IMPROVEMENT OF THE QUALITY AND SHELF LIFE OF TRADITIONALLY PRODUCED SORGHUM JUICE BY ADDITION OF ASHES, DRIED POWDERED LEAVES AND STEM OBTAINED FROM *COMBRETUM* SPP.

BY

MORONGWA MARY MATHIPA

[redacted]

A RESEARCH THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MICROBIOLOGY, IN THE DEPARTMENT OF BIOCHEMISTRY, MICROBIOLOGY AND BIOTECHNOLOGY, SCHOOL OF MOLECULAR AND LIFE SCIENCES, FACULTY OF SCIENCE AND AGRICULTURE, UNIVERSITY OF LIMPOPO, SOUTH AFRICA

SUPERVISOR: PROF. P. MASOKO

CO-SUPERVISOR: PROF. M.S. MPHOSI

2022

DECLARATION

I declare that the thesis titled: **IMPROVEMENT OF THE QUALITY AND SHELF LIFE OF TRADITIONALLY PRODUCED SORGHUM JUICE BY ADDITION OF ASHES, DRIED POWDERED LEAVES AND STEM OBTAINED FROM *COMBRETUM* SPP** hereby submitted to the University of Limpopo for the degree Doctor of Philosophy (PhD) (Microbiology) has not previously been submitted by me for a degree at this or any other University. I declare that it is my own work in design and in execution, and that all the material contained therein has been duly acknowledged.

_____

Morongwa M Mathipa,

_____ Day of _____ 2022

DEDICATION

I would like to dedicate this work to the most valuable women in my life: my mother (Welheminah Mathipa), my sisters (Nthabi and Linky Mathipa), my grandmothers (the late Lina Mmabjala Mathipa) and Maria Mothiba, and to all my aunts. Lastly, I dedicate this work to my beloved daughter, Kgethego Hope Mathipa. Thank you for your inspiration and support, ladies!

ACKNOWLEDGEMENTS

I would like to acknowledge the support, guidance and encouragement of my supervisor, **Prof. Peter Masoko**. Thank you for your positive comments and persistent encouragement. I am extremely grateful for your patience and constant checking in.

I also acknowledge sincerely **Prof. M.S. Mphosi**, my co-supervisor, for his constructive criticisms

I extend my special thanks to **Mr. M Matotoka** and **Mr. I. Ntlhamu**, for helping with plant collection.

I thank **Prof. Z. Mbita and Ms. L. Makgoo** for their assistance with the cytotoxicity assays. Thank you for your willingness to assist and your sacrifices.

My profound gratitude goes to **Mr. H. Bango and Ms Maluleke**, for helping me with mineral analysis.

My sincere thanks also goes to **Ms Z Ngubane**, for helping me with proximate analysis.

My profound gratitude goes to **Ms Anna Selane** and the **Matsheremare Group** for your participation in the sensory evaluation.

My gratitude goes to **Miss R Mofokeng** and **Miss P Rabekane** for helping me facilitate sensory evaluation.

I also appreciate the love and care my friends provided throughout this study. Thank you to every one of you. (**Sharon, Lerato, Valencia, Thembani** and **Mahlatse**). Your prayers and encouragements kept me going. Thank you.

A special thanks to **Prof. N.C. Hintsho-Mbita,** thank you for being my 'off the books' supervisor. Your encouragement and keen interest led to the satisfactory realisation of this study. I appreciate your contributions.

My gratitude goes to **Dr. Hlulani Archiebold Hlungwani**, Buddy, I appreciate the help with the Statistical analysis.

To everyone who helped, in one way or another during this work but have not been mentioned here, I sincerely extend my thanks.

This study has been made possible by the financial support of **Foodbev SETA** and the **University of Limpopo**.

I am thankful to all my family members, especially my parents **Petrus Malesela Mathipa** (my late father) for instilling the love of education in me and for always believing in me. To my mom, **Welheminah Mahlodi Mathipa** who has been there for me throughout my student life and for her never-ending motivation, encouragement, unreserved support and love in realising my dreams, I am grateful. To my sisters, **Nthabi** and **Linky Mathipa**, the sky is the limit, I appreciate you. To my daughter, **Kgethego Hope Mathipa**, I love you, thank you for your patience!

Lastly, all thanks to **Almighty God** for seeing me through. Had it not been for the **Lord** on my side, I would definitely have been swallowed alive. I am grateful for his grace, love and strength throughout this study (**Psalm, 27 and Psalm 124**).

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ATCC	American type culture collection
AOAC	Association of Official Analytical Chemists
CFU	Colony forming Units
dH2O	Distilled water
EOs	Essential oils
DMSO	Dimethyl sulphoxide
DPPH	2, 2, diphenyl-1-picrylhydrazyl
INT	Iodonitro-tetrazolium salts
MIC	Minimum inhibitory concentration
CLSI	Clinical and Laboratory Standards Institute
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye
XTT	2,3-bis{2-methoxy-4-nitro-5-[(sulfenylamino)carbonyl]-2H-tetrazolium hydroxide}
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
FDA	Food and Drug Administration
rpm	revolutions per minute
TLC	Thin layer chromatography
WHO	World Health Organization
ISO	International Organization for Standardization
UV	Ultra violet
SE	Staphylococcal enterotoxin

RDA	Recommended dietary allowance
MBC	Minimum Bactericidal Concentration
H_2SO_4	Sulphuric acid
ROS	Reactive oxygen stress
GAE/mg.	Gallic acid equivalents per milligrams
Ca	Calcium
Co	Cobalt
Cu	Copper
Fe	Iron
K	Potassium
Mg	Magnesium
Ni	Nickel
Zn	Zinc
Na	Sodium
Mn	Manganese
Cd	Cadmium
As	Arsenic
Pb	Lead
KJ/g	Kilojoulesper gram

ABSTRACT

Traditional sorghum juice is produced in many African countries for human consumption. The juice is very rich in calories, B-group vitamins including thiamine, folic acid, riboflavin, nicotinic acid, and essential amino acids such as lysine. Low earning income women at village level produce sorghum juice for home consumption and sale. The short shelf life (2 to 3 days) of sorghum juice is a major problem for both the brewers and consumers of this drink. The aim of the study was to use 12 *Combretum* plants to improve the microbiological quality and shelf life of sorghum juice.

Fresh stems and leaves of *C. caffrum, C. vendae, C. erythrophyllum, C. elaegnoides, C. apiculatum, C. imberbe, C. adenogdium, C. padoides, C. bracteosum, C. kraussii, C. mkuzens*e and *C. zeyherii* were collected at Nelspruit, National Botanical Gardens, Mpumalanga, South Africa. Voucher specimens and tree labels were used to verify the identity of the plants. The stems and bark collected were cut into pieces and air-dried for 30 days. When dried, the plant material was ground to a fine powder and stored in paper bags at room temperature. The wood was burnt in an open fire; fuel was not used to minimise contamination.

The qualitative phytochemical composition of both the leaves and stems of *Combretum* plants analysed in this study revealed the presence of saponins, tannins, terpenoids, steroids, cardiac glycosides and flavonoids. The following phytoconstituents were lost in the ashes; tannins with the exception of *C. mkuzense* and *C. padoides;* cardiac glycosides and flavonoids. The quantitative phytochemical analyses revealed that both the leaves, stems and some ashes such as *C. apiculatum* and *C. vendae* contained appreciable levels of phenolic compounds, tannins and flavonoids.

Quantitative analysis of antioxidant activity, the 2, 2, diphenyl-1-picrylhydrazyl (DPPH) assay was used as a screen test for the radical scavenging ability of the compounds present in the different 36 70% acetone extracts. DPPH screening method indicated great scavenging activity with the 70% acetone leaf extracts of *C. kraussii*, *C. zeyherii* and *C. mkuzense*. The leaf and stem extracts showed substantial great antioxidant activity in a concentration-dependent manner. There was a significant decrease in the

antioxidant activity in the ashes (p=001), when compared to both the leaves and the stems.

The proximate and nutritional analysis of the 70% acetone extracts were performed by AOAC and ICPE protocols, respectively. The results indicated that all the extracts had substantial amounts of ash, moisture, protein and energy. Mineral content of the plant parts was analysed as well, calcium had the highest concentration, while zinc was lowest in concentration. The mineral content decreased significantly in the stems ($p \leq 0.05$) when compared to the leaves. There was a further decrease in mineral content with regard to the ashes with the exception of calcium. Based on these findings, the leaves and ashes of *C. adenogonium* and *C. apiculatum* could provide a good source of calcium in the diet, while *C. adenogonium, C. bracteosum and C. apiculatum* had high levels of sodium.

A serial micro-dilution assay was used to determine the minimum inhibitory concentration (MIC) values for 70% plant extracts using tetrazolium violet reduction as an indicator of growth. Two Gram-positive (*Stapylococcus aureus* ATCC 29213 and *Enterobacter faecalis* ATCC 29212) and two Gram-negative (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) bacterial strains were used in this study. The leaves had good antibacterial properties with the lowest MIC value being 0.04 mg/ml against *E. coli* and *S. aureus. E. faecalis* was found to be resistant against all the leaves with the exception of *C. imberbe.* The stem extracts of *Combretum* spp. tested in the study showed antimicrobial properties with the lowest MIC value being 0.04 mg/ml against *E. coli* shown by *C. bracteosum.* However, *E. faecalis* was resistant against all the 12 plants tested. All the test microorganisms showed resistance to the ashes, with the exception of *S. aureus*, which was found to be susceptible to 75% of the test ash extracts with the lowest MIC value of 0.16 mg/ml.

Cytotoxicity and anticancer activity of the acetone extracts of *the 12* Combretum plants *were* evaluated using tetrazolium-based colorimetric assay (MTT assay) on A549 lung carcinoma cells. The assays revealed that 50% of the leaf extracts of tested plants showed cytotoxicity and cell proliferation inhibition in A549 lung carcinoma cells in a concentration-dependent manner. The A549 cells were more sensitive to the following plants: *C. elaegnoides, C. erythrophyllum C. imberbe, C. kraussii* and *C. mkuzense.* The following stems extract, *C. adenogdium and C. caffrum* did not have any

anticancer activity, whereas *C. apiculatum* and *C. bracteosum* were only able to reduce cell viability to less than 60%. *C. mkuzense, C. padoides, C. vendae* and *C. zeyherii* acted in a concentration-depended manner with the greatest activity seen at the highest concentration (1000 µg/ml). The plants had activity at concentrations between 31.25 and 1000 µg/ml allowing only 20% and 50%, respectively, of the cells to remain viable. Ashes from *C. mkuzense* showed good anti-cancer activity at the highest concentration (1000 µg/ml) reducing cell viability to around 10%.

Enterobacteriaceae, total coliform, *S. aureus*, *B. cereus, E. coli* and lactic acid bacteria viability were studied during the four weeks storage period of prepared sorghum juices. Juice samples were collected after preparation; the samples were serially diluted using peptone water. Tempo instrument (Biomereiux) was used to enumerate total coliform, total aerobic count, *E. coli, S. aureus*, lactic acid bacteria, enteric bacteria, yeast and mould using the most probable number following the manufacturers instruction. *C. mkuzense* and *C. padoides* plants were able to inhibit the growth of *B. cereus*, lactic acid bacteria and *S. aureus* during the first three weeks of storage. *E. coli* was not present throughout the four weeks storage time. Vitek 2 Compact (Biomereiux) was used for the characterisation and identification of the dominant bacterial isolates using biochemical reactions. The isolates were characterised by morphological differences. Sixty five percent of the isolates were the Enterobacter genus that are commonly found in soil, water, and sewage.

The nutritional composition and sensory properties of the prepared sorghum juices treated with *Combretum* plants were investigated. All the juices had appreciable amounts of protein, ash and energy. Sorghum juice treated with ashes of *C. caffrum, C. erythrophyllum* and *C. kraussii* had the highest levels of proteins when compared with other treated sorghum juices. The sorghum juices prepared in the study had varying levels of trace element or minerals with potassium -(3, 55 – 104 mg/l) and calcium (3.2-148 mg/l). Similarly, cobalt (-1.22), coppr (-0.99), iron(-0.962), magnesium (0.004), sodium (-0.145), nickel (-2.7)) and zinc (-1.2)are present in very low amounts. The juices treated with ashes had relatively higher levels of calcium, potassium, magnesium and sodium. Sorghum juices treated with the ashes had better sensory and organoleptic properties when compared with those treated with the leaves. The juices treated with the ashes of *C. caffrum* and *C. bracteseum* were more accepted by most of the panellist when compared with other treated juices.

Overall, this study presents valuable information on the phytochemical composition, nutritional composition and antioxidant properties of some *Combretum* species in South Africa. It recommended its use as food and in pharmaceutical preparations for the local industries. In addition, *Combretum* plants showing the effects tested in this study may be explored further for development into drugs. functional food as food preservatives and nutraceutical applications, beside their traditional use.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 6

CHAPTER 7

CHAPTER 8

LIST OF TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 7

CHAPTER 8

CHAPTER 1: INTRODUCTION

1.1 Introduction

In many African countries, cereals are used to produce indigenous fermented foods, non-alcoholic and alcoholic beverages. These beverages are popular because of the social, religious and therapeutic values associated with them (Aka *et al.,* 2008; Djè *et al.,* 2009; Nwachukwu *et al*., 2010). In most parts of the world, especially children, pregnant women, sick and old people, consume non-alcoholic beverages. These beverages are also used to wean infants. Men, on the other hand, usually prefer alcoholic beverages. These beverages have different names in different countries and regions where they are produced. Their production varies from one region to another; however, they essentially include malting, brewing and fermentation feedstock for millet, maize and mainly sorghum. They are managed by women at household and or small-scale levels and involve one or two steps of fermentation: lactic acid fermentation for non-alcoholic beverages, lactic acid fermentation, and alcoholic fermentation for alcoholic beverages (Maoura *et al*., 2005; Aka *et al.,* 2008; N'Guessan *et al.,* 2012).

Sorghum [(*Sorghum bicolor* (L.) Moench] is an indispensable and a vital staple food for millions of people and plays a crucial role in ensuring food security in developing countries (Drich and Pran 1987). In East Africa, for instance, it is a source of income to families, used as food in the form of thin and thick porridges and used to produce alcoholic and non-alcoholic beverages. Researchers (Board on Science and Technology for International Development, 1996) state that sorghum has not been developed into products and thus lacks markets. In Africa, it remains mostly a crop of small cultivators and is consumed locally where it is grown. Sorghum outperforms other cereals under various environmental conditions and is thus generally more economical to produce (Awika, 2017). Sorghum offers promise as a gluten-free, phytochemical-rich ingredient in functional food (Awika and Rooney, 2004).

Traditional sorghum juice is produced in many African countries for human consumption (Ekundayo, 1969; Ahmed *et al*., 1988; Chavan and Kadam, 1989; Steinkraus, 1996; Odunfa *et al.,* 1996; Usha *et al*., 1996). These juices are very rich

in calories, B-group vitamins, including thiamine, folic acid, riboflavin and nicotinic acid, and essential amino acids such as lysine (Lyumugabe *et al.,* 2010).

Bushera is a sweet and sour traditional non-alcoholic sorghum beverage consumed in the western, southwestern and central Uganda. This beverage is commonly consumed as a refreshing drink and to some extent, is used as a weaning food. In brief, to prepare bushera, sorghum flour is mixed with boiling water and left to cool at an ambient temperature (unpublished information). Germinated sorghum flour is then added and the mixture is left to blend at an ambient temperature for 1–3 days (unpublished information). Low-income women at a village level produce sorghum juice for home consumption and for sale. Both young children and adults (Muyanja et al., 2003), consume the product. The sorghum juice is consumed at various festivals and African ceremonies (e.g. marriage, birth, baptism, the handing over of a dowry, etc.) and is a source of economic return for female producers (Lyumugabe *et al*., 2010).

The short shelf life of sorghum juice is a major problem to both its brewers and consumers (Kutyauripo et al., 2009) Spoilage of sorghum juice is attributed to undesirable changes in sensory characteristics in terms of texture, smell, taste or appearance (Lyumugabe *et al*., 2010), which lead to the disposal of the whole product. Most traditional, African cereal-based juices deteriorate rapidly and become unacceptable to consumers within one to four days of production (Nout, 1980; Okafor, 1990). The deleterious changes are primarily due to the objectionable off-flavour or over-souring induced by continued microbial activities after production. The short shelf-life of sorghum food products is one of the major deterrents to their large-scale production and development as commercial products. Most contamination probably comes from the raw materials that do not go through any rigorous microbiological analysis and treatment before being used.

Plants, including many presently used as spices and culinary herbs, have been used as medicines, from prehistoric times. Spices are partly used to counter food spoilage microorganism, especially in hot climates (Tapsell *et al.,* 2006; Billing and Sherman, 1998), and especially in meat dishes that spoil more readily (Sherman and Hash, 2001). Plants synthesise hundreds of chemical and biochemical compounds for functions, including defence against insects, herbivorous mammals, fungi, and diseases. Numerous phytochemicals with established or potential biological activity

have been identified in plants. Similarly, a single plant has widely diverse phytochemicals and the effect of using a whole plant as medicine is not certain. Further, the pharmacological actions and phytochemical contents, if any, of several plants with medicinal potential remain untapped and unassessed to categorically define their safety and efficacy (Ahn, 2017).

Medicinal plants are used widely in non-industrialised societies and developing countries in Africa, Asia, and Southern America, mainly because they are thought to be very effective, cheaper than modern medicines, and readily available. The annual value of the global export of the thousands of plants with suspected medicinal characteristics and properties was projected to be 2.2 billion USD in 2012. In the year 2017, the potential global market for botanical medicines and extracts was projected at several hundred billion dollars (Ahn, 2017). In many nations, there is little or no regulations of traditional medicine practices, but the World health Organization (WHO) coordinates a network to encourage rational and safe use.

Combretum is the largest genus of the *Combretaceae* family with about 370 species (McGaw *et al.,* 2001). Several *Combretum* species have been used for several years in African traditional medical practices and as a condiment in soups (Onocha *et al*., 2005). Many species of *Combretum* have been found to possess powerful antibacterial and antifungal effects (Magwenzi *et al.,* 2014). The large number of antimicrobial compounds found in species of *Combretum* might explain why they are so widely used in African traditional medicine. In addition to their anthelmintic, antioxidant and antimicrobial properties, the plants are also used for the treatment of haemorrhoids, tuberculosis, toothache and male sterility (Burkill, 1985; Oliver-Bever, 1986). *Combretum* plants also possess nutritional components for energy metabolism and vital nutrients to maintain a state of optimal nutrition (Ujowundu *et al.,* 2015). The leaves of many *Combretum* plants such as *Combretum micranthum* are harvested from wild growing populations and used as popular traditional herbal tea in several tropical West African savannah countries (D'Agostino *et al.,* 1990). A study by Masoko *et al.* (2007) indicated that most of the *Combretum* species such as *C. moggii, C. petrophilum* and *C. nelsonii* possess substantial antifungal properties.

The use of wood ash extracts as a food additive and for medicinal purposes have been a continuous practice among Gbagyi people and other ethnic groups in the Middle-

Belt Region of Nigeria and Uganda, respectively. Wood ash is the inorganic and organic residue acquired after the combustion of wood. When wood is burned, the organic portion is converted to CO_2 and water while the inorganic portion remains as ash (Reimann *et al.*, 2008; Alberta Environment, 2002). Wood-ash is used widely across the globe for various purposes that range from washing cooking utensils, soap making, biodiesels, poultry and livestock feed processing (Kyarisiima *et al.,* 2004 and Rahman *et al.,* 2009). It is also used for improving soil fertility (Moyin-Jesu, 2012), food/seeds preservation fermentation processes (Pelig-Ba, 2009), insecticide (Famisa *et al.,* 2009), pest control and seed treatment to increase yield (Mochiah *et al.,* 2011; Moyin-Jesu *et al*., 2010; Moyin-Jesu, 2012).

Aqueous extracts of *Combretum imberbe* wood ash have been found to inhibit the growth of some phytopathogenic and mycotoxigenic fungi such as *Aspergillus flavus, Fusarium oxysporum, Penicillium italicum* and *Penicillium notatum* (Peloewetse *et al.,* 2008). A study by Wong and Selvam (2009) showed a reduction of indicator and pathogenic microorganisms such as *Salmonella,* faecal coliforms, *Escherichia coli* and faecal *Streptococci* in manure treated with fly ash. Since the *Combretum* plants possess vital nutrients and antimicrobial properties, this study aimed to use these characteristics to improve the microbiological quality, taste and shelf life of sorghum juice.

1.2 Aims and Objectives

1.2.1 Aim

The aim of the study was to investigate if ashes, powdered leaves and stems from *Combretum spp* as additives can improve the quality and shelf life of traditionally produced sorghum juice.

1.2.2 Objectives

The objectives of the study were to:

I. Screen for phytochemicals in the dried leaves and stems from 12 selected *Combretum* species.

II. Screen for minerals and heavy metals in the ashes, leaves and stems obtained from *Combretum* spp.
III. Assess the antimicrobial activity of the powdered leaves, stems and ashes of *Combretum* spp.
IV. Assess the cytotoxicity properties and anticancer activity of the powdered leaves, stems and ashes of *Combretum* spp. on lung cancer cell lines.
V. Prepare of the traditional sorghum juice using the powdered leaves and ashes of the *Combretum* spp.
VI. Assess the effect of the powdered leaves and ashes of the *Combretum spp.* on the nutritional properties of the prepared sorghum juices.
VII. Assess the sensory properties of prepared sorghum juices.
VIII. Assess the shelf life of produced juices.

1.3 Hypotheses

The hypotheses of the study were:

I. *Combretum* plants and ashes have the potential to reduce microbial levels in food products and beverages.
II. Addition of *Combretum* powdered leaves, stems and ashes will improve the quality and shelf life of the juice.

REFERENCES

Ahmed, A.R., Rao, A.G., and Ramanathan, G., 1988. Effect of auto fermentation on the physicochemical properties of proteins of sorghum-ground nut composite flour. *Journal of Agriculture and Food Chemistry* 36: 690–694.

Ahn, K., 2017. The worldwide trend of using botanical drugs and strategies for developing global drugs. *BMB Reports* 50 (3): 111–116. doi:10.5483/BMBRep.2017.50.3.221.

Aka, S., Djeni, N.T., N'guessan, K.F., Yao, K.C., and Dje, K.M., 2008a. Variability of physico-chemical properties and enumeration of the fermentary flora of tchapalo, a traditional sorghum beer in Côte d'Ivoire 04(2) : 274-286

Aka et al., 2009???

Alberta Environment. 2002. Standards and guidelines for the use of wood ash as a liming material for agricultural soils. http://www.gov.ab.ca/env/.

Awika, J.M., 2017. Chapter 3 - Sorghum: Its unique nutritional and health-promoting attributes. Woodhead Publishing Series in Food Science, Technology and Nutrition. 21–54.

Awika, J.M., and Rooney, L.W., 2004. Sorghum phytochemicals and their potential impact on human health. *Journal of Phytochemistry* 65: 1199–1221.

Billing, J., and Sherman, P.W., 1998. Antimicrobial functions of spices: why some like it hot. *Quarterly Review of Biology* 73 (1): 3–49. Doi: 10.1086/420058.

Board on Science and Technology for International Development. **1996.**, National Research Council: Lost crops of Africa volume I: Grains. ISBN 0-309-04990-3.

Burkill, H.M., 1985. Royal botanic gardens, Kew, Richmond, Surrey TW9 3AB. England (botany).

Chavan, J.K., and Kadam, S.S., 1989. Nutritional improvement of cereals by fermentation. *Critical Reviews in Food Science and Nutrition* 28: 349–400.

D'Agostino, M., Biagi, V.D., Feo, F., and Zollo, C., 1990. Flavonoids of *Combretum micranthum* Fitoterapia: 61.

Djè, M.K., Aka, S., Nanga, Y.Z., Yao, K.C., and Loukou, Y.G., 2009. Predominant lactic acid bacteria involved in the spontaneous fermentation step of tchapalo process, a traditional sorghum beer of Côte d'Ivoire. *International Research Journal of Biological Sciences* 4(7): 789-795.

Drich, B.C., and Pran, V., 1987. Nutritional evaluation of some varieties of *Sorghum bicolour* (L.) Moench. *Cereal Chemistry* 64: 413-417.

Ekundayo, J.A., 1969. The production of pito, a Nigerian fermented beverage. *Journal Food Technology* 4: 217–225.

Famisa, A.O., 2004. Evaluation of different levels of wood ash solution as insecticide on the pests infestation and maize yield. HND Project Work Agronomy Department, Federal College of Agriculture, Akure, Nigeria: 26–35.

Kutyauripo, K., Parawira W., Tinofablvy, S., Clement, K., and Ndengu, C.,2009 Investigation of shelf-life extension of sorghum beer (C*hibuku*) by removing the second conversion of malt. *International Journal of Food Microbiology* 129:271–276.

Kyarisiima, C.C., Okot, M.W., and Svihus, B., 2004. Use of wood ash in the treatment of high tannin sorghum for poultry feeding. *South African Journal of Animal Science* 34(2): 110–115.

Lyumugabe L., Kamaliza G., Bajyana E., and Thonart P., 2010. Microbiological and physico-chemical characteristics of Rwandese traditional beer "Ikigage". *African Journal of Biotechnology* 9: 4241–4246.

Magwenzi, R., Nyakunu, C., Mukanganyama, S., 2014. The effect of selected *Combretum* species from Zimbabwe on the growth and drug efflux systems of *Mycobacterium aurum* and *Mycobacterium smegmatis*. *Journal of Microbial, Biochemistry and Technology* S3: 003. doi:10.4172/1948-5948.S3-003.

Maoura, N., Mbaiguinam, M., Nguyen, H.V., Gaillardin, C., and Pourquie, J., 2005. Identification and typing of yeast strains isolated from bili bili, a traditional sorghum beer of Chad. *African Journal of Biotechnology* 4(7) 646-656.

Masoko, P., Picard, J., and Eloff, J.N., 2007. The antifungal activity of twenty-four southern African *Combretum* species (*Combretaceae)*. *South African Journal of Botany* 73:173–183.

McGaw, L.J., Rabe, T., Sarg, S.G., Jager, A.K., Eloff, J.N., Van Staden, J., 2001. An investigation on the biological activity of *Combretum* species. *Journal of Ethnopharmacology* 75: 45–50.

Mochiah, M.B., Banful, B., Fening, K.N., Amoabeng, B.W., Offei Bonsu, K., and Ekyem, S., 2011. Botanicals for the management of insect pests in organic vegetable production. *Journal of Entomology and Nematology* 3(6):85–97.

Moyin-Jesu, E.I., **2010.** Comparative evaluation of modified neem leaf, wood ash and neem leaf extracts for seed treatment and pest control in maize (*Zea mays* L.). *Emirates Journal of Food and Agriculture* 22(1):37–45.

Moyin-Jesu, E.I., 2012. Comparative evaluation of modified neem leaf, neem leaf and woodash extracts on soil fertility improvement, growth and yields of maize (*Zea mays* L.) and watermelon (*Citrullus lanatus*) (sole and intercrop). *Journal of Agricultural Science* 1:90–97.

Muyanja, C.M.B.K., Narvhus, J.A., Treimo, J., and Langsru, T., 2003. Isolation, characterisation and identification of lactic acid bacteria from bushera: A Ugandan traditional fermented beverage. *International Journal of Food Microbiology* 80: 201–210.

N'Guessan, K.F, Brou, K., Noémie, J., Casaregola, S. and Dje, K.M., 2012. Identification of yeasts during alcoholic fermentation of tchapalo, a traditional sorghum beer from Côte d'Ivoire. *Antonie van Leeuwenhoek* 99(4):855-864

Nout, M.J.R., 1980. Microbiological aspects of the traditional manufacture of Busaa, A Kenyan opaque beer. *Chemical, Microbiological and Technological Lebensm* 6:137–142.

Nwachukwu, E., Achi, O.K., and Ijeoma, I.O., 2010. Lactic acid bacteria in fermentation of cereals for the production of indigenous *Nigerian foods. African Journal of Food Science and Technology* 1(2): 021- 026.

Odunfa, S.A, Olasupo, N.A., and Olukayo, D.K., 1996. Potential of bacteriocins in food safety in lactic fermented cereal-ogi. In: Halm, M., Jakobsen, M. (Eds.), *Traditional Fermented Food Processing in Africa*. Proceedings of the Third Biennal Seminar on African Fermented Food, FRI, DANIDA, KVL, July. Accra, Ghana. 27–32.

Okafor, N., 1990. Traditional alcoholic beverages of tropical Africa-strategies for scale-up. *Process Biochemistry* 25:213–220.

Oliver-Bever, B., 1986. Medicinal plants in Tropical West Africa. Cambridge University Press, Cambridge.

Onocha, P.A., Audu, E.O. Ekundayo, O. and Dosumu, O.O., 2005. Phytochemical and antimicrobial properties of extracts of *Combretum racemosum*. *Acta Horticulturae* 675: 97–101.

Pelig-Ba, K.B., 2009. Effect of ash, KOH and millet on the fermentation of *Parkia biglobosa* seeds to form a condiment. *Pakistan Journal of Nutrition* 8(10):1548–1554.

Peloewetse, E., Thebe, M.M. Ngila, J.C., and Ekosse, G.E., 2008. Inhibition of growth of some phytopathogenic and mycotoxigenic fungi by aqueous extracts of *Combretum imberbe* (Wawra) wood. *African Journal of Biotechnology* 7 (60): 2934–2939.

Rahman, M.M., Akbar, M.A., Islam, K.M.S., Khaleduzzaman, A.B.M., and Bostami, A.B.M.R., 2009. Nutrient digestibility and growth rate of bull calves fed rice straw treated with wood ash extract. *Bangladesh Journal of Animal Science* 38:42–52.

Reimann, C., Ottesen, R.T., and Andersson, M., 2008. Element levels in birch and spruce wood ashes – green energy? *Journal of Science and Total Environment* 393(2-3):191–197.

Sherman, P.W., and Hash, G.A., 2001. Why vegetable recipes are not very spicy. *Evolution and Human Behavior* 22 (3): 147–163. doi:10.1016/S1090-5138(00)00068-4.

Steinkraus, K., 1996. Handbook of indigenous fermented foods. Marcel Dekker, New York, USA.

Tapsell, L. C., Hemphill, I. and Cobiac, L., 2006. Health benefits of herbs and spices: the past, the present, the future. *The Medical Journal of Australia* 185 (4 Suppl): S4–24

Ujowundu, F.N., Ukoha, A.I, Ojiako, A.O., and Nwaoguikpe, R.N., 2015. Nutritional characterization of *Combretum dolichopentalum* leaves. *Biochemistry & Analytical Biochemistry*.

Usha, A., Sripriya, G., and Chandra, T., 1996. The effect of fermentation on the primary nutrients in foxtail millet (*Setaria italica*). *Food Chemistry* 56: 381–384.

Wong, J. and Selvan, A, 2009. Reduction of indicator and pathogenic microorganisms in pig manure through fly ash and lime addition during alkaline stabilization. *Journal of Hazardous Materials* 169: 882–889.

CHAPTER 2: LITERATURE REVIEW

2.1 Traditional Beverages

Beverages are food-grade liquids mainly processed from animal or plant sources. They may be in the form of stimulants such as tea and coffee, as refreshers like soft drinks, juices, and water, or as nutritional drinks such as milk. Beverage processing could be by simple non-microbial processes (such as application of physical techniques) or may involve microbial fermentation and/or enzyme clarification (Tamang, 2010 Kubo *et al.*, 2014; Tafere 2015).

In Africa, diverse traditionally processed beverages exist; their processing methods as well as constituents and consumption patterns differ across ethnicities in countries and regions (Nikander *et al.,* 1991; Gaffa *et al.,* 2002; Gadaga and *et al.,* 2013; Aka *et al.,* 2014; Kubo *et al.,* 2014 Tafere, 2015). Every country has its own recipe for the local production of beverages and fermentation is the basic process utilised in more than 90% of these traditionally processed foods (Gaffa *et al.,* 2002; Amadou *et al.,* 2011; Aka *et al.,* 2014; Kubo *et al.,* 2014; Tafere 2015).

African traditionally processed beverages are made from single or mixed cereals/legumes, animal milk, and various plant parts (such as flowers, sap, and fruits). Cereal-based beverages are common and are constituted from grains such as maize (*Zea mays* L.), pearl millet (*Pennisetum glaucum* L.), finger millet (*Eleusine coracana*), and sorghum (*Sorghum bicolour* L. *Moench*; Gaffa *et al*., 2002; Sekwati-Monang, 2011; Aka *et al.,* 2014). In terms of consumption, traditionally processed beverages are popular because of the social, religious, nutritional, and therapeutic values that are associated with them, and both rural and urban populations (Aka *et al*., 2008) cherish them. In general, non-alcoholic beverages are widely consumed, especially by children, pregnant women, the sick, and the elderly. They are also used during the weaning of infants, whereas men mostly prefer alcoholic beverages.

2.2 Diversity of Traditionally Processed Beverages in Africa

African traditional beverage production dates back to the pre-historic era and has consistently been a home-made art involving an array of raw materials, including cereal grains, legumes, flowers and juices from plants, fruits, and milk (Amadou *et al.,*

2011). The beverages produced across Africa vary according to raw materials, origin, and processing techniques employed, and are usually unique to particular ethnic or cultural groups where they are relished (Obahiagbon, 2009; Kubo *et al.,* 2014; Tafere, 2015). Beverages also define, to some extent, the socioeconomic class and tribal identity of the consumers. For example, *areki*, a distilled product from maize, millet, and sorghum in rural and semi-urban areas of Ethiopia (East Africa) is widely consumed by farmers and the low-income class who either have become addicted to alcohol or cannot afford the finer industrial alcoholic products (Tafere, 2015). *Borde* (non-alcoholic), *keribo* (non-alcoholic), and *tella* (alcoholic) are popular traditional beverages that are consumed during traditional weddings and naming and rainmaking ceremonies (Tafere, 2015). In Nigeria (West Africa), *burukutu* (alcoholic), *kunu* (non-alcoholic), and *pito* (alcoholic), which can be made from single or mixed grains, are peculiar to the northern areas where they are commonly served at festivals and social events and are presently being commercialised on a small scale within villages (Gaffa *et al.,* 2002; Ezekiel *et al.,* 2015). Similarly, palm wine (non-alcoholic; from the sap of the *Rafia* tree) is popular in the eastern parts of Cameroon (Central Africa) and Nigeria and is the acceptable wine at festivals and culturally-related ceremonies like weddings and social events (Obahiagbon, 2009; Kubo *et al.,* 2014). In Namibia (Southern Africa), *oshikundu* (a non-alcoholic beverage from millet and sorghum) is served to visitors as a token of welcome and hospitality, and it is produced as part of the traditional initiation of young girls into womanhood (Mu Ashekele *et al.,* 2012).

In general, women and children in Africa produce African traditional beverages as a home art, and when commercialised at the local setting, they become a means of economic empowerment to the women (Abawari, 2013). Production of some traditional beverages, although not adequately accounted for across Africa, runs into million liters per annum, and generally per capita consumption data are lacking (Gensi *et al.,* 2000; Kanyana *et al.,* 2013).

