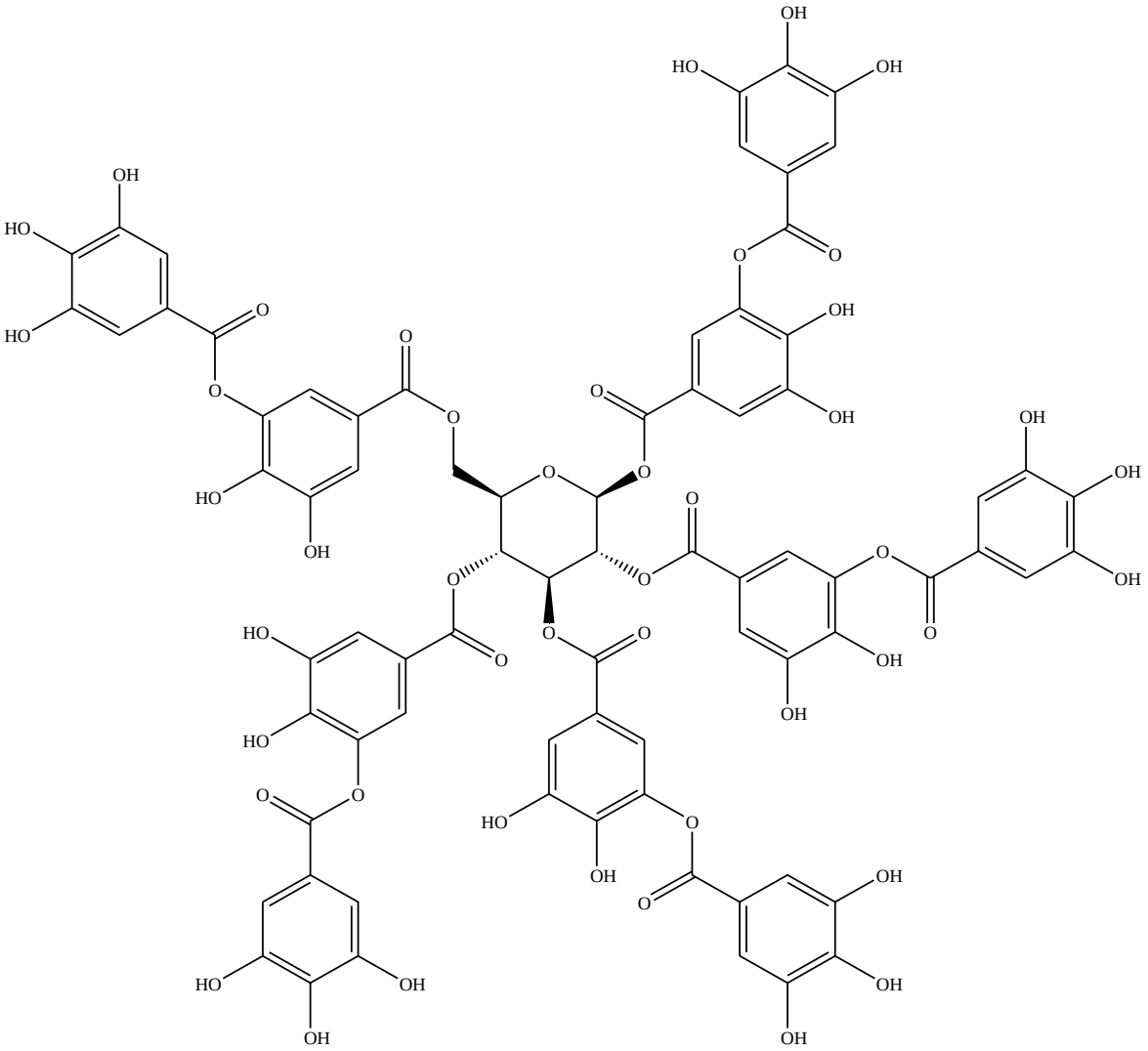


Figure 3.1.Chemical structure of tannic acid showing a central ß- D glucose molecule surrounded by several functional groups (**Cowan**, 1999).

Non-hydrolysed tannins (or condensed tannins) are structurally more complex than hydrolysable tannins and are mainly formed as a result of condensation reactions of benzopyran derivatives commonly known as flavans (**Cowan**, 1999, **Xie et al.**, 2003). Flavan-3-ols and flavan-3,4-diols form tannin oligomers and polymers either individually or in combinations (**Haslam**, 2007, **Aron and Kennedy**, 2008). Condensed tannins are also referred to as proanthocyanidins since they depolymerize and yield red anthocyanidins upon heating in acidic solutions (**Schofield et al.**, 2001). This chemical property forms the basis of

the colorimetric analysis technique popularly known as the acid-butanol assay (**Gessner and Steiner**, 2005).

Although implicated in poor protein digestibility, reduced energy metabolism and feed intake in livestock (**Chung et al.**, 1998, **Min et al.**, 2003), tannins have tremendous human health benefits due particularly to their outstanding astringent properties (**Manach et al.**, 2004). Carcinogens and mutagens produce oxygen free radicals capable of interacting with several cellular components often leading to mutations and cancerous growths in animals (**Valko et al.**, 2006). However, polar hydroxyl groups in tannins readily complex with paramagnetic hyperoxide radicals and hence protect cellular components from oxidative damages (**Zielińska-Przyjemska et al.**, 2015). Tannic acid has also be shown to inhibit the generation of superoxide radicals in some animal tissues (**Gülçin et al.**, 2010). These properties perhaps explain why tannins have, of late, been extensively investigated as potential anticarcinogens and antimutagens.

The antibacterial activities of tannins are well documented. Several researchers have demonstrated that some pathogenic Gram-negative and -positive bacterial strains are susceptible to the tannins (**Chung et al.**, 1993, **Mustapha et al.**, 2017, **Steiner et al.**, 2017, **Yang et al.**, 2017, **Dong et al.**, 2018). These polyphenols chelate nutrients and ions from bacterial growth media making them less available to bacteria (**Mila and Scalbert**, 1993, **Chung et al.**, 1998). They also inhibit enzyme catalysed reactions by either binding to enzymes or their substrates (**Akiyama et al.**, 2001). Tannins have also been shown to be responsible for antifungal, antioxidant anti-inflammatory and many healing properties exhibited by some medicinal plant extracts (**Moyo et al.**, 2010).

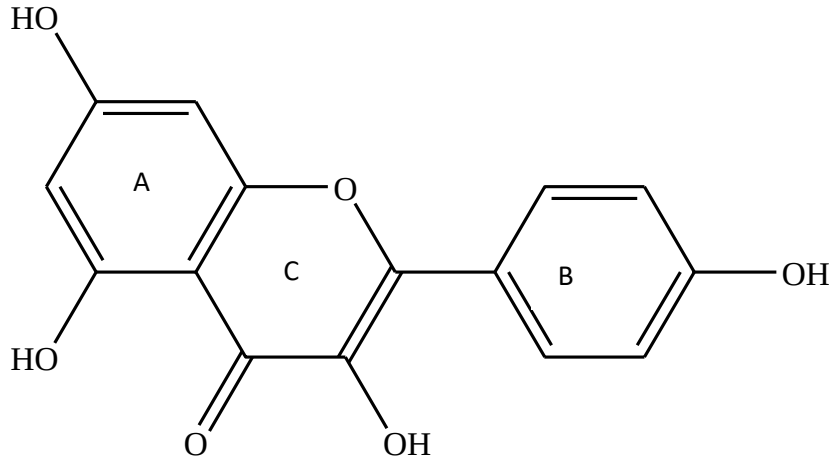
Other pharmacological activities associated with tannins include accelerated blood clotting, modulation of immune responses and reduced blood pressure (**Okuda**, 1995, **Chung et al.**, 1998).

3.3.2. Flavonoids

The term flavonoid is derived from the Latin term *flavus* which means yellow (**Cotelle**, 2001). Flavonoids (some of which are yellow in colour) are a large group of structurally diverse secondary metabolites found in ferns, bryophytes, algae, angiosperms and gymnosperms (**Iwashina**, 2000). Over 9 000 flavonoids are found in the plant kingdom (**Wang et al.**, 2011), and are based on their chemical properties, which can be subdivided into 15 classes including anthocyanins, flavones, flavonols, flavanones, flavan, proanthocyanidins, isoflavonoids, bioflavonoids, etc. (**Yao et al.**, 2004). Despite their manifold chemical diversity, all flavonoids are made up of a 15 carbon skeleton consisting of two aromatic rings (A and B) and one heterocyclic ring (C) (**Cushnie and Lamb**, 2005) as depicted in **Figure 3.2**. Numerous structurally diverse flavonoid compounds are formed by the addition of hydroxyl, methoxyl, methyl, glycosyl, sulafate, prenyl and isoprenyl, aromatic or aliphatic groups to the flavonoid carbon skeleton (**Iwashina**, 2000, **Kumar and Pandey**, 2013). Generally, the presence of hydroxyl groups and sugars makes flavonoids hydrophilic whereas prenyl units and methyl groups makes them lipophilic in nature **(Chebil et al., 2007)**. Apart from preventing microbial invasions, flavonoids are also involved in several other unrelated plant functions such as nitrogen fixation, ultra-violet filtration, chemotropism, cell cycle inhibition as well as floral, foliage and fruit pigmentation (**Woo et al.**, 2002).

Flavonoids have in recent years attracted much interest from several research groups around the world due to their numerous pharmacological properties. Their anti-inflammatory,

analgesic, anticancer, anti-HIV, antimicrobial, and antioxidant activities are well documented (**Lee et al.**, 2003, **Yao et al.**, 2004, **Ravishankar et al.**, 2013, **Xiao et al.**, 2016) . They are believed to exert their antibacterial activity through forming complexes with bacterial extracellular materials and proteins as described for tannins above (**Cowan**, 1999). Lipophilic flavonoids, however, rupture cell membranes resulting in the leakage of cytoplasm and the subsequent death of bacterial cells (**Taylor et al.**, 2005). Additional antibacterial mechanisms observed includes inhibition of cytoplasmic membrane formation, DNA gyrase and energy metabolism (**Cushnie and Lamb**, 2005). Catechins represent an interesting group of antibacterial flavonoids since they were demonstrated to inhibit the growth of *Vibrio cholerae* and to inactivate its toxins (**Borris**, 1996). Other studies revealed that catechin, inhibits the passage of hydrophilic molecules into bacteria cells (**Nakayama et al.**, 2013), damage bacterial cell walls and prevent the formation of peptidoglycan (**Taylor et al.**, 2005, **Shimamura et al.**, 2007).

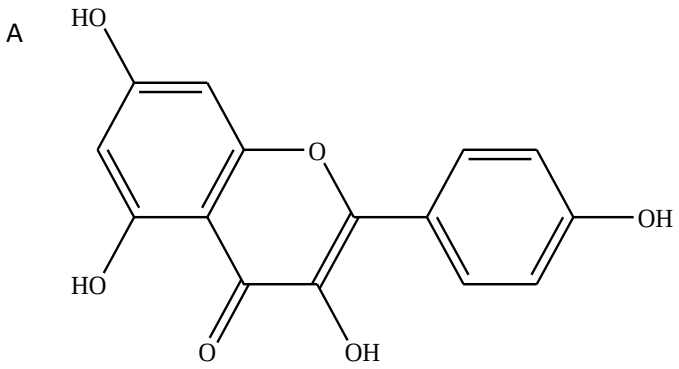


3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one

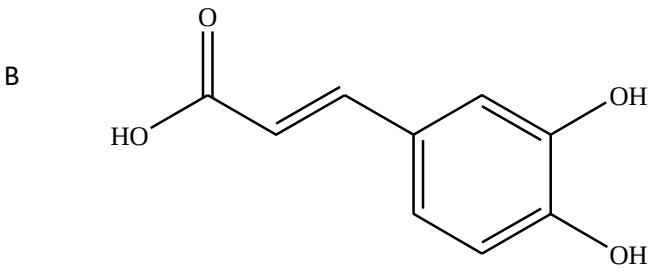
Figure 3.2: Chemical structure of a flavonoid (kaempferol). Letters A and B denote aromatic rings, while C is a heterocyclic ring (**Williams and Grayer**, 2004).

3.3.3. Phenolic acids

Phenolic acids form another group of plant based, pharmacologically important polyphenols. Two categorises of these phenolic compounds can be distinguished: hydrobenzoic and hydroxycinnamic acids (**Dykes and Rooney**, 2007). Hydrobenzoic acids are derivatives of benzoic acid and include gallic, *p*-hydrobenzoic, protocatechuic, syringic and vanillic acid (**Manach et al.**, 2004). The chemical structure of gallic acid is shown in **Figure 3.3**. These phenolic acids occur abundantly in red fruits, black radish, onions and tea leaves (**Shahidi and Naczk**, 1995). It is worth noting that some hydrobenzoic acids often form components of other complex polyphenolic structures such as gallotannins and ellagitannins (**Manach et al.**, 2004).



3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one



3-(3,4-dihydroxyphenyl)acrylic acid

Figure 3.3: Chemical structures of a hydroxybenzoic acid (A: gallic acid) and a hydroxycinnamic acid (B: caffeic acid) (**Cowan**, 1999).

Hydroxycinnamic acids are derivatives of cinnamic acid made up of a 9 carbon skeleton (**Khadem and Marles**, 2010). This group of phenolic acids consist of caffeic, chlorogenic,

coumaric, ferulic and sinapic acids (**Manach et al.**, 2004). The chemical structure of caffeic acid is shown in **Figure 3.3**. In plants, phenolic acids exist freely or bound to other compounds (**Bonoli et al.**, 2004). Hydroxycinnamic acids exist mainly in the bound form as glycosylated derivatives or esters of quinic, shikimic and tartaric acid (**Nardini et al.**, 2002, **Buiarelli et al.**, 2010). Chlorogenic acid for example is formed by the combination of caffeic and quinic acids (**Puupponen-Pimiä et al.**, 2005).

Some phenolic acids are known to have potent antibacterial properties. Studies by **Borges et al.** (2013) revealed that gallic acid and ferulic acids exert their bactericidal effects by binding to and rupturing bacterial cell membranes. **Lou et al.** (2011) demonstrated that chlorogenic acid binds to some Gram-positive and Gram-negative bacterial strains and increase their permeability. This in turn caused the leakage of both cytoplasmic and nuclei material leading to bacterial cell death. *P*-coumaric acid also causes intracellular material leakages and interferes with bacterial DNA replication and gene expression (**Lou et al.**, 2012). The activity of sinapic acid against a wide range of Gram-positive and Gram-negative bacterial strains was also demonstrated by several researchers (**Nowak et al.**, 1992, **Barber et al.**, 2000, **Johnson et al.**, 2008). However, most lactic acid bacteria such as *Lactobacillus plantarum* are resistant to sinapic acid (**Engels et al.**, 2012). This selective antibacterial activity suggests that sinapic acid can potentially be used to extend the shelf life of milk and its products.

The type and concentration of pharmacologically important secondary metabolites differ from one medicinal plant species to the other and within organs of the same plant species. The current study was therefore designed to determine the concentrations of condensed tannins, flavonoids, phenolic acids and total phenolic content in aqueous methanol extracts from the six selected *Croton* species. In this study the phytochemical properties from the various *Croton* species and their different organs were compared.

3.3.4. Alkaloids

Alkaloids are defined as heterocyclic nitrogen containing compounds commonly derived from amino acids (**Agostini-Costa et al.**, 2012, **Perviz et al.**, 2016). Other closely related compounds that contain neutral or weak acid properties are also included as alkaloids (**Kabera et al.**, 2014). All of these fall in one of the three different categories that alkaloids have been assigned to, which are based on the alkaloids' source or the initial substance of their biosynthetic pathway and physical structure (**Bennett and Wallsgrove**, 1994, **Eguchi et al.**, 2019). These categories include; true alkaloids, these are synthesized by amino acids, have nitrogen in heterocyclic rings and are basic, examples are atropine and nicotine; protoalkaloids; these are also synthesized by amino acids and are basic, however their nitrogen is not in a heterocycle, an example is mescaline. The final category are the pseudoalkaloids; these are not synthesized by amino acids examples are solanidine and caffeine (**Bennett and Wallsgrove**, 1994). Alkaloids are produced by living organisms such as bacteria, fungi, animals and more commonly plants (**Kabera et al.**, 2014, **Perviz et al.**, 2016). About 15% of these living organisms contain alkaloids and 12 000 has been identified in plants (**Wink**, 2003a). Many alkaloids are genus or species-specific (**Bourgaud et al.**, 2001). Many alkaloids may occur in any of plant organs but there are some that are organ-specific (**Cushnie et al.**, 2014, **Othman et al.**, 2019).