Beyond the cultural and socioeconomic usage and benefits of African traditional beverages are the nutritional and therapeutic values they offer, especially for the non-alcoholic grades (Aka *et al.,* 2014; Onuoha *et al.,* 2014). These beverages are rich in vitamins, minerals, and are easily utilisable carbohydrates (sugars) due to the mixtures of grains used and the fermentation process involved (Blandino *et al.,* 2003; Amadou *et al.,* 2011; Aka *et al.,* 2014). Supplementation of some of the beverages (such as

kunu gyada, a variety of *kunu* from Nigeria) with nuts, tubers, and spices has further boosted their protein and amino acid contents as well as the antioxidant properties of the drinks (Gaffa *et al.,* 2002; Blandino *et al.,* 2003).

Bushera is a collective name for popular traditional fermented cereal beverages consumed in western, southwestern and central Uganda. These beverages are commonly consumed as refreshing drinks and to some extent are used as a weaning food. Spontaneously fermenting gelatinised slurries of flour from malted or un-malted millet and/or sorghum (Mukisa *et al.,* 2012 Muyanja *et al.,* 2003) make *Bushera*.

Sorghum or millet flour from germinated sorghum and millet grains is mixed with boiling water and left to cool at an ambient temperature. Germinated millet or sorghum flour is then added and the mixture is left to ferment at an ambient temperature for 1 to 4 days (Vashudha and Mishra, 2013; Mishra and Mishra, 2012). The fermentation period depends on the type of *bushera* being produced and the targeted consumers or consumers' preference. For sweet *bushera*, the fermentation period ranges from 12 to 24 hours, whereas for sour *bushera* the fermentation time varies between 2 and 4 days (Muyanja *et al.,* 2012). Sour *bushera* is mostly for adults, while the sweet version is fed to children (Aka *et al.,* 2014). Back slopping is also practiced in the production of *bushera*, but this has been considered to lead to fast production of acid and hence excessive sourness. Therefore, back slopping is practiced in households where they prefer sour *bushera* to sweet (Muyanja, 2008; Aka *et al.,* 2014). Some of the beverages produced in different countries from varied agents are presented on table 2.1.

Table 2.1: Some of the most popular African non-alcoholic beverages.

Beverage	**Country of Origin**	**Ingredients**
Leite Azede	Angola	Fermented milk
Sobia	Egypt	Coconut milk, sugar, and ground rice.
Café Touba	Senegal	Coffee beans and (*Xylopia aethioopica*) also known as Guinea pepper
Nobo	Nigeria	water, dried roselle leaves, garlic, ginger, and pineapple

Sobolo	Ghana	roselle leaves or flowers
Oshikundu	Namibia	Water, pearl millet flour (*mahangu*), sorghum flour, and usually pearl millet bran.
Amasi	South Africa	fermented milk beverage containing numerous valuable probiotics
Mazagran	Algeria—that is now when granted to France by the Treaty of Tafna in 1837.	consists of strong coffee that is poured over ice
Bushera	Uganda	Sorghum millet, water and sugar
Mageu	South Africa	Fermented mealie pap

https://www.tasteatlas.com/most-popular-non-alcoholic-beverages-in-africa

2.3. Shelf life of Traditional Beverages and Soft Drinks

Soft drinks and fruit juices represent an important market within the food industry. The increasing variety of products being released at a bewildering rate has altered the potential for spoilage problems. Many microorganisms found in traditional beverages as environmental or raw material contaminants, but relatively few can grow within the acidic and low oxygen environment. Yeasts are the most significant group of microorganisms associated with the spoilage of soft drinks and traditional beverages. Spoilage is seen as the growth and production of metabolic by-products, for example, CO_2, acid, and tainting compounds (Hocking and Jensen, 2001; Jay and Anderson, 2001). Traditional beverages and soft drinks are commonly contaminated with yeasts and moulds.

It is important to realise that foods are diverse, complex and active systems in which microbiological, enzymatic and physicochemical reactions are simultaneously taking place (Singh and Cadwalleder, 2004). These reactions have major consequences in relation to flavour, texture and shelf life. Food preservation is dependent on the understanding of mechanisms of these reactions and the successful limitation of those most responsible for loss or spoilage of desirable characteristics and sometimes the channelling of other reactions towards beneficial changes. Essentially, the shelf life of a food can be defined as the period for which it will retain an acceptable level of eating quality, from a safety and sensory point of view (Singh and Cadwalleder, 2004). There

are four critical factors that determine that and these include formulation, processing, packaging and storage conditions. All the four factors are critical but their relative importance depends on the food. An understanding of the interplay between these factors is key to shelf-life estimation and testing. For example, a change in a single processing parameter may lead to undesirable chemical or physical changes in a product, or it may require reformulation or a change in packaging in order to attain the required shelf life (Singh and Cadwalleder , 2004). Similarly, the very act of processing may subject the formulated materials and ingredients to conditions that are unfavourable or inhibitory to undesirable deteriorative reactions and promote desirable physical and chemical changes, thus giving the food product its final form and characteristics (Singh, 1999). Some of the contributing factors in the search for improved shelf-life include increased consumer demand for fresh, convenient, safe and superior quality foods available year-round, and the continued globalisation of food distribution systems (Singh and Cadwalleder, 2004).

To attain knowledge about food expected shelf life, one must (1) understand the concerted series of biochemical/physicochemical reactions taking place in any given food, and (2) identify the mechanisms responsible for spoilage or loss of desirable characteristics such as texture, flavour, odour and/or nutrients. Food quality loss can be described in terms of a number of compositional factors, such as concentration of reactive species, microorganism levels, catalysts, reaction inhibitors, pH and water activity, as well as environmental factors, which include temperature, relative humidity, light, mechanical stress and total pressure (Labuza, 2000). According to Troller and Christian (1978) water activity, temperature and pH are the most important factors that control rates of deteriorative changes and microbial growth in foods.

2.4 Important Microorganisms that Commonly Affect Food Products

There are thousands of different types of microorganisms everywhere in the air, soil and water, and consequently on foods, and in the digestive tract of animals and human. Fortunately, the majority of microorganisms perform useful functions in the environment and in some branches of the food industry, such as the production of wine, beer, bakery products, dairy products, etc. On the other hand, microorganisms generally cause unwanted spoilage of foods and contamination of food with pathogens causes food safety problems (Doyle, 1989).

The microorganisms occurring on and/or in foods are, from a practical point of view, divided into three groups: moulds, yeast and bacteria. Moulds generally contribute to the spoilage of foods; their use in the food industry is limited (e.g. mould ripened cheese). Yeasts are the most widely used microorganisms in the food industry due to their ability to ferment sugars to ethanol and carbon dioxide (Halasz, and Lasztity, 1991). Some types of yeast, such as bakers' yeasts are grown industrially, and some may be used as protein sources, mainly in animal feed (Hui *et al.,* 2004).

2.4.1 Indicators of Food Microbial Quality and Safety

Microbiological indicator organisms are set to monitor hygienic conditions in food production. The determination of the microbiological quality of a food or food constituent may be required in order to estimate its shelf life or its suitability for human consumption. Food microbiology testing indicators include total bacterial count, coliform group and pathogenic bacteria. The main indicators are as follows:

2.4.1.1 Total bacterial count

The total bacterial count refers to the total number of bacterial colonies contained in 1g [1ml or 1cm (surface area)] food test samples after treatment and cultivation under certain conditions (Madigan *et al.,* 2009). The total bacterial count can reflect the freshness of the food, the degree of bacterial contamination and the general hygienic status of food production. Therefore, it is one of the important bases to judge the quality of food hygiene.

2.4.1.2 Coliform group

Coliform group refers to a group of aerobic or facultative anaerobic gram-negative spore less bacillus that can ferment lactose, produce acid and produce gas when cultured at 37℃ for 24 hours (Madigan *et al.,* 2009). These bacteria (figure 2.1) are resident flora in the intestines of human beings and warm-blooded animals, and are discharged from the body with their stools. The more the number of coliform groups in the food, the greater the degree of faecal contamination. Therefore, it is of paramount significance to evaluate the hygienic quality of food with coliform group as the hygienic indicator of faecal contaminated food.

Figure 2.1 Depiction of coliform bacteria on and agar plate (https://thumbs.dreamstime.com/b/food-safety-agar-plates-)

2.4.1.3 Pathogenic bacteria

Pathogenic bacteria are the bacteria that can cause people to get sick. It is one of the essential standards in the food hygiene quality standards that pathogenic bacteria should be prevented from getting into food. Various kinds of pathogenic bacteria, different food processing and different storage conditions lead to the different contamination situations, so generally targeted tests are made according to the possible contamination conditions of different foods. Certain indicator bacteria are selected for testing different foods (Madigan *et al.*, 2009).

2.4.1.3.1 *E. coli*

Escherichia coli is a Gram-negative, facultative anaerobic, rod-shaped bacterium found in the lower intestine of warm-blooded organisms (endotherms) (figure 2.2). Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in humans and are occasionally responsible for product recalls due to food contamination (Madigan *et al.,* 2009). The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K, and by preventing the establishment of pathogenic bacteria within the intestine. *E. coli* and related bacteria constitute gut flora, and faecal – oral transmission is the major route through which pathogenic strains of the bacterium cause disease (Madigan *et al.,* 2009). Cells are able to survive outside the body for a limited amount of time, which makes them ideal indicator organisms to test environmental samples for faecal contamination.

Figure 2.2. Depiction of *Escherichia. coli* on Brilliance selective chromogenic medium. https://www.fishersci.co.uk/shop/products/brilliance-e-coli-coliform-

2.4.1.3.2 *Bacillus cereus*

Bacillus cereus (figure 2.3) is a spore-forming bacterium that produces toxins that cause vomiting or diarrhoea. Symptoms are generally mild and short-lived (up to 24 hours). *B. cereus* is commonly found in the environment (e.g. soil) as well as a variety of foods. Spores are able to survive harsh environments, including normal cooking temperatures. *B. cereus* is a Gram-positive, motile (flagellated), spore-forming, rod-shaped bacterium that belongs to the *Bacillus genus*. Species within this genus include *B. anthracis, B. cereus, B. mycoides, B. thuringiensis, B. pseudomycoides and B. weihenstephanensis* (Rajkowski and Bennett 2003; Montville and Matthews 2005). Genomic sequencing data has shown *B. anthracis, B. cereus and B. thuringiensis* to be very closely related (Rasko *et al.*, 2004) with their 16S rRNA gene sequence sharing more than 99% similarity (Ash *et al.*, 1991). *B. cereus* is widespread in nature and readily found in soil, where it adopts a saprophytic life cycle; germinating, growing and sporulating in this environment (Vilain *et al.,* 2006). Spores are more resistant to environmental stress than vegetative cells due to their metabolic dormancy and tough physical nature (Jenson and Moir, 2003). *B. cereus* produces two types of toxins – emetic (vomiting) and diarrhoeal – causing two types of illness. The emetic syndrome is caused by emetic toxin produced by the bacteria during the growth phase in the food. The diarrhoeal syndrome is caused by diarrhoeal toxins produced during growth of the bacteria in the small intestine (Ehling-Schulz *et al.,* 2006).

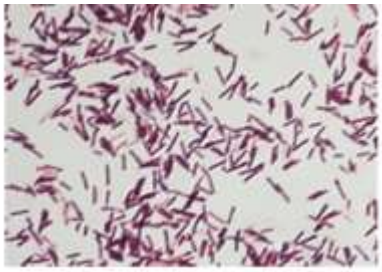


Figure 2.3.A photo of Gram stained *Bacillus cereus* under ×100 magnification. https://www.researchgate.net/publication/318434143/figure/fig2/AS:8644125629030.

2.4.1.3.3 *Staphylococcus aureus*

Staphylococcus aureus (figure 2.4) is a bacterium that causes staphylococcal food poisoning, a form of gastroenteritis with rapid onset of symptoms. *S. aureus* is commonly found in the environment (soil, water and air) and found in the nose and on the skin of humans. *S. aureus* is a Gram-positive, non-spore forming spherical bacterium that belongs to the *Staphylococcus genus*. The *Staphylococcus genus* is subdivided into 32 species and subspecies. *S. aureus* produces staphylococcal enterotoxin (SE) and is responsible for almost all-staphylococcal food poisoning (Montville and Matthews 2008; FDA 2012). *S. intermedius*, a Staphylococcus species which is commonly associated with dogs and other animals, can also produce SE and has been rarely associated with staphylococcal food poisoning (Talan *et al.,* 1989; Khambaty *et al.,* 1994; Le Loir *et al*., 2003). *S. aureus* is uniquely resistant to adverse conditions such as low water activity $_{aw}$, high salt content and osmotic stress. In response to low $_{aw,}$ several compounds accumulate in the bacterial cell, which lowers the intracellular $_{aw}$ to match the external $_{aw}$ (Montville and Matthews, 2008). As such, most *S. aureus* strains can grow over $_{aw}$ range of 0.83 to >0.99 (FDA, 2012). *S. aureus* is a poor competitor, but its ability to grow under osmotic and pH stress means that it is capable of thriving in a wide variety of foods, including cured meats that do not support the growth of other foodborne pathogens (Montville and Matthews 2008). *S. aureus* is a facultative anaerobe that can grow under both aerobic and anaerobic conditions. However, growth occurs at a much slower rate under anaerobic conditions (Stewart, 2003). For a non-sporing mesophilic bacterium, *S. aureus* has a relatively high heat resistance (Stewart, 2003). The bacteria have a higher heat resistance when it is encapsulated in oil, with a D-value at 60°C of 20.5 min for *S. aureus* in fish and oil (Gaze, 1985). An extremely heat resistant strain of *S. aureus* has been recovered from

a foodborne outbreak in India (Nema *et al.,* 2007). Several chemical preservatives, including sorbates and benzoates, inhibit the growth of *S. aureus.* The effectiveness of these preservatives increases as the pH is reduced. Methyl and propyl parabens are also effective (Stewart, 2003; Davidson and Taylor, 2007).

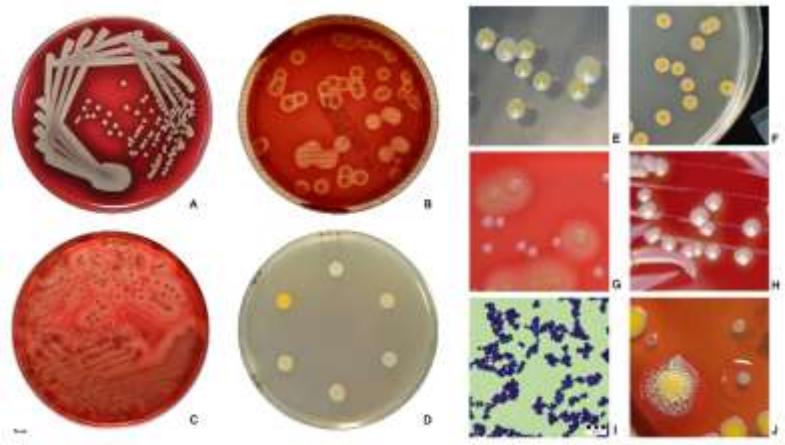


Figure 2.4. Depiction of *Staphyloccus aureus* on different agar plates.

2.4.1.4 Yeasts and moulds

Yeasts and moulds form an important group of organisms of great importance to the food industry. These groups are very different from the bacteria that are commonly associated with food, being eukaryotic organisms similar to cells found in plants and animals. The yeasts and moulds are widely dispersed, found in a variety of locations and are virtually ubiquitous in any environment (Hui *et al.,* 2004).

Yeasts

Yeasts (figure 2.5) are very important within food microbiology as they have both positive and negative effects. The origin of the positive effects of yeasts in food production probably came about by an accidental contamination of some raw materials with environmental yeasts. Mead, a fermented honey drink, is the oldest alcoholic beverage known to man and is believed to have been discovered during the Stone Age. A chance occurrence of honeycomb becoming wet from rain and then airborne yeasts fermenting the mixture is thought to have led to its discovery. Leavened bread first appeared in Egypt about 5,000 years ago, when flat bread dough became contaminated with wild yeasts which would have produced carbon dioxide, and 'raised' the bread. No doubt, an 'accidental' contamination of various fruit juices would have caused the production of wines. Contamination of raw materials with wild

environmental yeasts is still used to produce some foods such as specialist sourdough breads and lambic beers (Hui *et al.,* 2004). However, most food production that uses yeasts will now utilise specialist strains obtained from culture collections that are cultured and deliberately inoculated into their growth substrate to create the food required. Today in food production, yeasts are more usually linked with food spoilage. Yeasts are slow growing organisms when compared to bacteria (Yeast Book, 2011). If yeasts and bacteria were placed in the same optimum environment and both could grow, it is most likely that the faster growing bacteria would quickly outgrow and outcompete the slower growing yeast, becoming the dominant flora. Yeasts are generally associated with the fermentation of sugars such as glucose and sucrose, but they are able to utilise a variety of other compounds, such as alcohols, organic acids, hydrocarbons and aromatic compounds (Yeast Book, 2011). Some yeasts are also capable of utilising certain acid-based preservatives such as benzoic acid, propionic acid and sorbic acid, and this can make them a major issue in foods and drinks that rely on these preservatives for stability.

Figure 2.5. Depiction of yeasts on an agar plate
https://image.shutterstock.com/image-photo/colonies-yeasts-molds-fungal-testing.

Moulds

Like the yeasts, moulds (figure 2.6) can also produce both positive and negative effects in foods. Their negative effects are well known – mould contamination of

products containing high sugar or of low pH is obvious with the organisms tending to grow as colonies on the surface of such products. Moulds occur on the surface of mould-ripened cheeses such as brie and camembert, and within blue veined cheeses like Stilton and Danish Blue. Some species are a key part of some fermented food products from Japan, whilst the meat replacement known as Quorn, is produced from a *Fusarium* mould that is cultured and treated to form a 'meat-like' texture. On the negative side, mould-like yeasts can affect low pH and low water activity foods. Indeed, this group can grow at very low water activities (A_w) causing spoilage problems in products with A_w values below 0.75. Moulds are slow growing organisms and will be rapidly out competed by bacteria and even yeasts in normal conditions (Lodder, 1970). They come into their own when the pH and/or A_w is reduced and other organisms are unable to grow. Then the moulds can take over, forming hyphal mats or colonies on the surface of food products. It is important to realise that moulds are aerobic organisms; they need oxygen to grow, so they are confined to the surfaces of foods, forming easily visible, often coloured colonies. Moulds and some forms of yeast are able to produce spores, and these can be heat resistant. This makes the determination of any heat process used on food products very important (Lodder, 1970).

Figure 2.6. Image of mould on agar plates
https://mouldcleaningaustralia.com.au/wp-content/uploads/2019/10/Mold-768x384.

2.4.1.5 Enterobacteriaceae

The family *Enterobacteriaceae* (figure 2.7) is one of the most important bacterial families. It includes the plague bacillus *Yersinia pestis* and the typhoid *bacillus Salmonella* serotype *Typhi* (*Salmonella typhi)*, which are two of the most important bacterial pathogens in human history. It also includes two genera of intrinsic enteric pathogens, Shigella and Salmonella; essentially all strains in these two genera can cause diarrhoea or intestinal infections (Centers for Disease Control and Prevention

(Anonymous) 2003). Two other genera, *Escherichia* (Gamage *et al*., 2003; Misselwitz *et al.,* 2003; Naimi *et al.,* 2003; Riley *et al.,* 1983; Vallance *et al.,* 2002) and Yersinia, also include enteric pathogens; however, only a few of the many serotypes (strains) have the virulence factors that enable them to infect the intestinal tract or allow them to colonise it and produce enterotoxins. Many other species have an association with diarrhoea (Murata *et al.,* 2001), but their causal role is uncertain. Several other species of *Enterobacteriaceae* frequently cause extra intestinal human infections (Diekema *et al.,* 1999; Edmond *et al.,* 1999; O'Hara *et al*., 2000), and some have an association with chronic diseases such as arthritis (Yu and Kuipers, 2003). The family *Enterobacteriaceae* includes many bacteria that are found in the human or animal intestinal tract, including human pathogens such as Salmonella and Shigella. *Enterobacteriaceae* are useful indicators of hygiene and of post-processing contamination of heat processed foods. Their presence in high numbers (>104 per gram) in ready-to-eat foods indicates that an unacceptable level of contamination has occurred or there has been under processing. The group includes both pathogenic and non-pathogenic bacteria. In ready-to-eat foods that are fully cooked, *Enterobacteriaceae* are used as an indication of either post-processing contamination or inadequate cooking.

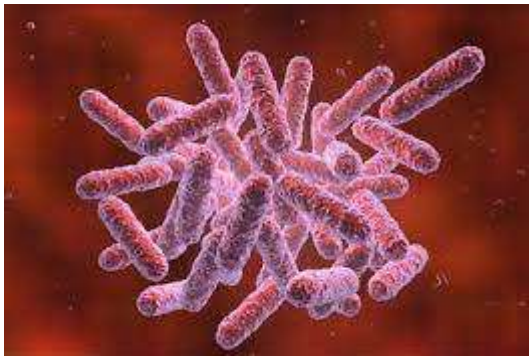


Figure 2.7: An image depicting the Enterobacteriaceae bacteria .

2.4.1.5 Lactic acid bacteria

Lactic acid bacteria (LAB) (figure 2.8) are among the important groups of bacteria providing health benefits to human, animal, and plant (Bintsis, 2018; Hati *et al.,* 2013) sing LAB in food fermentation is one of the ancient known food preserving techniques. Properties such as nutritional and environmental adaptations have provided LAB with

the ability to adapt and present in different environments ranging from food matrices such as dairy products, meats, vegetables, sourdough bread, and wine to human mucosal surfaces such as oral cavity, vagina, and gastrointestinal tract (Perez *et al*., 2014; Quinto, *et al.,* 2014). LAB are known for their fastidious nutritional requirements, which may vary among species and even among strains (Perez *et al*., 2014; Quinto, *et al*., 2014). Strains of LAB are also known as fast growing microorganisms that can explore different metabolic activities. Metabolic activities are associated with the production of many beneficial compounds such as organic acids and antimicrobial compounds, unique enzymes that can breakdown complex organic compounds into simple functional compounds (Parvez *et al.,* 2006). Thus, the fast-growing characteristics and the metabolic activity are the keys of LAB benefits and applications. Metabolic activities of LAB, which are necessary for survival and growth, are also important for any application. The primary metabolic activity in LAB is degradation of carbohydrates and related compounds to obtain mainly energy and carbon molecules (Mokoena, 2017; Salminen, 1998). However, proteinases and peptidases activities of LAB have gained much attention due to their importance in the accelerated maturation and enzyme modification of different food products especially cheese (Sharma *et al.,* 2020; Ni *et al.,* 2015).

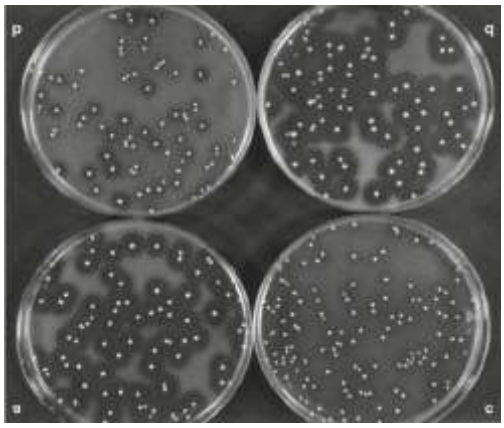


Figure 2.8: Colonies of lactic acid bacteria obtained from salt fermented cucumber https://www.researchgate.net/profile/Joginder-Duhan/publication/260694753/figure/fig

2.5 Preservation methods in the in the food industry

2.5.1 Food Preservation

Processing of food products involves numerous steps right from harvesting to consumption as products. The main aim of food processing is preservation; however, recent research is also focussing on retaining high quality and improving product functionality. Processing of raw food makes it more palatable, consumable and increases its shelf life.

Numerous forms of preservation techniques such as pasteurisation, freezing, drying and application of chemicals have been designed to extend the shelf-life of the food products, not only by reducing the microbial growth, but also to maintain the antioxidant potential to serve consumers needs (Yadav *et al.,* 2014; Sarkar *et al.,* 2014). To make the packaged food quality stable for a reasonable time, preservatives are used in different quantities and concentrations. Traditionally, food preservation has three goals; the preservation of appearance, the preservation of nutritional characteristics, and a prolongation of the time that the food can be stored. Hence, food preservatives can be defined as the "food additives used to inhibit the growth of microorganisms like yeast, moulds and bacteria and prevent the spoilage by different anti-oxidative reactions in order to maintain the quality, texture, consistency, taste, colour, alkalinity or acidity" (WHO, 1987; Tuormaa, 1994). Several forms of chemical preservatives are currently in use in food and beverage industries such as benzoate, sorbates, vitamins, fruit extracts, sodium salts, etc.

Antimicrobial preservatives reduce the microbial spoilage of foods by inhibiting the growth and proliferation of bacteria, yeasts and moulds. Benzoates, sorbates, nitrates, and sulphites are categorised under the group of antimicrobial preservatives (Abdulmumeen *et al.,* 2012). Sodium benzoate (produce benzoic acid when dissolved in water) and benzoic acids are the most common used preservative and widely used in acidic food products like fruit juice, carbonated drinks, pickles and jams (Mirza *et al.,* 2017). The maximum limit of concentration levels of benzoates approved by FDA is 0.2% and when used along with ascorbic acid, it is 0.1%. Sulphites like sodium bisulphide and potassium meta-bisulphites are used in food by dissolving in cold water. Upon dissolving, they produce sulphurous acid that inhibits the growth of bacteria and moulds and, to some extent, yeast. Sorbates like potassium sorbate, sodium sorbate are used as preservatives in products having a high pH value up to 6.5 (Hwang and Huang, 2014). Nitrites are mostly used to prevent the growth of yeast

and moulds in food products. The maximum limit of concentration level allowed is 0.1%.

Chemicals that prevent oxidation in other molecules are referred to as antioxidants. Ascorbates, tocopherols, erythorbates, Lactates, phosphates, succinates are effectively used as antioxidants for food and beverages. Ascorbic acid is a common antioxidant beverage and pickle. Foods containing unsaturated fats are easily attacked by oxidation. Oxidation causes them to turn rancid in order to prevent discolouring and unpleasant tastes like metallic or sulphuric (Toaima *et al*., 2015). Hence, the tocopherols (normally vitamin E) are used in rich fat foods for preservation.

2.5.2 Adverse effects of preservatives

Though preservatives are a beneficial to packaged food, they do have some negative effect on human health. All preservatives cause hyperactive activity on regular usage. Some of the common preservatives and their harmful effects on human health are listed below.

a) Nitrates and Nitrites: For curing of meat products, these additives are used. Nevertheless, sometimes, they react to cause urticarial, itching and anaphylaxis in human beings. Sodium nitrite is used in meat products during cooking to prevent botulism, but during high heat, it reacts with rich protein cancer cells such as liver, intestinal and oesophageal cancer cells (Anon, 1991; Theron and Lues, 2007).

b) Benzoates: Benzoate contained in foods are strictly restricted or prohibited for asthma patients because it worsens the condition. Benzoates are also reported to cause rhinitis, chronic urticarial and flushing in some cases (Sharma, 2015). Sodium benzoate that is used to enhance the shelf-life for a long time is found to form carcinogenic benzene when used with vitamin C or ascorbic acid. However, the amount of benzene form is low but is a risk factor in causing cancer (Jha *et al.,* 2013). It is also reported that benzoates can cause brain damage (McCann *et al.,* 2007).

c) Sorbates: Sorbates can cause urticarial and contact dermatitis in some cases (Kinderlerer *et al.,* 1990).

d) Sulphates: Copper sulphate is used in the colouring of peas and other vegetables. It has been found that copper, when added to the vegetables, forms a compound that is not easily soluble in the human body (Elhkim *et al.,* 2007).

2.6 The use of medicinal plants in food

Medicinal plants constitute an effective source of antimicrobial natural products. The use of medicinal plants all over the world predates the introduction of antibiotics and other modern drugs into the African continent (Haslam *et al*., 1989). Plants have been used in traditional medicine for many centuries as arbotifacients, contraceptives, for menstrual regulation, fertility control, as well for the treatment of ailments of both microbial and non-microbial origins (Gill and Akinwunmi, 1986).

The antimicrobial activity exhibited by plant extracts against bacteria that commonly contaminates food and drinks has been demonstrated by several workers (Delgado *et al.,* 2004; Alzoreky and Nakahara, 2003; Verma *et al.,* 2012; Akinpelu *et al.,* 2015). Gupta *et al.* (2010) investigated the antibacterial activity of five ethanolic and aqueous plant extracts against *S. aureus, P. aeruginosa* and *Bacillus subtilis.* their results showed that the ethanolic extracts of four plants *(Achyranthes aspera, Cynodon dacynodon dactylon, Lantana camara* and *Tagetes patula*) were effective against all tested microorganisms with minimum inhibitory concentrations (MIC) ranging between from 25 and 125 mg/ml. Sapkota *et al*. (2012) studied the antibacterial effect of guava leaves, garlic and ginger against some human microbial pathogens. They ascertained that ginger was only effective against *S. aureus* while guava and garlic were effective against all the tested microorganisms. Akinpelu *et al*. (2015) investigated the antibacterial potential of crude and butanolic extracts of *Persea americana* against *Bacillus cereus* implicated in food poisoning. The extracts exhibited antibacterial activity at concentrations of 25 and 10 mg/ml with minimum bactericidal concentrations (MBC) of both extracts ranging between 3.12 and 12.5 mg/ml. Moreover, the antimicrobial activity of different natural substances such as medicinal plant extracts have been investigated against food borne bacteria. For example; Ahmad and Beg (2001), Kokoska *et al.* (2002), Ateb and Erdo_Urul, (2003), and Rios and Recio (2005) tested the suppression of food borne bacteria and their diseases by medical plant extracts. The extract of three medicinal plants used in Nigerian folk medicine showed a highly antibacterial activity against some food borne pathogens. All extracts exhibited a strong antimicrobial activity against *Salmonella enteritidis, E. coli* and *S. aureus* but in variable degrees and with different MIC's depending upon the plant

extract and pathogenic organism (Ahmad *et al.,* 1998; Akinyemi *et al.,* 2006). In addition, Sher (2009), Venkatesan and Karrunakaran (2010) and Pirbalouti *et al*. (2010) investigated the antimicrobial activity of eight medicinal plants against *E. coli, B. cereus* and *Listeria monocytogenes*. The most effective extracts were those obtained from *Myrtus communis* and *Thymus daenensis* with MIC values ranging between 0.039 and 10 mg/ml. The antimicrobial activity of *Punica granatum* against food poisoning bacteria was proved by several investigators (Prashanth *et al.,* 2001; Negi and Jayaprakasha, 2003; Voravuthikunchai *et al.,* 2005; Naz *et al*., 2007; Nuamsetti et al., 2012). Verma *et al.* (2012). They investigated the antibacterial activity of Punica, Citrus and Allium extracts against food borne spoilage bacteria. All plant extracts were potentially effective against *S. typhi, E. coli, B. cereus* and *S. aureus* implicated in food spoilage, but the extract of *Punica granatum* was the most effective extract at concentration of 500 mg/ml. Ethanolic *P. granatum* peels extracts were found to be potentially effective against *Micrococcus luteus, S. aureus, Bacillus megaterium* and Gram negative bacteria like *E. coli* and *P. aeruginosa* in concentrations ranging between 30 and 50 mg/ml (Duman *et al.,* 2009; Sadeghian *et al.,* 2011; Dey *et al*., 2012). The antimicrobial activity of ethanolic *Punica granatum* extract and its fractions showed a highly antibacterial activity against Gram positive (S*. aureus and B. cereus*) and Gram-negative bacteria (*E. coli and S. typhi*) causing food poisoning. These extracts can be used for the prevention of food borne diseases or as preservatives in the food industry (Alzoreky, 2009; Mahboubi *et al.,* 2015). Spices extracts used as food additives were potentially effective against some food poisoning bacteria and their antibacterial activity was investigated by several workers (Ozcan and Erkmen, 2001; Nevas *et al.,* 2004; Parekh and Sumitra, 2007; Abdulrahman *et al.,* 2010).

2.7 Bioactive Compounds in Plant Materials

Phytoconstituents are natural bioactive compounds that are present in plants, which when combined with nutrients and fibers, form an integrated part of human defence mechanisms against diseases and stress conditions (Khandare, 2012). Phytochemicals are divided into two groups, namely, primary and secondary constituents according to their function in plant metabolism. Primary constituents comprise common sugars, carbohydrates, amino acids and proteins while secondary

constituents consist of alkaloids, flavonoids, phenolics, saponins, etc. (Dhawale, 2013).

2.7.1 Primary constituents

2.7.1.1 Proteins

Proteins are complex organic compounds; their basic structure is a chain of amino acids (figure 2.9) (Van Holde *et al.,* 2008). They provide energy for the body. Protein is an important component of every cell in the body. Hair and nails are mostly made of protein. The body uses protein to build and repair tissues (Beaton and Swiss, 1974). They are also used to make enzymes, hormones, and other body chemicals. Protein is an important building block of bones, muscles, cartilage, skin, and blood (Beaton and Swiss, 1974). Twenty different amino acids join to make all types of protein. Human bodies cannot synthesise some of these amino acids, so, these are known as essential amino acids. This type of amino acids is provided through diet.

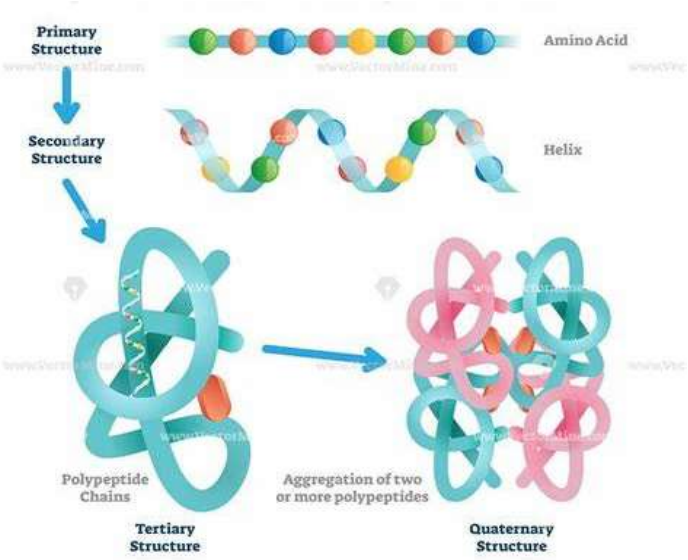


Figure 2.9 Generic and basic structure of a protein molecule. https://ulturfapp.ul.ac.za/pls/prodi02/web.w57pkg.w57_lst_frm2

2.7.1.2 Carbohydrates

Carbohydrates are a major class of naturally occurring organic compounds. Among the well-known carbohydrates are various sugars, starches, and cellulose (figure 2.10), all of which are important for the maintenance of life in both plants and animals. There are five primary functions of carbohydrates in the human body (Van Holde *et al.,* 2008), these include energy production, energy storage, building macromolecules, sparing protein, and assisting in lipid metabolism (Van Holde *et al.,* 2008).

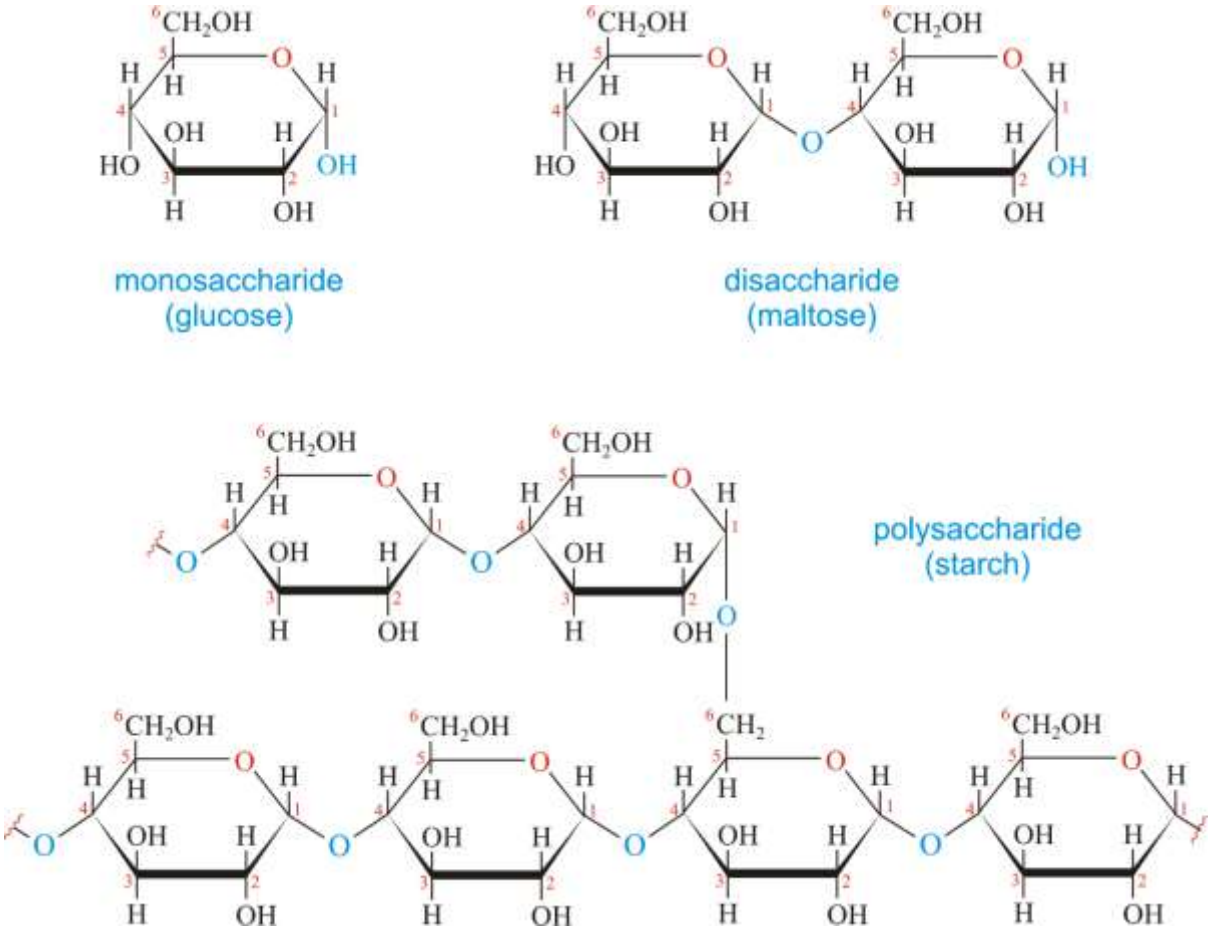


Figure 2.10 Generic and basic structure of a carbohydrate molecule.

https://www.google.com/search?q=Generic+and+basic+structure+of+a+carbohydrate+molecule

2.7.1.3 Sugars

Plant cells manufacture glucose (figure 2.11) through photosynthesis. When glucose is present in excess, plants store it by using it to synthesise chains of sugar molecules called starches. These starches form an important component of the human diet.

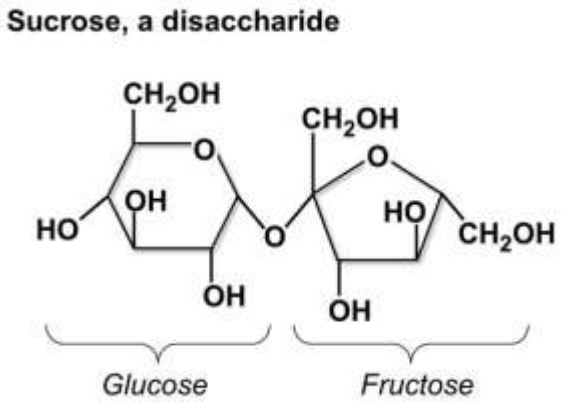


Figure 2.11 Generic and basic structure of a sugar molecule.

https://www.google.com/search?q=Generic+and+basic+structure+of+a+sugar+molecule

2.7.2 Secondary constituents

2.7.2.1 Phenolic compounds

Phenolic compounds (PCs) include a wide range of plant substances, which are recognised by their hydrophilic nature and their common origin from the aromatic precursor shikimic acid (Edeaga *et al.,* 2005). Phenolics are compounds bearing one or more aromatic rings, at least one hydroxyl group, and could contain a distinctive additional functional group (figure 2.12) (Ozcan *et al.,* 2014). More than 8000 different plant PCs have been identified so far (Pandey and Risvi, 2009). Phenolic compounds could perform different functions in plants, most of them related to plant protection; meanwhile, they contribute to flavour (odour, taste, and astringency), appearance (colour), and oxidative stability (Pandey and Risvi, 2009). Besides, phenols may exhibit relevant physiological activities including anti-inflammatory, anti-infective, anti-proliferative, and antioxidant. The latter is attributed to their capacity to retard or inhibit oxidation-related cell damage, such as lipid peroxidation and DNA oxidative decay, through their scavenging activity against free radicals. An imbalance between free radicals and antioxidants (both endogenous and dietary) has been linked to aging and CDDs associated with oxidative stress (Masibo *et al.,* 2008).

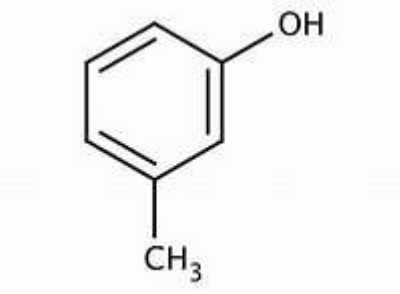


Figure 2.12 Generic and basic structure of a phenolic molecule.

https://www.google.com/search?q=Generic+and+basic+structure+of+a+phenolic+molecule

2.7.2.2 Terpenoids/Terpenes

Terpenoids (figure 2.13) are a large and diverse class of organic compounds and are characterised by being lipophilic in nature. In general, the term terpene is used to denote compounds containing an integral number of C_5 units and chemically all terpenoids can to be derived from the basic branched C_5 unit isoprene (2-methyl-1,3-butadiene,1) as indicated in Figure 2.14 (Zuanger and Basu, 2008). A number of terpenes or terpenoids are reported to be active against fungi (Cowan, 1999). The oxygen containing derivatives, i.e. terpenoids are known for their antiviral, anthelmintic, antibacterial, anti-cancer, antimalarial, and anti-inflammatory properties (Chen *et al*., 1993).

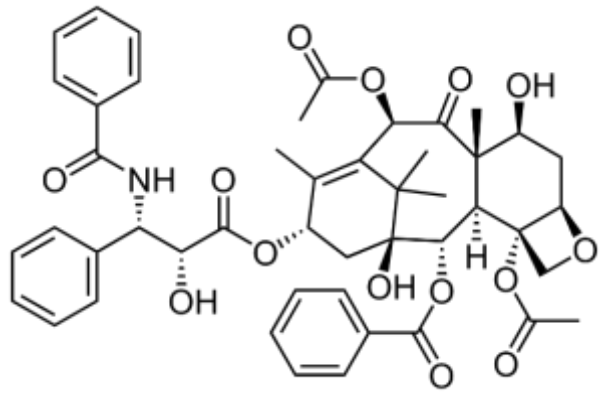


Figure 2.13 Generic and basic structure of a terpenoids molecule.

https://www.google.com/search?q=Generic+and+basic+structure+of+a+terpenoids+molecule&tbm

2.7.2.3 Glycosides

Glycosides are derived mostly from post modification of the secondary metabolites catalysed by plant enzymes, glycosyltransferases (Yu *et al.,* 2012; Blanchard and Thorson, 2006). They are stored and transported within the plant tissue and may play an important role in signalling, regulation of growth and development, and in an allelopathy (biological phenomenon where one plant inhibits the growth of another). They are also important in the plant's defence system against pathogens and herbivores. In many instances, glycosides (figure 2.14) are produced in response to some environmental conditions, such as abiotic (humidity, soil composition, sunlight, temperature) or biotic factors (e.g., plant herbivores, coexisting plants) (Bruneton, 1990; Evans *et al.,* 2009). Below is a structure of glycosides.

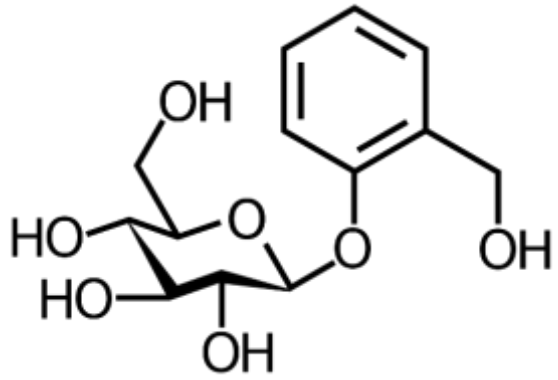


Figure 2.14 Generic and basic structure of a glycoside molecule. https://www.google.com/search?q=Generic+and+basic+structure+of+a+terpenoids+molecule&tbm

2.7.2.4 Alkaloids

Alkaloids are present in plant tissues as water-soluble salts of organic acids (tartaric, acetic, oxalic, citric, malic, and lactic acids), esters (or combined with tannins or sugars (Kar, 2003; Svendsen *et al*., 1983). Most alkaloids are isolated from plant matrices in the form of crystalline, amorphous, nonodorous, and non-volatile compounds. However, low molecular weight alkaloids, such as arecoline and pilocarpine, and alkaloids with no oxygen atom in their structure (e.g., sparteine and nicotine) occur in liquid form (Kar, 2003). Apart from the orange-yellow alkaloids berberine and colchicine, the red-coloured betaine, the brick red sanguinarine, or the orange-coloured canadine majority of alkaloids are colourless with a bitter taste. Indeed, quinine is still used as a bitter principle in tonic water (Drager, 2002). The free bases

of alkaloids are soluble in nonpolar organic solvents (chloroform, methylene chloride, ether), while their solubility in water is low (exceptions include caffeine and ephedrine). In contrast, the salts of alkaloids are soluble in water or dilute acids, whereas they are insoluble or sparingly soluble in organic solvents (Drager, 2002). Figure 2.15 shows the structure of an alkaloid.

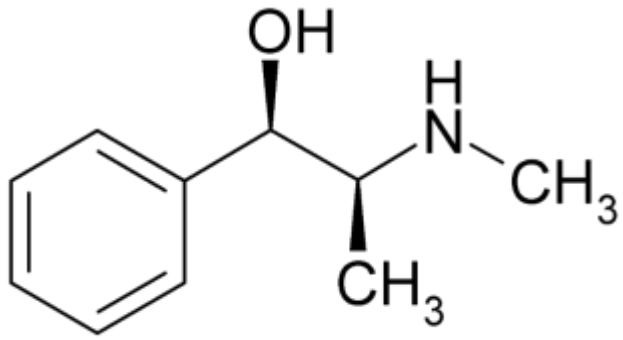


Figure 2.15 Generic and basic structure of an alkaloid molecule.

2.7.2.5 Tannins

Tannins are a group of plant secondary metabolites that have the ability to tan or convert animal skin into leather. These compounds are classified as being water soluble phenolics with a molar mass between 300 and 3000, and with the ability to precipitate alkaloids, gelatins, and other proteins (Khanbabee and Van Tee, 2001). The classical division of tannins was based on their resistance or not, to hydrolysis in the presence of hot water or the enzymes tannases (which catalyze hydrolysis reactions among the digallates). Tannins (figure 2.16) are categorised according to their structural characteristics, into four major groups: Gallotannins, ellagitannins, complex tannins and condensed tannins (Okuda and Ito, 2011). Some tannins derivatives are patented for the treatment of inflammation and inflammation related or associated diseases or conditions, and for the relief of pain, in individuals who are sensitive to selective cyclooxygenase-2 (COX-2) inhibitors,or are sensitive to COX-nonselective nonsteroidal anti-inflammatory drugs (Kwik-Uribe, 2007; Schmitz, 2002.

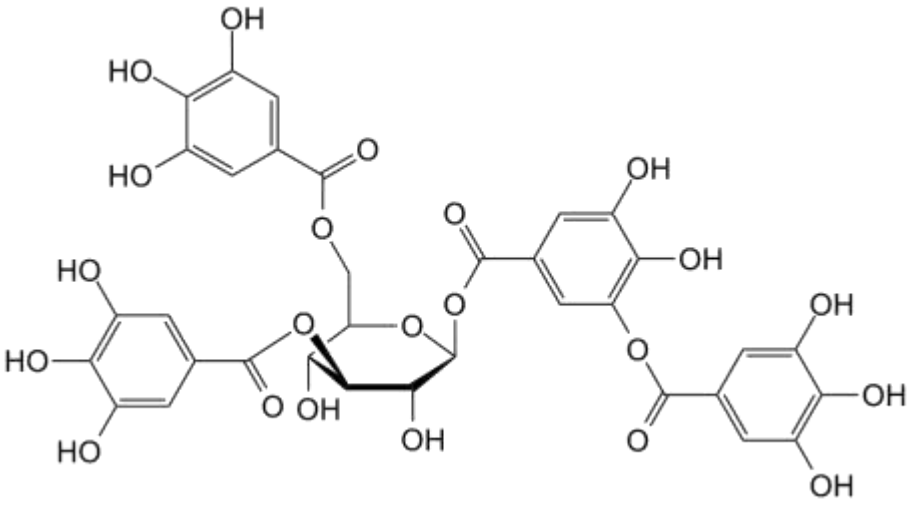


Figure 2.16 Generic and basic structure of a tannin molecule

https://www.google.com/search?q=Generic+and+basic+structure+of+a+tannin+molecule

2.7.2.6 Flavonoids

Flavonoids are phenolic compounds (figure 2.17) that continue to draw the interests of the scientific community because of their wide range bioactive properties. Flavonoids (figure 2.17) have an antioxidant, antiviral, anti-cancer, anti-allergic, antibacterial properties, among others (Formica and Regelson, 1995; Alvesalo *et al*., 2006). Apart from their physiological roles in the plants, flavonoids are important components of the human diet, although they are not considered as nutrients.

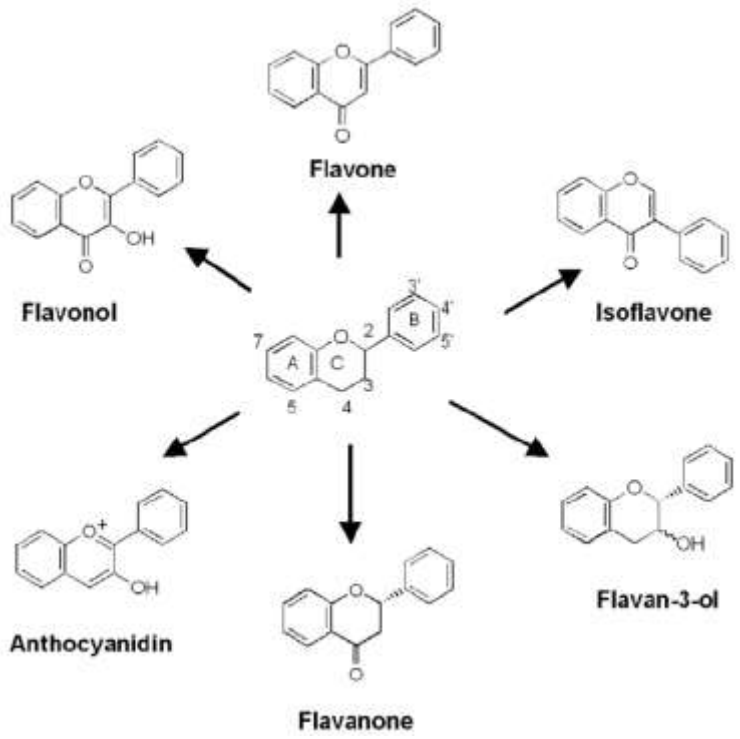


Figure 2.17: Structure of flavonoid molecules.

https://www.google.com/search?q=Generic+and+basic+structure+of+a+tflavonoid+molecule

2.7.2.7 Saponins

Saponins are a diverse group of compounds that are widely distributed in the plant kingdom, which are characterised by their structure containing a triterpene or steroid and one or more sugar chains (Figure 2.18). Their structural diversity is reflected in their physiochemical and biological properties, which are exploited in a number of traditional medicinal uses such as soaps, fish poison, and molluscicides and in industrial applications (Price *et al.,* 1987; Oakefull, 1981; Fenwick *et al*., 1991; Hostettmann and Marston, 1995; Oakenfull and Sidhu, 1989). Saponins are reported to have anti-inflammatory and antiviral activities (Chopra and Doiphode, 2002; Maurya *et al.,* 2008).

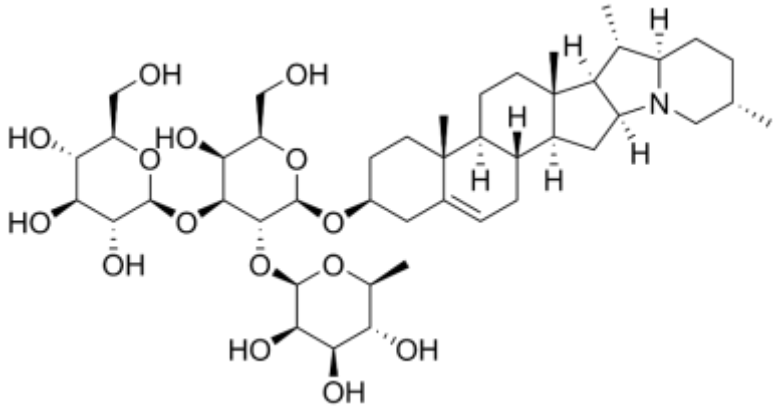


Figure 2.18: Generic and basic structure of a saponin molecule.

https://www.google.com/search?q=Generic+and+basic+structure+of+a+tsaponin+molecule

2.7.2.8 Steroids

Plant steroids are a unique class of chemical compounds that are found throughout the animal and plant kingdom. Steroids have the fundamental structure of four carbon rings called the steroid nucleus (figure 2.19). The addition of different chemical groups at different positions on backbone leads to the formation of many different types of steroidal compounds, including sex hormones; progesterone and testosterone, the antiinflammatory steroids like corticosteroids, cardiac steroids digoxin and digitoxin, animal steroid like cholesterol, steroidal glycosides. **(**Formica and Regelson, 1995; Alvesalo *et al*., 2006). Plant steroids possess many interesting medicinal, pharmaceutical and agrochemical activities like anti-tumor, immunosuppressive, hepato-protective, antibacterial, plant growth hormone regulator, sex hormone, anti-helminthic, cytotoxic and cardiotonic activity (Weber *et al*., 2003; Widlansky *et al.,* 2005).

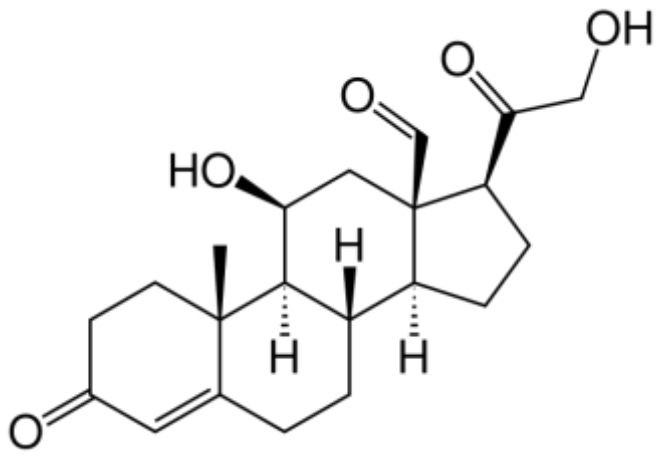


Figure 2.19: Generic and basic structure of a steroid molecule.

https://www.google.com/search?q=Generic+and+basic+structure+of+a+tsteroid+molecule

2.8 *Combretum* Plants Selected in the Study

Combretum is the largest and most widespread genus of Combretaceae. The genus comprises approximately 200-250 species being distributed throughout the tropical and subtropical regions mainly in Africa and Asia. *Combretum* plants have shown potential as a source of various secondary metabolites. Many of these indigenous plants are used as spices, medicine and food plants. Sometimes, they are also added to foods meant for pregnant and nursing mothers for medicinal purposes (Okwu, 1999; Okwu 2001).

2.8.1 *Combretum adenogonium*

C. adenogonium is a deciduous shrub or small tree growing to about 10 metres tall (figure 2.20). Leaves are usually arranged in whorls of 3 - 4, rarely opposite, ovate, ovate-elliptic, dark green, velvety when young, losing most hairs at maturity, 7-10 pairs of lateral veins prominent below; margin entire, sometimes wavy. Flowers are greenish cream to pale yellow, in dense, often branched spikes, appearing before or with the new leaves (http://tropical.theferns.info/viewtropical.php?id=Combretum+adenogonium). The fruit is ovoid 25 × 35 mm, 4-winged, yellow green flushed with reddish brown, drying to light brown. The plant is gathered from the wild for local use as a food and medicine, as well as for various commodities (Mmushi *et al.,* 2012) in the dry season, when the

trees are devoid of leaves, there is usually an intense burst of sweetly-scented yellow flowers, which attract many insects to the nectar.

The leaves are used medicinally for fevers, the branches and fruit are used to prepare an infusion in Liberia for washing the body to relieve pain. An infusion of the bark is taken with natron to relieve chest pains. The bark and roots provide a decoction used in treating abdominal pains and low backache (http://tropical.theferns.info/viewtropical.php?id=Combretum+adenogonium). The fresh roots, ground up and dried, are put on sores or prepared as a decoction for treating primary sores of syphilis. The wood is used as an incense and the smoke from the wood is used as a perfume (http://tropical.theferns.info/viewtropical.php?id=Combretum+adenogonium).

Figure 2.20: Depiction of the plant *Combretum adenogonium* showing the leaves.

2.8.2. Combretum apiculatum

C. apiculatum is a species of a tree in the family *Combretaceae* known by the common name red bushwillow (figure 2.21). It is native to the mesic to semi-arid savanna regions of Africa, southwards of the equator. This is a semi-deciduous tree growing up to 10 meters tall, or sometimes a shrub remaining shorter. It has a rough gray-black bark with fissures, and the smaller branches may be woolly in texture. The oppositely arranged leaves are up to 11 to 13 centimeters long (Schmidte, 2002). They are hairless or hairy. The tip of the leaf tapers abruptly to a twisted point. The foliage turns reddish or golden in the fall. The spike inflorescences emerge between the leaves and are up to 7 centimeters long. They bear yellow or greenish flowers with tiny sepals and petals, and with style and stamens about half a centimeter long (Van wyk and Van

wyk, 1997). The flowers have a strong scent. The reddish, winged fruit is 2 or 3 centimeters long (Coetes-Pelgrave, 2002). It occurs in South Africa, Swaziland, Botswana, Mozambique, Namibia, Zimbabwe, southern Angola, Zambia, Malawi, southeastern DRC, Tanzania and southern Kenya. This tree occurs in various ecosystems in southern Africa. It is the dominant tree on the savannah in many areas, including regions characterised as low-veld and mopane savannah (Van wyk and Van wyk, 1997). This tree has dense fine-grained, strong, dark brown to black heartwood, sometimes used as firewood or for making charcoal. It is hard, and termite-resistant. The tree responds well to coppicing, growing back with plentiful foliage. The bark has been used in leather tanning. Medicinal uses for the species include the treatment of conjunctivitis and stomach ailments. It contains a number of antioxidant compounds (Martini, 2001), such as cardamonin, pinocembrin, quercetin, and kaempferol. It is an appropriate garden tree, as it is tolerant of frost and drought and provides shade (Martini, 2001).

Figure 2.21: Depiction of the drought-tolerant plant *Combretum apiculatum*

2.8.3 *Combretum bracteosum*

C. bracteosum is a shrub (figure 2.22), which flowers during spring and summer, producing many deep orange to scarlet flowers in the form of densely packed racemes at branch tips. The stamens and styles protrude from the flower giving it a spike-like appearance (https://cjmgrowers.co.za/combretum-bracteosum/). It is different from other *Combretum* species in that its nut-like seed is wingless. The seed is coffee-coloured and looks like a small walnut. It is covered by a hard pericarp (I mm thick)

containing a softer fibrous inner lining (3 mm thick) which surrounds the embryo (Carr, 1988). The leaves contain antifungal, as well as cytotoxic compounds, and are used in traditional medicine to treat abdominal complaints, mental aberrations and skin disorders such as leprosy, snakebite, scorpion stings and brings relief from conjunctivitis. The roots, either pulverized or boiled, are taken to treat gonorrhoea, diarrhoea and infertility in women (https://cjmgrowers.co.za/combretum-bracteosum/).

Figure 2.22: Depiction of *Combretum bracteosum*, showing the leaves and fruit

2.8.4 Combretum caffrum

C. caffrum is found mainly in Eastern Cape and KwaZulu-Natal, South Africa. It is a deciduous, small to medium-sized spreading tree up to 10 meters in height; with young branches densely short-hairy, pinkish after shedding the bark. Leaves in whorls of 3, with no stipules, petiole long, densely short-hairy. The tree has bisexual flowers, regular, cream or yellow, usually congested; receptacle consisting of 2 parts, lower part 1.5–2.5 mm long, usually densely short-hairy (https://en.wikipedia.org/wiki/Combretum).

C. caffrum (Figure 2.23) occurs mainly along river and stream banks, in sand soil or alluvium, occasionally on hill and mountain slopes, in evergreen or coastal forest, from sea level up to 1100 m altitude, with at least 500 mm of annual rainfall. *C. caffrum* can also grow on degraded sandy, gravelly and even saline soil with good drainage. It tolerates rather dry conditions,but does not tolerate frost and waterlogging. Branch tips become damaged at temperatures below 5°C (https://en.wikipedia.org/wiki/Combretum).

The bark of *C. caffrum* is used in combination with a number of other herbs as an anti-cancer treatment (Katerere, 2001). The bark is widely used as a general tonic. A root decoction is added to bath water to treat general body pain. The Zulu people use the

plant extract as a spear poison. They also use the root bark as a charm against enemies (Cunningham, 1990). Leaf juice is used as eye drops to treat conjunctivitis in domestic animals. A stem bark decoction is given to cattle with red-water (babesiosis) disease (Cunningham, 1990). Honey of *C. caffrum* is very bitter, but no problems have been recorded from human consumption. The timber is yellow and dense and is used for poles and fuel wood. It is not very durable. The tree is left in the field as a shade tree. The bark leaves and roots of *C. caffrum* are commonly sold on local markets throughout South Africa. In South Africa, a formulation made from a bark decoction is produced commercially and sold as an anti-cancer remedy (https://en.wikipedia.org/wiki/Combretum). Studies have shown that this plant has antibacterial properties (Eloff, 1999).

Figure 2.23: Depiction of *Combretum caffrum*, showing the leaves and fruit

2.8.5 Combretum elaegnoides

Combretum elaegnoides, commonly known as Oleaster bush willow (figure 2.24) grows up to a height of 5 meters with light grey bark and many cream white to yellow flowers in spikes from spring to summer. The leaves opposite, rarely 3-whorled, narrowly elliptic, usually hairless but with minute silvery scales on both surfaces. Flowers in short, dense axillary spikes, creamy-white, flowering when the tree is leafless. Fruits 4-winged, 2-3.5 cm long, persitent on the tree (Michael, 2012). Studies have shown that the plant has antifungal properties and is traditionally used to treat malaria (Osborne and Pegel, 1984).

Figure 2.24 Depiction of *Combretum Elaegnoides* tree.

2.8.6 Combretum erythrophyllum

C. erythrophyllum (figure 2.25) is a member of Combretaceae family. The plant is widely used for the treatment of venereal diseases (Ruiters *et al.,* 2016). Roots are used as a purgative, while dried and powdered gum can be applied to sores (Sigidi *et al.,* 2016). *C. erythrophyllum* is widely distributed in the Southern Africa region, mostly found in South Africa along the coast in the Eastern Province, through Kwazulu- Natal. At Northern South Africa, this plant species is commonly found in Mpumalanga, Limpopo Province, Gauteng and the Eastern parts of North West, Zimbabwe, Swaziland and Mozambique and slightly into the eastern parts of Botswana (Ray *et al.,* 2016). Seven antibacterial phenolic compounds identified by Martini *et al.* (2010) including four flavonols, three flavones: 5, 7, 40-trihydroxyfavone (apigenin), 5, 40-dihydroxy-7-methoxyflavone (genkwanin) and 5-hydroxy-7, 40-dimethoxyflavone were isolated from *C. erythrophyllum.* The compounds exhibited good activity against *Vibrio cholera* and *Enterococcus faecalis.* Pharmacological studies conducted by different workers have revealed that *C. erythrophyllum* possesses antibacterial, antifungal, anti-inflammatory, genitor-urinary, cytotoxic and mutagenic properties (Martini *et al.*, 2010; Sigidi *et al.,* 2016).

Figure 2.25: Depiction of *Combretum erythrophyllum plant found on South Africa*

2.8.7 Combretum imberbe

C. imberbe is the largest sized tree of about 40 tree species in the *Combretum* genus occurring in southern Africa (Van Wyk, 1993). The growth form is generally small to large winter deciduous tree between 7 to 15 metres in height, and less commonly a shrub or multi-stemmed thicket in locations such as dry riverbeds (Coates Palgrave 1983; Viljoen and Bothma, 1990). *C. imberbe* (figure 2.26) has an average stem diameter of 16 cm (Clarke, 1997). It has a non-aggressive root system (Venter and Venter, 1996). The large taproots and well-developed lateral roots systems enable it to draw on ground water and to utilise the moisture held in surface soils after rains (Cole and Brown, 1976). Leaves are mainly oppositely arranged but may be in whorls of up to seven at the terminals of current laterals in axils of older growth. The bark of old trees is generally medium to light grey in colour on the main stem, and characteristically fissured longitudinally and split transversely to a depth of 5 to 10 mm (Carr, 1988).

C. imberbe is a commercially valuable species favoured primarily for its durable heartwood, which is much sought after in the woodcarving industry (Clarke, 1997). The wood is popular for sculpture- and lathe-work (Venter and Venter, 1996) and furniture production (Shackleton, 1993; Shackleton, 1998), the latter becoming increasing popular (Venter and Venter, 1996). In the Bushbuckridge District, the average woodcarver and furniture maker uses between 6 to 50 and 10 to 150 trees per annum, respectively, of several species, although the extent of species exploitation is still uncertain (Clarke, 1997). *C. imberbe* is considered an excellent fuelwood that

burns slowly with little smoke, but with a high calorific value (Liengme, 1983; Carr, 1988, McGregor, 1991; Venter and Venter, 1996).

C. imberbe is a preferred fuelwood species, among others, in the lowveld of the Limpopo Province of South Africa (Clarke, 1997), and in Zimbabwe (Sibanda, 1992). Fuelwood is used primarily for cooking, heating bath water and heating the home during winter (Mashabane *et al.,* 2001). In the low-veld, district of the Limpopo Province, South Africa, *C. imberbe* is mixed with other woods such as *Colophospermum mopane*, *Combretum* species, and *Grewia caffra*, among others, for cooking (Mashabane *et al.*, 2001).

Figure 2.26: Depiction of *Combretum imberbe* with image “A” showing the whole tree and image “B” depicting the fruit

2.8.8 *Combretum kraussii*

C. kraussii (figure 2.27) is indigenous to South Africa and is distributed on the coast through the midlands in the eastern regions and into neighbouring Swaziland. This forest plant can grow on rocky hillsides at altitudes up to 1,200 m. It grows in evergreen forests, forest margins or in dense woodland. It has adapted to wind dispersal by developing a wing-like fruit structure that can carry the seed with the help of air currents or wind. Some animals eat the fruit, which also helps with seed dispersal (http://pza.sanbi.org/combretum-kraussii).

Its range of seasonal features make this a good choice for the garden. In spring, it bears white flowers and an unusual flush of white leaves, the red fruit of late summer

is showy, and in winter, its leaves turn red before falling. The young stems are pliable and used in basket making. The wood is tough and yellowish in colour; the sawdust, however, can cause skin irritation. Certain parts from the tree are used to produce anti-diuretics, lotion for eye infections, as well as antiseptics (http://pza.sanbi.org/combretum-kraussii).

Figure 2.27: Depiction of plant *C. kraussii* with image "A" depicting branches and "B" depicting the fruit

2.8.9 *Combretum mkuzense*

C. mkuzense, commonly known as the Maputaland bush willow (figure 2.28), is a deciduous shrub or small tree that is native to a restricted area in the lowlands of eastern South Africa and southern Mozambique. It is similar in appearance to the large-fruited bush willow (https://en.wikipedia.org/wiki/Combretum_mkuzense). It is found at Maputaland to the eastern foothills of the Lobamba Mountains and in adjacent southern Mozambique. It occurs in closed woodland savannah and in sand forest, and is known from twelve locations. It has an extent of occurrence of 6,700 km², but is now declining, which is ascribed to clearing for subsistence crops and extraction of firewood (https://en.wikipedia.org/wiki/Combretum_mkuzense).

Figure 2.28: Depiction of the branches of Combretum mkuzense.

2.8.10 *Combretum padoides*

C. padoides is commonly known as Thicket bushwillow (figure 2.29), which is a South African indigenous shrub or small tree that grows up to 3 to 5 meters in height (https://toptropicals.com/catalog/uid/Combretum_padoides.htm). It has dark brownish grey rough bark and dull green leaves with yellowish veins. The plant grows in a range of habitats from muddy riverbanks to dry rocky hillsides. It possesses mostly opposite oval leaves that are carried on long slender branches. The tree flowers in profusion in mid-summer and the 4-winged fruits reach maturity from late summer to mid-winter (https://toptropicals.com/catalog/uid/Combretum_padoides.htm). Leaves of *C. padoides* have shown to have antimicrobial activity (Angeh, 2007).

Figure 2.29: Depiction of the plant *Combretum padoides*

2.8.11. Combretum vendae

C. vendae is a multi-stemmed, deciduous shrub of 1.5–3.0 m high, occasionally a small tree of 4–5 meters in height (figure 2.30). The main stems, widely spaced at ground level, arise from a substantial root system (http://pza.sanbi.org/combretum-vendae). The bark is smooth, with inconspicuous, longitudinal reticulation, medium grey with lighter grey areas arranged in circumferential bands. The leaves are simple, opposite, broadly elliptic to obovate, medium green above, grey-green below, velvety or hairless (http://pza.sanbi.org/combretum-vendae). The apex is rounded, forming a slender tip; the base is rounded to shallowly lobed; the margin is entire and the petiole 2 mm long. The upper surface is waxy; the lower surface has raised veining (Carr, 1988).