In plants, alkaloids are essential defence agents against pathogens and herbivores. Their potency has led to their prevalent use as pharmaceutical, narcotics, stimulants and poisons (**Facchini and St-Pierre**, 2005, **Richard et al.**, 2013). They are furthermore recognised for their efficacious antimicrobial properties; inhibiting various bacterial and fungal pathogens (**Shakeri et al.**, 2012, **Marutescu et al.**, 2017). Pyrrolizidine alkaloids, quinolizidine alkaloid extracts, furoquinoline alkaloids and isoquinoline alkaloids are some of the prevalent

biologically active alkaloids (**Marutescu et al.**, 2017). Alkaloids have a variety of mechanisms of action against microbes and these vary with different alkaloids. Some of them include inhibiting cell division of microbes, disruption of bacterial cell membranes, inhibition of virulence genes and inhibiting bacterial enzymes (**Othman et al.**, 2019) Alkaloids are a structural (**Cushnie et al.**, 2014) and chemically diverse group of compounds ranging, from the simple coniine compound to strychnine with a pentacyclic structure (**Makkar et al.**, 2007). Similarly alkaloids have a wide range of therapeutic functions in the medical sector this includes the analgesic codeine and morphine, the sedative scopolamine, muscle relaxants papaverine and (+) - tubocurarine, anti-cancerous vinblastine the anti-arrhythmic, ajmaline and the gout suppressant colchicine (**Facchini and St-Pierre**, 2005). Even with their wide clinical use some alkaloids are extremely toxic to humans, even in small quantities e.g. coniine and strychnine; other clinically used alkaloids are also toxic (**Bush and Fannin**, 2009).

3.3.5. Oxidative stress and antioxidant activity

Oxidative stress is a result of increased production of free radicals in the human body. This is due to the imbalance and low production of antioxidants by the cells (**Pham-Huy et al.**, 2008, **Aremu et al.**, 2011). Free radicals are described as unstable molecules that contain unpaired electrons on their outer shell. Their unpaired electrons are highly reactive, reacting with and altering organic molecules in an organism's body such as lipids, DNA and proteins (**Pham-Huy et al.**, 2008) therefore triggering various human diseases (**Lobo et al.**, 2010). Free radicals or reactive oxygenic species (ROS) produced as by-products of the human body's metabolism (**Amarowicz et al.**, 2004) are known to cause damage to cells, tissues and organs (**Gupta and Sharma**, 2006, **Prasad et al.**, 2010). These radicals have been implicated in the occurrence of many diseases in humans (**Amoo et al.**, 2011, **Aremu et al.**, 2011), such

as inflammation , cardiovascular diseases, diabetes and cancer (**Amoo et al.**, 2011). Free radicals may also occur as a result of external factors such as air pollution, smoking cigarettes, industrial chemicals, and exposure to X-rays (**Valko et al.**, 2007, **Lobo et al.**, 2010). Some major outcomes of oxidative stress include DNA damage, premature ageing and a reduced lifespan (**Blomhoff**, 2010). Antioxidants are the prime compounds used to counteract the effects of oxidative stress (**Gupta and Sharma**, 2006). Antioxidants are produced naturally or may be synthetic (**Gupta and Sharma**, 2006). Many synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanilosole (BHA) and tert-butylhydroquinone (TBHQ) have been used by humans and added to their food (**Amarowicz et al.**, 2004). However, due to recent discoveries and concerns about their toxicity to humans, new ventures and focus have been directed towards finding safer alternatives, of antioxidants (**Amarowicz et al.**, 2004, **Aremu et al.**, 2011). In this search for natural antioxidants, plants have been named as the best source of natural antioxidants, with possibly novel structures possessing unique effective mechanisms of action (**Prasad et al.**, 2010).

Plants produce antioxidants as natural chemical bioactive products that can reduce oxidative stress (**Aremu et al.**, 2011). Mechanisms of natural antioxidants includes their ability to act as reducing agents, free radical scavengers, singlet-oxygen quenchers, chain breakers peroxide decomposers metal-chelating agents and hydrogen donors (**Rice-Evans et al.**, 1996, **Amarowicz et al.**, 2004, **Lobo et al.**, 2010).

The use of a single assay to measure antioxidant activity has proven to be unreliable (**Mraihi et al.**, 2013), as the reaction of an antioxidant to different radicals and radical sources may vary (**Mikami et al.**, 2009). Thus, in this study two complementary and widely used assays were used to measure antioxidant activity of extracts from different organs of different

Croton species these are the DPPH Radical Scavenging Assay and β-carotene /Linoleic assay.

3.4. Materials and methods

3.4.1. Plant collection and preparation of samples

Croton species were selected and samples prepared as described in **Section 2.2.1** of **Chapter 2**.

3.4.2. Extraction of phenolic compounds from plant material

The extraction of phenolic compounds from dried plant materials was done according to the techniques described by **Makkar et al.** (2000). Two grams (2 g) of finely ground dry plant material from each collected plant species and their different organs was extracted with 10 ml of aqueous methanol (50%) in a sonication bath for 20 min. with ice added in the sonication bath to keep the temperature cool. The extracts were then filtered *in vacuo* through Whatman No. 1 filter papers and stored in air tight pill vials in the dark for further use.

3.4.3. Determination of condensed tannins with the Butanol – HCL assay

The concentration of condensed tannins in medicinal plant extracts was determined using the Butanol – HCL assay (**Makkar et al.**, 2000). In triplicates, 3 ml of the butanol - HCL reagent (95:5 v/v) was added to 500 µl of each methanolic extract followed by 100 µl ferric reagent (2% ferric ammonium sulphate in 2N HCl). The reaction mixtures were vortexed and incubated in a water bath at 100 C for 60 min. A blank containing 500 µl of 50% methanol instead of plant extract, mixed with 3 ml butanol-HCl reagent and 100 µl ferric reagent was

prepared but without heating. After 1 h, absorbance was measured at 550 nm using a UV-visible spectrophotometer (Varian Cary 50, Australia). Cyanidin chloride (0.1 mg/ml) was prepared in distilled water and used as a standard. The quantity of condensed tannins in each sample was then expressed as milligrams (mg) of cyanidin chloride equivalent (CCE) per one gram dry weight (g DW) of plant material.

3.4.4. Determination of flavonoid content using the Aluminium Chloride assay

The total flavonoid concentration of each plant extract was determined using the Aluminium Chloride assay as described in **Makkar et al.** (2000). In three replicates 250 µl of each plant extract was pipetted into test tubes and 1 ml of distilled water was added to all the test tubes, followed by 75 µl of 5% sodium nitrite ($NaNO_2$), 75 µl 10% aluminium chloride ($AlCl_3$), 0.5 ml 1 M sodium hydroxide (NaOH) and 0.6 ml distilled water. The mixture was vortexed and absorbance was read immediately (no incubation period) at 510 nm using a UV-visible spectrophotometer (Varian Cary 50, Australia). A similar preparation for the blank was made by adding 50% methanol instead of the plant extract. Catechin (0.1 mg/ml) (Sigma–Aldrich, USA) was used as a standard. The flavonoid content was then expressed as milligrams (mg) of catechin equivalents (CE) per one gram dry plant material.

3.4.5. Determination of total phenolics using the Folin Ciocalteu (Folin C) assay

Total phenolics concentration was quantified using the Folin Ciocalteu (Folin C) method as described by **Makkar et al.** (2000), with slight modifications**.** Folin C reagent (1N) was prepared from Folin C (2N) (Sigma - Aldrich, USA) by mixing equal volumes of the Folin C (2N) and distilled water in a dark (brown) bottle. Standard gallic acid (Sigma - Aldrich, USA) solution was prepared in distilled water at 0.1 mg/ml concentration. The reaction mixture

consisted of 50 µl triplicates of each plant extract in test tubes and 950 µl distilled water added to make up to 1ml, followed by 500 µl of 1 N Folin C reagent and 2.5 ml of 2% sodium carbonate. Similar preparation of a blank containing 50% aqueous methanol and different concentrations of gallic acid instead of a plant extract were also prepared. The test mixtures were incubated at room temperature for 40 min. Absorbance was then read at 725 nm using a UV-visible spectrophotometer (Varian Cary 50, Australia). Total phenolic concentrations were expressed as gallic acid (GAE) equivalents. The data was analysed using Graphpad Prism software (GraphPad version 5.02, San Diego, USA). .

3.4.6. Determination of alkaloids using Thin Layer Chromatography

Methanolic extracts (80%) were re-suspended in absolute methanol at 50 mg/ml, and 20 µl of each extract was spotted on thin layer chromatographic (TLC) plates (5×20 cm, silica gel 60 F_{254}, Merck, Germany). The plates were developed in ethyl acetate and methanol (90:10, v:v). After developing the TLC plates were air dried and viewed under ultraviolet light (254 and 366 nm), the florescence was noted and photographed. The TLC plates were then sprayed with Dragendorff reagent to determine the possible presence of alkaloids in the extracts. This was indicated by the formation of red colouration on the TLC plates (**Fawole**, 2009).

3.4.7. β-carotene /Linoleic assay

Antioxidant activity was measured following the method described by **Amarowicz et al.** (2004), with some modifications as described by **Moyo et al**. (2010). Dried plant extracts and the positive control, butylated hydroxytoluene (BHT), were re-dissolved in 50% aqueous methanol. Fifty percent aqueous methanol was designated as the negative control. β-carotene emulsion was prepared in the dark under a fume hood as follows: In a brown 500 ml Schott

bottle covered in foil, 5 mg of β-carotene was dissolved in 0.5 ml chloroform. Excessive chloroform was evaporated under vacuum, and a thin layer of β-carotene was left in which 50 µl linoleic acid and Tween 20 were immediately added. Aerated distilled (124 ml) water was added to the mixture to give a final volume of 125 ml. The mixture was vigorously agitated until an orange emulsion was formed, with a final β-carotene concentration of 20 µg/ml. The β-carotene emulsion (4.8 ml) was dispensed into test tubes, followed by 200 µl of either, plant extract, positive or negative controls, making the final concentration of the reaction mixture 250 µg/ml. The assay was done in triplicates per sample. The initial absorbance of the each reaction mixture was measured immediately with a UV-visible spectrophotometer (Varian Cary 50, Australia) at 470 nm (t=0). The tubes were covered with foil and incubated at 50°C in a water bath; in the dark; for 3 h. The absorbance for each mixture was measured every 30 min. Tween 20 was used as a blank. The rate of β-carotene bleaching was calculated according to first order kinetics using the formula:

$$\text{Rate of } \beta - \text{carotene bleaching} = \ln\left(\frac{A_{t=0}}{A_{t=t}}\right) \times 1/\text{t}$$

where At=0 is the absorbance of the emulsion at 0 min; and $A_{t=t}$ is the absorbance at time t (60, 90 and 120 min). The average rate of β-carotene bleaching was then calculated based on rates at 60, 90 and 120 min. The calculated average rates were used to determine the antioxidant activity (ANT) of the sample extracts, and expressed as percentage inhibition of the rate of β-carotene bleaching using the formula:

$$\%\text{ANT} = (\text{R}_{\text{control}} - \text{R}_{\text{sample}})/\,\text{R}_{\text{control}} \times 100$$

where $R_{control}$ and R_{sample} represent the respective average β-carotene bleaching rates for the standard antioxidant and plant extracts, respectively. Antioxidant activity was further expressed as the Oxidation rate ratio (ORR) based on the formula:

$$\mathrm{ORR} = \mathrm{R}_{sample}/\mathrm{R}_{control}$$

Antioxidant activity (AA) was calculated as described by **Braca et al.** (2003), based on the inhibition of coupled oxidation of β-carotene and linoleic acid against the negative control at t= 60 min, t= 90 min and t = 120 min using the formula with the formula:

$$\%AA = \left[1 - \left(\frac{A_0 - A_t}{A_{00-A_{0t}}}\right)\right] \times 100$$

Where A_0 is the absorbance of the sample extract at the beginning of incubation; A_t is the absorbance at t=60 and 120 min. for the sample extract and A_{00} and A_{0t} represent the absorbance of the negative control (without sample extract) at the beginning of incubation and at time t= 60, t= 60 min and 120 min., respectively.

3.4.8. Determining antioxidant potential using DPPH radical scavenging activity

The antioxidant activity of the different *Croton* species extracts was determined using the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay as described by **Xie et al**. (2010) and **Karioti et al.** (2004) with slight modifications. This assay was used to measure the free radical scavenging activity of methanolic extracts of the plants. The assay was performed in triplicate, in test tubes and in dim light. Dried methanolic extracts were re-dissolved in aqueous methanol. These extracts were diluted with 50% methanol to make a volume of 750 µl and a further 750 µl of DPPH (0.1 mM in methanol) was added to these diluted extracts making final concentrations of the reaction mixture at 6.7, 20, 33, 46.7 and 66.7 µg/ml. Similar mixtures were made, where all mentioned contents were added except that methanol was substituted for the plant extracts. To make a negative control and positive control the plant extracts were substituted with ascorbic (ASC) acid and butylated hydroxytoluene (BHT). The background control mixtures consisting of 50% methanol in place of DPPH were

made for each extract; these were used to correct any absorbance due to the colour of the extract. The mixtures were incubated for 30 min. in the dark at room temperature. After incubation, absorbance was read at 517 nm on a spectrophotometer (Varian Cary 50, Australia). The background for each extract was corrected by reading the background correction mixture after every reaction solution. The radical scavenging activity (RSA) was calculated using the following equation:

$$RSA\% = \left[1 - \left(\frac{A_{extract} - A_{background}}{A_{control}}\right)\right] \times 100$$

Where $A_{extract}$ was the the absorbance of the reaction mixture containing the plant extract or the standard antioxidant, $A_{background}$ was the absorbance of the background correction mixture and $A_{control}$ the absorbance of the negative control. The EC_{50}, which is the concentration of the plant extract required to scavenge 50% of the DPPH radicals, was determined using a linear regression curve from a graph showing antioxidant percentage (%) inhibition versus the concentration of the each sample.

3.4.9. Statistical analysis

Analysis of variance (ANOVA) was used to determine the level of significant difference (P=0.05) among means and the Duncan's multiple range test (DMRT) was used to further separate means. The analysis was performed using IBM SPSS (version 26) software. This was used to analyse the data from the condensed tannins, flavonoids, phenolics content assays as well as the antioxidant activity assays (β-carotene /Linoleic and DPPH Radical Scavenging Assays).

3.5. Results and discussion

3.5.1. Phytochemical evaluation

Previous studies have reported on the occurrence of a great variety phytochemicals in the *Croton* genus (**Ndhlala et al.**, 2013). These includes various diterpenes (pimarane, clerodane, labdane, phorbol esters, tracylobane) and diterpenoids (**Salatino et al.**, 2007, **Mukanganyama et al.**, 2012); cembranoids (**Salatino et al.**, 2007, **Langat et al.**, 2011); flavonols (**Aderogba et al.**, 2013, **Somit et al.**, 2013); alkaloids, tannins, phenolics, lignan, sterols and anthraquinones (**Salatino et al.**, 2007, **Maroyi**, 2017).

The phytochemical content of the Different *Croton* species collected in KZN and their different organs are represented in **Table 3.1**. **Figure 3.4**, **3.5 and 3.6 represent the variation in the concentrations of** condensed tannins, flavonoid, and total phenolics respectively across the different *Croton* species collected in KZN and their different organs.

3.5.2. Condensed tannins content

The condensed tannins content in evaluated extracts ranged between 0.3±0.1 and 31.3±0.1 mg CCE/g DW (**Fig 3.4 A-B** and **Table 3.1**). Higher quantities of condensed tannins were found in leaf extracts of *C. gratissimus* from Southport and Durban Botanic Gardens (31.3±0.1 and 29±0.7 mg CCE/g DW, respectively), as well as from *C. pseudopulchellus* from Mkuze (15.9±0.4 mg CCE/g DW). These and other leaf extracts with high concentrations of condensed tannins demonstrated broad-spectrum antibacterial activities (**Chapter 2, Table 2.2).** The current observations could be attributed to higher levels of condensed tannins within the assayed leaf extracts. The least condensed tannin content was observed in the stem bark extract of *C. steenkampianus* from Durban Botanic Gardens (0.3±0.1 mg CCE/g DW) and twig extracts of *C. pseudophulchellus* obtained from the same

location (0.4±0.1 mg CCE/g DW). Twig and stem bark extracts showed low condensed tannin content ranging between 0.3±0.1-5.7±0.3 mg CCE/g DW. Overall the stem bark extracts contained the lowest condensed tannins content with none above 5.7±0.3 mg CCE/g DW; which was from *C. gratissimus* from Southport. This is low compared to the content exhibited by leaf extracts.