The fruits are green, flushed pink or red at first, but turn wine red later. They are produced in autumn and winter. *C. vendae* is found in the South African Province of Limpopo,and is confined in a relatively small area of the Soutpansberg, from the Blouberg in the west, through the Soutpansberg, to the Thengwe area in the east. The plants are found mainly on acidic, sandy soils derived from quartzitic sandstone. *C. vendae* is used for the treatment of problems relating to the eyes, for blood purification and to treat bacterial infections and oxidative related diseases by indigenous people of Venda (Komape *et al.,* 2014).

Figure 2.30. Depiction of the plant *Combretum vendae*, with image “A” depicting the fruit while “B” depicts the braches.

2.8.12 *Combretum zeyherii*

C. zeyherii has a rounded crown and is multi-stemmed (figure 2.31). It reaches a height of up to 15 meters but is usually less than this. The trunk has a diameter of up to 38 cm and it may be twisted. The branches are slender, situated low down on the trunk,

and may be reddish in colour. Stems are hairy and have a smooth and the whitish bark, which becomes rough and mottled with age. The leaves are elliptic, oblong or obovate. Most of the hair present on young leaves is lost and adult leaves are almost hairless and leathery. The leaves are up to 14 × 9 cm, which are the biggest of the indigenous *Combretum* genus. The flowers are densely arranged in groups of round axillary spikes that are up to 8 cm long. Flowers usually appear before or with the new leaves and may possess an unpleasant smell. The fruit are initially green and turn light golden brown**.** It is a dry, indehiscent winged fruit. This fruit is the largest of the indigenous members of the *Combretum* genus – reaching between 5 and 10 cm, each fruit contains a single seed. Although they are individually of low density for dispersal, they occur in such large numbers that cause the branches to bend. The plant occurs naturally in South Africa in the following provinces: KwaZulu-Natal, Mpumalanga, Limpopo, Gauteng and North West. They also occur in Swaziland, Botswana, Zimbabwe, Namibia and northern parts of Africa. They grow in poor, sandy soils like sand dunes, soils with a low pH and at medium to low altitudes in summer rainfall areas. The tree is drought resistant (Michael, 2012).

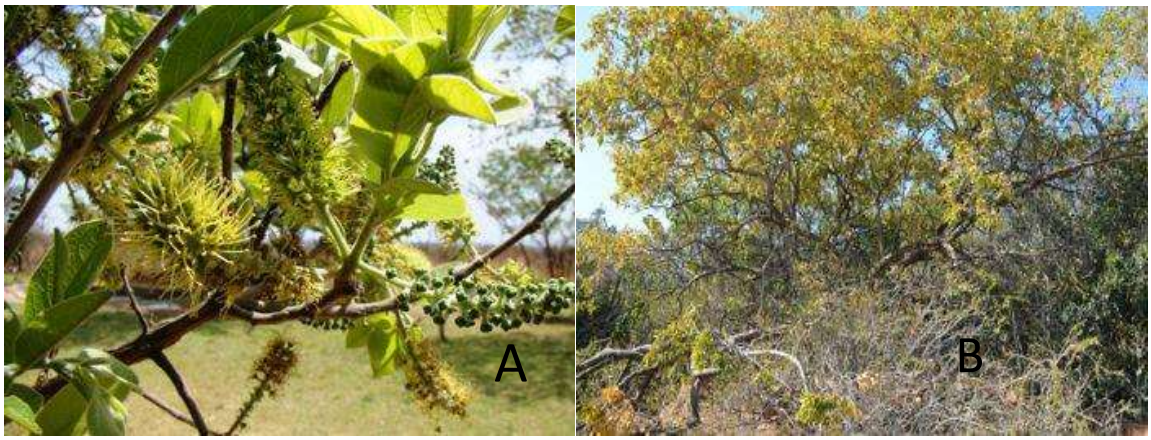


Figure 2.31: Depiction of the plant *Combretum zeyherii*, with image “A” depicting the fruit while “B” depicts the entire tree.

2.9 Wood Ash and Its Uses

Wood ash is the inorganic and organic residue remaining after the combustion of wood (Sidique, 2012). Because of the oxidation processes during combustion, the wood ash generated retains the overall composition of the mineral nutrients contained in the wood, with the exception of nitrogen compounds, which are mainly released into the gas phase. The physical and chemical composition of ash contents are variable

among tree species and depend on soil type and climate (Auta *et al.,* 2015). They vary significantly depending upon the method and manner of combustion, efficiency of the boiler, and other supplementary fuel used with wood. Wood ash is a by-product of wood burning, which is classed as a form of green energy production because it is both carbons neutral and renewable (Auta *et al.,* 2015). When wood is burned, the organic portion is converted to CO_2 and water while the inorganic portion remains as ash (Reimann *et al*., 2008). Wood-ash contains all the essential plant nutrients. Wood ash is a good source of potassium (K) ~5%, calcium (Ca) ~ 25%, phosphorous (P) ~2%, and magnesium (Mg) ~1%, which are essential plant nutrients. Crops respond positively to K and P from wood ash. Other micronutrients in wood ash include boron, copper, molybdenum, sulphur and zinc. In addition to the valuable mineral nutrients, wood-ash also contains heavy metals such as lead, zinc and cadmium.

Wood-ash is used widely across the globe for various purposes that range from washing cooking utensils, soap making, biodiesels, poultry and livestock feed processing, improving soil fertility. It is also used for food/seeds preservation, fermentation processes insecticide, pest control and seed treatment to increase yield, inhibition of growth of some phytopathogenic and mycotoxigenic fungi (Kyarisiima *et al.,* 2004; Moyin-Jesu 2012; Oyuntante and Adekunle, 2010;; Moyin-Jesu, 2012. Wood ash has been traditionally used as a grain protectant against storage pests in east and southern Africa (Golob *et al.,* 1982; Golob and Hanks, 1990; Katanga-Apuuli and Villet, 1996; Songa and Rono, 1998). In experiments carried out in Tanzania, treatment of maize with wood ash at between 5% and 30% w/w gave significant protection from damage by storage pests for up to 40 weeks (Golob and Hanks, 1990). Used alone, ash needs to be added at a level of at least 5% w/w of grain to provide effective protection against storage pests (Golob, 1997). In some areas, the ash is added to the hole-dug or pit toilets to reduce bad smell from the latrine. Additionally, ash is placed on the land as part of fertilizing the soil. Many African cultures believe that ash would stop hiccups in a person by tasting it two to three times with the tongue. In Namibia and the Northern part of Limpopo Province such as Venda, Giyani and Musina, ash is mixed with water; the mixture is given to a cow having problems of retaining the afterbirth. Wood ash is used as toothpaste. Some people use wood ash to whitewash their homes. In the northern rural areas of Mpumalanga Province, ash is also used as a replacement of liquid bath soaps to wash dishes and shine saucepans. The local

people of South-Western Uganda use it to reduce tannin content in the red, bird resistant sorghum that is cultivated in that region (Kyarisiima *et al*., 2004). This traditional technology involves soaking sorghum grain in wood ash slurry and then allowing it to germinate for four days.

2.10 Sensory Evaluation of Food Products

Sensory food science is a discipline dealing with human sensory perceptions of and affective responses to foods, beverages and their components. It is multidisciplinary by its nature, deriving research questions from food science and applying behavioural research methods to solve these questions. Sensory food science uses sensory evaluation as its central method of analysis. Sensory analysis involves the inspection of a product by the senses, i.e. sight, smell, taste, touch and hearing for various quality attributes like appearance, flavour, aroma, and texture (Chambers and Wolf, 1996; Kemp *et al.,* 2009; Kilcast, 2010; Hough, 2010).

Appearance is the first characteristic perceived by the human senses and plays an important role in the identification and final selection of food. This is the visual perception of food comprising colour, shape, size, gloss, dullness and transparency (Chambers and Wolf, 1996). The appearance of a meal has shown impact on appetite stimulation or depression resulting in pleasure or total depression. The look of a food or beverage influences acceptance before the product touches the lips. This is because people see with their eyes before they ever smell or taste food or beverage (Chambers and Wolf, 1996).

Flavour is a sensory phenomenon that is used to denote the sensations of odour, taste and mouthfeel. Flavouring substances are aromatic compounds that are conceived by the combination of taste and odour and perceived by the mouth and nose. Odour improves the delight of eating, e.g. aroma of freshly cooked rice and most of the baked products (Kemp *et al.,* 2009). Taste helps in the identification, acceptance and appreciation of food. The taste buds on the tongue perceive it. There are four types of taste perception: sweet, salty, sour and bitter. Sour and bitter are often confused. Lemon juice has a sour taste whereas coffee has a bitter taste. In case of mouthfeel, nerves present inside the mouth are enthused by chemical or thermal responses, e.g. coldness of ice cream or the fiery impression of pepper (Stone and Sidel, 1993).

Aroma is the first to be perceived before of taste. These are volatile compounds, which are perceived by the odour receptors of olfactory tissues of the nasal cavity. Aromatic compounds are released during the mastication process. Smell appraises the aroma of food that is important in appreciating flavour (Hough, 2010). A pleasant smell makes food delicious. To provoke a sensation of smell, the stuff must be in a gassy state. Furthermore, aroma is valuable in perceiving fresh, rancid or intermittently poisonous food (Lawless, 2013).

Texture is perceived by a combination of senses, i.e. touch, mouthfeel, sight and hearing. It is one of the most imperative feature of a food. Texture is prerequisite in the acceptance of numerous foodstuffs, e.g. tenderness of meat and softness of bread. It also includes the consistency, thickness, fragility, chewiness and the size and shape of particles in food (Kilcast, 2010).

Consumer testing methods described in the literature vary greatly. However, one common denominator of all methods is the use of some type of rating scale. The hedonic response scale is often used to assess the quality (Kemp *et al.,* 2009; Kilcast, 2010). The primary goal in designing scales for use with consumers has usually been to keep them easy to use and easily understandable by all. The most common examples of these scales are the nine-point hedonic scale and the relative to ideal or just right scales. Line scales have been used as hedonic scales (Pangborn *et al.,* 1989), or as just right scales but they can also be used to assess the intensity perceived by consumers for a specific attribute.

The 9-point hedonic scale

Since its development, the 9-point hedonic scale (Peryam and Girardot, 1952; Peryam and Pilgrim, 1957) has been the most commonly used scale for testing consumer preference and acceptability of foods. The 9-point hedonic scale (figure 2.32) is a balanced bipolar scale around neutral at the centre with four positive and four negative categories on each side. The categories are labelled with phrases representing various degrees of affect and those labels are arranged successively to suggest a single continuum of likes and dislikes (Peryam and Pilgrim, 1957). The descriptors are intended to help not only subjects to respond accordingly but also to help experimenters interpret the mean value of responses in terms of degree of liking/disliking. The primary reason for the wide acceptance of the 9-point hedonic

scale is that, compared to other scaling methods (e.g., magnitude estimation), its categorical nature and limited choices make it easy for both study participants and researchers to use. Its simplicity further makes the 9-point hedonic scale suitable for use by a wide range of populations without an extensive training (Lawless and Heymann, 1998). For researchers, data handling of the 9-point hedonic scale is also easier than other techniques, which require measuring lines or recording magnitude estimates that may include fractions, although this practical matter is of diminishing importance given the development of computerized programs (Lawless and Malone, 1986a, b). Therefore, when the primary concern of a study is measuring hedonic differences among foods, beverages, and consumer products and predicting their acceptance, the 9-point hedonic scale has proven itself to be a simple and effective measuring device.

Despite its wide use in the field of sensory science, various limitations of the 9-point hedonic scale have been reported. First, as noted above, due to its inequality of scale intervals and the lack of a zero point (Moskowitz 1971; Peryam and Pilgrim,

1957), the scale can yield only ordinal- or, at best, interval data (i.e., ordered metric). Thus, the scale cannot provide information about ratios of liking/disliking for stimuli (Moskowitz, 1971; Schutz and Cardello, 2001) or provide meaningful comparisons of hedonic perception between individuals and groups (Lim *et al.,* 2009). Nevertheless, this does not pose problems for measuring relative (ordinal) preferences among stimuli, which was its intended purpose. Second, due to its limited number of response categories, the 9-point hedonic scale offers little freedom for subjects to express the full range of their hedonic experiences (Marchisano *et al.,* 2003; Villanueva and Da Silva, 2009; Villegas-Ruiz, *et al.,* 2008). Third, because of both its small number of available categories and the general tendency of subjects to avoid using extreme categories (Hollingworth, 1910; Moskowitz, 1982; O'Mahony, 1982), the scale is highly vulnerable to ceiling effects (Schutz and Cardello, 2001; Stevens, 1957), one of the context effects that was described above (Section 2.3.4). The avoidance of the end categories effectively reduces the 9-point scale to a 7-point scale (Moskowitz, 1982; Moskowitz and Sidel, 1971) and limits its ability to discriminate among very well liked or very disliked stimuli (Lim and Fujimaru, 2010; Schutz and Cardello, 2001; Villanueva and Da Silva, 2009).

(a)

	FOOD ITEM	LIKE				INDIFFERENT	DISLIKE			
Not Tried	Cream Gravy	Like Extremely	Like Very Much	Like Moderately	Like Slightly	Neither Like Nor Dislike	Dislike Slightly	Dislike Moderately	Dislike Very Much	Dislike Extremely
Not Tried	Bread Pudding	Like Extremely	Like Very Much	Like Moderately	Like Slightly	Neither Like Nor Dislike	Dislike Slightly	Dislike Moderately	Dislike Very Much	Dislike Extremely
Not Tried	Cheese	Like Extremely	Like Very Much	Like Moderately	Like Slightly	Neither Like Nor Dislike	Dislike Slightly	Dislike Moderately	Dislike Very Much	Dislike Extremely
Not Tried	French Fried Onions	Like Extremely	Like Very Much	Like Moderately	Like Slightly	Neither Like Nor Dislike	Dislike Slightly	Dislike Moderately	Dislike Very Much	Dislike Extremely
Not Tried	Lettuce Wedges	Like Extremely	Like Very Much	Like Moderately	Like Slightly	Neither Like Nor Dislike	Dislike Slightly	Dislike Moderately	Dislike Very Much	Dislike Extremely

(b) Overall, how much do you like or dislike this juice sample?

Sample 351

- ☐ Like extremely
- ☐ Like very much
- ☐ Like moderately
- ☐ Like slightly
- ☐ Neither like nor dislike
- ☐ Dislike slightly
- ☐ Dislike moderately
- ☐ Dislike very much
- ☐ Dislike extremely

Figure 2.32 Example of the 9-point hedonic scale: (a) Questionnaire designed for studying soldier's preferences in the field (Peryam and Girardot, 1952); and (b) a sample ballot for a common consumer test used in a laboratory setting.

2.11. References

Abawari, R.A., 2013. Indigenous processing methods and raw material of *keribo*: an Ethiopian traditional fermented beverage. *Journal for Food Science of Animal Resources* 1:13–20.

Abdulmumeen HA, Risikat AN., and Sururah, A.R., 2012. Food: its preservatives, additives and applications. *International Journal of Chemical and Biochemical Science* 1:36-47. Academic Press.

Ahmad, I. and Beg, A.Z., 2001. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. Journal of ethnopharmacology, 74(2), pp.113-123.

Ahmad, I., Mehmood, Z., and Mohammad, F., 1998. Screening of some Indian medicinal plants for their antimicrobial properties. *Journal of Ethno Pharmacology* 62, 183–193.

Aka, S., Camara, F., Nanga, Y.Z., Loukou, Y.G., and Dje, K.N. 2008. Evaluation of organic acids and sugars contents during the production of *tchapalo* a traditional sorghum beer in Cote d'Ivorie. *Journal of Food Science and Technology* 6:189–95.

Aka, S., Konan, G., Fokou, G., Dje, K.M., and Bonfoh, B., 2014. Review on African traditional cereal beverages. *American Journal of Research Communication* 2:103–53.

Akinpelu, D.A., Aiyegoro, O.A., Akinpelu, O.F., and Okah, A.I., 2015. Stem bark extract and fraction of Persea americana (Mill) exhibits bactericidal activities against strains of Bacillus cereus associated with food poisoning. *Molecules* 20: 416–429.

Akinyemi, K.O., Oluwa, O.K., and Omomigbehin, E.O., 2006. Antimicrobial activity of crude extracts of three medicinal plants used in South-West Nigerian folk medicine on some food borne bacterial pathogens. *African Journal of Traditional, Complementary and Alternative Medicines* 3 (4): 13–22.

Alvesalo, J., Vuorela, H., Tammela, P., Leinonen, M., Saikku, P., and Vuorela, P., **2006.** Inhibitory effect of dietary phenolic compounds on *Chlamydia pneumoniae* in cell cultures. *Biochemistry and Pharmacology* 71:735–41.

Alzoreky, N.S., and Nakahara, K., 2003. Antibacterial activity of extracts from some edible plants commonly consumed in Asia. *International Journal of Food Microbiology* 80: 223–230.

Alzoreky, N.S., 2009. Antimicrobial activity of pomegranate (*Punica granatum L.)* fruit peels. *International Journal of Food Microbiology* 134: 244–248.

Amadou, I., Gbadamosi, O.S., and Le, G.W., 2011. Millet- based traditional processed foods and beverages. A review. *Cereal Foods World AACC International* 56:115–21.

Angeh, J.E., 2007. *Isolation and characterization of anti-bacterial compounds present in members of Combretum section, Hypocrateropsis* (Doctoral dissertation, University of Pretoria).

Anon, M.I., 1991. Food irradiation – a technique for preserving and improving the safety of food. WHO, Geneva.

Ash, C., Farrow, J.A., Dorsch, M., Stackbrandt, E., and Collins, M.D., 1991. Comparative analysis of Bacillus anthracis, Bacillus cereus, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. *International Journal of Systematic Bacteriology* 41:343–346.

Ateb, D.A., and Erdo_Urul, O.T., 2003. Antimicrobial activities of various medicinal and commercial plant extracts. *Turkish Journal of Biology* 27: 157–162

Auta, T., Otalu, E.J., and Hassan, A.T. Evaluation of chemical constituents in aqueous wood ash extracts of *Azadirachta indica* (neem) and *Parkia biglobosa* (locust bean). *Journal of Environmental Toxicology and Public Health* 1: 36-40.

Beaton, G.H., Swiss, L.D., 1974. Evaluation of the nutritional quality of food supplies: prediction of “desirable” or “safe” protein: calorie ratios. *American Journal of Clinical Nutrition* 27:485–504.

Bintsis, T., 2018. Lactic acid bacteria as starter cultures: an update in their metabolism and genetics. *AIMS Microbiology* 4: 665–684

Blanchard, S., and Thorson, J.S., 2006. Enzymatic tools for engineering natural product glycosylation. *Current Opinion in Chemical Biology* 10:263–71.

Blandino, A., Al-Aseeri, M.C., Pandiella, S., Cantero, D., and Webb, C., 2003. Cereal-based fermented foods and beverages. *Food Research International* 36:527–43.

Bruneton, J., 1999. Pharmacognosy. *Phytochemistry, medicinal plants*. 2nd Ed. Paris: Lavoisier Publishing; 1119.

Carr, T.P., Weller, C.L., Schlegel, V.L., Cuppett, S.L., Guderian, D.M., and Johnson, K.R., 2005. Grain sorghum lipid extract reduces cholesterol absorption and plasma non-HDL cholesterol concentration in hamsters. *Journal of Nutrition* 135: 2236–2240.

Centre for Disease Control and Prevention, (Anonymous)., 2003. Preliminary FoodNet data on the incidence of foodborne illnesses – selected sites, United States, 2002. *MMWR*, 52, 15, 340–343.

Chambers, E., M.B., Wolf., 1996. Sensory testing methods. 2nd edition. ASTM International West Conshohocken, PA, USA.

Chen et al., 2012.???

Chopra, A., and Doiphode. V.V., 2002. Ayurvedic medicine: core concept, therapeutic principles, and current relevance. *Medical Clinics of North America*, 86: 75–89.

Clarke, A.B., 1997. Sustainability of harvesting seven favoured plant species used in the indigenous wood carving industry in the Bushbuckridge district of the Limpopo Province Lowveld. Honours dissertation, University of the Witwatersrand, Johannesburg. 82.

Coates, P.K., **1983.** Trees of southern Africa. 2nd edition. Struik Publishers (Pty) Ltd, Cape Town.

Cole, M.M., and Brown, R.C., 1976. The vegetation of the Ghanzi area of western Botswana. *Journal of Biogeography* 3:169–196.

Cunningham, A.B., 1990. People and medicines: the exploitation and conservation of traditional Zulu medicinal plants. In: Ihlenfeldt, H.-D. (Ed.). Proceedings of the twelfth plenary meeting of AETFAT, 4–10 September 1988, Hamburg, Germany. Mitteilungen aus dem Institut für Allgemeine Botanik Hamburg. 23b: 979–990.

Delgado, B., Palop, A., Fernandez, P.S., and Periago, P.M., 2004. Combined effect of thymol and cymene to control the growth of Bacillus cereus vegetative cells. *The Journal European Food Research and Technology* 218, 188–193.

Dey, A., Seshasayee, D., Noubade, R., French, D.M., Liu, J., Chaurushiya, M.S., Kirkpatrick, D.S., Pham, V.C., Lill, J.R., Bakalarski, C.E. and Wu, J., 2012. Loss of the tumor suppressor BAP1 causes myeloid transformation. Science, 337(6101), pp.1541-1546.

Dhawale, P.G. 2013. Phytochemical Analysis of some medicinal plants from Yanatmal District (Ms) India. *The International Journal of Engineering and Science* 2 (1): 65–66.

Diekema, D.J., Pfaller, M.A., Jones, R.N. Doern, G.V. Winokur, P.L., Gales, A.C. Sader, H.S., and Kugler, K., 1999. Survey of bloodstream infections due to gram-negative bacilli: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, and Latin America for the SENTRY antimicrobial surveillance program, 1997. *Clinical Infectious Disease* 29, 595–607.

Doyle, M.P., 1989. Foodborne bacterial pathogens, Marcel Dekker Inc., New York, Basel. [A book giving detailed description of pathogens occurring on/in foods, including characteristics of microorganisms and diseases caused and control measures].

Drager, B., 2002. Analysis of tropane and related alkaloids. *Journal of Chromatography* 978:135.

Duman, A., Ozgen, M., Dayisoylu, K., Erbil, N., and Durgac, C., 2009. Antimicrobial activity of six pomegranate (*Punica granatum* L.) varieties and their relation to some of their pomological and phytonutrient characteristics. *Molecules* 14(5): 1808- 1817.

Edeoga, H.O., Okwu, D.E., and Mbacble, B.O., 2005. Phytochemical constituents of some Nigerian medicinal plants. *African Journal of Biotechnology* 4(7): 685–688.

Edmond, M.B., Wallace, S.E., McClish, D.K., Pfaller, M.A, Jones, R.N., and Wenzel, R.P., 1999. Nosocomial bloodstream infections in United States hospitals: a three-year analysis. *Clinical Infectious Disease* 29, 239–244.

Ehling-Schulz, M., Guinebretière, M., Monthan, A., Berge, O., Fricker, M., and Svensson, B. 2006. Toxin gene profiling of enterotoxic and emetic Bacillus cereus. *FEMS Microbiology Letters* 260(2):232–240.

Elhkim, M.O., Heraud, F., Bemrah, N., Tanaka T., and Ogata, A., 2007. New consideration regarding the risk assessment, intolerance reactions and maximum theoretical daily intake in France. *Regulatory Toxico Pharma* 43(3):308-16.

Evans, W.C., and Trease, E., 2009. Pharmacognosy. 16th edition. London: Saunders Elsevier: 385.

Ezekiel, C.N, Abia, W.A., Ogara, I.M., Sulyok, M., Warth, B., and Krska, R., 2015. Fate of mycotoxins in 2 popular traditional cereal-based beverages (kunu-zaki and pito) from rural Nigeria. *LWT Food Science and Technology* 60:137–41.

FDA., 2012. Bad bug book: Foodborne pathogenic microorganisms and natural toxins handbook, 2nd ed. US Food and Drug Administration, Silver Spring, 93-96.http://www.fda.gov/Food/FoodborneIllnessContaminants/CausesOfIllnessBadBug Book/ucm2 006773.htm.

Fenwick, G. R., Price, K.R., Tsukamoto, C., and Okubo, K., 1991. Saponins. In: J.P.F. D'Mello, C.M. Duffus, and J.H. Duffus, Eds. *Toxic Substances in Crop Plants*. The Royal Society of Chemistry, Cambridge. 285–327.

Formica, J.V., and Regelson, W., 1995. Review of the biology of quercetin and related bioflavonoids. *Food Chemistry and Toxicology* 33:1061–80.

Gadaga, T.H., Lehola, M., and Ntuli, V., 2013. Traditional fermented foods of Lesotho. *Journal of Microbiology, Biotechnology and Food Sciences* 2:2387–2391.

Gaffa, T., Jideani, I.A., and Nkama, I., 2002. Traditional production, consumption and storage of *kunu* – a non-alcoholic cereal beverage. *Plant Foods for Human Nutrition* 57:73–81.

Gamage, S.D., Strasser, J.E., Chalk C.L., and Weiss, A.A., 2003. Non-pathogenic *Escherichia coli* can contribute to the production of Shiga toxin. *Infection and Immunity Journal* 71: 3107–3015.

Gaze, J.E., 1985. The effect of oil on the heat resistant of *Staphylococcus aureus. Food Microbiology* 2:277–283.

Gensi, R.M., Kyamuhangire, W., and Carasco, J.F., 2000. Traditional production method and storage characteristics for banana beer (*tonto*) in Uganda. *Acta Horticulture* 540: 569–74.

Golob, P., 1997. Status and future perspectives for inert dusts for control of stored product insects. *Journal of Stored Products Research* 33: 69–79.

Golob, P., and Hanks, C., 1990. Protection of farm stored maize against infestation by *Prostephanus truncatus* (Horn) and *Sitophilus species* in Tanzania. J*ournal of Stored Products Research* 26, 187–198.

Golob, P., Mwambula, J., Mhango, V., and Ngulube, F., 1982. The use of locally available materials as protectants of maize grain against insect infestation during storage in Malawi. *Journal of Stored Products Research* 18: 67–74.

Gupta, R.N., Kartik, V., Manoj, P., Singh, P.S., and Alka, G., 2010. Antibacterial activities of ethanolic extracts of plants used in flok medicine. *International Journal of Research in Ayurveda and Pharmacy* (2), 529–535.

Halasz, A., Lasztity, R., Abonyi, T. and Bata, A., 2009. Decontamination of mycotoxin-containing food and feed by biodegradation. *Food Reviews International*, *25*(4),284-298.

Haslam, E., Lilley, T.H., Ya Cai, R., and Magnolato, M. 1989. Traditional herbal medicine. The role of polyphenols*. Planta Medica* 55: 1-8.

Hati, S., Mandal, S., and Prajapat, J.B., 2013 Novel Starters for Value Added Fermented Dairy Products. *Current Research in Food Science* 1: 83–91.

Hocking, A.D. and Jensen, N., 2001. Soft drinks, cordials, juices, bottled water and related products. Spoilage of processed foods: causes and diagnosis, .84-100.

Hollingworth, H. L., 1910. The central tendency of judgment. *Journal of Philosophical and Psychological Science Methods* 7:461–469.

Hostettmann, K., and Marston, A., 1995. *Saponins*. Cambridge University Press, Cambridge, New York.

https://www.researchgate.net/publication/318434143/figure/fig2/AS:8644125629030.

https://image.shutterstock.com/image-photo/colonies-yeasts-molds-fungal-testing.

https://mouldcleaningaustralia.com.au/wp-content/uploads/2019/10/Mold-768x384.

https://www.researchgate.net/profile/JoginderDuhan/publication/260694753/figure/fig

https://www.google.com/search?q=Generic+and+basic+structure+of+a+carbohydrate+molecule

https://www.google.com/search?q=Generic+and+basic+structure+of+a+sugar+molecule

https://www.google.com/search?q=Generic+and+basic+structure+of+a+phenolic+molecule

https://www.google.com/search?q=Generic+and+basic+structure+of+a+terpenoids+molecule&tbm

https://www.google.com/search?q=Generic+and+basic+structure+of+a+terpenoids+molecule&tbm

https://www.google.com/search?q=Generic+and+basic+structure+of+a+tannin+molecule

Hui Y.H., Goddick L.M., Hansen A.S., Josephsen J., Wai-Kit-Nip, Stanfield P.S., and Toldra, F., 2004. Handbook of food and beverage fermentation technology. CRC Press, Boca Raton. [An overview of microbiology and technology of production of fermented foods and beverages including alcoholic beverages, dairy, meat, bakery soy and vegetable food products].

Hwang, C., and Huang L. 2014. The effect of potassium sorbate and pH on the growth of *Listeria monocytogenes* in ham salad. *The Journal of Food Processing and Preservation* 38:1511–1516.

Jenson, M.C.J., 2003. *Bacillus cereus* and other Bacillus species. Ch 14 In: Hocking AD (Ed) *Foodborne microorganisms of public health significance*. 6th ed. Australian Institute of Food Science and Technology (NSW Branch), Sydney, pp. 445–478.

Jenson, I. and Moir, C.J., 2003. Bacillus cereus and other Bacillus species. Foodborne microorganisms of public health significance, (Ed. 6), 445-478.

Jha, K.H., Taneja, A., Kabra, K.K., and Sadiq, H.M., 2013. A study on consumer awareness, safety perceptions and practices about food preservatives and flavoring agents used in packed/canned foods from South India. National. *Journal of Community Medicine* 4(3):402-406.

Kanyana, I., Ouma, E., and Van Asten, P., 2013. Quality assessment of banana juice and beer in Rwanda. *Journal of Food Technology* 11:38–43.

Kar, A., 2003. Pharmacognosy and pharmaco-biotechnology. New Delhi: New Age International Ltd.: 436–448.

Katanga-Apuuli, J.K., and Villet, M.H., 1996. The use of wood ash for the protection of stored cowpea seed (*Vigna unguiculata* (L.) Walp.) against *Bruchidae (Coleoptera*). *African Entomology* 4: 97–99.

Katerere, D.R.P., 2001. Phytochemical and pharmacological studies of African Combretaceae. PhD Thesis. University of Strathclyde, Glasgow, United Kingdom.

Kemp, S.E., Hollowood, T., and Hort., J., 2009. Sensory evaluation: a practical handbook. John Wiley & Sons Ltd, Chichester, West Sussex, U.K.

Khambaty, F.M., Bennett, R.W., and Shah, D.B. 1994. Application of pulsed-field gel electrophoresis to the epidemiological characterization of Staphylococcus intermedius implicated in a food related outbreak. *Epidemiology and Infection* 113:75–81.

Khanbabaee, K., and Van Ree, T., 2001. Tannins: classification and definition. *Natural Product Reports*, 18:641–649.

Khandare, N.A., 2012. Qualitative poytopchemical analysis of ethanomedicinally important plant Cappairis Aphyla Roth (Capparidaceae) from Akola District, Maharashtra, India. *International Research Journal of Pharmacy* 3(4): 206–207.

Kilcast, D., 2010. Sensory analysis for food and beverage quality control: a practical guide. Wood head Publishing Limited, Abington Hall, Great Abington, Cambridge, U.K.

Kinderlerer, J.L., and Hatton P. 1990. Fungal metabolites of sorbic acid. *Food Additives & Contaminants*, *7*(5): 657-669.

Kokoska, L., Polesny, Z., Rada, V., Nepovim, A., and Vanek, T., 2002. Screening of some Siberian medicinal plants for antimicrobial activity. *Journal of Ethno Pharmacology* 82: 51– 53.

Komape, N.P.M., Aderogba, M., Bagla, V.P., Masoko, P., and Eloff, J.N., 2014. Anti-bacterial and anti-oxidant activities of leaf extracts of *Combretum vendae (Combretacea)* and the isolation of an anti-bacterial compound. *African Journal of Traditional Complementary Alternative Medicines* 11(5): 73– 77.

Kwik-Uribe, C.L., and Schmitz, H.H., 2007. Patent no: WO2007053641 A2.

Kyarisiima, C.C., Okot, M.W., and Svihus, B., 2004. Use of wood ash in the treatment of high tannin sorghum for poultry feeding. *South African Journal of Science* 34(2).

Labuza, T.P., 2000. The search for shelf life. *Food Testing and Analysis*: 26–36.

Lawless, H.T., and Malone, G.J., 1986a. The discriminative efficiency of common scaling methods. *Journal of Sensory Studies* 1(1): 85–98.

Lawless, H.T., and Malone, G.J., 1986b. A comparison of rating scales: sensitivity, replicates and relative measurement. *Journal of Sensory Studies* 1(2): 155–174.

Lawless, H. T., and H. Heymann. 1998. Pages 606–608 in Sensory Evaluation of Food: Principles and Practices. Chapman & Hall, New York, NY

Lawless, H.T., 2013. Laboratory exercises for sensory evaluation. Springer, New York.

Le Loir, Y., Baron, F., and Gautier, M., 2003. *Staphylococcus aureus* and food poisoning. *Genetics and Molecular Research* 2(1):63–76.

Liengme, C.A., 1983. A study of wood use for fuel and building in an area of Gazankulu. *Bothalia* 14:245–257.

Lim, J., and Fujimaru, T. 2010. Evaluation of the labelled hedonic scale under different experimental conditions. *Food Quality and Preference* 21: 521–530.

Lim, J., Wood, A., and Green, B.G., 2009. Derivation and evaluation of a labelled hedonic scale. *Chemical Senses* 34: 739–751.

Lodder, J., 1970. The yeasts: a taxonomic study. 2nd edition. North-Holland Publishing Co., Amsterdam, the Netherlands.

Madigan, M.T., Martinko, J.M., Stahl, D.A. and Clark, D.P., 2009. Brock Biology of microorganisms.

Mahboubi, A., Asgarpanah, J., Sadaghiqani, P.N., and Faizi, M., 2015*.* Total phenolic and flavonoid content and antibacterial activity of *Punica granatum L.* Var. pleniflora flower (Golnar) against bacterial strains causing food borne diseases. *BMC Complementary Medicine and Therapies* 15: 366–373.

Marchisano, C., Lim, J., Cho, H.S., Suh, D.S., Jeon, S.Y., and Kim, K.O., 2003. Consumers report preference when they should not: a cross-cultural study. *Journal of Sensory Studies* 18: 487–516.

Martini, N.D., 2001. The isolation and characterisation of antibacterial compounds from *Combretum erythrophyllum* [burch.] PhD Dissertation submitted to the Faculty of Health Sciences, Department of Pharmacology, and University of Pretoria, South Africa.

Martini, M., Ericson, M., Chanfray, G. and Marteau, J., 2010. Neutrino and antineutrino quasielastic interactions with nuclei. Physical Review C, 81(4), p.045502.

Mashabane, L.G., Wessels, D.C.J., and Potgieter, M.J., 2001. The utilisation of Colophospermum mopane by the Vatsonga in the Gazankulu region (eastern Limpopo Province, South Africa). *South African Journal of Botany* 67: 199-205.

Masibo, M., He, Q 2008. Comprehensive reviews in food science and food safety 7: 309–319.

Masoko, P., Picard, J., and Eloff, J.N., 2007. The antifungal activity of twenty-four southern African *Combretum* species (*Combretaceae)*. *South African Journal of Botany* 73:173–183.

McCann, D., Barrett, A., Cooper, A., Crumpler, D., Dalen, L., Grimshaw, K., Kitchin, E., Lok, K., Porteous, L., Prince, E., Sonuga-Barke, E., Warner., and Stevenson, J.O., 2007. Journal of food additives and hyperactive behaviour in 3-year-old and 8/9-year-old children in the community: a randomised, double-blinded, placebo-controlled trial. *The Lancet* 370(9598):1560-67.

McGregor, J. 1991. Woodland resources; ecology, policy and ideology: an historical case study of woodland use in the Shurugwi Communal Area, Zimbabwe. Unpublished PhD thesis, Loughborough University of Technology.

Michael, C.H., 2012. Kunene River. Topic ed. P. Saundry. Encyclopedia of Earth. National Council for Science and the Environment. Washington DC.

Mirza, S.K., Asema, U.K., and Kasim, S.S., 2017. To study the harmful effects of food preservatives on human health. *Journal of Medicinal Chemistry* 2(2):610-616.

Mishra, S., and Mishra, H.N., 2012. Technological aspects of probiotic functional food development. *Nutrafoods* 11:17–130.

Misselwitz, J., Karch, H., Bielazewska M, John U, Ringelmann F, Rönnefarth G., and Patzer, L., 2003. Cluster of haemolytic-uremic syndrome caused by Shiga toxin-producing *Escherichia coli* O26:H11. *The Paediatric Infectious Disease Journal* 22 (4): 349–354.

Mokoena, M.P., 2017. Lactic acid bacteria and their bacteriocins: classification, biosynthesis and applications against uropathogens: a mini-review. *Molecules* 22: 125511.

Montville, T.J., and Matthews, K.R., 2005. Food microbiology: an introduction. ASM Press, Washington D.C.

Montville TJ, Matthews KR. 2008. Food microbiology: an introduction. 2nd ed. Washington, USA: ASM Press\

.**Moskowitz, H.R., 1982**. Utilitarian benefits of magnitude estimation scaling for testing product acceptability. In J.T. kuznicki, R.A. Johnson and A.F. Rutkiewic (Eds.), *Selected sensory methods: problems and approaches to measuring hedonics, ASTM STP 773*. Philadelphia, PA: American society for testing and materials.

Moyin-Jesu, 2010.???

Moyin-Jesu, E.I., 2012. Comparative evaluation of modified neem leaf, neem leaf and woodash extracts on soil fertility improvement, growth and yields of maize (Zea mays L.) and watermelon (Citrullus lanatus) (sole and intercrop). *Journal of Agricultural Science* 1:90-97.

Mu Ashekele, H., Embashu, W., and Cheikhyoussef, A., 2012. Indigenous knowledge system best practice from Namibia: the case of *Oshikundu* processing methods. *Trends Applied Sciences* 7:913–21.

Murata, T., Iida, T., Tagomori, K., Akeda, Y., Yanagihara, I., Mushiake, S., Ishiguro, F., and Honda, T., 2001. A large outbreak of foodborne infection attributed to *Providencia alcalifaciens. Journal of the Infectious Diseases* 84(8): 1050–1055.

Muyanja, C.M.B.K., Narvhus, J.A., and Langsrud, T., 2012. Organic acids and volatile organic compounds produced during traditional and starter culture

fermentation of bushera, a Ugandan fermented cereal beverage, food Biotechnology 1: 26–28.

Muyanja, C.M.B.K., Narvhus, J.A., Treimo, J., and Langsrud, T., 2003. Isolation, characterisation and identification of Lactic acid bacteria from *bushera*: A Ugandan traditional fermented beverage. *International Journal of Food Microbiology* 80:201–10.

Naimi, T.S., Wicklud, J.H., Olsen, S.J, Krause, G, Wells, J.G, Bartkus, J.M., Boxrud D.J., Sullivan, M., Kassenborg, H., Besser, J.M, Mintz, E.D., Osterholm, M.T., and Hedberg, C.W. 2003. Concurrent outbreaks of *Shigella sonnei* and enterotoxigenic *Escherichia coli* infections associated with parsley: implications for surveillance and control of foodborne illness. *Journal of Food Protection* 66 (4): 535–541.

Naz, S., Siddiqi, R., Ahmad, S., Rasool, S.A., Sayeed, S.A., 2007. Antibacterial activity directed isolation of compounds from Punica granatum. *Journal of Food Science and Nutrition* 72, 341–349.

Negi, P.S., and Jayaprakasha, G.K., 2003. Antioxidant and antibacterial activities of Punica granatum peel extracts. *Journal of Food Science and Nutrition* 68: 1473–1477.

Nevas, M., Korhonen, A.R., Lindström, M., Turkki, P. and Korkeala, H., 2004. Antibacterial efficiency of Finnish spice essential oils against pathogenic and spoilage bacteria. Journal of food protection, 67(1), pp.199-202.

Ni, K., Wang, Y., Li, D., Cai, Y., Pang, H., 2015. Characterization, identification and application of lactic acid bacteria isolated from forage paddy rice silage. *PLoS ONE*, 10, e0121967.

Nikander, P., Seppala, T., Kilonzo, G.P., Huttunen, P., Saarinen, L., Kilima, E., and Pitkanen, T., 1991. Ingredients and contaminants of traditional alcoholic beverages in Tanzania. *Transact Royal Society of Tropical Medicine and Hygiene* 85:133–135.

Nuamsetti, T., Dechayuenyong, P., Tantipailbulvut, S., 2012. Antibacterial activity of pomegranate fruit peels and arils. *Journal of the Science Society of Thailand* 38: 319–322.

O'Hara, C.M., Brenner, F.W., and Miller, J.M., 2000. Classification, identification, and clinical significance of *Proteus, Providencia, and Morganella. Clinical Microbiology Review* 13(4): 534–546.

Oakenfull, D., and Sidhu, G.S. 1989. Saponins. In: Cheeke, P.R. (ed.), *Toxicants of plant origin, Vol II Glycosides*. CRC. Boca Raton, Florida. 97–141.

Obahiagbon, F.I., 2009. A review of origin, morphology, cultivation, economic products, health, and physiological implications of *Raphia* palm. *African Journal of Food Science* 3:447–53.

Oguntade, T.O., and Adekunle, A.A., 2010. Preservation of seeds against fungi using wood-ash of some tropical forest trees in Nigeria. *African Journal of Microbiology Research* 4(4): 279-288.

Okuda, T., and Ito, H., 2011. Tannins of constant structure in medicinal and food plants hydrolyzable tannins and polyphenols related to tannins. *Molecules* 16:2191–217.

Okwu, D.E., 1999. Flavouring properties of spices on cassava Fulu. *African Journal of Root Tuber Crops* 3(2): 19–21.

Okwu, D.E., 2001. Evaluation of the chemical composition of indigenous spices flavouring agents. *Global Journal of Pure and Applied Science* 7(3): 455–459.

Onuoha, O.G., Haruna, U.S., Yelmi, B.M., Samuel, E., Uhiara, N.S., and Ngwu, P.C., 2014. Storage study on color retention in *zobo* concentrates by increasing concentration of ginger (*Zingiber officinale)*. *African Journal of Food Science* 8:292–6.

Özcan, M. and Erkmen, O., 2001. Antimicrobial activity of the essential oils of Turkish plant spices. European Food Research and Technology, 212(6), pp.658-660.

Ozcan, T., Akpinar-Bayizit, A., Yilmaz-Ersan, L., and Delikanli, B., 2014. *International Journal of Chemical Engineering and Applications* 5: 393–396.

Pandey, K.B. and Rizvi, S.I., 2009. Plant polyphenols as dietary antioxidants in human health and disease. Oxidative medicine and cellular longevity, 2(5), pp.270-278.

PANGBORN, R.M., GUINARD, J.X. and MEISELMAN, H.L., 1989. Evaluation of bitterness of caffeine in hot chocolate drink by category, graphic, and ratio scaling. Journal of Sensory Studies, 4(1), pp.31-53.

Parekh, J. and Chanda, S., 2007. Antibacterial and phytochemical studies on twelve species of Indian medicinal plants. African Journal of Biomedical Research, 10(2).

Parvez, S., Malik, K.A., Ah Kang, S., and Kim, H.Y., 2006. Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology* 100: 1171–1185.

Perez, R.H., Zendo, T. and Sonomoto, K. 2014. Novel bacteriocins from lactic acid bacteria (LAB), various structures and applications. Microbiology, Cell Factories 13 (Suppl. 1), S3.

Peryam, D. R., Girardot, N. F. 1952. Advanced taste-test method. *Food Engineering* 24: 58–61.

Peryam, D.R., and Pilgrim, F.J., 1957. Hedonic scale method of measuring food preference. *Food Technology* 11:9–14.

Pirbalouti, A.G., Jahanbazi, P., Enteshari, S., Malekpoor, F., and Hamedi, B., 2010. Antimicrobial activity of some Iranian medicinal plants. Archives of Biological Sciences. *Belgrade* 62 (3): 633–642.

Prashanth, D.J., Asha, M.K., and Amit, A., 2001. Antibacterial activity of *Punica granatum*. *Fitoterapia* 72: 171–173.

Price, K.R., Johnson, I.T., and Fenwick, G.R., 1987. The chemistry and biological significance of saponins in foods and feeding stuffs. *Critical Reviews in Food Science and Nutrition* 26:27–135.

Quinto, E.J., Jimenez, P., Caro, I., Tejero, J., Mateo, J., and Girbes, T., 2014. Probiotic lactic acid bacteria: A review. Food and Nutrition Sciences 5: 1765–1775.

Rahman, 2004.???

Rajkowski, K.T., and Bennett, R.W., 2003. *Bacillus cereus*. In: Miliotis MD, Bier JW (eds.), *International handbook of foodborne pathogens*. Marcel Dekker, New York, 27–39.

Rasko, D.A., Ravel, J., Okstad, O.A., Helgasen, E., Cer, R.Z., Jiang, L., Shores, K.A., Fouts, D.E., Tourasse, N.J., Angiuoli, S.V., Kolonay, J., Nelson, W.C., Kolsto, A., Fraser, C.M., and Read, T.D., 2004. The genome sequence of *Bacillus cereus* ATCC10987 reveals metabolic adaptations and a large plasmid related to *Bacillus anthracis* pXO1. *Nucleic acids Research* 32(3):977–988.

Ray, I., Wren, B.T., and Bowers, E.J., 2016. Documentation of plant consumption by Galago moholi in South Africa. *Journal of African Primates* 11(1): 45–48.

Reimann, C., Ottesen, R.T., and Andersson., 2008. Element levels in birch and spruce wood ashes – green energy. *Science of the Total Environment* 393(2-3):191-197.

Riley, L.W., Remis, R.S., Helgerson, S.D., McGee, H.B., Wells, J.G., Davis, B.R., Hebert, R.J., Olcott, E.S., Johnson, L.M., Hargrett, N.T., Blake, P.A., and Cohen, M.L., 1983. Haemorrhagic colitis associated with a rare *Escherichia coli* serotype. The New England Journal of Medicine 308: 681–685.

Rios, J.L., and Recio, M.C., 2005. Medicinal plants and antimicrobial activity*. Journal of Ethno Pharmacology* 4: 80–100.

Ruiters, A.K., Gericke, N., Stander, M., and Van Wyk, B.E., 2016. The apocynaceae as a major source of functional foods in Southern Africa. *Planta Medica* 81(S 01): S1-S381.

Sadeghian et al., 2011

Salminen, S., Bouley, C., Boutron-Ruault, M.C., Cummings, J.H., Franck, A., Gibson, G.R., Isolauri, E., Moreau, M.C., Roberfroid, M., and Rowland, I., 1998. Functional food science and gastrointestinal physiology and function. *British Journal of Nutrition* 80 (Suppl. 1): S147–S171.

Sapkota, R., Dasgupta, R., and Nancy, D.S., 2012. Antibacterial effects of plants extracts on human microbial pathogens & microbial limit tests. *International Journal of Research in Pharmacy and Chemistry* 2 (4): 926–936.

Sarkar, S., Saha, S., Rai. C., and Bhattacharyya, S., 2014. Effect of storage and preservatives on antioxidant status of some refrigerated fruit juices. *International Journal of Current Microbiology and Applied Sciences* 3(7):1007-1013.

Schmidt, E., 2002. Trees and shrubs of Mpumalanga and Kruger National Park. Jacana Media. 2002. 456. *Combretum apiculatum*. (http://apps.kew.org/efloras/namedetail.do?flora=fz&taxon=3348&nameid=8091) Flora ZambesiacaVolume 4 Part 0 (1978). *Combretaceae* by A. W. Exell. Kew Royal Botanic Gardens.

Schutz, H. G., and Cardello, A.V., 2001. A labeled affective magnitude (LAM) scale for assessing food liking/disliking. *Journal of Sensory Studies* 16: 117–159.

Sekwati-Monang, B., 2011. Microbiological and chemical characterization of *ting*, a sorghum-based gluten-free fermented cereal product from Botswana. Ph.D. Thesis. University of Alberta, Edmonton, Canada.

Shackleton, C.M., 1993. Demography and dynamics of the dominant woody species in a communal and protected area of the eastern Transvaal Lowveld. *South African Journal of Botany* 59(6): 569–574.

Shackleton, C.M., 1998. Annual production of harvestable deadwood in semi-arid savannas, South Africa. *Forest Ecology and Management* 112: 139–144.

Sharma, A., Kaur, J., Lee, S., and Park, Y.S., 2020. Tracking of intentionally inoculated lactic acid bacteria strains in yogurt and probiotic powder. *Microorganisms* 8, 5. [CrossRef] [PubMed]

Sharma, S., 2015. Food preservatives and their harmful effects. *International Journal of Scientific and Research Publication* 5 (4):1-2.

Sher, A., 2009. Antimicrobial activity of natural products from medicinal plants. *Gomal Journal of Medical Sciences* 7 (1): 72–78.

Sibanda, H.M., 1992. Livestock-based agroforestry systems in Gwanda District. In: *Forest research in Zimbabwe*, Piearce, G.D. and Shaw, P. (eds.), The Forestry Commission, Harare, Zimbabwe.

Sidique, R., 2012. Utilization of wood ash in concrete manufacturing. *Resources, Conservation and Recycling* 67: 27–33.

Sigidi, M.T., Anokwuru, C.P., Zininga, T., Tshisikhawe, M.P., Shonhal, A., and Ramaite, I.D.I., 2016. Comparative in vitro cytotoxic, anti- Venda medicinal plants. *Translational Medicine Communications* 1(1): 9.

Singh, R.P., 1999. Scientific principles of shelf life evaluation. In Man, M.D., Jones A. A., *Shelf life evaluation of food*. Gaithersburg, MD: Aspen Publications. 3–26.

Singh, T. K., and Cadwallader K.R., 2004. Understanding and measuring the shelf life of foods. In *ways of measuring shelf life and spoilage*. Wood Head Publishing, 165–170.

Songa, J.M., and Rono, W., 1998. Indigenous methods for bruchid beetle (*Coleoptera: Bruchidae*) control in stored beans (*Phaseolus vulgaris L.). International Journal of Pest Management* 44: 1–4.

Stevens, S.S., 1957. On the psychophysical law. *Psychological Review* 64(3): 153–181.

Stewart, C.M., 2003. Staphylococcus aureus and staphylococcal enterotoxins. Ch 12 In: Hocking AD (Ed) Foodborne microorganisms of public health significance. 6th edition. Australian Institute of Food Science and Technology (NSW Branch), Sydney. 359–380.

Stone, H., and Sidel, J.L., 1993. Sensory evaluation practices. 2nd edition. Academic Press Inc., San Diego, CA.

Svendsen, A.B., and Verpoorte, R., 1983. Chromatography of alkaloids. Part A: thin-layer chromatography, vol. 23A. Amsterdam Oxford New York: Elsevier Scientific Publishing Company.

Tafere, G., 2015. A review on traditional fermented beverages of Ethiopia. *Journal of Natural Science Research* 5:94–102.

Talan, D.A., Staatz, D., Staatz, A., Goldstein, E.J.C., Singer, K., and Overturf, G.D., 1989. Staphylococcus intermedius in canine gingiva and canine-inflicted human wound infections: Laboratory characterization of a newly recognized zoonotic pathogen. *Journal of Clinical Microbiology* 27(1):78–81.

Tamang, P., and Kailasapathy, K., 2010. Fermented foods and beverages of the world. USA: CRC Press Taylor and Francis Group. 434.

Theron, M.M., and Lues J.F., 2007. Organic acids and meat preservation: a review**.** *Food Reviews International* 23:141-158.

Toaima, W., Trak, J., and Alkowwatly, K.A., 2015. Nisin peptide as promising natural food preservative for food. *Journal of Chemical and Pharmaceutical Research* 7(4):11-14.

Troller, J.A., and Christian, J.H.B., 1978. Water activity and food, New York.

Tuormaa, T.E., 1994. The adverse effects of food additives on health: A review of the literature with special emphasis on childhood hyperactivity. *The Journal of Orthomolecular Medicine* 9(4): 225-243.

Vallance, B.A., Chan, C., Robertson, M.L., and Finlay, B.B., 2002. Enter pathogenic and enterohemorrhagic *Escherichia coli* infections: emerging themes in pathogenesis and prevention. *Canadian Journal of Gastroenterology and Herpetology* 16: 771–778.

Van Holde et al., 2008.???

Van Wyk, B. A.E., and Van Wyk, P., 1997. Field guide to trees of southern Africa. Cape Town: Struik.

Van Wyk, P., 1993. Southern African trees: A photographic guide. Cape Town: Struik Publishers.

Vashudha, S.H., and Mishra, N., 2013. Non-dairy probiotic beverages. *International Food Research Journal* 20: 7–15.

Venkatesan, D., and Karrunakaran, C.M., 2010. Antimicrobial activity of selected Indian medicinal plants. *Journal of Phytology* 2(2): 44–48.

Venter, F., and Venter, J.A., 1996. Making the most of indigenous trees. Briza Publications, Pretoria.

Verma, V., Singh, R., Tiwari, R.K., Srivastava, N., and Verma, S., 2012. Antibacterial activity of extracts of Citrus, Allium and Punica against food borne spoilage. *The Asian Journal of Plant Science and Research* 2(4): 503–509.

Vilain, S., Luo, Y., Hildreth, M., and Brözel, V., 2006. Analysis of the life cycle of the soil saprophyte Bacillus cereus in liquid soil extract and in soil. *Applied and Environmental Microbiology* 72:4970–4977.

Viljoen, P.J., and Bothma, J., 1990. The influence of desert-dwelling elephants on vegetation in the northern Namibia Desert, South West Africa/Namibia. *Journal of Arid Environments* 18(1): 85–96.

Villanueva, N.D.M., and Da Silva, M.A. A.P., 2009. Comparative performance of the nine-point hedonic, hybrid and self-adjusting scales in the generation of internal preference maps. *Food Quality and Preference* 20: 1–12.

Villegas-Ruiz, X., Angulo, O., and O'Mahony, M. 2008. Hidden and false ''preferences'' on the structured 9-point hedonic scale. *Journal of Sensory Studies* 23: 780–790.

Voravuthikunchai, S.P., Sririrak, T., Limsuwan, S., Supawita, T., Iida, T., and Hond, T., 2005. Inhibitory effect of active compounds from *Punica granatum* pericarp on verocytotoxin production by Enterohemorrhagic *Escherichia coli* O157: H7. J*ournal of Health Science* 51:590–596.

Weber, J.M., Ruzindana-Umunyana, A., Imbeault, L., and Sircar, S., 2003. Inhibition of adenovirus infection and adenain by green tea catechins. *Antiviral Research* 58:167–73.

Widlansky, M.E., Duffy, S.J., Hamburg, N.M., Gokce, N., Warden, B.A., and Wiseman, S., 2005. Effects of black tea consumption on plasma catechins and markers of oxidative stress and inflammation in patients with coronary artery disease. *Free Radical Biology and Medicine* 38:499–506.

World Health Organization (WHO), 1987. Principles for the safety assessment of food additives and contaminants in food. *Environmental Health Criteria* Available:http://www.who.int/iris/handle/10

Yadav, P., Garg, N., and Kumar S., 2014. Improved shelf stability of mulberry juice by combination of preservatives. *Indian Journal of Natural Products and Resources* 5(1):62-66.

Yamamura, A., Murai, A., Takamatsu, H. and Watabe, K., 2000. Antimicrobial effect of chemical preservatives on enterohemorrhagic *Escherichia coli* O157: H7. J*ournal of Health Science* 46, 204–208.

Yeast Book, 2011. A comprehensive compendium of reviews that presents the current state of knowledge of the molecular biology, cellular biology, and genetics of the yeast *Saccharomyces cerevisiae,* Genetics.

Yu, D. and Kuipers, J.G., 2003. Role of bacteria and HLA-B27 in the pathogenesis of reactive arthritis. *Rheumatic Disease Clinics*, *29*(1),.21-36.

Yu, B., Sun, J., and Yang X., 2012. Assembly of naturally occurring glycosides, evolved tactics, and glycosylation methods. *Accounts of Chemical Research* 45(8):122–736.

Chapter 3: Phytochemical composition and antioxidant activity

3. 1 Introduction

Phytochemistry is a discipline concerned with the enormous variety of organic substances that are biosynthesised and stored by the plants. These substances have been known to reduce the risk of many human diseases, including cardiovascular disease, hepatorenal diseases, diabetes, cancers and neurodegenerative disorders (Modak *et al.*, 2007; Shakya and Shukla, 2011). Phytochemicals can be classified based on their chemical composition or functional group; these plant constituents are the sources of basic raw material for the establishment of pharmaceutical industries (Mothana and Lindequist, 2005). Phytochemical screening plays a vital role in identifying new sources of pharmacologically active compounds, such as alkaloids, anthraquinones, flavonoids, phenolic compounds, saponins, steroids, tannins and terpenoids (Akindele and Adeyemi, 2007; Shakya, 2016). Most of these phytochemical constituents are potent bioactive compounds found in parts of medicinal plants that serve as leads for modern drugs (Sofowora, 1993).

Large numbers of plants have been investigated for their bioactive constituents and antioxidant properties. Natural antioxidants either in the form of raw extracts or in their chemical constituents are very effective in preventing the destructive processes caused by oxidative stress (Zengin *et al*., 2011). Substantial evidence has accumulated and indicated key roles for reactive oxygen stress (ROS) and other oxidants in causing numerous disorders and diseases. The evidence has brought the attention of scientists to an appreciation of antioxidants for the prevention and treatment of diseases, and maintenance of human health (Gulcin, 2012). Antioxidants stabilise or deactivate free radicals, often before they attack targets in biological cells (Nunes *et al.,* 2012). Recently, interest in naturally occurring antioxidants has considerably increased for use in food, cosmetic and pharmaceutical products, because they possess multifacetedness in their multitude and magnitude of activity and provide an enormous scope in correcting imbalance(s) (Djeridane *et al.,* 2006; Wannes *et al.,* 2010). The role of free radical reactions in disease pathology is well established and is known to be involved in many acute and chronic disorders in human

beings, such as diabetes, atherosclerosis, aging, immunosuppression and neurodegeneration (Haman, 1998). Studies on herbal plants, vegetables, and fruits have indicated the presence of antioxidants such as phenolics, flavonoids, tannins, and proanthocyanidins. Antioxidant agents of natural origin have attracted special interest because of their free radical scavenging abilities (Osawa *et al*., 1990; Govind, 2011). The search for novel natural antioxidants of plant origin has ever increased. It is unknown which constituents of plants are associated with reducing the risk of chronic diseases, but antioxidants appear to play a major role in the protective effect of plant medicine. The present chapter was designed to evaluate the antioxidant activities of the 12 *Combretum* plants and the wood ashes and to determine the presence of phytochemicals such as alkaloids, cardiac glycosides, flavonoids, saponins, phlobotannins, tannins and terpenoids.

3.2 Methods and Materials

3.2.1 Collection and extraction of plant materials

Fresh stems and leaves of *Combretum caffrum, C. vendae, C. erythrophyllum, C. elaegnoides, C. apiculatum, C. imberbe, C. adenogdium, C. padoides, C. bracteosum, C. kraussii, C. mkuzens*e and *C. zeyherii* were collected at Nelspruit, National Botanical Gardens, Mpumalanga, South Africa. Voucher specimens and tree labels were used to verify the identity of the plants. The voucher specimens were deposited at the Larry Leach Herbarium (UNIN) for confirmation. The leaves were air-dried for 30 days. The stems, including bark collected, were cut into pieces and also air-dried for 30 days. The dried plant materials were ground to a fine powder and stored in paper bags at room temperature. The wood was burnt in an open fire; fuel was not used to minimise contamination. The ash collected from the burned wood was stored in paper bags. Preparation of ashes is depicted below:

Figure 3.1: A depiction of the systematic preparation of ashes

The combretum plants were extracted by weighing 1 g of finely ground plant material and extracting it with 10 ml of 70 % acetone in different centrifuge tubes. Tubes were vigorously shaken for 10 minutes in series 25 shaking incubator machine (New Brunswick Scientific Co., Inc.) at a high speed (200 rpm). There after the extracts were filtered into labelled bottles. The process was repeated three times to exhaustively extract constituents of the plant material and the extracts were combined. The solvent was removed under a stream of cold air at room temperature. The final extracts were reconstituted in 70 % acetone to a concentration of 10 mg/ml.

3.2.2 Phytochemical Constituents Screening

3.2.2.1 Saponins

The persistent frothing test was used to test for saponins by weighing 1 g of plant-powdered leaves and stems and mixed with 30 ml of tap water. The mixture was vigorously shaken and heated at 100°C. The sample was observed for the formation of persistent froth. (Odebiyi and Sofowora, 1978).

3.2.2.2 Tannins

The presence of tannins was tested by boiling 0.5 g of powdered leaves and stems in 5 ml of distilled water in a test tube, then cooled and filtered. A few or three drops of 0.1% ferric chloride was added to 1 ml of the solution in a test tube and observed for brownish green or a blue-black colouration (Trease and Evans, 1989).

3.2.2.3 Phlobotannins

Phlobotannins were tested by weighing 0.2 g of powdered leaves and stems of *R. communis* into 10 ml of distilled water and filtered. The filtrate was boiled with 2% hydrochloric acid solution. The sample was observed for the formation of red colour of precipitate (Borokini and Omotayo, 2012).

3.2.2.4 Terpenes/ terpenoids

The Salkowski's test was used. 5 mg of the leaves and powders were mixed in 2 ml of chloroform and 3 ml concentrated sulphuric acid (H_2SO_4) was carefully added to form a layer. The appearance of a reddish-brown colour indicates the presence of terpenes (Borokini and Omotayo, 2012).

3.2.2.5 Steroids

About 2 ml of acetic anhydride was added to 0.5 g of the powdered leaves and stems, followed by an addition of 2 ml of H_2SO_4. Blue colour was observed to draw an inference, indicating the presence of steroids (Borokini and Omotayo, 2012).

3.2.2.6 Cardiac glycosides

The Keller-Killani's test was used. About 5 ml of the leaves and stem powders of the plant parts studied were treated with 2 ml of glacial acetic acid, containing one drop of ferric chloride solution. This was followed by an addition of 1 ml concentrated H_2SO_4. The colour changes (brown interface, violet ring below and greenish ring at the lowest) were observed to draw inference, indicating the presence of cardiac glycosides (Borokini and Omotayo, 2012).

3.2.2.7 Flavonoids

About 5 ml of diluted ammonia solution was added to a portion of the filtrate of each plant extract, followed by the addition of concentrated H_2SO_4. Yellow colour change

was observed to draw an inference, indicating the presence of flavonoids (Borokini and Omotayo, 2012).

3.2.2.8 Alkaloids

Drangendorff's reagent was used to test for alkaloids by weighing 0.2 g of ground powdered leaves and stems with 95% ethanol using soxhlet extractor. The extracting solvent was evaporated to dryness using a vacuum evaporator at 45°C. The plant residues were dissolved in 5 ml of 1% hydrochloric acid and 5 drops of Drangendorff's reagent was added. Reddish-brown colour change was observed to draw an inference (Harborne, 1973).

3.2.3 Total Phenolic Content Determination

The total phenolic content of the 70% aqueous acetone extracts was determined by Folin-Ciocalteau assay (Humadi and Istudor, 2008). Aliquots of extracts (1 mg/ml) or a standard solution of tannic acid (0.063, 0.125, 0.25, 0.5, and 1 mg/ml) of about 0.9 ml was added to a 25 ml volumetric flask, containing 0.9 ml of distilled water. Folin-Ciocalteau reagent (0.1 ml) was added to the mixture and shaken, then incubated for 5 minutesSeven percent of sodium carbonate solution (1 ml) was added to the mixture and diluted with distilled water to 25 ml followed by mixing. Reagent blank containing everything except extracts was also prepared. After 90 minutes incubation at room temperature, the absorbance of the standards and samples against the prepared reagent blank was determined at 550 nm using a UV-VIS Spectrophotometer. The results were expressed as milligrams of gallic acid equivalents per milligrams (GAE/mg). All samples were analysed in triplicates.

3.2.4 The Tannin Content Determination

The tannin content was determined using Folin-Ciocalteau method described by Tambe and Bhambar (2014). About 0.1 ml of the 70% aqueous acetone extracts of the dried leaves and stems was added to a 25-ml volumetric flask with 5 ml of distilled water. To this mixture, 0.2 ml of 2 M Folin-Ciocalteau phenol reagent and 1ml of 35% Na_2CO_3 solution was added and this was made up to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 minutes. A set of standard solutions of gallic acid (0.063, 0.125, 0.25, 0.5, and 1 mg/ml) were prepared in the same manner as described above. Absorbance for test samples and standard

solutions were measured against the blank at 725 nm with a UV/visible spectrophotometer. The tannin content was expressed as mg of GAE/g of extract.

3.2.5 Total Flavonoid Content Determination

Total flavonoid content was determined by the aluminium chloride colorimetric assay. One millilitre of 70% aqueous acetone extracts of the selected plants was mixed with 4 ml of distilled water in a 25 ml volumetric flask. To the flask, 0.30 ml of 5% sodium nitrite was added. About 0.3 ml of 10% aluminium chloride was added to the mixture after 5 minutes; this was mixed. After 5 minutes, 2 ml of 1 M sodium hydroxide was added; it was made up to 10 ml with distilled water. A set of reference standard solutions of quercetin (0.0625, 0.125, 0.25, 0.5 and 1 mg/ml) were prepared in the same manner as described above. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with a UV/visible spectrophotometer. The total flavonoid content was expressed as mg of quercetin equivalent (Tambe and Bhambar, 2014).

3.2.6 Quantitative Antioxidant Activity Assay

Free radical scavenging activity of the plants was quantified and compared using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma) method reported by Chigayo *et al.* (2016) with modifications. Briefly, different concentrations of the dried leaves and stem (250, 125, 62.5, 31.25 and 15.63 µg/ml) were prepared to a volume of 1 ml of the solution. To this 1 mL solution, 2 mL of 0.2 mmol/L DPPH solution dissolved in methanol was added and vortexed thoroughly. All the prepared mixtures were left to stand in the dark for 30 min. The control solution was prepared by adding 2 ml of 0.2 mmol/l DPPH to 1 ml of distilled water. After the elapsed time, the solutions were analysed with a UV/VIS spectrophotometer at a 517nm wavelength.

3.2.7 Data analysis

Statistical analysis of results was performed using Statistix 10 data analysis software, completely randomised test and the Welch's Test was used for comparison of any significant differences between the means. Statistical analysis was performed to determine variation between the juices in terms of proximate, nutritional and sensory evaluation. Results were considered significantly different when $p < 0.05$.

3.3 Results

Phytochemicals (saponins, tannins, terpenoids, steroids, cardiac glycosides and flavonoids) were found to be present in all the leaves tested in this study. However, phlobatannins and alkaloids were not detected in the leaves (Table 3.1).

Table 3.1: Qualitative test for phytochemicals in the leaves of twelve *Combretum* species.

Plant	Saponins	Tannins	Phlobatannins	Terpenoids	Steroids	Cardiac glycosides	flavonoids	Alkaloids
C. adenogdnium	+	+	-	+	+	+	+	-
C. apiculatum	+	+	-	+	+	+	+	-
C. bracteosum	+	+	-	+	+	+	+	-
C. caffrum	+	+	-	+	+	+	+	-
C. elaeagnoides	+	+	-	+	+	+	+	-
C. erythrophyllum	+	+	-	+	+	+	+	-
C. imberbe	+	+	-	+	+	+	+	-
C. krausii	-	+	-	+	+	+	+	-
C. mkuzense	+	+	-	+	+	+	+	-
C. padoides	+	+	-	+	+	+	+	-
C. vendae	+	+	-	+	+	+	+	-
C. zeyherii	+	+	-	+	+	+	+	-

Key: + present; - absent

The stems of the plants contain the same phytochemicals as those present in the leaves, as shown in table 3.2. Alkaloids and phlobatanninins were not detected in all the plants while the rest of the phytochemicals were detected.

Table 3.2: Qualitative test for phytochemicals in the stems of twelve *Combretum* species.

Plant	**Saponins**	**Tannins**	**Phlobatannins**	**Terpenoids**	**Steroids**	**Cardiac glyocosides**	**flavonoids**	**Alkaloids**
C. adenogdnium	+	+	-	+	+	+	+	-
C. apiculatum	+	+	-	+	+	+	+	-
C. bracteosum	+	+	-	+	+	+	+	-
C. caffrum	+	+	-	+	+	+	+	-
C. elaeagnoides	+	+	-	+	+	+	+	-
C. erythrophyllum	+	+	-	+	+	+	+	-
C. imberbe	+	+	-	+	+	+	+	-
C. krausii	-	+	-	+	+	+	+	-
C. mkuzense	+	+	-	+	+	+	+	-
C. padoides	+	+	-	+	+	+	+	-
C. vendae	+	+	-	+	+	+	+	-
C. zeyherii	+	+	-	+	+	+	+	-

Key: + present; - absent

The ashes of the plants contain only saponins, terpenoids and steroids. However, tannins were also detected in the ashes of *C. mkuzense* and *C. padoides;* Cardiac glycosides and flavonoids (Table 3.3).

Table 3.3: Qualitative test for phytochemicals in the ashes of twelve *Combretum* species.