3.5.3. Flavonoid content

The flavonoid content in evaluated plant extracts ranged from 1.3±0.1-31.1±0.7 mg CE/g DW. Represented in **Table 3.1**. The leaf extracts contained significantly higher concentration of flavonoids than other organs, as depicted in **Figures 3.5 A- B, and Table 3.1**. Leaf extracts with highest flavonoid content being *C. gratissimus* from Southport 31.1±0.7 mg CE/g DW, *C. sylvaticus* from Vernon Crookes N.R. 28.1±0.3 mg CE/g DW, *C rivularis* from Umzumkhulu Valley 27.4±0.2 mg CE/g DW, *C. gratissimus* from Muden 28±1.0 mg CE/g DW and *C. menyhartii* from Kranskop 26.6±0.6 mg CE/g DW. The antioxidant activity (**Table 3.2 and 3.3**) shown by these plant extracts could be attributed to the flavonoids therein. Flavonoids contain a benzopyrone, which is known to be a free radical scavenger (**Somit et al.**, 2013). Both twig and stem bark extracts had comparatively lower flavonoid content than the other plant organs. The lowest flavonoid content was observed from the stem bark and twig extracts of *C. steenkampianus* obtained from the Durban Botanic Gardens (1.3±0.1 and 1.3±0.01 mg CE/g DW respectively). However the twig extracts of *C. steenkampianus* obtained from the Durban Botanic Gardens exhibited noteworthy antibacterial activity of 0.31-0.63 mg/ml against *E. faecalis* and *S. aureus* (**Table 2.2**). The twigs also showed noteworthy antioxidant activity 63.10±0.88 ANT% (**Table 3.2**). A similar trend was reported in a study conducted by **Bojase et al.** (2002) and **Mulaudzi et al.** (2011) on *Bolusanthus speciosus* (Bolus) Harms, where its leaf, stem and bark contained low

amounts of flavonoids but exhibited high antibacterial activity against various bacteria. This suggests that even in small quantities flavonoids can inhibit bacterial growth.

3.5.4. Total phenolics content

The highest phenolic content was recorded in *C. pseudopulchellus* (Mkuze) leaf extracts (28.3±1.1 mg GAE/g DW) whereas the lowest content was observed from *C. gratissimus* (Durban Botanic Gardens) stem bark (1.8±0.1 mg GAE/g DW) as shown in **Figure 3.6**, and **Table 3.1**. Notably a large number of leaf extracts contained the highest phenolic acids content than other plant organs. A significant difference in the total phenolic content and general phytochemical content was observed among similar organ extracts of the same species. This variation may be due to environmental factors and varying external stress experienced by plants in different regions. The yield of biological chemical compounds produced is related to type and amount of stress they experience; this includes stresses such as drought, sunlight and microorganism. Thus, plants with high stress levels produce high concentrations of secondary metabolites (**Blomhoff**, 2010).

3.5.5. Alkaloid detection

Plant extracts containing alkaloids are presented in **Figures 3.7 and 3.8**. Ten out of the 40 plant extracts evaluated indicated the presence of alkaloids. Alkaloids were detected in the following plant parts, leaves of *C. gratissimus* (Durban Botanic Gardens), twigs of *C. gratissimus* (Southport and Muden), twigs of *C. sylvaticus* (Southport), leaves and stem bark of *C. steenkampianus* (Durban Botanic Gardens), leaves of *C. pseudopulchellus* (Durban Botanic Gardens), leaves and twigs of *C. menyhartii* (Kranskop and Mkuze), twigs of *C. menyhartii* (Mkuze) as well as twigs of *C. rivularis* (Umzumkhulu). Most extracts from the

above-mentioned plant parts exhibited noteworthy antibacterial activities (**Chapter 2, Table 2.2**), which could, to some extent, be attributed to the presence of alkaloids in the extracts.

As anticipated several plant extracts that contained higher concentrations of bioactive phytocompounds (**Figures 3.4,-3.6**) exhibited noteworthy antibacterial activities (**Chapter 2, Table 2.2**). Notably the leaves of *C. peudopulchellus* (Mkuze) and *C. gratissimus* (Durban Botanic Gardens) with MIC values ranging between 0.16–0.63 mg/ml and 0.08–0.94 mg/ml, respectively. Certain plants such as the twigs of *C. gratissimus* (Southport and Durban Botanic Gardens), twigs and stem bark of *C peudopulchellus* (Durban Botanic Gardens) exhibited noteworthy antibacterial activity of MIC values ranging between 0.47–0.63 mg/ml, 0.31–0.63 mg/ml, 0.16–0.63 mg/ml and 0.08–0.63 mg/ml, respectively (**Chapter 2, Table 2.2**). These plant extracts, however had low to moderate phytochemical content (**Figures 3.4-3.6**). The antibacterial activity of these extracts may be due to other phytochemical compounds not screened for in this study or the present phytochemicals may also be highly active even in small quantities. Antibacterial activity may also be due to the actions of a combination of the available phytochemicals.

Table 3.1: Phytochemical properties of *Croton* species occurring in KZN

Plant species and locality	**Plant part**	**Phenolics (**mg GAE/g DW)	**Flavonoids (**mg CE/g DW**,)**	**Condenced tannins (**mg CCE/g DW)
C. gratissimus Southport	Leaves	10,4±0,1	**31,1±0,7**	**31,3±0,1**
	Twigs	3,1±0,1	5,7±0,3	5,1±0,5
	Stem bark	4±,03	6,5±0,2	5,7±0,4
C. gratissimus D.B.G.	Leaves	7,1±1,1	22,9±0,7	29,1±0,7
	Twigs	2,8±0,1	2,9±0,1	1,4±0,1
	Stem bark	1,8±0,1	2,8±0,1	0,6±0,1
C.gratissimus Muden	Leaves	10±0,5	**28±1,0**	11,3±0,4
	Twigs	3±0,1	3,9±0,1	2,9±0,4
	Stem bark	2,7±0,4	4,4±0,1	1,2±0,1
C. sylvaticus Southport	Leaves	14,4±0,1	25,5±0,4	3,5±0,1
	Twigs	4,9±0,1	6,0±0,1	0,9±0,1
C. sylvaticus Umdoni Park	Leaves	10,1±0,1	14,6±0,4	2,0±0,1
	Twigs	5,9±0,2	4,6±0,1	0,8±0,1
	Stem bark	6,2±0,3	7,8±0,3	0,9±0,1
C. sylvaticus Ntunjambili	Leaves	16,4±0,6	6,1±0,1	**7,5±0,2**
	Twigs	5,4±0,2	5,5±0,1	1,1±0,1
C. syvaticus Vernon Crookes	Leaves	**17,3±0,5**	**28,1±0,3**	2,4±0,1
	Twigs	7,8±0,5	7,6±0,1	0,9±0,1
	Stem bark	8,3±0,2	7,5±0,1	1,4±0,1
C. steenkampianus D.B.G.	Leaves	13,7±1,1	**3,4±0,1**	**2,6±0,1**
	Twigs	6±0,8	1,3±0,1	0,5±0,1
	Stem bark	3,2±0,2	1,3±0,1	0,3±0,1
C. steenkampianus Kosi Bay	Leaves	**20,3±2,5**	3,3±0,1	1,8±0,1
	Twigs	8,1±0,4	1,7±0,1	0,7±0,1

Table 3.1 Continued…..

Plant species and locality	Plant part	**Phenolics (**mg GAE/g DW)	**Flavonoids (**mg CE/g DW**,)**	**Condenced tannins (**mg CCE/g DW)
	Stem bark	12,7±0,8	2,9±0,1	1,6±0,1
C. pseudopulchellus Southport	Twigs	4,1±0,1	2,1±0,1	0,5±0,1
C. pseudopulchellus D. B. G.	Leaves	11,5±0,6	3,9±0,1	2±0,1
	Twigs	5,8±0,5	1,8±0,1	0,4±0,1
	Stem Bark	3,6±0,3	1,6±0,1	0,5±0,1
C. pseudopulchellus Mkuze	Leaves	**23,8±1,1**	**23,5±0,4**	**15,9±0,4**
	Twigs	8,8±0,8	2,9±0,1	1,3±0,1
	Stem bark	10,3±1,4	2,8±0,1	1,7±0,3
C. menyhartii Mkuze	Leaves	7,5±0,6	11,0±0,2	1,9±0,1
	Twigs	2,9±0,2	3,5±0,2	0,6±0,1
	Stem bark	4,7±0,2	4,3±0,2	1,1±0,1
C. menyhartii Kranskop	Leaves	**8,9±0,2**	**26,6±0,6**	**8,8±0,2**
	Twigs	3,2±0,2	4,4±0,1	1±0,1
	Stem Bark	2,7±0,1	3,1±0,1	0,5±0,1
C. rivularis Umzimkhulu Valley	Leaves	**14,6±0,2**	**27,4±02**	2,8±0,4
	Twigs	4,4±0,1	5,6±0,2	0,7±0,1

Values are the mean ± standard error (n = 3), GAE = gallic acid equivalents, CE = Catechin equivalents, CCE = Cyanide chloride equivalents, and, DW = dry weight, High phytochemical content is represented in bold.

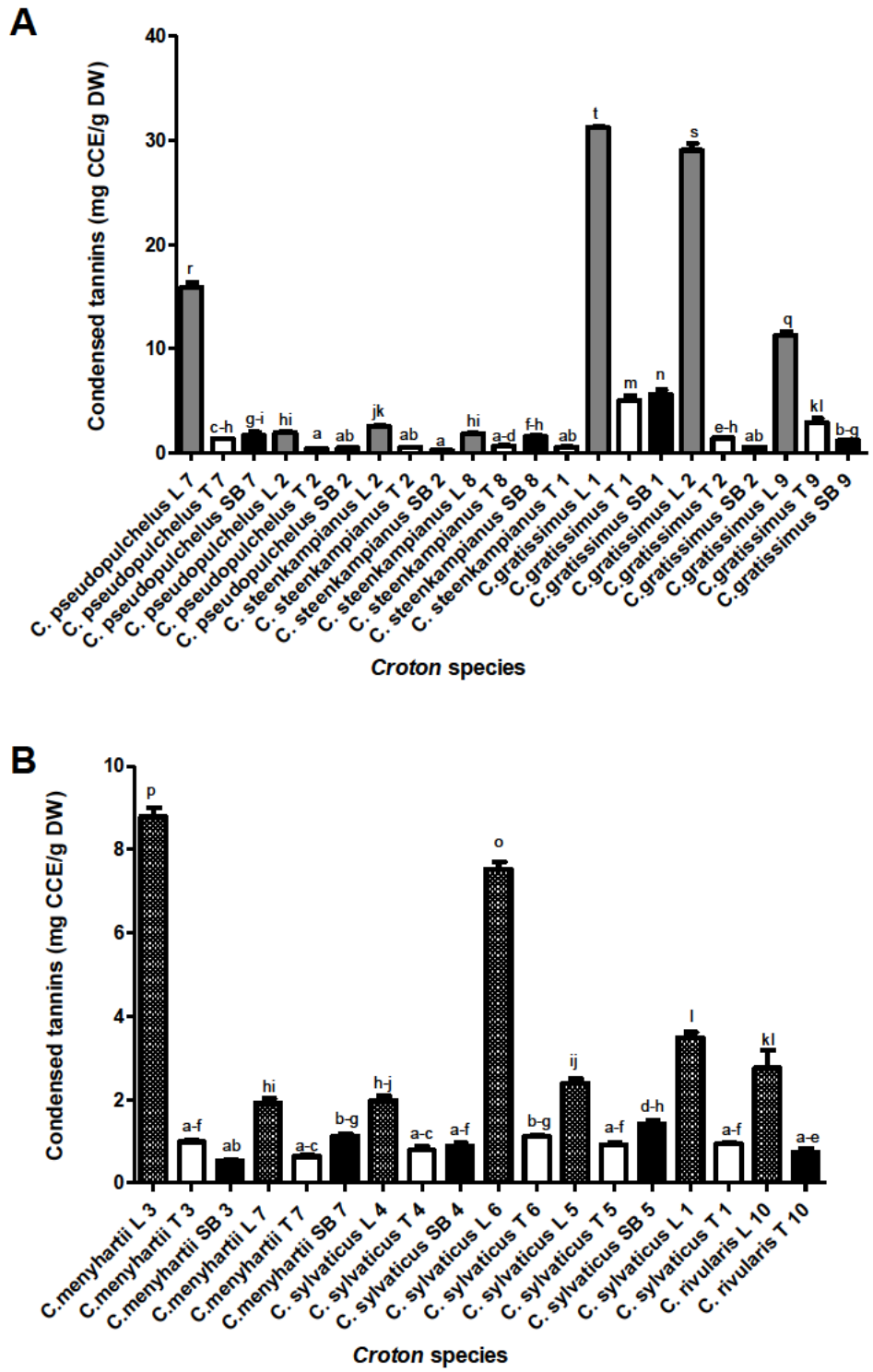


Figure 3.4: Quantity of condensed tannins (CCE: Cyanide chloride equivalents) in 80% methanol extracts of six *Croton* species, where CCE = Cyanide chloride equivalents, DW = dry weight. Bars represent mean± standard error, n=3. The different letter(s) on the bars represent significantly ($p \leq 0.05$) based on Duncan's multiple range test (DMRT).

1. Southport, 2. Durban Botanic Gardens, 3. Kranskop, 4. Umdoni Park, 5. Vernon Crookes, 6. Ntunjambili, 7. Mkuze, 8. Kosi Bay, 9. Muden, 10. Umzumkhulu

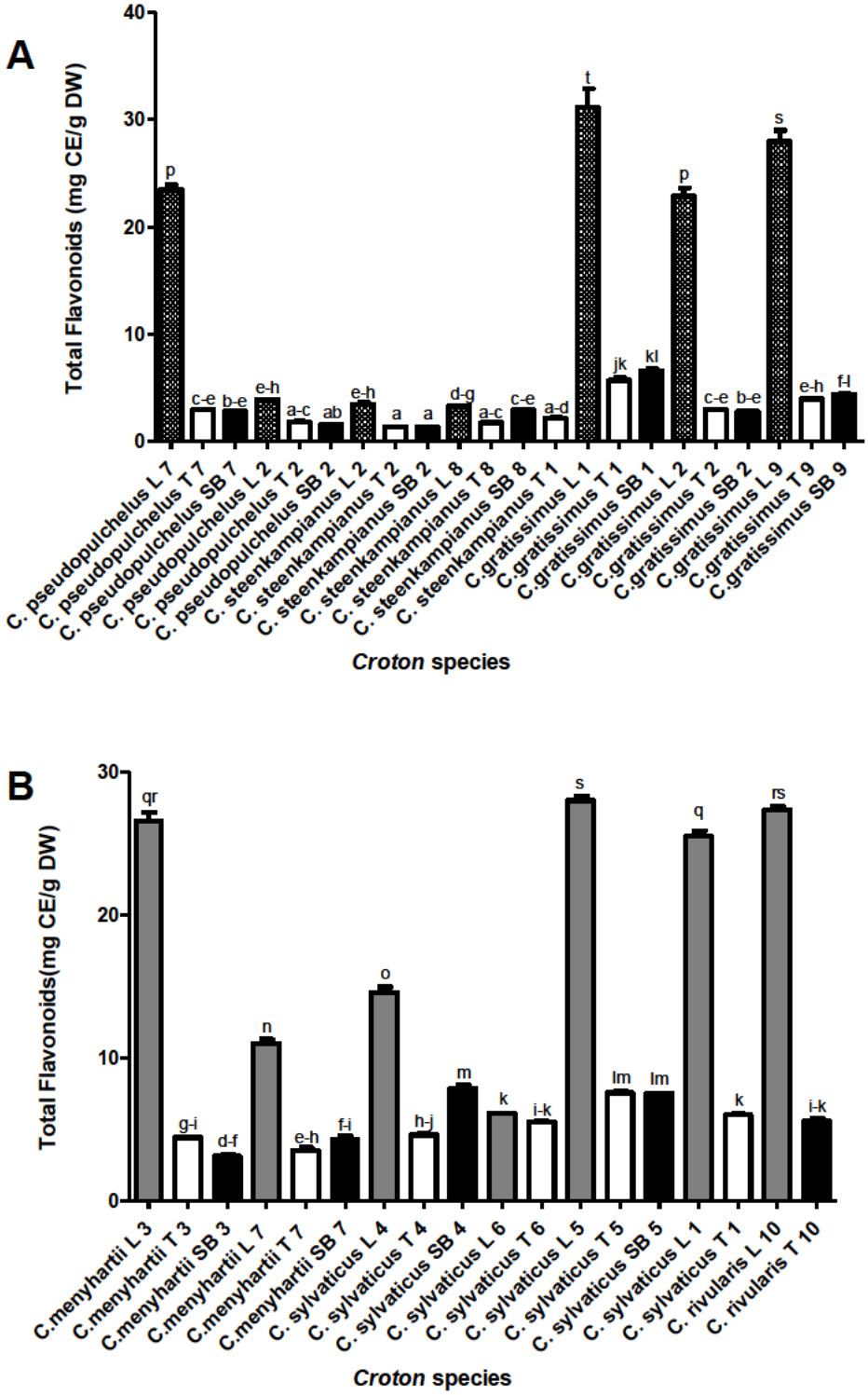


Figure 3.5: Quantity of flavonoids (CE: Catechin equivalents of total flavonoids) in 50% methanol extracts of six *Croton* species. CE = Catechin equivalents, DW = dry weight. Bars represent mean± standard error, n=3. The different letter(s) are on the bars represent significantly ($p \leq 0.05$) based on Duncan's multiple range test (DMRT).