Plant	Saponins	Tannins	Phlabatannins	Terpenoids	Steroids	Cardiac glyocosides	flavonoids	Alkaloids
C. adenogdnium	+	-	-	+	+	-	-	-
C. apiculatum	+	-	-	+	+	-	-	-
C. bracteosum	+	-	-	+	+	-	-	-
C. caffrum	+	-	-	+	+	-	-	-
C. elaeagnoides	+	-	-	+	+	-	-	-
C. erythrophyllum	+	-	-	+	+	-	-	-
C. imberbe	+	-	-	+	+	-	-	-
C. krausii	-	-	-	+	+	-	-	-
C. mkuzense	+	+	-	+	+	-	-	-
C. padoides	+	+	-	+	+	-	-	-
C. vendae	+	-	-	+	+	-	-	-
C. zeyherii	+	-	-	+	+	-	-	-

Key: + present; - absent

Quantitative phytochemical composition of the *Combretum* plants

The leaves showed the presence of high amounts of phenolic compounds when compared to the stems with *C. adenogdnium* having the highest concentration of

phenols. The phenolic compounds of the leaves, stems and ashes of the plants ranged between 172- 2164, 35-350 and 0-43 mg of GAE/g of sample, respectively. (Figure3.2).

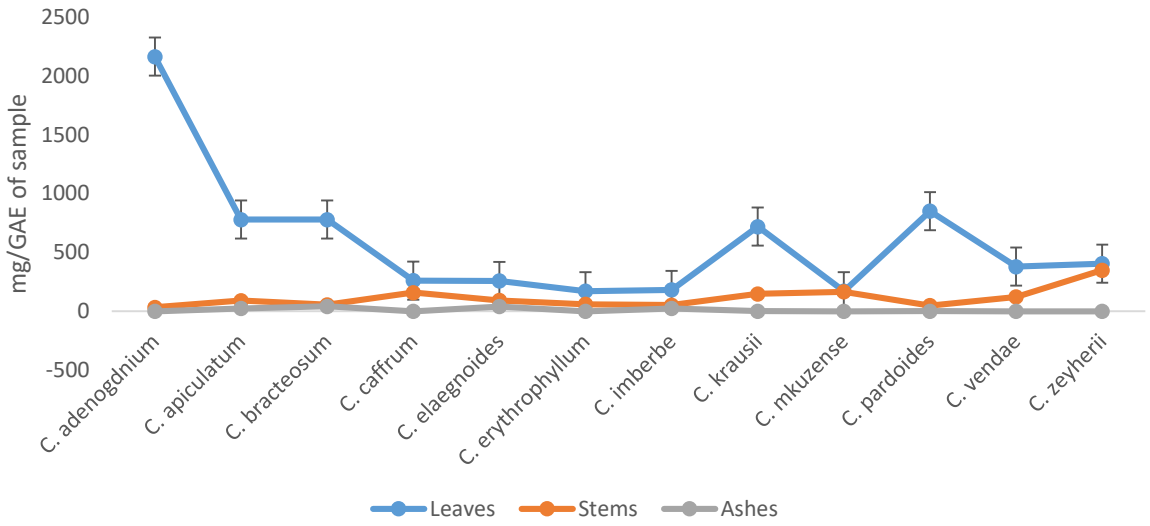


Figure 3.2: Evaluation of the phenolic compounds in the leaves, stems and ashes of twelve *Combretum* species.

The concentration of tannins in leaves, stems and ashes of the plants ranged from 172- 604,1, 93,3-508,3 and 0-50 mg of GAE/g of sample, respectively. The ashes of *C. apiculatum* had the highest concetration of phenols when compared to the stems (Figure 3.3).

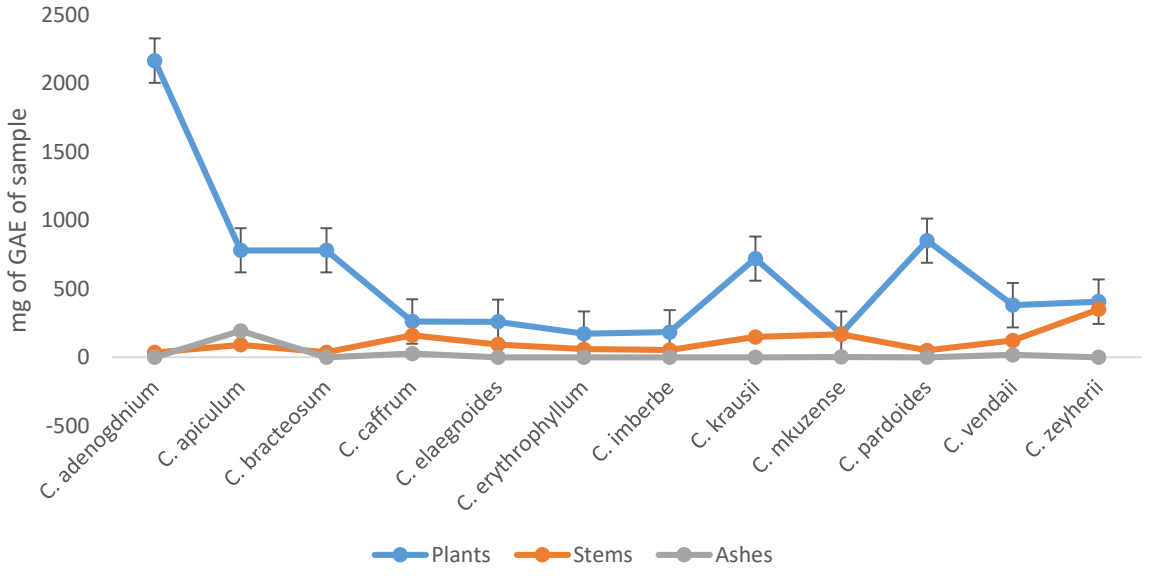


Figure 3.3: Total tannins content concentrations of the 70% aqueous acetone extracts of twelve *Combretum* species.

Most of the leaves had the highest flavonoids concentration when compared to the stems with the exception of *C. kraussii*. Interestingly, ashes of *C. apiculatum, C. elaegnoides* and *C. vendae* had the highest concentrations of the flavonoids when compared with other species of *Combretum*. (Figure 3.4).

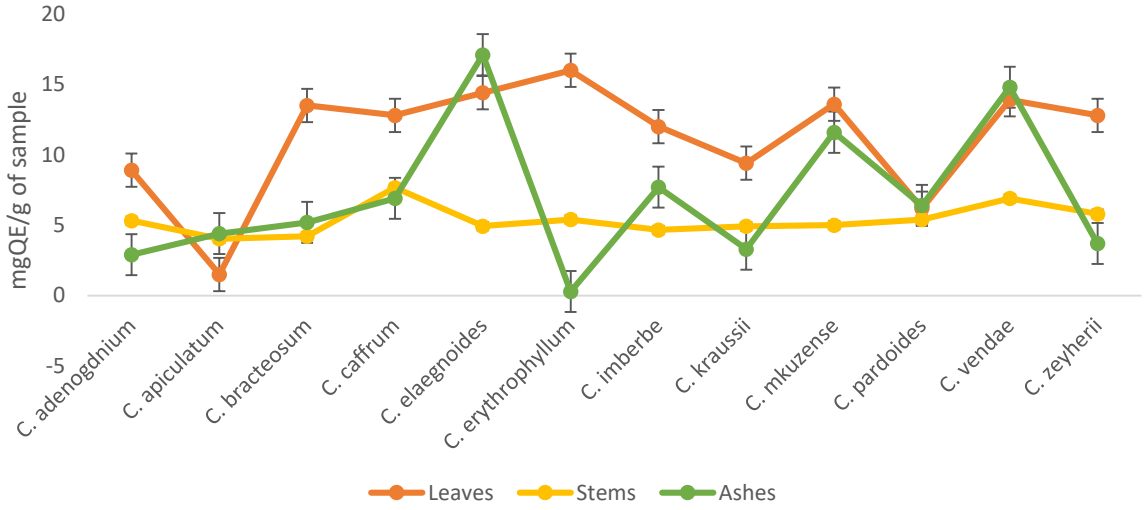


Figure 3.4: Total flavonoids content concentrations of the 70% aqueous acetone extracts of twelve *Combretum* species.

Free radical scavenging properties of 70 % leaf extracts are presented in figure 3.5. Most of the leaves tested exhibited a dose-dependent manner of antioxidant activity. The following leaf extracts showed highest scavenging activity C *mkuzense, C. zeyherii, C. kraussii and C. padoides.* Statistically, there was no significant difference (p=001) in the scavenging activity of the following plants: *C. mkuzense* (0. 0625; 0,125; 0, 25 and 0.5 mg/ml); *C. zeyherii* (0, 25 and 0.5 mg/ml); *C. kraussii* (25 and 0.5 mg/ml) *and C. padoides* (1 mg/ml). *C. elaegnoides, C. erythrophyllum* and *C. bracteosum* had the lowest radical scavenging potential when compared to other leaves in the study, as depicted in Figure 3.5.

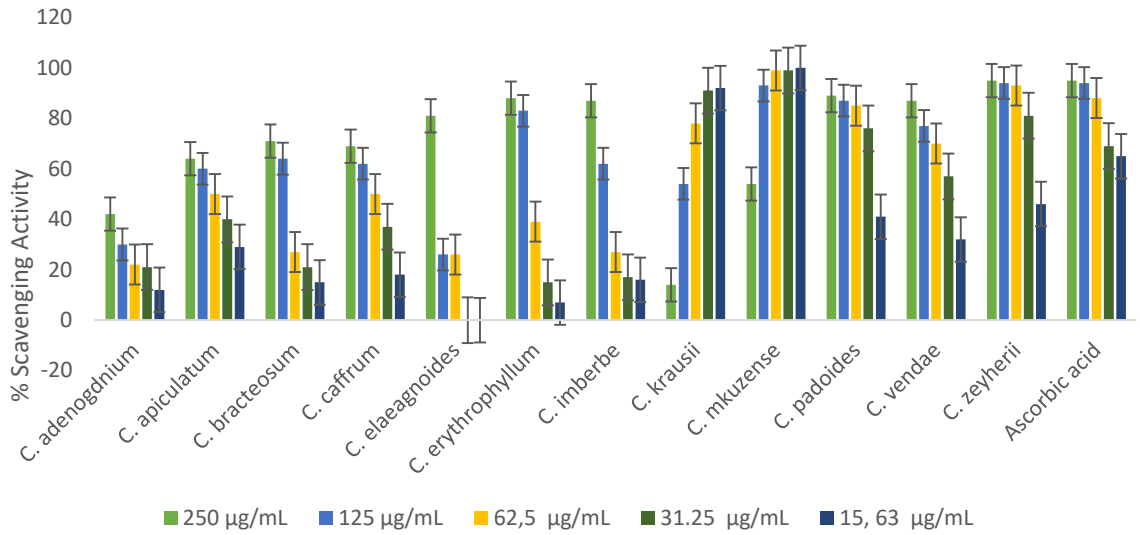


Figure 3.5: Evaluation of the antioxidant activity of the 70% acetone leaf extracts of the twelve *Combretum* species.

Figure 3.5 shows the concentration-dependent activity of the leaf extracts of the plants on DPPH radical scavenging by stems of *Combretum* spp. with the exception of *C. kraussii*. As the concentration of sample increased, the percentage inhibition of DPPH radical also increased. In the plants that acted in a concentration-dependent manner, the following stems: *C. zeyherii, C. bracteosum, C. imberbe and C. adenogonium*, showed the greatest percentage scavenging activity (Figure 3.5).

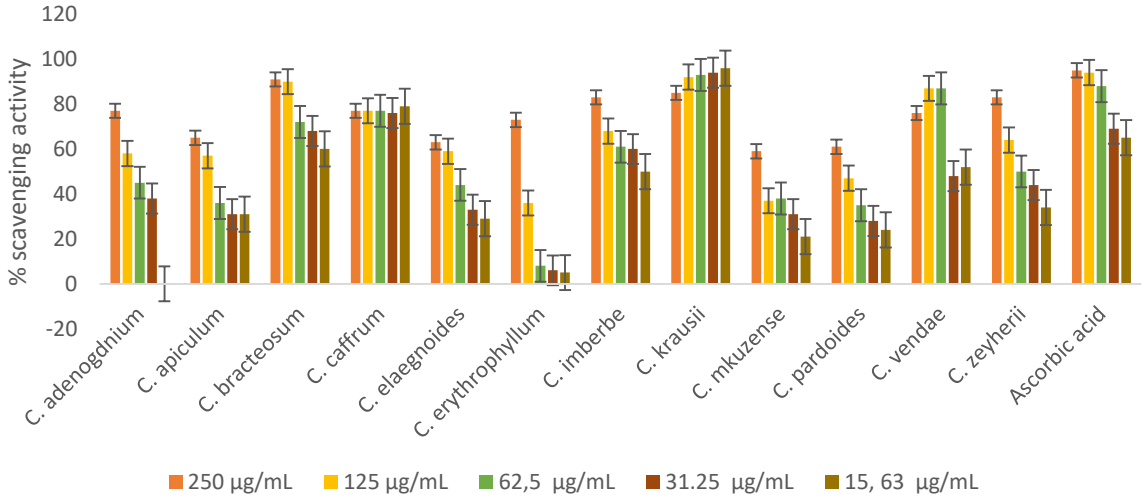


Figure 3.6: Evaluation of the antioxidant activity of the stems of the twelve *Combretum* species.

Majority of the *Combretum* ash extracts such as *C. elaegnoides* and *C. zeyherii* did not possess any antioxidant activity. Ashes from *C. apiculatum, C. caffrum, C padoides* and *C. vendae* showed some antioxidant activity in a dose dependent manner while the activity of *C. imberbe* was observed to be opposite, as depicted in figure 3.7.

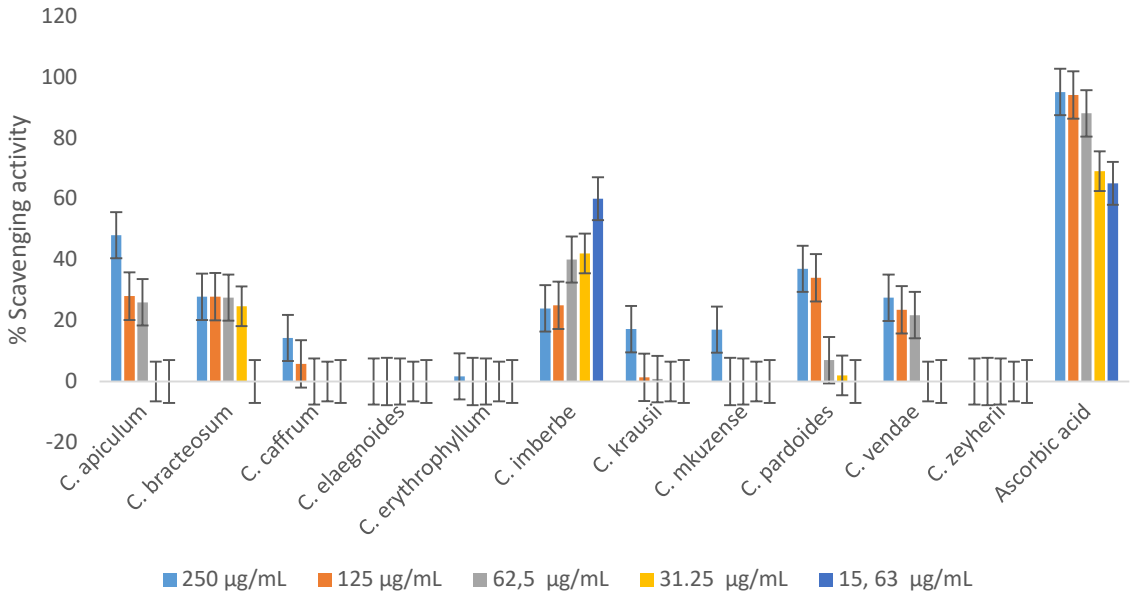


Figure 3.7: DPPH radical scavenging activity of 70% acetone ash extracts of twelve *Combretum* species..

3.4 Discussion

Phytochemicals are secondary metabolites of plants known to exhibit diverse pharmacological and biochemical effects on living organisms (Trease and Evans, 1989). The qualitative phytochemical composition of both the leaves and stems of some *Combretum* species analysed in this study revealed the presence of saponins, tannins, terpenoids, steroids, cardiac glycosides and flavonoids. These phytoconstituents were not detected in the ashes of the plants. However, tannins were present in *C. mkuzense*, while cardiac glycosides and flavonoids were contain in *C. padoides;*. These phytoconstituents, which were not detected in the ashes may have been destroyed by heat. The quantitative phytochemical analyses revealed that both the leaves, stems and some ashes such as *C. apiculatum* and *C. vendae* contained appreciable levels of phenolic compounds, tannins and flavonoids. These secondary

metabolites as detected in this study have been associated with antimicrobial activities and numerous physiological activities in mammalian cells in various studies (Sofowora 1993; Abo *et al.,* 1999; Nweze *et al.,* 2004; Mishra *et al*., 2009). Generally, the leaves showed to have higher concentrations of the phytoconstituents when compared to the stems. The phenolic compounds contain in the leaves of the plants were found to vary in the following order *C. apiculatum> C. padoides> C. bracteosum=C. adenogdnium> C. krausii> C. zeyheri> C. vendae> C. caffrum> C. elaeagnoides> C. imberbe> C. mkuzense > C*. *erythrophyllum.* These indicates that the leaves of *C. apiculatum* are a good source of phenolic compounds when compared to other plant leaves in the study. These results are in line with those of Masoko and Eloff (2007) who investigated the qualitative antioxidant activity and phytochemical properties of 30 members of the Combretaceae. The phenolic compounds of the stems were found to be in the following order *C. mkuzense> C. caffrum> C. krausii> C. vendae> C. apiculum= C. elaeagnoides> C. bracteosum> C. imberbe> C. padoides> C. zeyheri> C. adenogdnium> C. erythrophyllum.* Although there was a significant decrease (p=001) in the concentration of phenolic compounds in the stems when compared to the leaves, they still possessed substantial amounts and can still be used during seasons when leaves are scarce. Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardio protective and vasodilatory effects (Manach *et al.,* 2004 Middleton *et al.,* 1998, Puupponen-Pimia *et al.,* 2001). Phenolic compounds have been associated with the health benefits derived from consuming high levels of fruits and vegetables (Hertog *et al.,* 1993; Parr and Bolwell, 2000). The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity (Heim *et al.,* 2002). In the ashes, notable amounts of phenolic compounds and tannins were only observed in *C. apiculatum, C. bracteosum* and *C. caffrum.* Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure and are ubiquitously present in plants (Kumar and Pandey, 2012). The study revealed that the all the *Combretum* plants tested possess substantial concentrations of flavonoids, with their leaves having high concentrations than the stem. It was interesting to observe that the ashes *C. apiculatum, C*. *mkuzense* and *C*. *vendae* were significantly higher than those of the leaves and stems (p=001). Currently, there is little or no studies that report the presence of phytoconstituents in the ashes of these plants. The flavonoids in the leaves were found to be in the following order: *C. elaeagnoides> C. vendae> C.*

padoides> C. imberbe> C. apiculatum > C. bracteosum> C. erythrophyllum> C. caffrum> C. zeyheri> C. krausii> C. mkuzense. Several studies; Rogers and Verotta (1996), Pettit *et al.,* (1987) and Schwikkard *et al.,* (2000) have reported the presence of flavonoids in *C. apiculatum, C. caffrum, C. kraussii* and *C. erythrophyllum,* supporting the findings of this study. Several reports have revealed that other *Combretum* plants that were not included such as *C. hereroense* (Letcher and Nhamo, 1973), *C. nigricans* (Jossang *et al.,* 1996), *C. leprosum* (Facundo *et al.,* 1993) and *C. micranthum (*Masoko *et al.,* 2005; Masoko *et al*., 2007; Masoko and Eloff, 2007) contain flavonoids. These reports, together with the findings of the these study, indicate that *Combretum* plants are a good source of flavonoids. Flavonoids have potential health benefits arising from the antioxidant activities of these polyphenolic compounds. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions (Kumar *et al.,* 2013; Kumar and Padey, 2012. As a dietary component, flavonoids are thought to have health-promoting properties due to their high antioxidant capacity both *in vivo* and in *in vitro* systems (Rice-Evans *et al.*, 1996) Cook and Sammon, 1996). Saponins are used to treat the following medical conditions: hypercholesterolemia, hyperglycaemia, antioxidant, anticancer, anti-inflammatory and weight loss. Reports have shown that they also possess antifungal properties (Tijjani *et al.,* 2012). Saponins exhibit a cytotoxic effect and growth inhibition against a variety of cell lines, making them potential anti-inflammatory and anticancer agents (Iniaghe *et al.,* 2009). Terpenes are a crucial group of organic compounds that have been reported as potent drugs used in the treatment of a wide range of ailments. The most rapidly acting anti-malarial Artemisin and its derivate are terpenes (Tijjani *et al.,* 2012). Phenols, which are also found in plant sources, are a major group of compounds acting as primary antioxidant or free radical scavenger (Adesuyi *et al.,* 2011).

The antioxidant activities of many plants are of great interest in the food, cosmetics and pharmaceutical industries, since their possible use as natural additives emerged from a growing tendency to replace synthetic preservatives with natural ones (Ref!). DPPH assay is widely used for the evaluation of the antioxidant activity of biological samples. DPPH is a stable free radical with characteristic absorption at 520 nm, and antioxidants react with DPPH radical and convert it to diamagnetic 2,2- diphenyl-1-picrylhydrazine molecule. The degree of discolouration indicates the scavenging

potential of the antioxidant extract, which is due to the hydrogen donating ability (Von Gadow *et al*., 1997; Jaitak *et al.,* 2010). In this study as the concentration of sample increased, the percentage inhibition of DPPH radical also increased. However, in the case of *C. kraussii* and *C. mkuzense*, the opposite was observed *i.e.,* at the lowest concentration, the scavenging activity was the highest. This means that, out of all the leaves tested, the *C. kraussii* and *C. mkuzense* are good sources of antioxidants. Concentration-dependently, C. *zeyherii* leaves showed the overall highest scavenging activity when compared to the other leaves. It was observed to have scavenging activity, which was comparable to that of the control agent (ascorbic acid) which was tested at concentrations of 250 µg/ml and 125 µg/ml.. The methanol and acetone extracts of the leaves of *C. zeyherii* were found to possess antioxidant activity . (Masoko and Eloff, 2007). The stems of the plants had a relatively good antioxidant activity, which was found to be concentration-dependent, except for *C. kraussii* (Figure 3.6). There was a significant decrease in the antioxidant activity in the ashes (p=001), when compared to both the leaves and the stems. This may be due to the loss of some phytoconstituents, (tannins cardiac glycosides and flavonoids). Flavonoids and tannins have been shown to act as secondary antioxidant defence system in plant tissues exposed to different abiotic and biotic stresses (Agati *et al.,* 2012). Ashes from *C. imberbe, C. apiculatum and C. padoides* still possessed antioxidant activity after the burning process. Overall, *Combretum* plants are a good source of phytochemicals that possess antioxidant capacity.

Conclusion: The pharmacological effect of the phytochemical constituents such as alkaloids, glycoside, tannins and flavonoids as well as the antioxidant activity of the plants and ashes in the study explains the rationale for the use of these plants in traditional medicine and their use as food additives. The outcome of this study suggests that the selected plant and ashes could probably be a veritable and cheaper substitute for conventional drugs and preservatives since the plants are easily obtainable, can be cultivated on a sustainable basis and the extract can easily be made through a simple process. The *Combretum* plants in the study have great potential to be used in the food industry as antimicrobial agents and in medicine as food additives because of the health benefits associated with the presence of phytoconstituents.

3.5 References

Abo, K.A., Ogunleye, V.O. and Ashidi, J.S., 1999. Antimicrobial potential of Spondias mombin, Croton zambesicus and Zygotritonia crocea. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 13(6), pp.494-497.

Aderogba, M.A., Kgatle, D.T., McGaw, L,.J, and Eloff. J.N., 2012. Isolation of antioxidant constituents from *Combretum apiculatum subsp. apiculatum*. *South African Journal of Botany* 79(1): 125-131.

Adesuyi, A.O., Awosanya, O. A., Adaramola F. B., and Om eonu A.I., 2011. Nutritional and phytochemical screening of *Aloe barbadensis*. *Current Research Journal of Biological Sciences* 4(1), 4 – 9.

Agati, G., Azzarello, E., Pollastri, S., and Tattini, M., 2012. Flavonoids as antioxidants in plants: location and functional significance. *Plant Science* 196: 67–76.

Akindele, A.J., and Adeyemi, O.O., 2007. Anti-inflammatory activity of the aqueous leaf extracts of *Byrsocarpus coccineus*. *Fitoterapia* 7: 25–28.

Borokini, T.I., and Omotayo, F.O., 2012. Phytochemical and ethnobotanical study of some selected medicinal plants from Nigeria. *Journal of Medicinal Plants Research* 6: 1106–1118.

Chigayo, K., Mojapelo, P.E.L. and Moleele, S.M., 2016. Phytochemical and antioxidant properties of different solvent extracts of *Kirkia wilmsii* tubers. *Asian Pacific Journal of Tropical Biomedicine* 6:1037–1043.

Cook, N. C. and S. Samman, 1996. Review: flavonoids-chemistry, metabolism, cardio protective effects and dietary sources. *Journal of Nutritional Biochemistry* 7(2): 66–76.

Djeridane, A., Yousfi, M., Nadjemi, B., Boutassouna, D., Stocker, P., and Vidal, N., **2006.** Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. *Food Chemistry* 97:654–660.

Facundo, V.A., Andrade, C.H.S., Silveira, E.R., Braz-Filho, R. and Hufford, C.D., 1993. Triterpenes and flavonoids from *Combretum leprosum. Phytochemistry* 32: 411–415.

Gadow, A., Joubert, E. and Hansmann, C.F., 1997. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (Aspalathus linearis), α-tocopherol, BHT, and BHA. Journal of agricultural and food chemistry, 45(3), pp.632-638.

Govind, P., 2011. Medicinal plants against liver diseases. *Iranian Journal of Pharmaceutical Research* 2:115–121.

Gulcin, I., 2012. Antioxidant activity of food constituents: an overview. *Archives of Toxicology* 86: 345–391.

Harborne, J.B., 1973. Phytochemical methods. 3rd edition. Chapman and Hall Limited, London. 135–203.

Harman, D., 1998. Free radical theory of aging. Status. Amsterdam. Elsevier; 3–7.

Heim, K. E., Tagliaferro, A. R., and Bobilya, D.J., 2002. Flavonoid antioxidants: chemistry, metabolism and structure–activity relationships. *The Journal of Nutritional Biochemistry* 13, 572–584.

Hertog, M.G.L., Hollman, P.C.H., Katan, M. B., and Kromhout, D. 1993. Intake of potentially anti carcinogenic flavonoids and their determinants in adults in the Netherlands. *Nutrition and Cancer* 20: 21–29.

Humadi, S.S. and Istudor, V., 2008. Quantitative analysis of bioactive compound *Hibiscus sabadariffa L*. extracts note 1: quantitative analysis of flavonoids. *Farmácia* 6:699–707.

Iniaghe, O.M., Malomo S.O., and Adebayo. J.O., 2009. Proximate composition and phytochemical constituents of leaves of some Acalypha species. *Pakistan Journal of Nutrition* 8(3): 256-258.

Jaitak, V., Sharma, K., Kalia, K., Kumar, N., Singh, H.P., Kaul, V.K., Singh, B., 2010. Antioxidant activity of Potentilla fulgens: an alpine plant of western Himalaya. *Journal of Food Composition and Analysis* 23: 142–147.

Jossang, A., Seuleiman, M., Maidou, E., and Bodo B .1996. Pentacyclic triterpenes from *Combretum nigricans*. *Phytochemistry* 41: 591–594.

Kumar, A.A., Reddy, B.V., Ramaiah, B., Sahrawat, K.L., and Pfeiffer, W.H., 2013. Gene effects and heterosis for grain iron and zinc concentration in sorghum [Sorghum bicolor (L.) Moench]. *Field Crops Research*, 146: 86-95.

Kumar, S., and Pandey, A.K., 2013 Antioxidant, lipo-protective and antibacterial activities of phytoconstituents present in *Solanum xanthocarpum* root. International Review of Biophysical Chemistry 3(3), 42-47

Letcher, R.M., and Nhamo, L.R.M., 1973. Chemical constituents of the Combretaceae. Part IV. Phenanthrene derivatives fromthe heartwood of Combretum hereroense. *Journal of the Chemical Society Perkin Transactions* I: 1179–1181.

Manach, C., Scalbert, A, Morand, A., Rémésy, C., and Jiménez, L., 2004. Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition* 79(5):727-747.

Modak, M., Dixit, P., Londhe, J., Ghaskadbi, S. and Devasagayam, T.P.A., 2007. Indian herbs and herbal drugs used for the treatment of diabetes. *Journal of clinical biochemistry and nutrition*, *40*(3), pp.163-173.

Masoko, P., and Eloff, J.N., 2007. Screening of twenty-four South African Combretum and six Terminalia species (Combretaceae) for antioxidant activities. *African Journal of Traditional Complementary and Alternative Medicines* 4, 231–239.

Masoko, P., Picard., J and Eloff, J.N., 2005. Antifungal activities of six South African Terminalia species (Combretaceae). *Journal of Ethno Pharmacology* 99: 301–308.

Masoko, P., Picard, J., and Eloff, J.N., 2007. The antifungal activity of twenty-four southern African Combretum species (Combretaceae). *South African Journal of Botany* 73: 173–183.

Middleton, E.J., 1998. Effect of plant flavonoids on immune and inflammatory cell function. *Advances in Experimental Medicine and Biology* 439:175–182.

Mishra, A.K., Kehri, H.K., Sharma, B. and Pandey, A.K. 2009. Inhibitory activity of Indian spice plant *Cinnamomum zeylanicum* extracts against *Alternaria solani* and *Curvularia lunata*, the pathogenic dematiaceous moulds, doi: 10.1186/1476-0711-8-9. *Annals of Clinical Microbiology and Antimicrobials* 8:9.

Mothana, R.A. and Lindequist, U., 2005. Antimicrobial activity of some medicinal plants of the island Soqotra. Journal of ethnopharmacology, 96(1-2), pp.177-181.

Mothana, R.A., Lindequist, U., Gruenert, R., and Bednarski, P., 2009. Studies of the *in vitro* anticancer, antimicrobial, and antioxidant potentials of selected Yemeni medicinal plants from the island Sogotra. *BMC Complementary and Alternative Medicine*, doi: 10.1186/1472-6882-9-7.

Nunes, P.X, Silva, S.F., Guedes, R.J. and Almeida, S., 2012. Biological oxidations and antioxidant activity of natural products, phytochemicals as nutraceuticals - Global Approaches to Their Role in Nutrition and Health. Intech Open, London. 10.5772/26956.

Nweze, E.I., Okafor, J.I., and Njoku, O., 2004. Methabolic extracts of *Treme guineenes* (Schumm and thorn) and *Morinda lucida* Benth used in Nigeria herbal medicinal practice. *Biological Research* 2(1): 39-48.

Odebiyi, O.O. and Sofowora, E.A., 1987. Phytochemical screening of Nigerian medicinal plants II. *Lloydia* 41 (6): 234–246.

Osawa, T., Kavakishi, S., Namiki, M., Kuroda, Y., Shankal, D.M., and Waters, M.D., 1990. Antimutagenesis and anticarcinogenesis mechanisms II. New York: Plenum; 1990:139–153.

Parr, A. J., and Bolwell, G. P. 2000. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *Journal of the Science of Food and Agriculture* 80: 985–1012.

Pettit, G.R., Smith, C.R., and Singh, S.B., 1987. Recent advances in the chemistry of plant antineoplastic constituents. In: Hostettmann, K., Lea, P.J. (eds.), *Biologically active natural products*. Proceedings of the Phytochemical Society of Europe. Oxford Science Publications, UK.

Puupponen-Pimia¨, R., Nohynek, L., Meier, C., Ka¨hko¨nen, M., Heinonen, M., and Hopia, A., 2001. Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology* 90: 494–507.

Rice-Evans, C.A, Miller, N.J. and Paganga G. 1996. Structure antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical*, *Biology and Medicine* 20: 933-956.

Rogers, C.B. and Verotta, L. 1996. Chemistry and biological properties of the African Combretaceae. *Chemistry, Biological and Pharmacological Properties of African Medicinal Plants*: 121–141.

Shakya, A.K. and Shukla, S., 2011. Evaluation of hepatoprotective efficacy of Majoon-e-Dabeed-ul-ward against acetaminophen-induced liver damage: A Unani herbal formulation. *Drug development Research*, *72*(4), pp.346-352.

Schwikkard, S., Zhou, B.N., Glass, T.E., Sharp, J.L., Mattern, M.R., Johnson, R.K. and Kingston, D.G.I., 2000. Bioactive compounds from *Combretum erythrophyllum. Journal of Natural Products* 63: 457–460.

Sofowora, L.A., 1993. Medicinal plants and traditional medicine in Africa. Spectrum Books Ltd, Ibaban. 55–71.

Tambe, V.D. and Bhambar, R.S., 2014. Estimation of total phenol, tannin, alkaloid and flavonoid in Hibiscus Tiliaceus Linn. wood extracts. Research and reviews. *Journal of Pharmacognosy and Phytotherapy* 2:2321–6182.

Tijjani, M.A., Abdurahman, F.I., Buba, S.W., Mala, G. I., Akan, J.C., Aji, B.M., and Abdullahi. A.S., 2012. Chemical and proximate contents of methanolic leaf extract of *Piliostigma thonningii* schum (Camel Foot). *Journal of Chemical and Pharmaceutical Research* 4(5): 2409 – 2414.

Trease, G.E., and Evans, W.C., 1989. Pharmacognosy. 14th edition. W.B. Sanders, London. 288.

Von Gadow, A., Joubert, E., and Hansmann, C.F., 1997. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of Rooibos tea (*Aspalathus linearis*), α- tocopherol, BHT, and BHA. *Journal of Agricultural and Food Chemistry* 45: 632-638.

Wannes, W.A., Mhamdi, B., Sriti, J., Jemia, M.B., Ouchikh, O., Hamdaoui, G., Kchouk, M.E., Marzouk, B., 2010. Antioxidant activities of the essential oil and

methanol extracts from myrtle (*Myrtus communis var. italica* L.) leaf, stem and flower. *Food and Chemical Toxicology* 48:1362–1370.

Zengin, G., Cakmak, Y.S., Guler, G.O., Aktumsek, A., 2011. Antioxidant properties of methanolic extract and fatty acid composition of *Centaurea urvillei* DC. Subspecies hayekiana Wagenitz. *Records of Natural Products* 5:123–132.

CHAPTER 4: PROXIMATE AND MINERAL COMPOSITION

4.1 Introduction

Plants are a good source of energy and supply nutrients to the consumers, including people. Over the past two decades, there has been an increased recognition of the importance of wild or locally cultivated food plants as sources of micronutrients and plant secondary metabolites (Scoones *et al.,* 1992). More recently, the role of these biologically diverse species in maintaining human and environmental health has been highlighted, particularly in relation to global food security, sustainable development and the United Nations Millennium Development Goals (Frison *et al.,* 2006; Johns and Eyzaguirre, 2006).

Plants are a rich source of organic and inorganic compounds. The body needs to obtain appropriate nutrients from food to maintain the normal functions of cells and organs, and to promote growth and development (Clemens *et al.,* 2004). Any lack or excess amounts of vitamins in the body may have side effects. In human metabolic reactions, macro, micro and trace elements play an important role. Trace elements play a combating role in curing of various diseases. In medicinal plants, these elements form active compounds that are responsible for both their medicinal and toxic properties (Rajurkar and Damame, 1998). Some medicinal plants contain toxic elements such as (Pb) and cadmium (Cd), which are detrimental to health (Garcia *et al.,* 2000; Lekouch *et al*., 2001; Lo´pez *et al*., 2000). Proximate and nutritional analysis of plants plays an important role in assessing their nutritional significance. Evaluating the nutritional significance of medicinal plants helps to understand the use of such plants (Pandey, 2006). The basic nutrients of food include macronutrients (carbohydrate, protein, fats) and micronutrients (vitamins, and minerals).

4.1.1 Macronutrients

Macronutrients are needed in larger quantities (in gram range). They normally include water, carbohydrates, fat and protein. Macronutrients are also called energy-providing nutrients. Energy is measured in calories and is essential for the body to grow, repair and develop new tissues, conduct nerve impulses and regulate life processes (www.fao.org/elearning/Course/NFSLBC/en/story_content/external_files/Essential_Nutrients.pdf).

Carbohydrates

Carbohydrates are a major class of naturally occurring organic compounds. Among the well-known carbohydrates are various sugars, starches, and cellulose, all of which are important for the maintenance of life in both plants and animals. There are five primary functions of carbohydrates in the human body. These are energy production, energy storage, building macromolecules, sparing protein, and assisting in lipid metabolism.

Protein

Proteins are the most abundant biological macromolecules, occurring in all cells and all parts of cells. Amino acids are the building blocks of proteins. All proteins, whether from the most ancient lines of bacteria or from the most complex forms of life, are constructed from the same set of 20 amino acids. What is most remarkable is that cells can produce proteins with strikingly different properties and activities by joining the same 20 amino acids in many different combinations and sequences. From these building blocks, different organisms can make such widely diverse products as enzymes, hormones, antibodies, transporters, muscle fibers, the lens protein of the eye, feathers, spider webs, rhinoceros horn, milk proteins, antibiotics, and mushroom poisons and other substances having distinct biological activities (http://www.fao.org/3/i3261e/i3261e05.pdf).

Fats

Fats are used in making steroids and hormones and serve as solvents for hormones and fat-soluble vitamins. Fats have the highest caloric content and provide the largest amount of energy when burnt. When measured by a calorimeter, fats provide about 9 calories per gram of fat, making them twice as energy-rich than protein and carbohydrates. Extra fat is stored in adipose tissue and is burnt when the body has run out of carbohydrates (Gurr,, 1984).

4.1.2 Micronutrients

Vitamins have various functions that help to regulate metabolism, to prevent chronic diseases (such as heart disease and cancer), and to maintain normal appetite, mental health, and immunity. Vitamins can basically be classified into the following two categories: (1) Fat-soluble vitamins, which include vitamins A, D, E and K; they dissolve in fats and are absorbed with the help of fats that are in the diet; (2) Water-

soluble vitamins, which include Vitamins B and C; they dissolve in water. Vitamins and minerals are essential for many biochemical and physiologic functions in our bodies (www.fao.org/elearning/Course/NFSLBC/en/story_content/external_files/Essential_Nutrients.pdf).

Minerals are divided into two categories, namely, major minerals and trace minerals based on the amounts one needs to stay healthy. Major minerals, also referred to as macro-minerals, are named thus because more of them are needed in the diet. The daily needs for major minerals range from hundreds of milligrams to over a thousand, depending on the specific mineral. Major minerals include sodium, potassium, magnesium, calcium, phosphorus, chloride and sulphur. Trace minerals are named because less quantities are needed to stay healthy; usually less than 20 milligrams per day. Iron, copper, iodine, manganese, molybdenum, zinc, selenium, fluoride and chromium are trace minerals. Minerals are inorganic, which means they are not produced by living things. Instead, minerals in plants come from the soil in which they are grown. Animals get their minerals from eating plants grown in mineral-rich sources. Because of this, the mineral content of foods can vary widely based on the quality of their soil. Micro-elements have been found to play an important biological role in human, animal and plant health (Abu-Darwish and Abu-Dieyeh, 2009; Slam *et al.,* 2001). The aim of this study was to determine the proximate and mineral composition of the *Combretum* plant extracts.

4.2 Methods and Materials

4.2.1 Ash content

Two grams of the dried sample was weighed into a dry porcelain dish and then heated in the maffle furnace at 600 ^{o}C for 6 hours. It was cooled in desiccators and weighed. The percentage ash content was calculated by using the following formula:

$$\% \text{ Ash} = \frac{weight\ of\ ash - weight\ of\ crusible}{weight\ of\ the\ original\ sample\ x\ dry\ coefficient\ factor} \text{x}100$$

4.2.2 Moisture content

Two grams of the dried sample was put in the moisture analyser. The equipment used the basic “loss-on-drying” technique to simultaneously weigh and heat the sample. Moisture was recorded as percentage moisture.

4.2.3 Mineral analysis

4.2.3.1 Digestion of the dried leaves and stems

Four hundred milligrams of the sample were weighed into the digestion vessels. Five millilitres of HNO_3 and 3 ml of H_2O_2 was added and the mixture was shaken. A waiting period of 10 min was observed before closing the vessel. The microwave heating program is shown in table 4.1.

Table 4.1: Microwave digester conditions

Step	*Target temp ^oC*	*Pressure Max [bar]*	*Ramp Time*	*Hold time Min*	*% Power*
1	150	30	10	5	50
2	150	35	5	15	80
3	50	35	1	10	0
4	-				

Following digestion, the mineral analysis was performed by ICPE 9000 (Shimadzu).

4.2.4 Data analysis

Descriptive statistics were computed using ANalysis Of VAriance (ANOVA) programmed into the R statistical software. Tukey and Dunnett's T3 post-hoc tests were run for equal and un-equal variances, respectively. Means were considered significantly different at $p < 0.05$.

4.3 Results

4.3.1 Proximate analysis

4.3.1.1 Ash

Ash percentage ranged from 1-15 in the leaves and 4-35 in the stems. The leaves of *C. vendae* and *C. adenogdnium* had the highest levels of ash . Generally, the stems (83%) had the highest ash percentage levels when compared to the leaves (17%), as shown in figure 4.1.

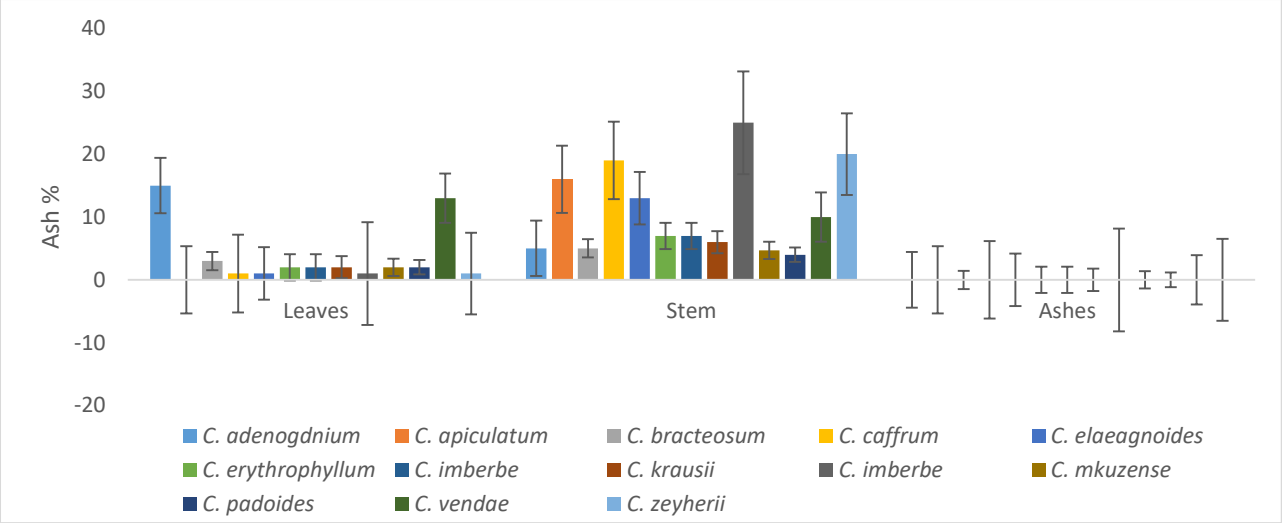


Figure 4.1: Ash percentage of the leaves and stems of the *Combretum* species

4.3.1.2 Moisture

Moisture content of the plants ranged from 0-9%in both the ash, leaves and stems. The leaves of *C. vendae* and *C. adenogonium* had no moisture; it was only detected in the stems. The leaves of *C. caffrum* had the highest moisture content, however; the stems had 0% of content moisture, as depicted in figure 4.2.

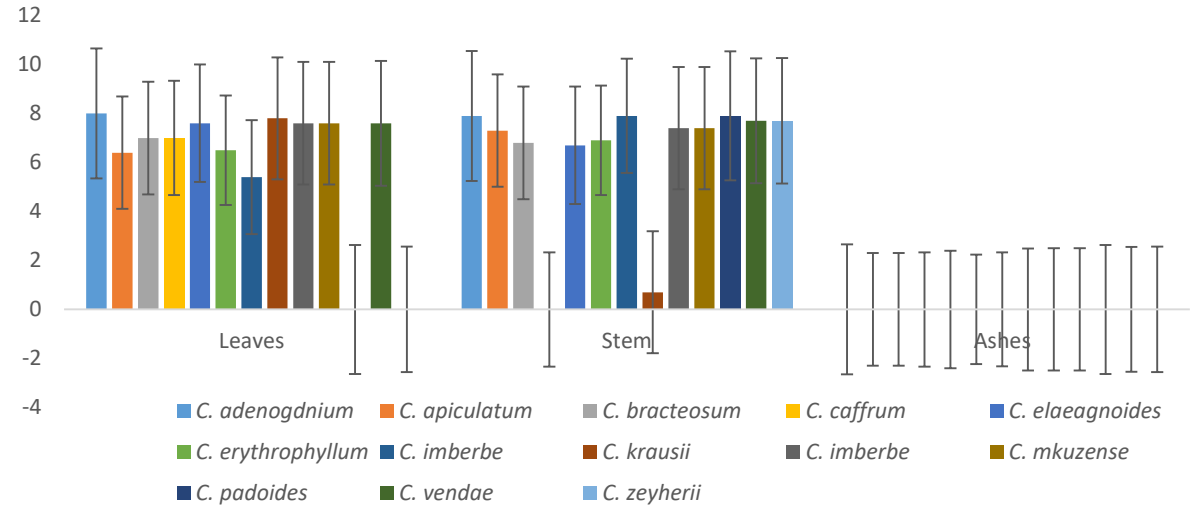


Figure 4.2: Moisture content (%)of the leaves and stems of the *Combretum* plants

4.3.1.3 Protein percentage

The protein content in the leaves ranged between, 2 and 14.4 %, stems 4.04 and 7.66 %, while the ashes contain protein in the range of 0.29-17.1 %, as depicted in figure 4.3. Generally, the leaves had higher protein content compared to the stems and ashes of other species. However, the protein contents of the *C. elaegnoides* and *C. padoides* were interestingly high.

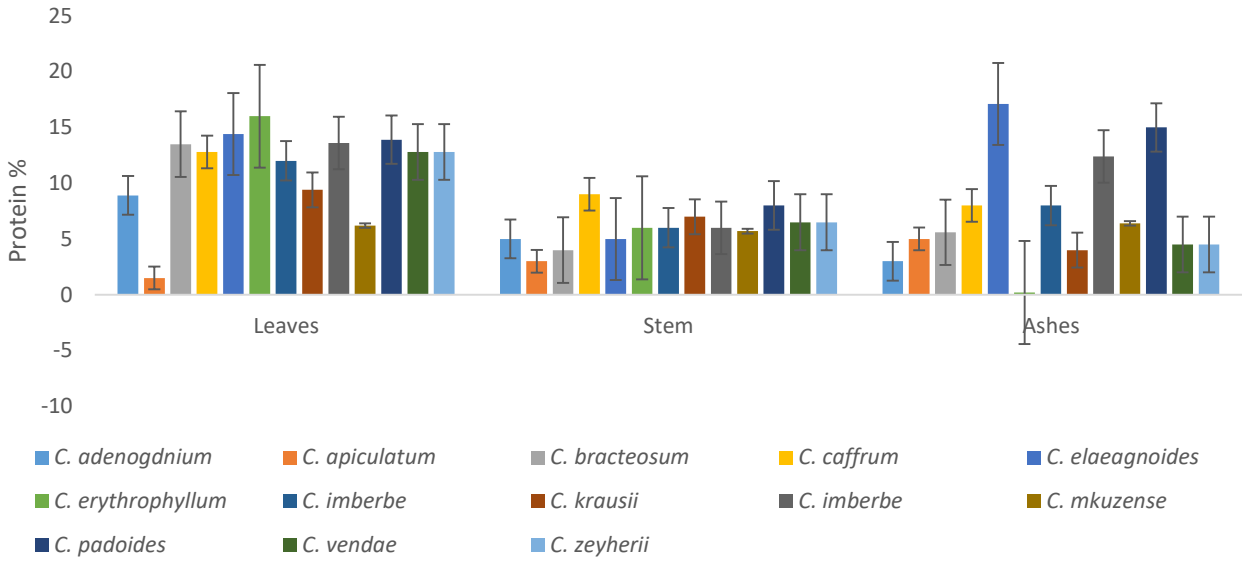


Figure 4.3: Protein percentage of the leaves, stems and ashes of the *Combretum* species.

All the plants had appreciable amounts of energy with *C. adenogdnium* exhibiting the highest concentrations for both leaves and stems, as illustrated in figure 4.4. The ashes *C. erythrophyllum* had the lowest energy overall.

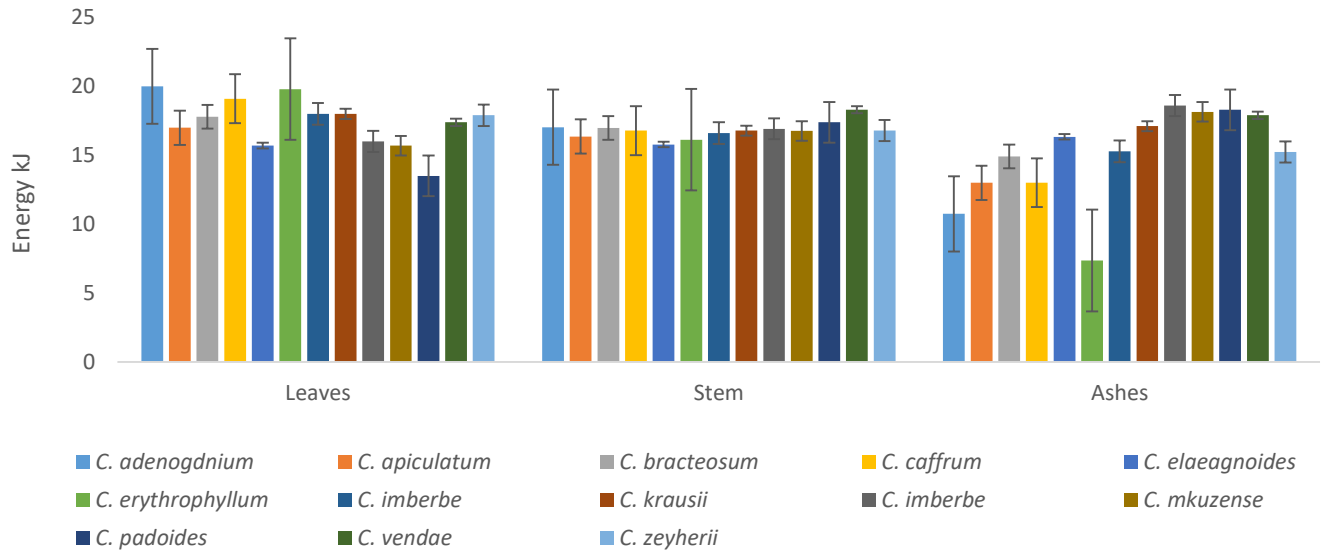


Figure 4.4: Energy content of the leaves, stems and ashes of the *Combretum* plants tested in the study

4.3.2 Mineral and trace metals composition of the leaves.

The minerals detected in the leaves ranged from 0,979-817 (As); 4,86-283 (Ca); 0,962-9,95 (Cd); 0,114-166 (Co); 0,15-0,661 (Cu); 0,209-3,19 (Fe); 2,98-65,5 (K); 1.03-9,55 (Li); 2,03-51,2 (Mg) 0,0319-3,98 (Mn); 1,84-13,7 (Na); 0,0139-3,5 (Ni); 0,729-17,6 (Pb) and 0,006-1,04 (Zn). *C. elaeagnoides* had the lowest concentrations of minerals with the exception of Mg and Zn. *C. adenogonium* had the highest concentration of Ca, Cu and Fe while *C. apiculatum* had the highest concentration of Co, K, Mg and Mn (Table 4.2).

Table 4.2: Concentration (mg/ml) of trace minerals in the leaves of some *Combretum* species

Plant	**Calcium**	**Cobalt**	**Copper**	**Iron**	**Potassium**	**Magnesium**	**Manganese**	**Sodium**	**Nikel**	**Zinc**
C. adenogonium	241	1,03	0,661	3,14	59,9	23,1	1,67	0[f]	3,5	0,941
C. apiculatum	198	1,66	0,508	2,07	65,5	51,2	3,98	12	1,45	0,801
C. bracteosum	28,3	1,54	0,584	3,19	63,4	28,7	1,43	14	1,61	1,03
C. caffrum	119	1,14	0,444	2,63	63,3	37,7	2,96	9,5	1,01	0,688

C. elaegnoides	4,86	0,114	0,15	0,209	2,98	2,03	0,0319	1,8	0,0139	0,177
C. erythrophyllum	131	1,02	0,432	1,71	35,1	26,3	1,32	9,1	0,983	1,04
C. imberbe	46,6	0,816	0,328	0,586	16,7	18,8	0,23	6,6	0,751	0,279
C. kraussii	117	1,11	0,423	1,49	39	27,4	1,69	9,7	1,07	0,599
C. mkuzense	176	1,36	0,509	2,74	63,2	24,3	2,26	11	1,25	0,792
C. padoides	132	1,26	0,467	1,98	37,1	34,7	2,57	10	1,17	0,601
C. vendae	3,9	0,701	0,367	2,51	51,7	22,3	1,84	6,7	0,693	0,351
C. zeyherii	36,1	0,456	0,258	0,955	23,2	8,53	0,929	4,3	0,36	0,006
P value	P=001	P=001	P=001	P=001	P=001	P=001	P=001	P=001	P=001	P=001

C. krausii had the lowest concentration of the following minerals: Ca, Co, Cu, K and Mn while *C. erythrophyllum* had the highest concentration of Cu and Zn. The mineral that was detected in high concentrations was Ca whereas Zn is lowest as shown in table 4.3.

Table 4.3: Concentration (mg/ml) of trace minerals in the stem of some Combretum species

Plant	**Trace elements/minerals**									
	Ca	**Co**	**Cu**	**Fe**	**K**	**Mg**	**Mn**	**Na**	**Ni**	**Zn**
C. apiculatum	**253**	1,15	0,784	1,2	**30**	**25**	2	**10,5**	3,7	0,968
C. bracteosum	**80**	0,526	0,45	1,52	**28**	**11**	0,798	**12,5**	2,88	0,472
C. caffrum	**95**	0,782	0,527	2	**88**	**18**	3,4	6,8	3,1	0,63
C. elaegnoides	**176**	0,81	0,524	1,41	**26**	**14**	0,995	5	3,34	0,782
C. erythrophyllum	**284**	0,978	0,679	2,1	**31**	**19**	1,19	9,1	3,43	1,09
C. imberbe	**235**	1,08	0,678	2,1	**33**	**22**	1,45	5,8	3,56	0,877
C. kraussii	2,67	0,13	0,0547	0,145	**2,89**	**2,61**	0,307	8,7	1,99	0,53
C. mkuzense	**85,1**	0,546	0,386	2,37	**16,1**	**7,6**	0,997	8	2,87	0,387
C. padoides	**104**	0,7	0,44	1,18	**26,1**	**13,5**	1,23	5,1	2,98	0,397
C. vendae	8,08	0,156	0,0707	0,117	**11,6**	**2,19**	0,428	3,5	1,89	0,567
C. zeyherii	**94,5**	0,623	0,523	1,92	**31**	**16,1**	2,17	7,8	2,9	0,342

The mineral compositions in percentage in the ashes ranged from 38.9-980 (Ca); 0.185-0.107 (Co), 0.186-25,4 (Fe); 8.66-25.1(K); 2.08-57,9 (Mg); 0.119-2.71 (Mn); 2.14-24.4 (Na); 0.267-2.22 (Ni); 0.360-18,7 (Zn). Overall *C. zeyherii* had the highest concentration of all the minerals, as highlighted in Table 4.4

Table 4.4: Concentration (mg/ml) of trace minerals in the leaves of some Combretum species

Plant	**Trace elements/minerals**									
	Ca	**Co**	**Cu**	**Fe**	**K**	**Mg**	**Mn**	**Na**	**Ni**	**Zn**
C. adenogonium	**869**	1,13	0,53	0,8	**29**	**22**	0,867	18,8	1,57	2,93
C. apiculatum	**804**	1,15	0,5	2,32	**93**	**35,1**	2,19	18,01	1,66	3,4
C. bracteosum	0	0	0	0	0	0	0	0	0	0
C. caffrum	38,9	0,312	0,208	0,211	36,2	2,58	0,172	3,75	0,386	0,36
C. elaegnoides	**579**	0,92	0,375	0,766	**69,9**	30,9	1,15	12,4	1,22	1,58
C. erythrophyllum	**516**	0,944	0,614	3,17	**78,8**	19,1	0,735	16,2	1,25	3,14
C. imberbe	**0**	0	0	0	0	0	0	0	0	0
C. kraussii	**231**	0,669	0,338	3,17	**70,3**	20	1,03	8,48	0,874	5,88
C. padoides	0	0	0	0	0	0	0	0	0	0
C. mkuzense	**61**	0,245	0,19	0,188	8,67	3,2	0,143	2,35	0,268	0,586
C. vendae	**26,3**	0,248	0,185	0,315	8,67	2,08	0,119	2,14	0,284	0,411
C. zeyherii	**980**	1,73	1,07	25,1	**196**	57,9	2,71	24,4	2,22	18,7

The experimental results regarding the elemental composition of the *Combretum* leaves are presented in Table 4.5. The highest concentrations of Arsenic (As), cadmium (Cd) and lead (Pb) were obtained in the leaves of *C. bracteosum, C. adenogonium. bracteosum (p=001)* and *C. mkuzense*; *C. padoides (p=001)*, respectively. Lowest concentrations of As, Cd and Pb were detected in *C. elaegnoides.*

Table 4.5: Trace metals in the leaves, stems and ashes (mg/ml).

Plant	**Arsenic**			**Cadmium**			**Lead**		
	Leaf	**Stem**	**Ash**	**Leaf**	**Stem**	**Ash**	**Leaf**	**Stem**	**Ash**
C. adenogonium	7,3	0,302	0,752	9,1	0,482	3,1	4,91	0,325	4,92
C. apiculatum	7,44	7,49	0,798	7,87	6,79	3,6	14,9	4,01	5,08
C. bracteosum	8,17	4,31	0	9,95	4,89	0	15,3	2,58	0
C. caffrum	5,07	5,81	0,221	6,2	6,2	0	12,8	3,4	0,968

C. elaegnoides	0,979	5,8	0,589	0,962	7,43	2,49	0,729	4,13	3,72
C. erythrophyllum	4,89	7,44	0,618	5,63	8,45	2,33	13	4,45	3,75
C. imberbe	4,31	8,7	0	4,92	9,07	0	7,32	5,02	0
C. kraussii	6,38	1,92	0,431	6,14	2,201	1,42	16,6	1,45	2,35
C. mkuzense	7,01	3,45	0	7,37	3,98	0	17,6	2,44	0
C. padoides	5,25	3,99	0,174	6,32	5,01	0	11,9	2,77	0,735
C. vendae	3,9	1,4	0,177	3,81	2,25	0	11,7	1,28	0,699
C. zeyherii	2,54	3,7	0,998	1,97	3,61	5,1	7,48	2,4	6,35
P value	P= 001			P=001			P= 001		

Key: L-leaves, S-stem and A-ashes

4.4 Discussion

All the plants investigated are traditionally used for medicinal purposes in South Africa and other African countries. The majority of the plants are indigenous to South Africa. The study of the proximate content, for instance, the moisture content presented in the different parts of the plants, could reflect the plant's ability to resist harsh environmental condition like drought. Reports have shown that most *Combretum spp*, such as *C. erythrophyllum* and *C. zeyherii*, are drought resistant (Michael, 2012). Moisture content is the quantity of water in a material. Water is an essential compound of many foods. About 20% of the total water is consumed through food (FNB, 2005).

The percentage ash content of the plants could be important reflection of the nutritional mineral contents (Raman *et al.,* 2010). Ash contains inorganic material of the plant, which includes oxides and salts containing anions such as phosphates, sulphates, chlorides and other halides and cations such as sodium, potassium, calcium, magnesium, iron, and manganese (Gopalan *et al.,* 2004). The ash content indicates the amount of minerals in that food. Generally, the leaves had the lowest Ash percentage when compared to the stems, with the exception of *C. adenogdium* and *C. vendae. C. kraussii*, which had the highest Ash percentage, followed by *C. zeyherii.* This means that these plants should possess the highest concentration of mineral content. High ash content indicates the presence of heavy amounts of inorganic nutrients in plant material (Odhav *et al.*, 2007). Proteins are large biomolecules or macromolecules that comprise one or more long chains of amino acid residues. Proteins perform a vast array of functions within organisms, including catalysing metabolic reactions, DNA replication, responding to stimuli, providing structure to cells and organisms, and transporting molecules from one location to another (Perrett, 2007; Keskin *et al.,* 2008). The protein contents in the plants were observed in the

following order: leaves > stem > ashes for the following plants (*C. adenogdium, C. caffrum, C. erythrophyllum, krausii and C. zeyherii); Ashes > leaves > stem (C. elaegnoides, C. apiculatum* and *C. vendae); leaves > ashes > stem (C. bracteosum, C. mkuzense* and C. *padoides).* Overall, the leaves had the highest protein content with the exception of *C. elaegnoides* at 17.1%. Foods that provide more than 12 % of their calorific value from proteins are considered a good source of proteins (Ali, 2009). Since many of the plants tested in the study had appreciable levels of protein content, they can be considered for use in the food industry. These plants showed appreciable levels of energy content within them. Plant-based proteins are considered as functional ingredients with various roles in food formulations, including thickening and gelling agents, stabilisers of emulsions and foams, binding agents for fat and water. Moreover, some proteins have biological activities such as antioxidant or antimicrobial characteristics (Jafari *et al*., 2020; Sedaghat Doost, *et al.,* 2019; Warnakulasuriya and Nickerson, 2018).

Trace elements (also known as trace minerals) are dietary minerals that are essential for proper growth, development, maintaining and recovering the health of the organism (Aliasgharpour and Marjan, 2013). Some trace elements control important biological processes through such actions as facilitating the binding of molecules to receptor sites on cell membranes, altering the structure or ionic nature of membranes to prevent or allow specific molecules to enter or leave a cell, and inducing gene expression resulting in the formation of proteins involved in life processes. In this study, the leaves contain the highest concentration of calcium, while zinc has the lowest concentration. *C. bracteosum* had the lowest levels of minerals such as calcium, copper, nickel and zinc when compared to leaves of other Combretum species used in this study *C. adenogonium* had the highest levels of copper and iron, while *C. caffrum* had highest levels magnesium and manganese*. C. elaegnoides* had the lowest concentrations of the following minerals: Ca, Cd, Co, Cu, Fe, K, Mg, Mn and Ni.

The variation of elemental content from plant to plant was mainly attributed to the differences between the botanical structure and the mineral composition of the soil in which plants are cultivated. Other factors responsible for the variation include: absorbability of the plants, use of fertilizers, irrigation water and climatic conditions (Masson *et al.,* 2010). Compared to other foods, the leaves analysed could be considered all as good sources of minerals. They were found to have higher

concentrations of minerals when compared to consumable vegetables such as *Allium sativum* and *Allium tuberosum (*Khalid *et al., 2014).* In addition,, they were found to have higher concentrations than some wild plants that are used as spices such as *F. xanthoxyloïdes, H. gabonii* (bark and fruit), *M. myristica, M. whitei, P. brazzeana, P. guineense, P. umbellatum, S. melongena, S. striatinux, S. zenkeri* (fruit) *S zenkeri (bark) T. tetraptera X. aethiopica* (Bouba *et al.,* 2012). With regards to stems, the mineral content was found to have decreased significantly when compared to the leaves. There was a further decrease in mineral content with regard to the ashes, with the exception of calcium. It was interesting to observe that the concentration of calcium in all the ashes increased significantly, that is, approximately four times more than the leaves and stems. Based on this findings, it can be concluded that the leaves and ashes of *C. adenogonium* and *C. apiculatum* could provide a good source of calcium. Calcium is also important for blood coagulation and normal functioning of the cardiac muscles (Sundriyal and Sundriyal, 2004). It also noteworthy to highlight that *C. zeyherii* has high mineral content. These minerals are necessary for the the maintainance of good health in both animals and humans. Furthermore, the mineral elements affect biochemical processes and play crucial roles in living organisms, specifically the biological, metabolic and enzymatic reactions leading to the development of active organic components (Serfor-Armah *et al.,* 2002). Sodium and potassium maintain the ionic balance of the human body and maintain tissue excitability. Sodium plays an important role in the transport of metabolites (Sinha *et al.*, 2019). The ratio of potassium/sodium in any food is an important factor associated with hypertension and arteriosclerosis. Sodium enhances and potassium depresses blood pressure (Saupi *et al.,* 2009). In this study, the leaves and ashes of *C. adenogonium, C. bracteosum and C. apiculatum* proved to be a good source of sodium . Iron is essential in oxygen binding to haemoglobin and acts as a catalyst for many enzymes like cytochrome oxidase (Geissler and Powers, 2005). In the current study, the leaves of *C. adenogonium*; *C. bracteosum* and ashes of *C. zeyherii* possessed high levels concentrations of iron. Thus, these plants can be recommended against anaemia. Magnesium helps to prevent muscle degeneration, growth retardation, cardiomyopathy, immunologic dysfunction, impaired spermatogenesis and bleeding disorders (Chaturvedi *et al.,* 2004). The leaves of *C. bracteosum*; stems of *C. apiculatum* and ashes of *C. caffrum, C. bracteosum* possessed appreciable amounts when compared to other plants in the study. The recommended dietary

allowance (RDA) for calcium (1000 mg/day), magnesium (400 mg/day) and iron (8 mg/day) suggests that these plants contribute substantially to improving the diet in terms of mineral requirement. The manganese concentration ranged between 0.00 and 3.9 mg/mg, which is higher than the values obtained from wild edible plants such as *Aegle marmelos (L.) Corrêa, Argyreia speciosa (L. f.) Sweet, Butea monosperma (Lam.) Taub)* reported by Seal and Chaudhury (2016) and Seal *et al.* (2017. Zn concentration ranged between 0.006 and1, 04mg/ml, which is similar to the levels reported in some wild and leafy vegetables in India (Salkia and Deka, 2013), Bangladesh (Abdus Satter *et al.,* 2016) and Nigeria (Mohammed and Sharif, 2011), and higher than those reports from Cameroon (Mih *et al.,* 2017). According to Shirwaikar *et al.* (2004), minerals such as copper, manganese and zinc are well-known antioxidants. The presence of these minerals might be attributed to the increase in antioxidant activity of certain plants. Metals and other elements can be naturally present in food or can enter food because of human activities such as industrial and agricultural processes. Arsenic, cadmium and lead are the main heavy metals that are closely monitored by food manufacturers and producers due to their toxicity. These heavy metals can end up in a food for several reasons. Heavy metals food testing is the only way to ensure that one's products are within normal range and meet regulatory requirements. Arsenic is a naturally occurring toxic metal found in almost all environments. Its presence in food could be a potential risk to both humans and animals (Al Ramali, 2005). High levels of As, Cd and Pb were obtained in *C. bracteosum, C. adenogonium, C. bracteosum (p=001) and C. mkuzense; C. padoides (p=001)*, respectively. Arsenic concentrations ranged from 0.979-8, 17, cadmium 0.962- 9, 95 and lead 0,729-17, 6 mg/ml. Similar results were reported by Hussain *et al.* (2019), who investigated plant-based foods. Although these heavy metals were detected in the plants tested in the study, they were found to be within the limits regulated in food in South Africa (Regulations of Metals in Food, 2016).

Conclusion: Based on findings from this study, the selected plants and ashes contained appreciable amounts of nutrients such as protein, energy and mineral elements that could enhance the nutrition of both humans and livestock. This further suggest that these plants and their ashes could serve as feed supplement to improve health and growth performance in humans and livestocks. It can be ascertained that the *Combretum* extracts, together with the ashes, can be exploited as a source of

natural nutrients and minerals, especially *C. adenogonium, C. bracteosum and C. apiculatum. Combretum* species could be potential nutraceuticals as nutritional supplements. This indicates that the *Combretum* plants will offer both nutritional and medicinal benefits to its users.

4.5 References

Abdus Satter, M.M., Khan, M.M.R.R.L., Jabin, S.A., Abedin, N., Islam, M.F., and Shaha, B., 2016. Nutritional quality and safety aspects of wild vegetables consume in Bangladesh. *Asian Pacific Journal of Tropical Biomedicine* 6 (2): 125-131.

Abu-Darwish, M. S. and Abu-Dieyeh, Z.H., 2009. Essential oil content and heavy metals composition of thymus vulgaris cultivated in various climatic regions of Jordan. *International Journal of Agriculture and Biology* 11: 59–63.

Aliasgharpour, M. and Rahnamaye Farzami, M., 2013. Trace elements in human nutrition: A review. International journal of medical investigation, 2(3), pp.0-0.

Ali, A., 2009. Proximate and mineral composition of the marchubeh (*Asparagus officinalis)*. World Journal of Dairy Food Science 4:142-149.

Bouba, A.A., Njintang, N.Y., Foyet, H.S., Scher, J., Montet, D. and Mbofung, C.M.F. Year??? 2012?? Agricultural systems, a literature review and annotated bibliography. *International* Journal of Food Sciences and Nutrition, ????, ???.

Chaturvedi, V.C., Shrivastava, R. and Upreti, R.K., 2004. Viral infections and trace elements: a complex trace element. *Current Science* 87.

Clemens, S., Palmgren, M.G. and Kramer, U., 2004. A long way ahead: understanding and engineering plant metal accumulation. Department of Political Science at the University of Roma Tre. 7: 309–315.

FNB, 2005. Food and Nutrition Board Dietary Reference Intakes for Water Potassium, Sodium, Chloride and Sulphate. Institute of Medicine, National Academies, Washington, DC: National Academies Press.