1. Southport, 2. Durban Botanic Gardens, 3. Kranskop, 4. Umdoni Park, 5. Vernon Crookes, 6. Ntunjambili, 7. Mkuze, 8. Kosi Bay, 9. Muden, 10. Umzumkhulu

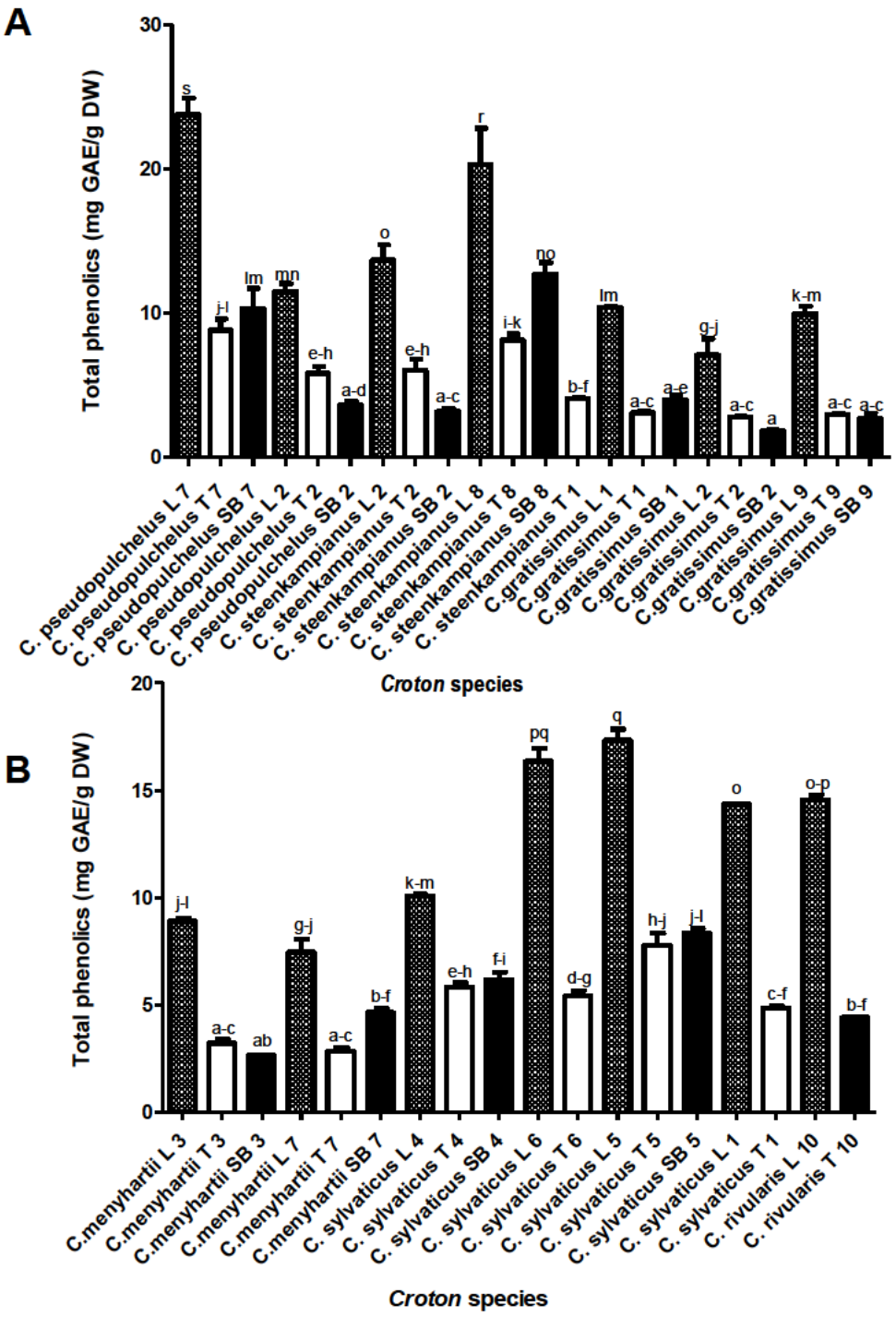
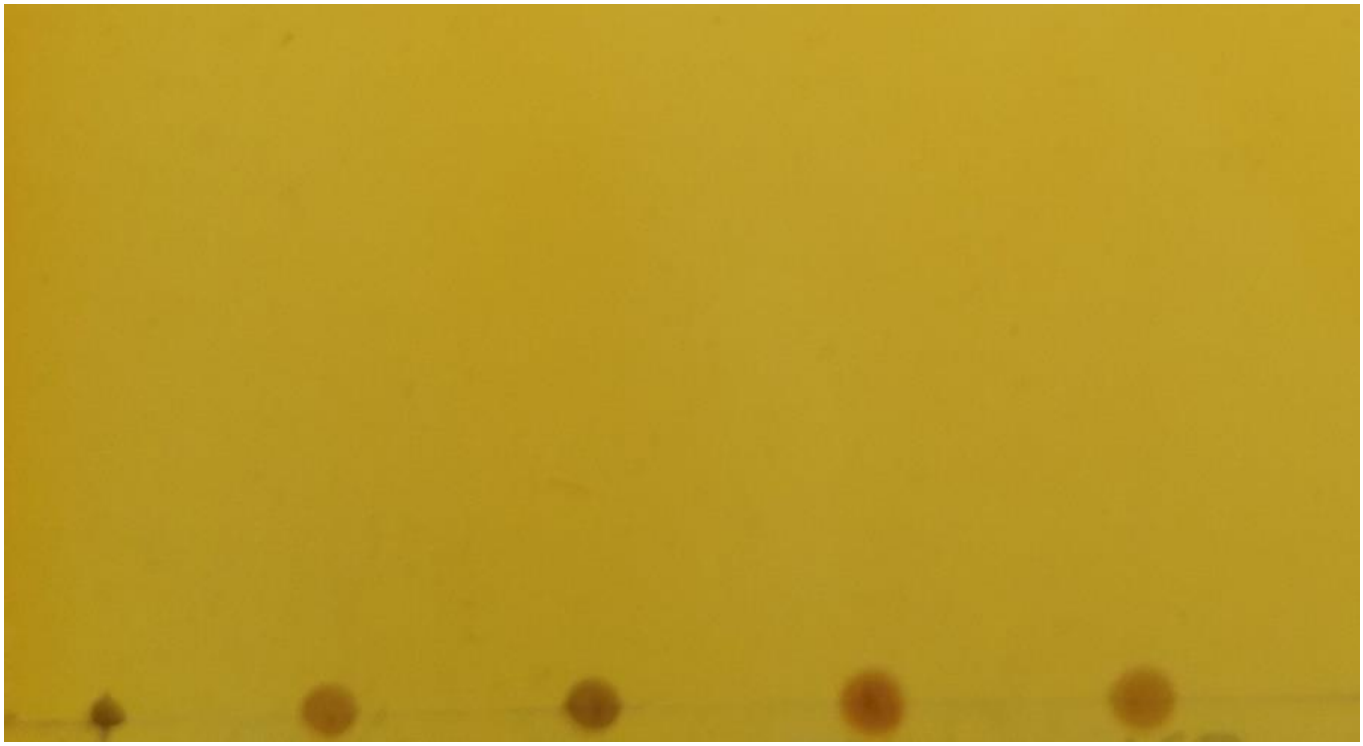


Figure 3.6: Quantity of phenolics in six *Croton* species extracts where GAE = gallic acid equivalents, DW = dry weight. Bars represent mean± standard error, n=3. The different letter(s) are on the bars represent significantly ($p \leq 0.05$) based on Duncan's multiple range test (DMRT).

1. Southport, 2. Durban Botanic Gardens, 3. Kranskop, 4. Umdoni Park, 5. Vernon Crookes, 6. Ntunjambili, 7. Mkuze, 8. Kosi Bay, 9. Muden, 10. Umzumkhulu



1 2 3 4 5

Figure 3.7: TLC plate presenting the red colouration of Dragendorff reagent indicating alkaloids in methanolic plant extracts. 1-*C gratissimus* (leaf) from Durban Botanic Gardens, 2- *C gratissimus* (twigs) from Southport, *3-C gratissimus* (twigs) from Muden, 4- *C. sylvaticus* (twigs) from Southport, 5-*C. steenkampianus* (leaves) from Durban Botanic Gardens.

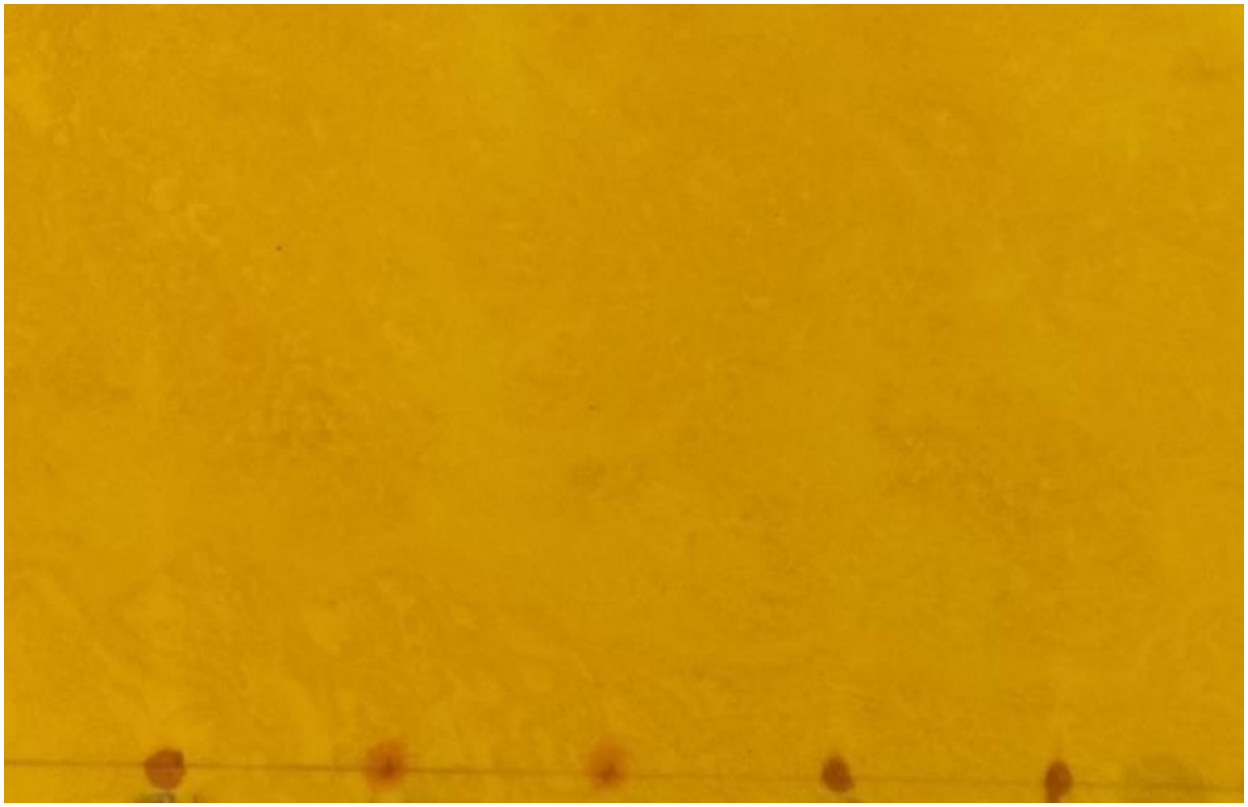


Figure 3.8: TLC plate presenting the red colouration of Dragendorff reagent indicating the presence of alkaloids in methanolic plant extracts. 6- *C. steenkampianus* (stem bark) from Durban Botanic Gardens, *7- C. pseudopulchellus* (leaves) from Durban Botanic Gardens, 8- *C. menyhartii* (leaves) from Kranskop, 9- *C. menyhartii (*twigs) from Mkuze 10- *C. rivularis* (twigs) from Umzimkhulu.

3.5.6 β-carotene-Linoleic Acid Model System

The β-carotene-linoleic acid model system assay is used to determine the antioxidant activity of plant extracts by measuring their ability to decrease or inhibit the oxidation of β-carotene-linoleic acid in an emulsified model system (**Shakeri et al.**, 2012, **Ndhlala et al.**, 2013). Oxidation in the β-carotene-linoleic acid emulsion model is heat (50°C) induced. During oxidation a hydrogen atom is removed from an active methylene group of linoleic acid, resulting in the formation of pentadienyl free radicals (**Amarowicz et al.**, 2004, **Moyo et al.**, 2010). The free radicals attack the unsaturated β-carotene in order to recover the lost hydrogen atom (**Ndhlala et al.**, 2013). As β-carotene reacts with the free radicals it loses its hydrogen atom and its orange colour is bleached; a process which could be prevented in the presence of antioxidants (**Amarowicz et al.**, 2004, **Moyo et al.**, 2010). As antioxidants have the ability to neutralize free radicals, they delay and inhibit bleaching of β-carotene (**Moyo et al.**, 2010, **Ndhlala et al.**, 2013). The process can be observed by periodically (every 30 min. for 2 h) monitoring the absorbance of a β-carotene-linoleic acid emulsion containing a plant extract in a spectrophotometer. The change in the absorbance (decreasing absorbance) with time represents the bleaching and oxidation of β-carotene-linoleic acid. The slower it takes for β-carotene-linoleic be bleached the higher antioxidant activity is exhibited by the plant extract (**Amarowicz et al.**, 2004, **Moyo et al.**, 2010, **Ndhlala et al.**, 2013).

Table 3.2 presents the antioxidant activities of the evaluated methanolic plant extracts. Antioxidant activity of *C. gratissimus* extract ranged between 63.03 to 81.97% and an ORR ranging between 0.18 to 0.37, with *C. gratissimus* leaf extracts from Muden showing the highest ANT% of 81.97% and an ORR of 0.18. The leaf extracts of *C. gratissimus* showed better results when compared with other plant organs of this species, with the exception to of *C. gratissimus* leaf extracts from Southport which exhibited lower antioxidant activity than that of the twigs and stem bark. However, all the extracts within this species displayed

substantial antioxidant activities. *C. gratissimus* (Crude 20% MeOH, hexane ethyle acetate, butanol) leaf extracts were reported by **Ndhlala et al.** (2013) to possess the capacity to inhibit the oxidation of β-carotene-linoleic acid.

The ANT % of all *C. sylvaticus* extracts ranged from 48.66 to 75.75% and ORR ranged from 0.24 to 0.51. The most potent extract being twig extracts from Ntunjambili and the lowest being the leaf extract collected at Vernon Crookes. Antioxidant activity of *C. sylvaticus* extracts was generally high. A modest decrease in antioxidant activity AA was observed over time from t=60, 90 and 120 min.

There were two samples of *C. steenkampianus* collected from different regions with antioxidant activity ranging between 47.49 to 73.62% and ORR from 0.26–0.53. Within this species C. *steenkampianus* stem bark extracts from Durban Botanic Gardens exhibited the highest antioxidant activity (73.62%) and the best ORR (0.26). *C. steenkampianus* Kosi Bay leaf extract showed the least antioxidant activity with antioxidant of 47.49% and an ORR of 0.53. These two *C. steenkampianus* samples showed a great difference in their antioxidant activity. This suggests that species from different localities may not have the same phytochemical content and that there are various factors affecting phytochemical content.

All of the *C. pseudopulchellus* extracts showed good antioxidant activities which had ANT% at 51.58 to 68.11% and ORR from 0.32 to 0.48. The twig extracts of this species exhibited the most antioxidant activity compared to the leaf and stem bark extract of the same species.

C. menyharitii extracts generally yielded high levels of antioxidant activity with ANT% ranging between 53.32 to 78.20% and ORR ranging between 0.22 - 0.47. The lowest and highest antioxidant activities of this species were both from stem bark extracts of two samples from two different regions. The lowest was *C. menyhartii* stem bark extract from

Mkuze and the highest was *C. menyhartii* stem bark extract from Kranskop. This species did not have a definite pattern showing what plant organs were the most active.

C. rivularis from Umzimkhulu Valley was the only sample of this species that was collected. This species showed high antioxidant activity ranging between 80.24% from leaf extract and 70.40% from the twig extract.

Table 3.2: Antioxidant activity of different *Croton* species collected in KwaZulu-Natal as determined by the β-carotene–linoleic acid model system

Species and locality	Plant part	%ANT	ORR	AA60min	AA90min	AA120min
C. gratissimus Southport	Leaves	63,03±11,28^{c-j}	0,37±0,11^{f-m}	41,50±20,70^{e-k}	47,48±16,62^{cd}	46,88±18,43^{kl}
	Twigs	70,48±1,06^{g-n}	0,30±0,01^{b-i}	42,27±1,15^{e-k}	39,18±1,69^{a-d}	35,25±1,98^{g-k}
	Stem bark	70,73±1,53^{g-n}	0,29±0,02^{b-i}	41,90±3,25^{e-k}	39,63±3,07^{a-d}	36,11±3,05^{g-k}
C. gratissimus D. B.G.	Leaves	79,09±0,48^{i-n}	0,21±0,00^{b-d}	23,97±1,42^{a-e}	22,13±1,69^{a-d}	18,90±1,65^{d-i}
	Twigs	72,88±0,26^{h-n}	0,27±0,00^{b-h}	45,38±1,04^{f-k}	43,73±0,79^{cd}	41,24±0,81^{j-l}
	Stem bark	76,83±0,89^{k-n}	0,23±0,01^{b-e}	49,23±1,19^{h-k}	32,91±1,49^{a-d}	21,03±1,91^{e-j}
C. gratissimus Muden	Leaves	**81,97±1,32^{n}**	0,18±0,01^{b}	47,62±2,14^{h-k}	34,01±3,44^{a-d}	24,78±4,14^{e-j}
	Twigs	78,83±1,98^{l-n}	0,21±0,02^{b-d}	52,38±2,63^{i-k}	38,18±3,39^{a-d}	28,27±3,79^{e-k}
	Stem bark	74,81±0,75^{i-n}	0,25±0,01^{b-g}	41,08±2,23^{d-k}	25,17±2,15^{a-d}	14,83±2,46^{c-g}
C. sylvaticus Southport	Leaves	74,12±5,57^{h-n}	0,26±0,06^{b-h}	56,47±19,25^{k}	49,83±21,51^{d}	57,56±10,74^{l}
	Twigs	62,43±4,29^{c-j}	0,38±0,04^{g-m}	41,85±3,66^{e-k}	39,18±3,99^{a-d}	35,77±4,17^{g-k}
C. sylvaticus Umdoni Park	Leaves	57,95±7,90^{a-g}	0,42±0,08^{i-o}	47,02±10,23^{g-k}	43,39±10,13^{cd}	39,22±9,83^{i-l}
	Twigs	61,83±0,67^{b-h}	0,38±0,01^{h-n}	32,43±0,78^{b-h}	25,61±0,77^{a-d}	6,82±27,4^{ab}
	Stem bark	61,93±0,98^{b-h}	0,38±0,01^{h-n}	31,69±0,61^{a-h}	27,03±0,80^{a-d}	22,23±1,03^{e-j}
C. sylvaticus Vernon Crookes	Leaves	48,66±4,83^{a}	0,51±0,05^{o}	37,85±3,71^{b-j}	33,20±3,40^{a-d}	28,60±2,89^{e-k}
	Twigs	63,08±2,38^{c-j}	0,37±0,02^{f-m}	40,50±1,89^{d-k}	37,83±2,04^{a-d}	33,72±2,15^{f-k}
	Stem bark	65,08±1,08^{e-k}	0,35±0,01^{e-k}	25,10±1,27^{a-e}	7,99±1,71^{a}	2,18±1,27^{bc}
C. sylvaticus Ntunjambili	Leaves	53,56±2,00^{a-e}	0,46±0,02^{k-o}	24,59±0,50^{a-e}	20,20±0,51^{a-d}	17,44±0,63^{c-i}
	Twigs	**75,75±2,76^{j-n}**	0,24±0,03^{b-f}	54,40±2,95^{jk}	40,05±4,46^{a-d}	31,24±5,48^{e-k}

Table 3.2 continued…

Species and locality	Plant part	%ANT	ORR	AA60min	AA90min	AA120min
C. steenkampianus D.B.G.	Leaves	61,37±1,54^{b-h}	0,39±0,02^{h-n}	28,55±2,32^{a-g}	24,32±2,34^{a-d}	20,31±2,43^{e-j}
	Twigs	58,30±3,58^{a-g}	0,42±0,04^{i-o}	42,45±3,99^{e-k}	37,72±4,05^{a-d}	33,33±3,87^{e-k}
	Stem bark	**73,62±1,11^{h-n}**	0,26±0,01^{b-h}	41,81±1,77^{e-k}	26,02±2,61^{a-d}	16.75±2,58^{c-h}
C. steenkampianus Kosi Bay	Leaves	47,49±13,64^{a}	0,53±0,14^{o}	35,26±0,77^{b-i}	30,76±0,30^{a-d}	26,43±0,41^{e-k}
	Twigs	66,44±3,35^{f-l}	0,34±0,05^{d-j}	14,04±0,87^{a}	38,78±46,70^{a-d}	20,94±0,54^{a}
	Stem bark	49,46±2,25^{ab}	0,51±0,02^{no}	27,01±1,67^{a-f}	22,87±1,72^{a-d}	19,36±1,60^{e-j}
C. pseudopulchellus D.B.G.	Leaves	52,21±1,79^{a-d}	0,48±0,02^{l-o}	19,98±1,84^{a-c}	18,19±2,02^{a-d}	16,23±2,34^{c-h}
	Twigs	63,10±0,88^{c-j}	0,37±0,01^{f-m}	38,35±4,67^{c-k}	29,55±3,10^{a-d}	25,09±3,22^{e-j}
	Stem bark	51,58±2,15^{a-c}	0,48±0,02^{m-o}	27,92±1,24^{a-f}	23,94±1,51^{a-d}	20,44±1,50^{e-j}
C. pseudopulchellus Mkuze	Leaves	56,62±1,14^{a-f}	0,43±0,01^{j-o}	19,72±2,48^{ab}	17,53±2,18^{a-c}	15,38±2,29^{c-h}
	Twigs	64,73±0,98^{d-k}	0,35±0,01^{e-l}	42,35±0,52^{e-k}	40,16±0,72^{b-d}	36,98±0,81^{h-k}
	Stem bark	53,34±0,97^{a-e}	0,47±0,01^{k-o}	32,58±0,61^{b-h}	28,33±0,63^{a-d}	24,61±0,67^{e-j}
C. pseudopulchellus Southport	Twigs	**68,11±3,68^{f-m}**	0,32±0,04^{c-i}	40,36±4,60^{d-k}	22,45±5,32^{a-d}	11,52±5,26^{b-e}
C. menyhartii Mkuze	Leaves	66,61±0,64^{f-l}	0,33±0,01^{d-j}	38,08±2,69^{b-k}	34,38±1,69^{a-d}	32,22±2,49^{e-k}
	Twigs	74,91±1,28^{i-n}	0,25±0,01^{b-g}	27,04±5,78^{a-f}	9,78±5,78^{ab}	1,75±5,67^{b-d}
	Stem bark	53,32±1,98^{a-e}	0,47±0,02^{k-o}	22,81±2,03^{a-d}	17,33±1,85^{a-c}	11,86±1,72^{b-f}
C. menyhartii Kranskop	Leaves	75,25±0,07^{i-n}	0,25±0,00^{b-g}	32,03±0,93^{a-h}	30,06±1,07^{a-d}	28,50±0,67^{e-k}
	Twigs	66,36±0,37^{f-l}	0,34±0,00^{d-j}	35,26±0,89^{b-i}	31,87±0,93^{a-d}	28,08±1,00^{e-k}
	Stem bark	**78,20±1,01^{l-n}**	0,22±0,01^{b-d}	49,64±2,11^{h-k}	34,95±2,30^{a-d}	26,61±2,67^{e-k}
C. rivularis Umzimkhulu Valley	Leaves	**80,24±1,38^{mn}**	0,20±0,01^{bc}	48,91±1,88^{h-k}	33,93±2,81^{a-d}	23,82±3,67^{e-j}
	Twigs	70,40±1,66^{g-n}	0,30±0,02^{b-i}	45,60±2,28^{f-k}	42,61±2,38^{cd}	39,03±2,54^{i-l}
BHT		96,09±1.81^{o}	0,04±0,02^{a}	92,36±6,59^{c}	83,83±5,95^{e}	75,82±5,65^{n}

Values are the mean ± standard error (n = 3), the different letters in a column represent significant differences between the means (P=0.05) according to Duncan's multiple range test (DMRT). ANT % is antioxidant activity calculated based on the rate at which β-carotene was bleached at t=30, 60 and 90 min., this is represented as the percent of bleach inhibition. ORR is oxidation rate ratio, this is the average β-carotene bleaching rates as compared to the negative control. The lower the ratio the more active the plant extracts. AA60 and AA120 are antioxidant activities of the plant extracts or BHT at t= 60 min and t=120, respectively. Values in bold represent the highest ANT% per species

3.5.7. DPPH radical scavenging assay

Table 3.3 presents EC_{50} (scavenging activity) of MeOH extracts of different *Croton* species and their different plant parts collected from different localities within KZN. The EC_{50} values were calculated from the dosed dependent radical scavenging activities of these *Croton* extracts, which showed an increase in DPPH radical scavenging activity with an increase in extract concentration. The overall EC_{50} values of the different *Croton* species and plant part ranged from 1.76 to 5.35 µg/ml. The EC_{50} of the standard antioxidant BHT and ASC used as positive control were 1.66 and 1.42, respectively.

Ndhlala et al. (2013) reported on the radical scavenging activity of the leaf extracts of *C. gratissimus* against DPPH radicals. The results displayed both potency and no radical scavenging activity, depending on the solvents used during extraction. Potency was observed in *C. gratissimus* crude extract (20% MeOH), *C. gratissimus* ethyl acetate, *C. gratissimus* butanol, wheras extracts from solvents hexane and DCM showed no activity. This emphasises the significance and effects on plant extract potency that the solvents used for extraction have.

The EC_{50} values for *C. gratissimus* in this study ranged from 1.76 to 5.08 µg/ml. The lowest EC_{50} value was from *C. gratissimus* leaf extract from Southport (1.76 µg/ml) which was not significantly different to the positive controls' EC_{50}s, and the highest was from *C. gratissimus* stem bark from Muden (5.08 µg/ml). In general, *C. gratissimus* leaf extracts showed the better EC_{50} compared to the other plant parts ranging between 1.76 to 2.34 µg/ml.

Scavenging activity of all the *C. sylvaticus* showed EC_{50} ranges between 2.71 to 5.35 µg/ml. *C. sylvaticus* twigs showed the highest EC_{50} values (lowest scavenging activity) of analysed organs ranging between 4.24 to 5.35 µg/ml.

Leaf extracts of *C. sylvaticus* showed low to moderate scavenging activity with EC_{50} ranging between 2.71 to 4.48 µg/ml. The highest EC_{50} value (5.35 µg/ml) of *C. sylvaticus* was from twig extracts from Vernon Crookes. It was also the overall lowest free radical scavenging activity in this study.

For the two *C. steenkampianus* plants tested the EC_{50} ranged from 3.42 to 4.85 µg/ml. *C. steenkampianus* stem bark extracts from Kosi Bay had the lower EC_{50} of 3.42 µg/ml in this species. The scavenging activities of the different organs in this species yielded no significant difference.

The EC_{50} of *C. pseudopulchellus* ranged from 2.67 to 5.18 µg/ml. *C. pseudopulchellus* leaf extract from Mkuze showed the highest free radical scavenging activity in this species with its EC_{50} of 2.67 µg/ml. However, the other leaf extract for this species, from Durban Botanic Gardens species, showed the lowest free radical scavenging activity with an EC_{50} of 5.18 µg/ml.

The EC_{50} values of *C. menyhartii* ranged from 2.66 to 4.26 µg/ml, where the lowest was EC_{50} from *C. menyhartii* leaf extract from Kranskop (2.66 µg/ml) and the highest from *C. menyhartii* stem bark extracts from Mkuze (4.26 µg/ml). There were no significant differences in the EC_{50} of the plant parts of this species and the EC_{50} between the plants collected in different localities were very similar.

Only one sample of *C. rivularis* was analysed in the present study. The EC_{50} of the two organs of this species were 3.03 µg/ml for the leaf extract and 3.74 µg/ml for the twig extract. Generally most of the species and their organs had good antioxidant activities; whereas there were some that had poor activities. *C. gratissimus* showed higher antioxidant activity in most of its organs when compared with the other species. Leaves and twigs showed antioxidant activity that was equal or higher than the commonly used stem bark. This supports the notion

to consider the use of aerial parts of the plants in efforts to promote sustainable use and conservation of plants and other natural resources.

Table 3.3: 1.1-Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity (EC_{50}) of methanol extracts of various *Croton* species occurring in KwaZulu-Natal province, South Africa

Species and locality	**Plant part**	**EC_{50}(µg/ml)**	**R^2**
C. gratissimus- Southport	Leaves	**1,76±0,14^{a}**	0,46±0,04^{a}
	Twigs	2,91±0,03^{def}	0,89±0,01^{e-i}
	Stem bark	2,94±0,66^{def}	0,84±0,06^{ef}
C. gratissimus -D.B.G.	Leaves	2,25±0,07^{b}	0,64±0,02^{b}
	Twigs	3,82±0,03^{i-n}	0,94±0,01^{i-l}
	Stem bark	3,57±0,01^{i-m}	0,91±0,02^{g-k}
C.gratissimus Muden	Leaves	2,34±0,04^{bc}	0,72±0,01^{c}
	Twigs	2,98±0,04^{def}	0,82±0,01^{de}
	Stem bark	5,08±0,05^{tu}	0,98±0,01^{kl}
C. sylvaticus Southport	Leaves	**2,71±0,12^{de}**	0,74±0,04^{c}
	Twigs	4,24±0,05^{o-r}	0,97±0,01^{jkl}
C. sylvaticus Umdoni Park	Leaves	4,48±0,11^{r}	0,97±0,01^{jkl}
	Twigs	4,38±0,01^{qr}	0,95±0,01^{i-l}
	Stem bark	3,21±0,10^{f-i}	0,87±0,05^{e-h}
C. sylvaticus Vernon Crookes	Leaves	3,10±0,05^{e-h}	0,84±0,05^{efg}
	Twigs	5,35±0,12^{u}	0,99±0,01^{l}
	Stem bark	4,42±0,06^{qr}	0,90±0,01^{f-j}
C. sylvaticus Ntunjambili	Leaves	2,78±0,04^{de}	0,78±0,02^{cd}
	Twigs	3,75±0,04^{j-n}	0,94±0,01^{i-l}

Table 3.3 continued….

Species and locality	Plant part	EC_{50}(µg/ml)	R^2
C. steenkampianus D.B.G.	Leaves	$4,52\pm0,11^{rs}$	$0,98\pm0,01^{l}$
	Twigs	$4,06\pm0,02^{n-q}$	$0,91\pm0,01^{f-k}$
	Stem bark	$4,31\pm0,02^{p-r}$	$0,93\pm0,01^{h-l}$
C. steenkampianus Kosi Bay	Leaves	$3,69\pm0,07^{j-n}$	$0,97\pm0,01^{kl}$
	Twigs	$4,85\pm0,05^{st}$	$0,94\pm0,01^{i-l}$
	Stem bark	**$3,42\pm0,06^{h-k}$**	$0,92\pm0,01^{c}$
C. pseudopulchellus D.B.G.	Leaves	$5,18\pm0,12^{tu}$	$0,98\pm0,01^{l}$
	Twigs	$3,52\pm0,01^{ijk}$	$0,90\pm0,01^{f-i}$
	Stem bark	$3,94\pm0,02^{m-p}$	$0,92\pm0,01^{h-l}$
C. pseudopulchellus Mkuze	Leaves	**$2,67\pm0,02^{cd}$**	$0,74\pm0,01^{c}$
	Twigs	$3,77\pm0,01^{l-n}$	$0,93\pm0,01^{h-l}$
	Stem bark	$3,85\pm0,04^{l-n}$	$0,91\pm0,02^{g-k}$
C. pseudopulchellus Southport	Twigs	$3,37\pm0,07^{g-j}$	$0,91\pm0,02^{g-k}$
C. menyhartii Mkuze	Leaves	$3,90\pm0,01^{l-o}$	$0,99\pm0,01^{l}$
	Twigs	$3,27\pm0,09^{f-i}$	$0,95\pm0,02^{i-l}$
	Stem bark	$4,26\pm0,07^{o-r}$	$0,96\pm0,01^{i-l}$
C. menyhartii Kranskop	Leaves	**$2,66\pm0,06^{cd}$**	$0,74\pm0,01^{c}$
	Twigs	$3,27\pm0,07^{f-i}$	$0,92\pm0,02^{h-l}$
	Stem bark	$3,91\pm0,01^{l-o}$	$0,94\pm0,01^{i-l}$
C. rivularis Umzimkhulu Valley	Leaves	**$3,03\pm0,02^{d-g}$**	$0,84\pm0,01^{efg}$
	Twigs	$3,74\pm0,04^{j-n}$	$0,85\pm0,04^{efg}$
BHT		$1,66\pm0,01^{a}$	$0,44\pm0,01^{a}$
ASC		$1,42\pm0,04^{a}$	$0,40\pm0,01^{a}$

Values are the mean ± standard error (n = 3), the different letters on the values represent significant differences between the means (P=0.05) according to Duncan's multiple range test (DMRT). R^2 correlation coefficient BHT=butylated hydroxytoluene and ASC=ascorbic acid. The values in bold represent the lowest EC_{50} values (highest scavenging activity) from that species

3.6. Conclusions

Croton species show great variation in their phytochemical constituency and pharmacological activities. The plant extracts in the current study generally exhibited activity that validated their use in traditional medicine. The species and plant parts that showed low activity may require further research and investigation. Some plant extracts such as the stem bark of *C. gratissimus* (Durban Botanic Gardens) and *C. menyhartii* Kranskop contained the lowest amount of phenolic compounds but had previously showed noteworthy antibacterial (**Chapter 2, Table 2.2**) and antioxidant (**Table 3.2**) activities. **Mulaudzi et al**. (2011) attributed the potency of such plant extracts to the quality of the phenolic compounds and suggested that other secondary metabolites may be responsible for the activity. In another study a different *Croton* species, *Croton bonplandianus* Baill. exhibited a broad spectrum of antimicrobial activity which **Jeeshna et al**. (2011) attributed to the variety of secondary metabolites contained by its plant extracts. This included the secondary metabolites screened in this study and others which were not screened in this study such as glycosides, steroids, saponins and resins, which are known for their therapeutic properties. *Croton* species are known to be rich in secondary metabolites (**Rizk**, 1987, **Jeeshna et al.**, 2011). For a better understanding of the pharmacological activity of the species it would be advantageous ~~wiser~~ to screen for a wider variety of secondary metabolites, particularly those that have therapeutic activity.

Further investigation measures to be considered include the use of different solvents for extracting plant compounds. It may be beneficial to consider using plants extracted with DCM and PE to conduct phytochemical and antioxidant assays as they yielded noteworthy activity in antibacterial studies. Methanol was used because it is widely used and has in previous studies proven to exhibit high extraction yield for phenolic compounds and antioxidants (**Yen et al.**, 1996, **Barchan et al.**, 2014, **Truong et al.**, 2019). Leaf extracts

yielded considerable phytochemical and pharmacological activity. This suggests the possibility of leaves becoming sources of therapeutic drugs and as possible substitutes for bark.

Chemical composition and quality of the secondary metabolites are not only compromised by environmental factors, the degradation and modification of compounds may occur during processing, isolation, and storage (**Atanasov et al.**, 2015). For plant extracts that showed low secondary metabolites and had previously exhibited noteworthy antimicrobial activity it should be considered that these may have been compromised during the processing and storage stages. This means careful consideration and caution should be taken during these stages

CHAPTER 4

Genotoxicity test for bioactive plant extracts

4.1 Introduction

An estimated 10^7 cells divide per second in a human body and a third of these divided cells spontaneously mutate (**Marnett and Plastaras**, 2001). Mutation is the initial and fundamental stage in the development of cancer in humans (**Lin et al.**, 1999). Mutation occurs when there is an alteration, insertion or deletion of one or a few bases in a gene. It may also occur when chromosomes are rearranged or break, or when whole chromosomes are lost or gained. Mutation may likewise result when large rearrangement and deletions of DNA occur (**Mortelmans and Zeiger**, 2000). Many mutations in human tissue occur as a result of DNA damage usually caused by endogenous processes, such as DNA hydrolysis, exposure of DNA to reactive oxygen substances (ROS) and other biological reactive metabolites such as oestrogen metabolites and S-adenosylmethionine (SAM) (**De Bont and Van Larebeke**, 2004). Most carcinogenic mutations have the capacity to activate oncogenes or deactivate tumour suppressor genes (**Lin et al.**, 1999). Mutation are not induced by endogenous metabolic processes alone; there are other exogenous factors that humans are exposed to or ingest as part of their diet or for therapeutic purposes that have mutagenic and carcinogenic potentials. Plants are known to produce a variety of natural chemical compounds, which may be cytotoxic and or mutagenic (**Ames**, 1989).

Plants are widely used medicinally and have generally been assumed to be safe, particularly because they are natural (**Fennell et al.**, 2004, **Awodele et al.**, 2012). However there are growing concerns that the misadministration, misidentification and prolonged use of medicinal plants and some herbs may pose a serious threat to consumers. It is important to note that while secondary phyto-metabolites may possess therapeutic properties they are synthesized by plants as part of their innate defence mechanism. Among other things, these compounds poison, deter and/ or kill herbivores and pathogens (**Street et al.**, 2008, **Demma et al.**, 2009). It is therefore unreasonable to assume that plants are safe. Scientific researchers have documented evidence suggesting that some ethnobotanically significant plants possess toxic, mutagenic and/or carcinogenic potentials (**Fernandes de Sá Ferreira and Ferrão Vargas**, 1999, **Fennell et al.**, 2004). Some examples include the phorbol ester found in the plant family Euphorbiaceae. This family contains species that are widely used for medicinal purposes and herbal teas. However they have been suspected of causing oesophageal cancer in Curacao and nasopharyngeal cancer in China (**Ames et al.**, 1990). *Aloe vera* (L.) Burm.f., a widely used herb for bruised skin and internal consumption has likewise been found to be toxic to humans. The latex produced by the plant is known to lead to potassium deficiency when consumed over a long time, which in turn results in electrolyte imbalances (**Haq**, 2004).

Some compounds produced by plants may have contradictory activities. Examples would include phenolics, which are said to be responsible for double-strand DNA breakages, chromosome aberrations, DNA and adducts, mutations. They have also been proven to produce precancerous lesions, papillomas and act as carcinogens in rodent experiments (**George**, 2011). Phenolics have however, shown actions of carcinogen suppression on various carcinogenic compounds (**George**, 2011). There is however, a relationship to be considered between toxicology and pharmacology of plants, Many plants and chemical

compounds show therapeutic efficacy at low dosages, but are however toxic when consumed at high dosages (**Botha and Penrith**, 2008, **Fred-Jaiyesimi and Ajibesin**, 2012). For this reason there is a need to further investigate the effects and actions of natural plant material. Under certain conditions chemical compounds synthesized by plants are known to react with biological metabolites in the human body and may induce mutagenic, cytotoxic and genotoxic effects. This is due to their numerous biological properties (**Sarkar et al.**, 1996, **Celik**, 2012).

The Ames test is a widely used method of examining the mutagenic potential of various novel chemical substances. This method has the capacity to detect chemical compounds that are responsible for genetic damage which results in gene mutations. Different histidine dependent *Salmonella* tester strains, each containing different mutations in different genes in the histidine operon are used to determine the presence of mutagens that produce cells which have reverted into histidine independent cells. *Salmonella* is known to be dependent on histidine for its growth. In the assay tester strains are grown in the presence of minimal histidine. This is to determine their ability to mutate or revert to histidine independence. This is evident by the large number of colonies formed in the Petri-dish (**Margolin et al.**, 1989). Plant extracts used in the assay were previously screened for anti-microbial and anti-oxidant activity in this research. This section of the study aims to determine the genotoxicity of these effective samples.

4.2. Materials and methods

4.2.1. Plant collection and preparation of samples

Croton species were collected, samples prepared and extracted as described in **Sections 2.2.1 and 2.2.2** of **Chapter 2**. On account of limited financial resources, only plant extracts that

exhibited noteworthy anti-bacterial activity (MIC < 1mg / ml) were selected to be screened for mutagenic properties (**Chapter 2, Table 2.2**). The biologically active plant extracts were re-dissolved in 10% DMSO to make three concentrations of 0.05, 0.5 and 5 mg/ml, respectively.

4.2.3. Ames mutagenic test

Mutagenic properties of plant extracts that exhibited noteworthy anti-bacterial activity with minimum inhibitory activity at concentrations of <1mg/ml (**Chapter 2**, **Table 2.2**) were tested using the Ames mutagenic test assay. The assay described by **Maron and Ames** (1983) and **Elgorashi** et al. (2003) uses two *Salmonella* tester strains (TA98 and TA102), in the absence of S9 metabolic activation to detect carcinogens and mutagens in plant extracts. One hundred microliters of each tester bacterial strain were grown in 10 ml of Oxoid nutrient broth No. 2 for 16 h at 37 C on a shaker (100 rpm) to achieve a cell density of approximately 1 x 10^9 cfu/ml. In triplicates, 100 µl of different concentrations (0.05, 0.5 and 5mg/ml) of each plant extract were dispensed in sterile test tubes, followed by 500 µl of phosphate buffer (0.1 mM, pH 7.4), along with 100 µl of the bacterial strain and then 2 ml of sterile melted top agar, containing 0.5 mM of biotin and 0.5 mM histidine were added to the mixture. The mixture was vortexed and poured onto labelled minimal agar plates and allowed to solidify for 2-3 min. One hundred microliters of 10% DMSO served as the negative control and 2 µg/ml of 4-Nitroquinoline-N-oxide (4-NQO) as a positive control. After solidifying, the plates where inverted and incubated at 37°C for 48h. The number of viable colonies was counted using a colony counter. The assay was conducted twice and results expressed as mean ± standard error number of reverted colonies per plate. Plant samples that produced more than twice the number of His+ revertants produced by the negative control were considered to be mutagenic (**Maron and Ames**, 1983). Samples that exhibited a dose-

dependent increase in the number of His+ revertants in one or two strains were considered to be mutagenic (**Cariello and Piegorsch**, 1996).

4.3. Results and discussion

Table 4.1 presents the average His+ revertant colonies induced during the Ames mutagenic test, where different concentrations (0.05, 0.5 and 5 mg/ml) of crude extracts from *Croton* species were tested for their production of revertant colonies in two *S. typhimurium* strains (TA98 and TA102). The average number of His+ revertant colonies produced by the *Croton* species extracts across all three concentrations ranged from 12.7-68 for TA98 and ranged from 201-436 for TA102. The corresponding average number His+ revertant colonies produced by the negative control were 34.3 for TA98 and 446.3 for TA102. These results suggest that none of the extracts showed mutagenic activity as none of the produced His+ revertant colonies were more than twice the number of His+ revertant produced by the negative control (10% DMSO). According to the dose dependent growth principle none of the extracts showed mutagenic activity in the TA98 strain, however in TA102 three *Croton* extracts showed weak mutagenic activity because there was dose dependent growth across the evaluated concentrations (0.05, 0.5 and 5mg/ml). These extracts were from the leaf DCM extract of *C. steenkampianus* (Durban Botanic Gardens), twig PE extracts of C. *sylvaticus* (Southport) and stem bark DCM extract of *C. menyhartii* (Mkuze). The rest of the extracts were non-mutagenic.

In study conducted by **Ndhlala et al.** (2013) three compounds isolated from *C. sylvaticus* leaves were tested for mutagenic properties and were found to be non-mutagenic against *S. typhimurium* strains (TA98 and TA100). A similar study was conducted by **Verschaeve and Van Staden** (2008) on the DCM and MeOH leaf and stem extracts of *C. sylvaticus* where

these extracts were found to have no mutagenic activity. While most of the extracts in this study did not show mutagenic activity, further investigations are necessary, where different toxicological tests will be used to determine mutagenesis of the species as plant extracts are complex mixtures and their toxicity may vary with evaluation criteria (**Verschaeve and Van Staden**, 2008). This is also important because *Croton* species are from the Euphorbiaceae family known to produce a wide variety of mammalian toxins, carcinogens and irritants (**Croteau et al.**, 2000, **Rossi et al.**, 2003).

Table 4.1 Mutagenic evaluation of *Croton* species extracts using *Salmonella typhimurium* tester strains (TA98 and TA102) without S9 metabolic activation. The data is reported as number of histidine independent (His^+) revertant colonies per plate.

		Number of His+ revertants/plate (mg/ml)					
		TA98			**TA102**		
Plant species and locality	**Plant extract**	**0,05 mg/ml**	**0,5 mg/ml**	**5 mg/ml**	**0,05 mg/ml**	**0,5 mg/ml**	**5 mg/ml**
C. pseudopulchellus Southport	twigs (MeOH)	13,3±1,9	23,3±11,6	12,7±4,4	366,7±9,8	315±11,0	378,0±11,5
C. pseudopulchellus Southport	twigs (DCM)	13,7±2,7	17,3±2,7	16,0±4,0	344,3±11,3	349,7±4,9	349,3±14,9
C. pseudopulchellus Southport	twigs(PE)	18,7±3,2	33,0±4,0	18,7±3,8	325,3±28,9	285,7±5,0	294,7±0,9
C. gratissimus D.B.G.	leaves (DCM)	45,0±4,6	33,7±0,9	17,3±5,0	325,3±14,3	436,7±1,5	275,3±45,0
C. gratissimus D.B.G.	leaves (PE)	51,3±2,4	43,0±2,3	16,3±1,9	349,7±16,3	245±5,5	348,7±28,8
C. gratissimus D.B.G.	twigs(DCM)	44,3±11,7	38,3±3,3	21,0±1,2	333±1,5	279,7±13,9	231,0±20,6
C. menyhartii Kranskop	twigs(PE)	51,0±1,0	31,7±3,8	19,3±2,6	369,3±13,4	369±3,2	201,0±3,5
C. sylvaticus Vernon Crookes	leaves (DCM)	29,7±1,2	33,3±2,2	20,0±3,1	272±13,3	302,0±23,2	295,7±28,7
C. steenkampianus D.B.G.	leaves (DCM)	33,7±1,5	26,0±3,1	14,3±2,0	**259,0±11,5**	**278,3±9,6**	**299,0±21,8**
C. sylvaticus Southport	twigs(DCM)	31,0±1,5	29,3±1,5	29,3±1,5	254±6,8	280,7±9,4	267,0±7,5
C. sylvaticus Southport	twig(PE)	33,3±2,4	26,3±3,0	16,7±0,7	**254,0±18,2**	**283,3±20,3**	**304,3±4,4**
C. steenkampianus D.B.G.	leaves (PE)	40,7±8,7	18,7±2,9	23,7±4,9	403±4,6	244,7±14,1	407,3±11,9
C. pseudopulchellus Mkuze	leaves (DCM)	46,3±7,5	19,3±1,5	20,7±1,5	413±11,5	322,3±12,8	375,0±4,6
C. pseudopulchellus Mkuze	leaves (PE)	49,3±8,7	42,3±4,9	33,0±3,5	315,3±11,3	305,7±1,8	366,7±12,4
C. steenkampianus D.B.G.	twigs(DCM)	68,0±14,4	45,7±3,5	20,3±1,7	308,3±33,2	218,0±20,6	252,0±7,5
C. menyhartii Mkuze	stem bark (DCM)	68,0±15,4	20,3±2,2	19,7±2,0	**242,7±24,9**	**243,7±14,7**	**358,3±4,9**
4-nitroquinoline-1-oxide (4-NQO)	Positive control		50,7±2,9				352,3±25,5
(10% DMSO)	Negative control		34,3±3,2				446,3±13,7

Values presented as mean±standard error (n=3). DCM = Dichloromethane, MeOH = Methanol, PE = Petroleum ether, 4-NQO = 4-nitroquinoline-oxide. Value in bold represent extracts with weak mutagenic activity.

4.4. Conclusions

Literature has declared some *Croton* species toxic (**Maroyi**, 2017). However results in this study have shown that they are not mutagenic. This leaves room for further analysis, perhaps using alternative methods of genotoxicity testing. Whereas the results obtained from the experiment answer the main question of the safety of the selected *Croton* species, it also emphasizes the significance of evaluating and validating the safety of traditional medicinal plants. These plants are used by people for long periods of time and may be the reason for development of certain conditions in the long run. Knowing toxicity and mutagenic potential plays a crucial role in determining the dosages at which the plants are safe and therapeutic to humans. Many of the *Croton* species in this study have little to no mutagenesis and toxicity reports. It is therefore imperative that more safety evaluations be done on this genus. The genotoxicity report of this study is based on one test method (Ames test). The Ames test in a study by **Elgorashi et al**. (2002) showed no mutagenic activity in C. *sylvaticus* extracts contrary to the reports of the micronucleus test and comet assay tests which found C. *sylvaticus* to possess mutagenic activity in the same study. This may be due to the complexity of the mutation mechanisms and may require technologically advanced tools to be able to obtain accurate results (**Debnath et al.**, 1991, **Eldeen and Van Staden**, 2007).

CHAPTER 5

DNA barcoding and molecular phylogeny

5.1. Introduction

DNA barcoding is a modern technique used to establish the identity of organisms through short, standardized DNA sequences of 400-800 bp (base pairs). The application of the DNA barcoding technique requires each sequence to be sufficiently variable to distinguish individual species, however not too variable within the same species such that a consistent boundary can be established between intra- and inter-specific diversities (**Savolainen et al.**, 2005, **Ganie et al.**, 2015). This analysis of DNA sequences has been a powerful tool in reconstructing the phylogenies of many lower and higher plants (**Wink**, 2003b). It is, however, not without its challenges. The greatest challenge with DNA barcoding of plants occurs when trying to differentiate between taxa of extremely speciose genera, where species recognition rates are rarely greater than 70% when using a range of reputed barcodes. In some instances, DNA barcoding groups of diverse newly evolved species can be inadequate as an identification method (**Kress et al.**, 2009, **Spooner**, 2009). The severest cases of this challenge are observed in situations where certain aspects of life history have influenced the rates of molecular evolution in a population, which may in turn affect species assignment rates through DNA barcodes; this includes generation times and age of crown group diversification (**Kress et al.**, 2009).

Nonetheless, DNA barcoding remains the best and most powerful tool for identifying and authenticating plants and animals. This is due to DNA being an exceedingly stable micromolecule that is seldom affected by external factors, hence can be extracted from

biological material of varying states such as fresh, dried, and processed. Furthermore, the markers are not tissue-specific and can be identified at any point in the organism's growth. Only a small volume of sample is required for analysis. (**Heubl**, 2010, **Tripathi et al.**, 2011, **Techen et al.**, 2014). DNA barcoding is often used by plant taxonomists or systematists and is also a valuable tool for forensics experts, customs agents, and traditional drug suppliers (**Chen et al.**, 2010). DNA barcoding is a suitable support method of taxonomic identification as it may assist in instances where no taxon or biodiversity experts are available. It may also help in cases where there is a high level of morphological similarity between closely related species such that it is almost impossible to distinguish them without their reproductive organs such as flowers and fruits and these are not always presented during field surveys (**Parmentier et al.**, 2013).

5.2. Phylogeny and secondary metabolites

Researchers have studied the connection between plant species evolution and their secondary metabolites for almost 200 years. Because of their nature and function as natural chemical defences of plants, it is believed that they are characters that underwent natural selection during evolution (**Wink**, 2003b). The variation and distribution of secondary metabolites among plant species is part of the evolution of plants. Different plants developed various chemical compounds as a result of herbivores and herbivorous insects that target certain plants (**Nyman and Julkunen-Tiitto**, 2005, **Rønsted et al.**, 2012). Many researchers have used chemical similarities to deduce relationship between plant species (**Wink**, 2003b, **Nyman and Julkunen-Tiitto**, 2005, **Maldonado et al.**, 2017). However, there have been concerns about the reliability of the chemical data in phylogenetic inference due to the sporadic occurrence of specific compounds in totally unrelated plant species (**Nyman and**

Julkunen-Tiitto, 2005). In a study conducted by **Maldonado et al.** (2017), genetic variation was the leading cause of alkaloid content variation within *Cinchona calisaya*. DNA based phylogenies have proven to be the best classification tools and could be diversified for use in ethnobotany by improving medicinal plant identification and bio-prospecting through further investigation of the correlation of medicinal plant phytochemistry or secondary metabolites and their DNA based phylogenies (**Rønsted et al.**, 2008).

5.3 Phylogenetic study of th *Croton* species of KwaZulu-Natal

Croton is one of the largest genera of flowering plants, consisting of herbs, shrubs, trees, and rarely lianas (**Berry et al.**, 2005). It belongs to the family Euphorbiaceae, known for its species richness, morphological and phytochemical diversity and commercial importance (**Wurdack et al.**, 2005). The high diversity and its global distribution have proven to be a challenge for the subgeneric classification of *Croton* (**Webster**, 1993, **Webster et al.**, 1996). Diagnostic morphological characters for *Croton* include conspicuous scalelike or starlike trichomes, narrow or condensed inflorescence with unisexual flowers, clear to coloured latex, frequent petiolar glands, and senescent leaves that turn orange before abscising (**Berry et al**. 2005).

The current study aimed to determine barcodes for and the phylogenetic affinities of all *Croton* species naturally occurring in KwaZulu-Natal. DNA barcodes of properly vouchered and locally sourced specimens were produced in order to allow future characterisation of material that cannot easily be identified morphologically such as bark, leaves or roots. The phylogenetic relationships were compared with their pharmacological and phytochemical properties. They were used to determine whether closely related *Croton* species contain similar phytochemical compounds and whether they occur in similar quantities. Previous

researchers have suggested that the lineage distribution of plants is shaped by bioactivity. Furthermore, studies have demonstrated the presence of similar medicinal and chemical properties (**Saslis-Lagoudakis et al.**, 2012). This has led to the use of plant phylogenies in bio-prospecting, with the expectation that plants that occur in the same clade in a phylogeny would have similar medicinal properties (**Hawkins and Teixidor Toneu**, 2017).

In the current study, two regions were sequenced and used in a phylogenetic analysis of *Croton* as described by **van Ee et al.** (2015), the nuclear ribosomal Internal Transcribed Spacer (ITS) and the chloroplast *trn*L-*trn*F intergenic spacer (*trn*L-F). These markers have been previously used in several *Croton* analyses and have proven suitable for phylogenetic study at species level (**Berry et al.**, 2005, **van Ee and Berry**, 2009, **van Ee et al.**, 2015). The nuclear and chloroplast regions are often used to study the evolutional relationship between related species. The current study further aimed to determine the phylogenetic relationship of the KZN *Croton* genus and extend on the work done by **Haber et al**. (2017), who focused on the African and Western Indian Ocean (WIO) species. Phylogenetic placement of the KZN species will allow us to assess whether the phytochemical and secondary metabolite profile of *Croton* species is clade-specific.

5.4. Material and methods

5.4.1. DNA extraction, amplification, and sequencing

DNA of six representative *Croton* species was extracted from silica-dried leaf tissue using the Zymo Research Quick-DNA^{TM} Plant/Seed Miniprep Kit, following the manufacturer's protocol. The quality and concentration of the extracted DNA was examined with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA). The ITS and *trn*L-F regions were amplified by the Polymerase chain reaction (PCR)

using a Veriti 96-Well Thermal Cycler (Applied Biosystems, Life Technologies, Carlsbad, California, USA).

5.4.2. Amplification of the nuclear ribosomal internal spacer (ITS) marker

The ITS region was amplified using the primer pair ITS4 and ITS5 (**White et al.**, 1990) to sequence in both directions. The PCR reaction contained: 12.5 µl of the One Taq Quick-load 2X Master mix (New England BiolLabs$_{Inc.}$), 0.5 µM forward primer, 0.5 µM reverse primer, 10.5 µl nuclease-free water, and 1 µl of DNA, making a total of 25 µl. The PCR thermo-cycling conditions were: 30 seconds initial denaturation at 94°C; 35 cycles of 30 seconds denaturation at 94°C; 1-min. annealing at 53°C and 1 min. of elongation at 68°C; followed by 5 min. of final elongation at 68°C.

5.4.3. Amplification of plastid marker *trnL-F*

The plastid marker *trn*L-F was amplified using the e and f primer pair (**Taberlet et al.**, 1991) sequencing from both directions. The PCR reaction was as above (**5.4.2**) except that the primers e and f were used here. The PCR thermo-cycling conditions were: 30 seconds initial denaturation at 94°C; 35 cycles of 30 seconds denaturation at 94°C; 1-min. annealing at 55°C and 1 min. of elongation at 68°C.; followed by 5 min. of final elongation at 68°C.

5.4.4 Gel electrophoresis

PCR products (1µL) were visualised by electrophoresis with 1% agarose gels run in 1X TBE buffer at 100V for 45 min. DNA was visualised by adding Gel Red nucleic acid gel stain (Biotium Inc., Fremont, California, USA) to the PCR sample.

5.4.5 Sequencing

The PCR products and the respective primers (1.1µM) used for amplification were sent to the DNA Sequencer Unit at the Stellenbosch University Central Analytical Facility (CAF) for sequencing.

5.5. Phylogenetic analyses

Upon receiving sequence chromatograms from CAF, they were assembled and edited in Sequencher v4.1.4 (Gene Codes Corp., Ann Arbor, Michigan, USA). The sequences were then inserted into the alignment from **Haber et al.** (2017) provided to for this study van Ee (personal communication). The ClustalW Multiple alignment function in BioEdit v.7.2.5 was used to align the sequences followed by manual alignment where necessary (**Hall**, 1999). Only the ITS and *trn*LF parts of the **Haber et al.** (2017) alignment were used.

Phylogenetic relationships were inferred using Bayesian Inference performed under best model parameters using MRBAYES on XSEDE (v3.2.1a) available in the CIPRES Science Gateway V. 3.3 (**Miller et al.**, 2010). The analysis was performed using two independent parallel runs, consisted of 10 million MCMC generations, and was sampled for every 1000 generations. Convergence between runs was assessed by checking the effective sample size (ESS > 200) of each parameter and the log-likelihood (LnL) values, using Tracer v1.7 (**Rambaut et al.**, 2018). The first 25% of generations were discarded from the burn-in, and majority-rule consensus trees with posterior probability (PP) were generated. Clades with PP ≥ 95% are strongly supported (**Huelsenbeck and Ronquist**, 2001).

Additionally, a maximum likelihood (ML) analysis was conducted using RAxML (**Stamatakis**, 2014) in the CIPRES Science Gateway, using the GTRCAT substitution model and 1000 bootstrap replicates for statistical support. The ML and Bayesian trees were then

visualized and edited using FigTree v 1.4.2. (**Rambaut**, 2012) Clades with bootstrap support (BS) ≥ 70% were considered strongly supported (**Hillis and Bull**, 1993).

5.6. Results and discussion

Six ITS and six *trn*L-F sequences from six different *Croton* species occurring in KZN were newly generated and incorporated into the ITS and *trn*L-F matrix of **Haber et al**. (2017) bringing the total number of terminals to 129 *Croton* taxa and two outgroups. The aligned and partitioned dataset contained 1793 base pairs (bp), in which the ITS sequences contributed 704 bp and the *trn*L-F sequences contributed 1089 bp.

Figure 5.1 shows the majority rule consensus Bayesian inference (BI) tree. **Figure 5.2** presents the maximum likelihood tree. The majority rule Bayesian inference tree of *Croton* from the study of Haber et al. (2017) is presented in **Figure 5.3**. It should be noted that the latter shows more resolution as it is based of four DNA regions.

The phylogenetic tree revealed that sequences generated for *C. sylvaticus*, *C. gratissimus*, and *C. menyhartii* from KwaZulu-Natal match the sequences generated for the same species from a different geographic region in the study of **Haber et al.** (2017). However, this is not the case for *C. pseudopulchellus* from KZN, which is not sister to *C. pseudopulchellus* from **Haber et al.** (2017). When comparing the neighbouring species of the two *C. pseudopulchellus* specimens in **Figure 5.1** and **Figure 5.3**, the two specimens were found to be relatively closely related; however, they are clearly different. Several explanations are possible. One possibility is that the Haber specimens may have been misidentified. The identity of the specimen used in this study was confirmed by G. Nichols and B. Bytebier and was confirmed to be *C. pseudopuclhellus*. The specimen in **Haber et al.** (2017) came from Tanzania and an alternative explanation could perhaps be that the genetic variation in *C.*

pseudopulchellus may have been underestimated, such that cryptic species may be hidden in the current concept of the *C. pseudopulchellus*. Nevertheless, the difference in the barcode sequences for two specimens supposedly belonging to the same species highlights the need for having regionally acquired material as the source of DNA sequences if misidentification on the basis of the barcode is to be avoided.

C. rivularis and *C. steenkampianus* appear to be sister species. These two species were not similar in their antimicrobial activity. *C. steenkampianus* showed some notable activity whereas *C. rivularis* showed no activity (**Table 2.2**). On the other hand, *C. rivularis* showed noteworthy antioxidant activity, whereas *C. steenkampianus* only moderate antioxidant activity (**Table 3.2).** Phytochemicals contained in these species were similar for some compounds and different for others. For example, they contained similar amounts of condensed tannins and phenolics in their leaves and stem barks but different quantities of flavonoids (**Figures 3.4-3.6**). There have been some similarities observed in the phytochemistry of these two sister species. However, the dissimilarities that occur suggest that although some species are closely related, they may not always contain the same phytochemical quantity and quality.

Because our phylogenetic analysis was only based on two gene regions (instead of four in (**Haber et al.**, 2017)), our tree is less resolved. As a result, all the species from KZN, South Africa, appeared in the same clade (**Clade V, Figure 5.1**) apart for *C. sylvaticus*, which appeared in a sister clade (**Clade IV, Figure 5.1**). It was further observed that even though these *Croton* species are in the same clade, they may not be closely related, particularly not if we consider where they would be placed in the better resolved phylogeny of **Haber et al.** (2017). Therefore, they may not be expected to have the same phytochemical properties. Pharmacological and phytochemical investigations conducted on *Croton* species from KZN, South Africa, in **Chapters 2** and **3** revealed that all the species contained the same

phytochemical properties. These include phenolics, flavonoids, condensed, and tannins (**Chapter 3**). However, they varied in their quantity and distribution across the various plant parts. These species also demonstrated antioxidant activity.

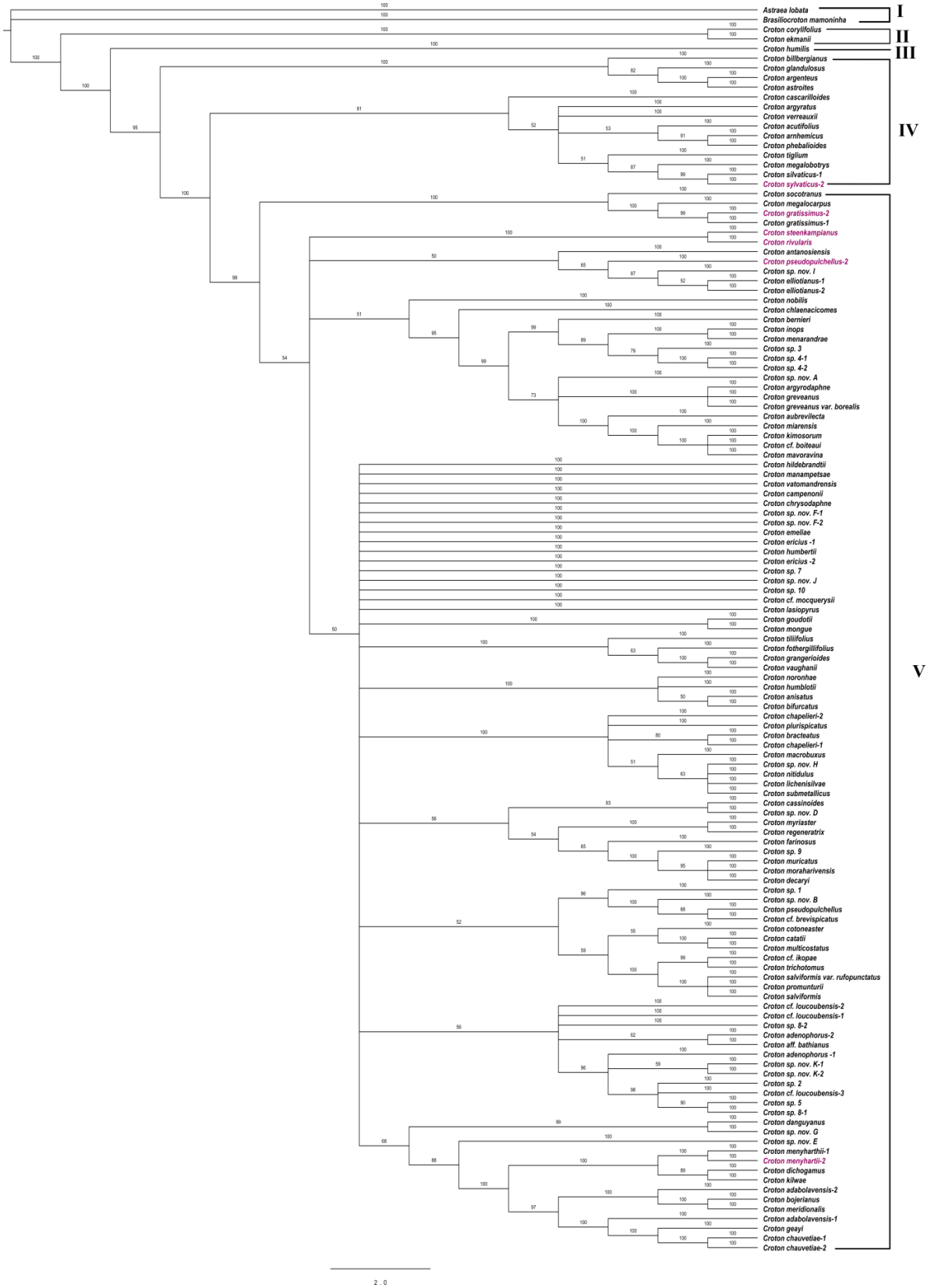


Figure 5.1: Majority rule consensus tree from Bayesian MCMC inference from combined ITS and *trn*L-F sequences of *Croton* species and two outgroups. Values above the branches represent posterior probability (PP), strongly supported branches (PP ≥0.95). I-V=clades.

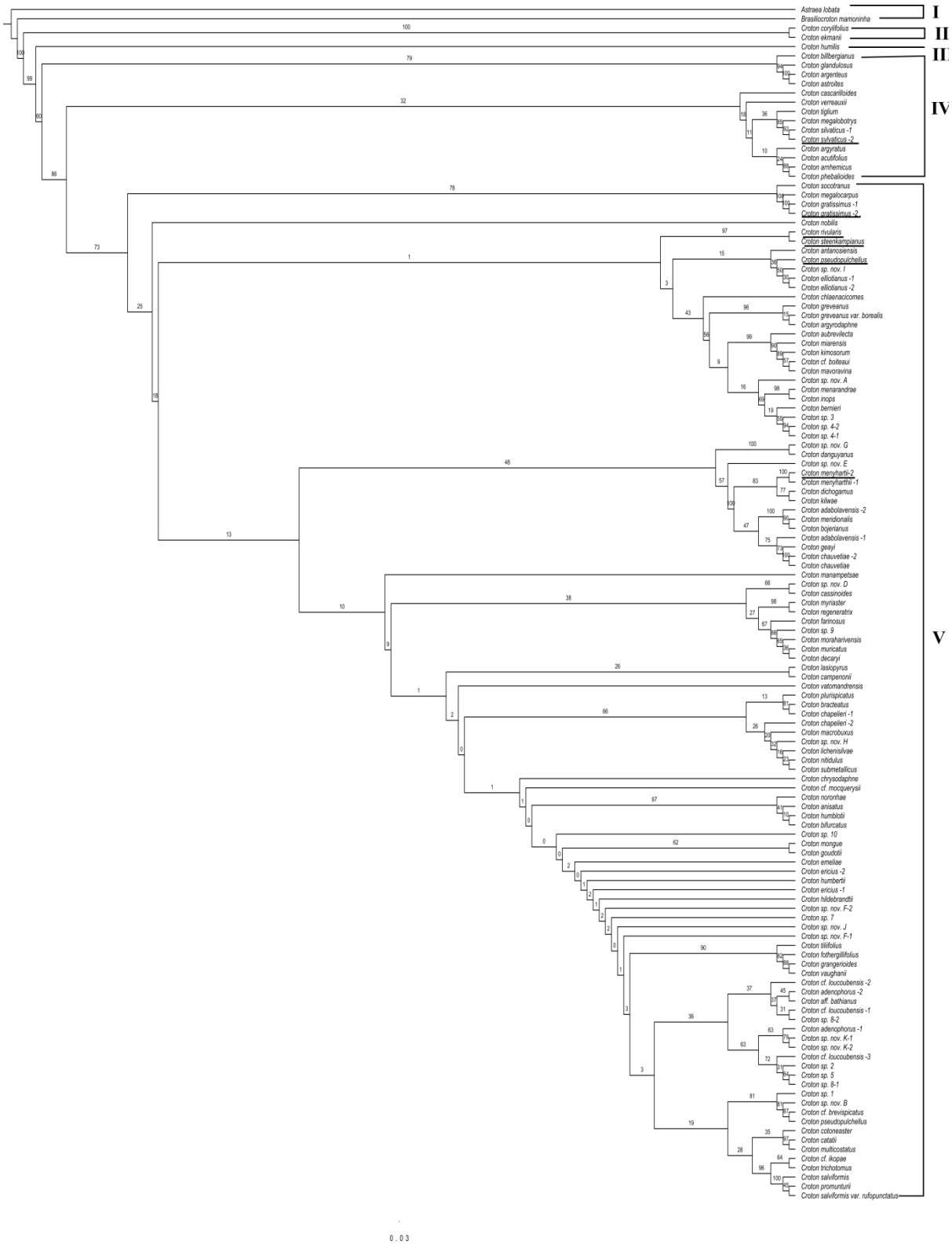


Figure 5.2: Maximum Likelihood phylogenetic tree *Croton* species constructed with RAxML, with DNA markers ITS and *trn*L-F. The numbers above the branches represent bootstrap support percentages. On the right of the tree is the distribution of the main clades. I-V=clades.

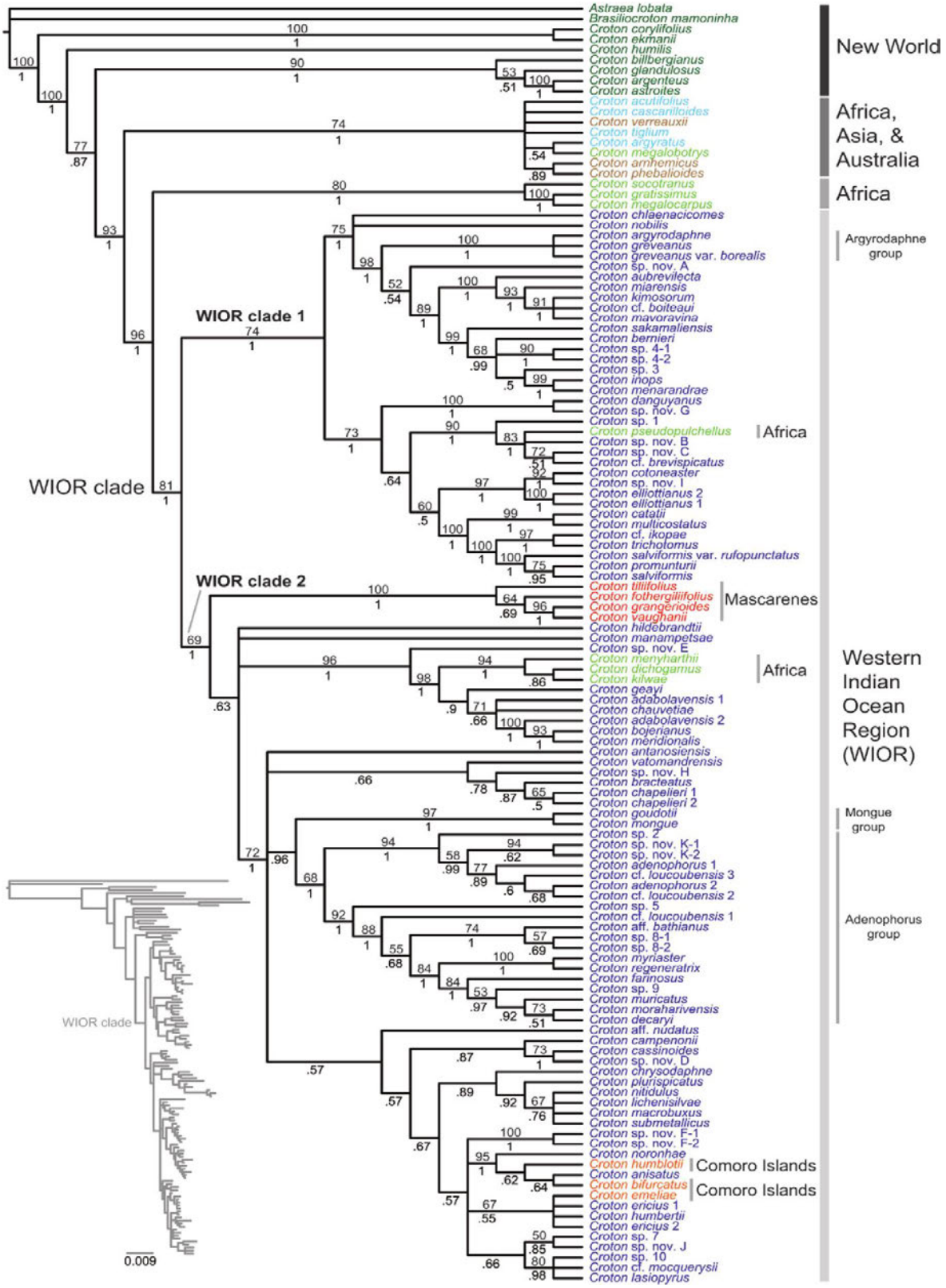


Figure 5.3: Majority rule consensus tree from the Bayesian MCMC inference for *Croton* WIO spp. of the combined ITS and plastid results. Above the branches, bootstrap values from ML analyses are indicated, and corresponding posterior probabilities below the branches are provided (**Haber et al.**, 2017).

5.7. Conclusions

Studies of this nature are conducted for various reasons, such as determining plant identification, plant authentication, and plant relationship determination. The current research has shown that DNA barcodes may not always be the best tool to identify medicinal plants with potentially valuable phytochemicals. The phylogenetic tree revealed that the *Croton* species from KZN are not closely related except for *C. steenkampianus* and *C. rivularis*. Though results in **Chapter 3** revealed that the different *Croton* species contained the same phytochemicals, there was high variation in the levels. Phytochemical quantities and quality may vary due to different environmental stimuli that trigger specific phytochemical production. There was also variation in the phytochemical distribution in the different plant organs. A close phylogenetic relationship between species does not signify the same phytochemistry but a similarity. This means more assays need to be conducted to confirm phytochemical properties.

Several other *Croton* species closely related to the *Croton* species from KZN have been used for medicinal purposes in their areas. Many of the uses listed for these species are similar to those of the *Croton* species from KZN. These species include *Croton megalocarpus* Hutch.- which is used as an anthelmintic agent and to treat whooping cough (**Addae-Mensah et al.**, 1992), coryza sinusitis, and pneumonia (**Matu and Van Staden**, 2003, **Schmelzer and Gurib-Fakim**, 2008). *Croton socotranus* Balf.f. is used to heal wounds and is used as an anthelmintic agent (**Bakthir et al.**, 2011, **Mothana et al.**, 2012). *Croton tiglium* L. is used as a purgative, is used to treat fever, sore throat, dysentery, and cancerous tumors (**Schmelzer and Gurib-Fakim**, 2008). The evidence gathered in this study suggests that *Croton* species may sometimes be used as substitutes of one another for therapeutic use. However, efficacy might not be the same.

DNA barcodes for KZN *Croton* species have been developed to be deposited in the Genbank for future use as references. It is vital to have regional representative DNA for widespread species. This was seen in the DNA results for *C. pseudopulchellus,* which revealed that the same species in different geographic regions might not be genetically identical. This may require further investigation to determine whether these are indeed the same species. DNA barcodes are useful in medicinal plant authentication.

CHAPTER 6

General conclusions

Many researchers have studied medicinal plants, their use, and their contribution to the livelihoods of people all over the world (**Shinwari and Qaiser**, 2011). However, concerns about the loss of medicinal plants in their natural habitat remain. Studies focused on the conservation and sustainable use of these valuable renewable resources and their habitat have fallen behind increasing demand for medicinal plant research (**Hamilton**, 2004). To ensure a continuous supply of herbs from nature, as well as maintaining medicinal plant species diversity,ethnobotanical studies should integrate a conservation and sustainability approach.

This study aimed at finding possible solutions for plant exploitation studying and comparing phytochemical and pharmacological properties of six *Croton* species occurring in KwaZulu-Natal; as well as testing and comparing the phytochemical and pharmacological activities of various plants organs of these six species. This was done to determine whether the aerial organs such as leaves and twigs could be used instead of organs such as the bark which involves destructive harvesting. Additionally the study compared the phytochemical and pharmacological properties of the different *Croton* species to determine whether they could be substituted for one another, thus trying to reduce destructive sampling of any given species. Phytochemicals such as condensed tannins, phenolics, flavonoids, and alkaloids were tested for and the former three were quantified and compared to find out whether they occur in similar amounts in closely related *Croton* species. To some extent, the current research findings validated the use of *Croton* species in traditional medicine as almost all of the *Croton* species tested, apart from *C. rivularis*, showed noteworthy inhibitory activity against

various test microbes (*S. aureus, E. faecalis,* and *K. pneumonia*). The results also showed that a more sustainable use of the species is possible as most of the leaves and twigs of the various *Croton* species showed higher inhibitory activity than the stem bark, which is the most commonly used part of the plant in traditional medicine. Only one species showed similar or higher inhibitory activity from its stem bark than that exhibited by leaves and twigs. These noteworthy antibacterial activities displayed by the stem bark were from the DCM and PE extracts of *C. pseudopulchellus* collected from the Durban Botanic Gardens. Their MIC was 0.08 and 0.04 mg/ml, respectively, against *S. aureus* and *E. faecalis*. Only one extract showed noteworthy inhibitory activity against the fungus *C. albicans*. This was from the MeOH leaf extracts of *C. steenkampianus* collected at Kosi Bay with a MIC of 0.6 mg/ml.

The study further revealed variations in the pharmacological and phytochemical properties of the same *Croton* species collected from different localities. This was most likely attributed to the variation in the environmental stimuli experienced by species in different regions.

Phytochemical studies revealed that the *Croton* species under investigation contained varying concentrations of secondary metabolites. These included condensed tannins, flavonoids, phenolics and alkaloids. Here, a similar pattern as in the antimicrobial assay was observed, where leaves and twigs displayed higher compound content than stem bark. Variation in the phytochemical profiles of the same species collected from different regions was also observed. This suggests that the phytochemicals present in the plant extracts vary according to the habitat in which they grow.

The two antioxidant activity assays, β-carotene-Linoleic Acid Model System and DPPH radical scavenging assay tested on the *Croton* species demonstrated the presence of antioxidant activity for all these species (**Table 3.2**). Some of the antioxidant activity was

noteworthy, for other species moderate and few were low, with ANT% ranging between 47.49 to 81.97 %**.**

Croton species have proven worthy of their popularity and are commonly used by traditional healers. However, efficacy alone does not make a good medicinal plant. Further investigations were performed to determine the safety of these medicinal plants. The plant extracts that showed noteworthy biological activities were further tested for mutagenic activity. The results of the Ames mutagenic test conducted on the *Croton* extracts demonstrated no evidence of mutagenic behaviour on two *Salmonella* test strains (TA98 and TA102). This shows the plants to be safe but only in terms of mutagenic potential. More toxicity tests need to be conducted as there are various ways in which a plant may be harmful. This is more especially with previous reports stating *Croton* species to be toxic (**Selowa et al.**, 2010).

Many researchers believe that related species ought to have similar chemical properties. Against this expectation, a phylogenetic tree was constructed with sequences of the *Croton* species from KwaZulu-Natal and the sequence of *Croton* species from a study by **Haber et al.** (2017). The tree was studied to determine how closely related the KZN *Croton* species are and whether they possessed the same chemical properties. The results revealed that even though the species were related, they did have similar chemical properties; however, they were not the same quantities. Thus the species did not exhibit the same pharmacological activity.

Overall findings of the study showed that *Croton* species are possible candidates for medicines; however, further research is required to fully understand the potential of this genus. It is important to consider that traditional healers use a combination of various medicinal plants to maximise the effectiveness of their therapeutic medicines, and this is

where plant extracts perform more effectively. Future studies of medicinal plants should include research into synergy of plant extract.

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