Frison, E.A., Smith, I.F., Johns, T., Cherfas, J. and Eyzaguirre, P., 2006. Agricultural biodiversity, nutrition, and health: making a difference to hunger and nutrition in the developing world. *Food and Nutrition Bulletin* 27:167–179.

Garcia, E., Cabrera, C., Lorenzo, M.L. and Lo´pez M.C., 2000. Chromium levels in spices and aromatic herbs. *Science of the Total Environment* 247: 51– 56.

Geissler, C.A., and Powers, H.J., 2005. Human nutrition. 11th edition. Elsevier Churchill Livingstone.

Gopalan, C., Rama Sastri, B.V., and Balasubramanian, S.C., 2004. Nutritive value of Indian foods. Printed by National Institute of Nutrition, Indian Council of Medical Research, Hyderabad-500 007, India, 2-58.

Johns, T.E. and Eyzaguirre, P.B., 2006. Linking biodiversity, diet and health in policy and practice. *Proceedings of the Nutrition Society* 65:182–189.

Keskin, O., Tuncbag, N., Gursoy, A., 2008. Characterization and prediction of protein interfaces to infer protein-protein interaction networks. *Current Pharmaceutical Biotechnology* 9 (2): 67–76.

Khalid, N., Ahmed, I., Latif, M.S.Z., Rafique, T. and Fawad, S.A., 2014. Comparison of antimicrobial activity, phytochemical profile and minerals composition of garlic *Allium sativum* and *Allium tuberosum. Journal of the Korean Society for Applied Biological Chemistry* 57(3): 311-317.

Lekouch, N., Sedki, A., Nejmeddine, A. and Gamon, S. 2001. Lead and traditional Moroccan pharmacopoeia. Science of the Total Environment: 1-3 39–43.

Lo´pez, F.F., Cabrera, C., Lorenzo, M.L., Lo´pez, M.C., 2000. Aluminium levels in spices and aromatic herbs. Science of the Total Environment 257:191–197.

Michael, 2012.???

Mih, A.M., Ngone, A.M. and Ndam, L.M., 2017. Assessment of nutritional composition of wild vegetables consumed by the people of Lebialem Highlands, South Western Cameroon. Food Science and Nutrition 8 (6): 647-657.

Mohammed, M.I., and Sharif, N., 2011. Mineral composition of some leafy vegetables consumed in Kano, Nigeria. Niger. Journal of Basic and Applied Sciences19 (2): 208 –212.

Odhav, B., Beekrum, S., Akula, U. and Baijnath, H. 2007. Preliminary assessment of nutritional value of traditional leafy vegetables in KwaZulu-Natal, South Africa. *Journal of Food Composition and Analysis* 20: 430–435.

Pandey, M., Abidi. A.B., Singh, S. and Singh, R.P., 2006. Nutritional evaluation of leafy vegetable paratha. *Journal of Human Ecology* 19:155–156.

Perrett, D., 2007. From 'protein' to the beginnings of clinical proteomics. *Proteomics: Clinical Applications* 1 (8): 720–738.

Rajurkar, N.S., and Damame, M.M., 1998 Elemental analysis of some herbal plants used in the treatment of cardiovascular diseases by NAA and AAS. *Journal of Radio Analytical Nuclear Chemistry* 219: 77–80.

Raman, R. C. Ipper, G. B. and Subhash, J. D. 2010. Preliminary phytochemical investigation of extract of leaves of Pergularia daemia Linn. *International Journal of Pharmaceutical Sciences and Research*: 111–116.

Saikia, P., and Deka, D.C., 2013. Mineral content of some wild green leafy vegetables of North-East India. *Journal of Chemical and Pharmaceutical Research* 5 (3): 117-121.

Saupi, N., Zakaria, M.H., and Bujang, J.S., 2009. Analytic chemical composition and mineral content of yellow velvet leaf (*Limnocharis flava L. Buchenau*)'s edible parts. *Journal of Applied Science* 9 (16): 2969e2974.

Scoones, I., Melnyk, M., and Pretty, J.N., 1992. The hidden harvest: wild foods and compounds from *Combretum erythrophyllum* (Combretaceae). *Journal of Ethnopharmacology* 62(3): 255 -263.

Seal, T., and Chaudhuri, K., 2016. Nutritional analysis of some selected wild edible plants consumed by the tribal people of Meghalaya state in India. *The International Journal of Food Sciences and Nutrition* 1 (6): 39-43.

Sedaghat Doost et al., 2019.???

Senthilkumar, M., Gurumurthi, P., Janardhanan, K., 2006. Some medicinal plants used by Irular, the tribal people of Marudhamalai Hills, Coimbatore, Tamil Nadu. *Journal of Natural Products Radiance* 5 (5): 382-388.

Serfor-Armah, Y., Nyarko, B.J.B., Akaho, E.H.K., Kyere, A.W.K., Osae, S., and Oppong-Boachie, K., 2002. Multielemental analysis of some traditional plant medicines used in Ghana. *Journal of Trace and Microprobe Techniques*, 20: 419–427.

Shinwari, Z.K., 2011. Determination of macro and micronutrients and nutritional prospects of six vegetable species of Mardan, Pakistan. *Journal of Botany* 43 (6): 2829-2833.

Shirwaikar, A., Rajendran, K., and Kumar, C.D., 2004. *In vitro* antioxidant studies of *Annona squamosa* Linn. *Leaves, Indian Journal of Experimental Biology* 42: 803-807.

Sinha, B.K. Bhattacharjee, S., and Tapan, S., 2019. Nutritional composition, mineral content, antioxidant activity and quantitative estimation of water-soluble vitamins and phenolics by RP-HPLC in some lesser-used wild edible plants. Heliyon e01431.

Slam, M.A., Travis, R.L., and Rains, D.W., 2001. Differential effect of amino acids on nitrate uptake and reduction systems in barley roots. *Plant Science* 160 (2): 219-228.

Sundriyal, M., and Sundriyal, R.C., 2004. Wild edible plants of the Sikkim Himalaya: nutritive values of selected species. *The Society for Economic Botany* 58 (2): 286-299.

Warnakulasuriya and Nickerson, 2018.???

CHAPTER 5. ANTIMICROBIAL EFFECTS OF THE LEAVES, STEM AND WOOD ASHES OF COMBRETUM SPECIES

5.1 Introduction

Natural products are still one of the major sources of new drug and food preservatives molecules today. Plants and other natural sources can provide a wide range of complex and structurally diverse compounds (Balouiri *et al.,* 2016). Recently, many workers have focused on the investigation of plant and microbial extracts, essential oils, pure secondary metabolites and new synthetised molecules as potential antimicrobial agents (Runyoro *et al.,* 2006; Mabona *et al.,* 2013; Nazzaro *et al.,* 2013).

Some of the commonly encountered opportunistic and entero-pathogenic microbes include *Escherichia coli,* a Gram-negative bacterium present in normal human and animal flora. However, the pathogenic strains are implicated in causing serious diseases or symptoms such as diarrhoea, haemorrhagic colitis, haemolytic uraemic syndrome and thrombocytopenic purpura (Bell and Kyriakides, 1998). *P. aeruginosa* is a Gram-negative opportunistic highly antibiotic resistant bacterial pathogen responsible for infections in the urinary tract, respiratory system, soft tissue, bone and joints, gastrointestinal and a variety of systemic infections, dermatitis and bacteremia, particularly in patients with severe burns, cancer and AIDS patients (Andualem, 2012). *S. aureus*, a Gram-positive bacterium, causes mastitis, toxic shock syndrome (TSS) and staphylococcal food-poisoning (SFP) in humans and animals. SFP symptoms caused by ingestion of food containing heat-stable staphylococcal enterotoxins (SETs) include nausea, vomiting, abdominal cramps and diarrhoea (Rosengren *et al.,* 2010). The microbe has also been implicated in causing skin infections such as boils, abscesses, carbuncles and sepsis of wounds. *E. faecalis* is another Gram-positive bacterium and natural inhabitant of the gastrointestinal tract of humans and is usually disseminated from the gastrointestinal tract to cause cholangitis, peritonitis, and intra-abdominal abscess. *Enterococci* are a leading cause of nosocomial infection and an infrequent cause of pneumonia, meningitis and osteomyelitis, usually in the immunocompromised host (Butler, 2006).

Pseudomonas aeruginosa is a common bacterium, Gram-negative opportunistic pathogen capable of infecting humans with compromised natural defences and causing severe pulmonary disease. It is one of the leading pathogens associated with

nosocomial infections. It has a vast arsenal of pathogenicity factors that are used to interfere with host defences. *P. aeruginosa* is a motile, non-fermenting, Gram-negative organism belonging to the family *Pseudomonadaceae* (Alhazmi, 2015). It is one of the leading pathogens associated with nosocomial infections andits pathogenicity interferes with host defenses. The propensity of P. aeruginosa to form biofilmsfurther protects it from antibiotics and the host immune system. P. aeruginosa is intri nsically resistant to a large number of antibiotics and can be acquired resistance to many others, making treatment difficult (Alhazmi, 2015).

E. faecalis is a Gram-positive, commensal bacterium inhabiting the gastrointestinal tracts of humans and other mammals. Like other species in the *genus Enterococcus*, *E. faecalis* can cause life-threatening infections in humans, especially in the nosocomial (hospital) environment where the naturally high levels of antibiotic resistance in *E. faecalis* contribute to its pathogenicity (http://www.microbiologyinpictures.com/bacteria-photos/enterococcus-faecalis images.html). *E. faecalis* has been frequently found in root canal-treated teeth in prevalence values ranging from 30% to 90% of the cases. Root canal-treated teeth are about nine times more likely to harbour *E. faecalis* than other primary infections. *E. faecalis* is resistant to many commonly used antimicrobial agents (aminoglycosides, aztreonam, cephalosporins, clindamycin, the semisynthetic penicillins (nafcillin and oxacillin).

The fact that a plant extract exhibits antimicrobial activity is of interest. A variety of laboratory methods can be used to evaluate or screen the *in vitro* antimicrobial activity of an extract or pure compound. The most common and basic methods are disc-diffusion and broth or agar dilution methods.

Disc diffusion or the Kirby–Bauer test is one of the classic microbiology techniques, and is still commonly used (Christenson *et al.,* 2018). Because of convenience, efficiency, and cost, the disc diffusion method is probably the most widely used method for determining antimicrobial resistance around the world. A suspension of the isolate (of approximately 1–2 × 10^8 CFU/ml) is prepared to a particular McFarland standard, then spread evenly onto an appropriate agar (such as Müller-Hinton agar) in a Petri dish. With the test, the discs are impregnated with various defined concentrations of different anti-microbial agent and are placed onto the surface of the agar. A

multichannel disc dispenser can speed up the placement of the discs. After incubation (16–24 h at 37 °C), zones of growth inhibition around each of the anti-microbial agent discs are measured to the nearest millimetre. A clear circular zone of no growth in the immediate vicinity of a disc indicates susceptibility to that antimicrobial (CLSI, 2012a).

Dilution methods are the most appropriate ones for the determination of MIC values, since they offer the possibility of estimating the concentration of the tested antimicrobial agent in the agar (agar dilution) or broth medium (macro-dilution or micro-dilution). Either broth or agar dilution method may be used to quantitatively measure the *in vitro* antimicrobial activity against bacteria and fungi. MIC value recorded is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the microorganism tested, and it is usually expressed in mg/ml or mg/l.

The agar dilution method involves the incorporation of varying desired concentrations of the antimicrobial agent into an agar medium (molten agar medium), habitually using serial two-fold dilutions, followed by the inoculation of a defined microbial inoculum onto the agar plate surface. The minimum inhibitory concentration (MIC) end point is recorded as the lowest concentration of antimicrobial agent that completely inhibits growth under suitable incubation conditions. This technique is suitable for both antibacterial and antifungal susceptibility testing. If multiple isolates are being tested against a single compound, or if the compound (or extract) tested masks the detection of microbial growth in the liquid medium with its colouring, agar dilution method is often preferred to broth dilution for the MIC determination. Nowadays, commercially produced inoculum replicators are available and can transfer between 32 and 60 different bacterial inoculators of each agar plate. Agar dilution is often recommended as a standardised method for fastidious organisms (CLSI, 2012b).

Broth micro-dilution is one of the most basic antimicrobial susceptibility testing methods. The procedure involves preparing two-fold dilutions of the antimicrobial agent in a liquid growth medium dispensed in tubes containing smaller volumes using 96-well micro titration plate. Then, each well is inoculated with a microbial inoculum prepared in the same medium after dilution of a standardised microbial suspension adjusted to 0.5 McFarland scale. After well mixing, the inoculated 96-well micro titration

plates were incubated under suitable conditions depending upon the test microorganism.

The MIC is the lowest concentration of antimicrobial agent that completely inhibits the growth of the organism in micro-dilution wells as detected by the unaided eye (CLSI, 2012a). For the determination of MIC endpoint, viewing devices can facilitate reading micro-dilution tests and recording results with high ability to discern growth in the wells. Moreover, several colorimetric methods based on the use of dye reagents have been developed. Tetrazolium salts 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) and 2,3-bis{2-methoxy-4-nitro-5-[(sulfenylamino)carbonyl]-2H-tetrazolium hydroxide} (XTT), are often used in the MIC endpoint determination for both antifungal and antibacterial micro-dilution assays (Liang *et al*., 2012; Monteiro *et al.*, 2012; Kuhn *et al.,* 2003). The Alamar blue dye (resazurin), an effective growth indicator, can also be used for this purpose (Reis *et al.,* 2004; Ouedrhiri *et al.,* 2015; Bouhdid, *et al.,* 2009; Castilho *et al.,* 2015). This method offers advantages over diffusion methods in that it has increased sensitivity even on smaller quantities, reproducibility and convenience (Reller *et al.,* 2009). The broth micro-dilution method is considered the most sensitive method for screening antimicrobial activity in plant extracts (Eloff, 1998). The aim of this chapter was to evaluate the antibacterial properties of the leaves, stems and ashes used in preparation of sorghum juice.

5.2 Methods and Materials

5.2.1 Test organisms

The test organisms were supplied by the Department of Biochemistry, Microbiology and Biotechnology section of the University of Limpopo (Turfloop Campus). Two Gram-positive (*S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212) and two Gram-negative (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) bacterial strains were used in this study. The organisms were sub-cultured on nutrients broth, incubated at 37°C for 24 h and stored at 4°C in the refrigerator as stock cultures.

5.2.2 Plant extraction

The combretum plants were extracted by weighing 1 g of finely ground plant material and extracting it with 10 ml, acetone in different polyester centrifuge tubes. Tubes were vigorously shaken for 10 minutes in series 25 shaking incubator machine (New Brunswick Scientific Co., Inc.) at a high speed (200 rpm). There after the extracts were

filtered into labelled bottles. The process was repeated three times to exhaustively extract constituents of the plant material and the extracts were combined. The solvent was removed under a stream of cold air at room temperature. The final extracts were reconstituted in 70% acetone to a concentration of 10 mg/ml

5.2.3 Broth micro-dilution assay

The minimum inhibitory concentration (MIC) values were determined using the serial microplate broth dilution methods developed by Eloff (1998). The plant extracts were dissolved in acetone to give a final concentration of 10 mg/ml. The bacterial species were inoculated into 150 ml nutrient broth and incubated at 37 °C for 24 hours, this served as the stock culture. From the stock culture, 10 ml was removed and inoculated in 150 ml nutrient broth and incubated at 37 °C for 24 hours. Hundred microlitres of the plant extract was serially diluted (50%) with sterile distilled water in 96-well microtitre plates, and 100 µl of the bacterial culture was added into each well. Acetone was used as a negative control; the microtitre plates were covered and incubated at 37 °C for 24 hours. Following incubation, 40 µl of 0.2% ρ-iodonitrotetrazolium chloride (INT) (sigma) dissolved in water was added to each well as an indicator. The covered plates were further incubated for 30 minutes at 37 °C at relative humidity. The plates were observed for clear wells (activity), which resulted from reduction of the purple colour and the MIC values were recorded as the lowest concentration that inhibited bacterial growth. The tests were done in triplicates. Total activity of the extracts was calculated by dividing the MIC values with the mass extracted from 1 g of the plant material. The resultant values indicated the volume to which the amount obtained from 1 g of the plant material could be diluted to and still inhibit growth of the test microorganisms (Eloff, 2001).

5.3 Results

5.3.1 MIC of the Combretum leaves stems and ashes

In this study, the antimicrobial activity of the 70% acetone extracts of 12 *Combretum* leaves, stems and ashes were investigated. The leaves tested in the study showed great antibacterial properties with the lowest MIC value being 0.04 mg/ml against *E. coli* and *S. aureus. E. faecalis* was found to be resistant against all the leaves with the exception of *C. Imberbe,* as indicated in the table 5.1.

Table 5.1: The minimum inhibitory concentrations (mg/ml) of 70% acetone leaf extracts of *Combretum* species against some bacterial isolates.

Microbes	**Plants (leaves)**												**Ampicillin**
	CAd	**CAp**	**CB**	**CC**	**CEl**	**CEr**	**CI**	**CK**	**CM**	**CP**	**CV**	**CZ**	
E. coli	>2.5	>2.5	**0.04**	>2.5	0.16	2.5	2.5	0.31	2.5	>2.5	0.63	2.5	0.03
P. aeruginosa	1.25	1.25	2.5	1.25	1.25	1.25	**0.63**	1.25	2.5	>2.5	**0.31**	**0.63**	0.02
S. aureus	0.16	0.16	**0.04**	0.16	0.08	0.16	0.16	0.08	0.16	0.16	0.63	0.63	0.03
E. faecalis	>2.5	2.5	>2.5	2.5	2.5	2.5	**1.25**	>2.5	>2.5	>2.5	2.5	>2.5	0.08

Key: (CAd) *C. adenogdium*, (CAp) *C. apiculatum,* (CB) *C. bracteosum,* (CC) *C. caffrum, (CEl) C. elaegnoides, (CEr) C. erythrophyllum,* (CI), *C. imberbe,* (CK) *C. kraussii,* (CM) *C. mkuzense,* (CP) *C. padoides*, (CV) *C. vendae and* (CZ) *C. zeyherii*

Most of the stems of *Combretum* spp. tested in the study showed antimicrobial properties with the lowest MIC value being 0.04 mg/ml against *E coli.* However, *E. faecalis* showed to be resistant against all the 12 plants tested as shown in Table 5.2

Table 5.2: The minimum inhibitory concentrations (mg/ml) of 70% acetone extracts the stems of *Combretum* species against some bacterial isolates.

Microbes	**Plants(stem)**												**Ampicillin**
	CAd	**CAp**	**CB**	**CC**	**CEl**	**CEr**	**CI**	**CK**	**CM**	**CP**	**CV**	**CZ**	
E. coli	2.5	2.5	**0.04**	2.5	**0.16**	2.5	2.5	**0.31**	2.5	2.5	**0.63**	2.5	0.03
P. aeruginosa	1.25	1.25	2.5	1.25	1.25	1.25	0.63	1.25	2.5	>2.5	**0.31**	0.63	0.02
S. aureus	0.31	0.16	**0.04**	0.16	**0.08**	0.16	0.16	**0.08**	0.16	0.16	0.63	0.63	0.03
E. faecalis	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.08

Key: (CAd) *C. adenogdium*, (CAp) *C. apiculatum,* (CB) *C. bracteosum,* (CC) *C. caffrum, (CEl) C. elaegnoides, (CEr) C. erythrophyllum,* (CI), *C. imberbe,* (CK) *C. kraussii,* (CM) *C. mkuzense,* (CP) *C. padoides*, (CV) *C. vendae and* (CZ) *C. zeyherii*

All the test microorganisms showed resistance to the ashes of *Combretum* species, with the exception of *S aureus*, which was found to be susceptible to 75% of the test ashes, as shown in table 5.3 where 0.16 mg/mL was the lowest MIC.

Table 5.3: The minimum inhibitory concentrations (mg/ml) of the ashes of *Combretum* spp. against some bacterial isolates.

Microbes	**Ashes**												**Ampicillin**
	CAd	**CAp**	**CB**	**CC**	**CEl**	**CEr**	**CI**	**CK**	**CM**	**CP**	**CV**	**CZ**	
E. coli	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.03
P. aeruginosa	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.02
S. aureus	2.5	2.5	0.63	**0.16**	**0.16**	**0.16**	**0.16**	**0.16**	**0.16**	0.31	1.25	2.5	0.03
E. faecalis	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	0.08

Key: (CAd) *C. adenogdium*, (CAp) *C. apiculatum,* (CB) *C. bracteosum,* (CC) *C. caffrum, (CEl) C. elaegnoides, (CEr) C. erythrophyllum,* (CI), *C. imberbe,* (CK) *C. kraussii,* (CM) *C. mkuzense,* (CP) *C. padoides*, (CV) *C. vendae and* (CZ) *C. zeyherii*

5.4 Discussion

Plants have an impressive ability to produce a myriad of bioactive secondary metabolites like flavonoids, tannins, alkaloids, glycosides, terpenoids, saponin, steroids, quinones and coumarins (Das *et al.,* 2010). These bioactive molecules are the source of plant-derived antimicrobial substances and many have been reported to be highly efficient in treating bacterial infections (Srivastava *et al.,* 2015). After assessing the antibacterial properties of the leaves in the study, the following was observed; *C. bracteseum* was found to be more active against *E. coli* with the lowest MIC value of 0.04mg/ml. It was followed by *C. elaegnoides* with the MIC value of (0.16 mg/ml) and *C. kraussii* at 0.63 mg/ml. Not all the leaves tested were active against *E. coli.* Although, *C. bracteseum* was the most active leave extract against *E. coli,* in comparison to the control (Ampicilin), it was found to have the higher MIC value. *P. aeruginosa* was susceptible to *C. vendae* with the MIC value of 0.31 mg/mL, followed by *C. zeyherii* and *C. imberbe* with the MIC value of 0.63 mg/ml. *C. adegnonium, C. apiculatum, C. caffrum, C. elaegnoides and C. kraussii*, had MIC value of 1.25 mg/ml, while *C. padoides, C. mkuzense* and C. *bracteseum* did not inhibit *P. aeruginosa*. It was interesting to observe that plants such as *C. elaegnoides and C. kraussii* inhibited

E. coli at low concentrations of 0.31 and 0.63 mg/mL, respectively, while a higher concentration (1.25 mg/ml) inhibited the growth of *P. aeruginosa*. The activity of these plants may be due to the presence of several classes of secondary metabolites, including triterpenoids, flavonoids, stilbenes, tannins and lignans (Lima De Morais *et al.,* 2012; Zhang *et al.,* 2019). The same compounds have been isolated from different species of the genus *Combretum*. For example, a series of unique stilbenes (combretstatins) were isolated from *C. kraussii, C. caffrum,* and *C. erythrophyllum* (Pettit *et al.,* 1987; Rogers and Verotta, 1996; Brookes *et al.,* 1999; Schwikkard *et al.,* 2000; Eloff *et al.,* 2005; Famakin *et al.,* 2005). It also important to note that although *C. bracteseum* was highly active against *E. coli* with lowest MIC value (0.04 mg/ml), it was not active against *P. aeruginosa* (2.5 mg/ml). Most *E. coli* bacteria are harmless and exist in the intestines of people and warm-blooded animals. However, some strains can cause illness (Madigan *et al.,* 2009). *E. coli* is used as a pathogen indicator in water and food products. *S aureus* was susceptible to all the leaf extracts in this study with the lowest MIC value of 0.04 mg/ml (*C. bracteseum)* and the highest MIC of 0.63 mg/ml (*C. vendae* and *C. zeyherii).* Several pharmacological activities of *Combretum* species and some of the isolated compounds have been reported from South Africa, Democratic Republic of Congo and Burkina Faso. The plants have demonstrated higher antimicrobial activities when compared with currently used antibiotics like chloramphenicol and ampicillin (Martini and Eloff, 1998; Eloff, 1999; McGaw *et al.,* 2001; Atindehou *et al.,* 2004; Masoko and Eloff, 2005; Eloff and McGaw, 2006; Gansan´e *et al.,* 2010; Manga *et al*., 2012). However, in this study, although great activity was observed, it was found to be lower than ampicillin. *E. faecalis* was found to be resistant to all the leave extracts with the exception *of C. Imberbe* that had and MIC of 1.25 mg/ml. *E. faecalis* is Gram-positive cocci that often in the form of diplococci or short chains. *E. faecalis* can cause endocarditis and bacteraemia, urinary tract infections (UTI), meningitis, and other infections in humans (Kousedghi *et al.,* 2012). Recent reports show that resistance of these bacteria to commonly used antibiotics is increasing worldwide, even vancomycin-resistant and gentamicin-resistant species of *E. faecalis* were reported (Aligholi *et al.,* 2011; Furustrand Tafin *et al.,* 2011). When it comes to the activity of the stems against the bacterial cultures, a similar trend shown by the leaves were observed. The only difference was observed in the case of *E. faecalis,* the leaves *of C. Imberbe* had activity with MIC value of 1.25 mg/ml while the stem had no activityAll the bacterial cultures were resistant to the

ashes with the exception of *S. aureus. S. aureus* was resistant to most ash extracts with the exception of *C. adegnonium* and *C. apiculatum C. caffrum, C. elaegnoides, C. erythrophyllum, C. imberbe, C. kraussii* and *C. mkuzense* had the lowest MIC of 0.16 mg/ml. Currently, there are no reports on the antimicrobial activity of the ashes obtained from *Combretum* plants. Therefore, there is the need to isolate compounds from the ashes that exhibited promising effects. Overall, most of the plant extracts (leaves, stems and ashes) were found to be active against *E. coli and S. aureus. C. bracteosum* was found to be the most active extract against *E. coli* and *S. aureus* while *C. vendae* was active against *P. aeruginosa*. Several workers investigated the efficiency of plant extracts and their effective compounds as antimicrobial agents to control the growth of food borne and spoilage bacteria. Some workers have suggested that antimicrobial components of the plant extracts (terpenoids, alkaloids and phenolic compounds) interact with enzymes and proteins of the microbial cell membrane causing its disruption to disperse a flux of protons towards cell exterior, which induces cell death or may inhibit enzymes necessary for amino acids biosynthesis (Burt, 2004; Gill and Holley, 2006). In addition, the inhibitory effects of these plant extracts on bacteria have been attributed to hydrophobicity characters of these plants extracts that enable them to react with protein of microbial cell membrane and mitochondria disturbing their structures and changing their permeability (Friedman *et al*., 2004; Tiwari *et al.,* 2009). The present study suggested that plant extracts, which proved to be potentially effective, can be used as natural preservatives to control food poisoning and preserve food this could lead to the avoidance of application of chemical preservatives that are harzadous to health..

Conclusion: The broth microdilution assay demonstrated significant antimicrobial activity against some of the pathogens in most of the 12 *Combretum* plant species and ashes that were tested. Wild edible *Combretum* plants such as *C. bractesoum*, *C. adegnonium* and *C. apiculatum C. caffrum, C. elaegnoides, C. erythrophyllum, C. imberbe, C. kraussii and C. mkuzense* could be considered a promising source of preservatives in the food industry as well as perhaps new drug candidates. Further in-depth studies need to include *in vivo* tests to determine the effectiveness, stability and impact of the studied extracts (and their bioactive compounds) on controlling food pathogens, and to evaluate their potential as preservatives to prolong the shelf life of food.

5.5 References

Aligholi, M., Emaneini, M., Taherikalani, M., Shahsavan, S., Ja-balameli, F., and Asadollahi, P., 2011. Time-kill study and synergisttic activity of cell-wall inhibitor antibiotics in combination with gentamicin against *Enterococcus faecalis* and *Enterococcus faecium. Acta Microbiologica et Immunologica Hungarica* 58:219-26.

Alhazmi, A., 2015. Pseudomonas aeruginosa-pathogenesis and pathogenic mechanisms. International Journal of Biology, 7(2), p.44.

Andualem, B., 2012. The isolation rate of Pseudomonas aeruginosa opportunistic pathogen and their antimicrobial responses in HIV-1positive and negative diarrhoea patients at north-west part of Ethiopia. *Journal of AIDS Clinical Research* 3: 148.

Balouiri, M., Sadiki, M., and Ibnsouda, S.K., 2016. Methods for in vitro evaluating antimicrobial activity: a review. *Journal of Pharmaceutical Analysis* 6(2):71-79.

Bell, C., and Kyriakides, A., 1998. *E. coli:* a practical approach to the organism and its control in Foods Blackie Academic and Professional, London.

Bouhdid, S., Abrini, J. and Zhiri, A., 2009. Investigation of functional and morphological changes in Pseudomonas aeruginosa and Staphylococcus aureus cells induced by Origanum compactum essential oil. Journal of Applied Microbiology 106: 1558–1568.

Brookes, K.B., Doudoukina, O.V., Katsoulis, L.C., and Veale, D.J.H., 1999. Uteroactive constituents from Combretum kraussii. *South African Journal of Chemistry* 52: 127–132.

Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods – a review. *International Journal of Food Microbiology* 94: 223–253.

Butler, 2006.???

Castilho, A.L., Caleffi-Ferracioli, K.R., and Canezin, P.H., 2015. Detection of drug susceptibility in rapidly growing mycobacteria by Resazurin broth micro-dilution assay. *Journal of Microbiology* 111: 119–121.

Christenson, J.C., Korgenski, K.E., and Relich, R.F., 2018. Principles and practice of paediatric infectious diseases. 5th edition. 286 - Laboratory Diagnosis of Infection Due to Bacteria, Fungi, Parasites, and Rickettsiae: 1422-1434.

CLSI, 2010. Methods for antimicrobial dilution and disk susceptibility of infrequently isolated or fastidious bacteria, approved guideline. 2nd edition. CLSI document M45-A2. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087, USA.

CLSI, 2012a Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, approved standard. 9th edition. CLSI document M07-A9. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087, USA.

CLSI, 2012b. Performance standards for antimicrobial disk susceptibility tests, approved standard. 7th edition. CLSI document M02-A11. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087, USA.

Eloff, J.N., 1998. A sensitive and quick method to determine the minimum inhibitory concentration of plant extracts for bacteria. Planta Medica 64: 711–713.

Eloff, J.N., 1999. It is possible to use herbarium specimens to screen for antibacterial components in some plants. Journal of Ethnopharmacology 67:355–360.

Eloff, 2001.???

Eloff, J.N., Famakin, J.O., and Katerere, D.R.P., 2005. Isolation of an antibacterial stilbene from Combretum woodii (Combretaceae) leaves. African Journal of Biotechnology 4: 1167–1171. https://doi.org/10.5897/AJB2005.000-3232.

Eloff, J.N., and McGaw, L.J., 2006. Plant extracts use to manage bacterial, fungal and parasitic infection in South Africa. In: Ahmad, 1, Aqil, F., Owais, M. (Eds.), *Modern Phytomedicine*. Wiley- VCH Verlag GmbH and Co., KGaA, pp. 71–121.

Famakin, J.O., Eloff, J.N., Katerere, D.R.P., 2005. Isolation of an antibacterial stilbene from *Combretum woodii* (Combretaceae) leaves. *African Journal of Biotechnology* 4: 1167–1171.

Friedman, M., Henika, P.R., Levin, C.E., and Mandrell, R.E., 2004. Antibacterial activities of plant essential oils and their components against *Escherichia coli O157:H7* and *Salmonella enterica* in apple juice. Journal of Agricultural and Food Chemistry 52: 6042–6048.

Furustrand Tafin, U., Majic, I., Zalila Belkhodja, C., Betrisey, B., Corvec, S., and Zimmerli, W., 2011. Gentamicin improves the ac-tivities of daptomycin and vancomycin against Enterococcus faecalis in vitro and in an experimental foreign-body infection model. *Antimicrobial Agents and Chemotherapy* 55:4821-7.

Gansané, A., Nébié, I., Soulama, I., Tiono, A., and Diarra, A., 2009. Change of antimalarial first line treatment in Burkina Faso in 2005. Le *Bulletin de la Société de Pathologie Exotique* 102 (1):31–35

Gill, A.O., and Holley, R.A., 2006. Disruption of *Escherichia coli, Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. *International Journal of Food Microbiology* 108: 1–9.

Kousedghi, H., Ahangari, Z., Eslami, A., and Ayatolahi, A., 2012. Antibacterial activity of propolis and Ca (OH) 2 against Lactobacillus, *Enterococcus faecalis,* Peptostreptococus and *Candida albicans*. *African Journal of Microbiology Research* 14:3510-5.

Kuhn, D.M., Balkis, M., and Chandra, J., 2003. Uses and limitations of the XTT assay in studies of Candida growth and metabolism. *Journal of Clinical Microbiology* 41: 506–508.

Liang, H., Y. Xing, Y., and Chen, J., 2012. Antimicrobial activities of endophytic fungi isolated from *Ophiopogon japonicus (Liliaceae),* BMC Complementary *Alternative Medicine* 12:238.

Lima De Morais, G.R., De Sales, I.R.P., Filho, M.R.D.C., De Jesus, N.Z.T., De Sousa Falcao, H., Barbosa-Filho, J.M., Cabral, A.G.S., Souto, A.L., Tavares, J.F., and Batista, L.M., 2012. Bioactivities of the genus *Combretum (Combretaceae)*: a review. Molecules 17. https://doi.org/10.3390/molecules17089142, 9142–7206.

Mabona, U., Viljoen, A., and Shikanga, E., 2013. Antimicrobial activity of Southern African medicinal plants with dermatological relevance: from anethno-pharmacological

screening approach to combination studies and the Isolation of a bioactive compound. *Journal of Ethnopharmacology* 148: 45–55.

Madighan et al., 2009.???

Manga, F.N., Khattabi, C.E.I., Fontaine, J., Berkenboom, G., Duez, P., Nzunzu, J.L. and Pochet, S., 2012. Vascular effects and antioxidant activity of two *Combretum* species from Democratic Republic of Congo. *Journal of Ethnopharmacology* 142: 194–200.

Martini, N.D., and Eloff, J.N., 1998. The preliminary isolation and of several antibacterial compounds from Combretum erythrophyllum (Combretaceae). *Journal of Ethnopharmacology* 62: 255–263.

Masoko, P., and Eloff J.N., 2005. The diversity of antifungal compounds in six South African Terminalia species (Combretaceae) determined by bio-autography. *African Journal of Biotechnology* 14: 1425–1431.

McGaw, L.J., Steenkamp, V., and Eloff, J.N., 2007. Evaluation of Athrixia bush tea for cytotoxicity, antioxidant activity, caffeine content and presence of pyrrolizidine alkaloids. *Journal of Ethnopharmacology* 110: 16–22.

Monteiro, M.C., delaCruz, M., and Cantizani, J., 2012. A new approach to drug discovery: high-throughput screening of microbial natural extracts against Aspergillus fumigatus using resazurin. *Journal of Biomolecular Screening* 17: 524–529.

Nazzaro, F., Fratianni, F., and DeMartino, L., 2013. Effect of essential oils on pathogenic bacteria. *Pharmaceuticals* 6:1451–1474.

Ouedrhiri, W., Bouhdid, S., and Balouiri, M., 2015. Chemical composition of Citrusaurantium L. Leaves and zest essential oils, their antioxidant, antibacterial single and combined effects. *Journal of Chemical and Pharmaceutical Research* 7: 78–84.

Pettit, G.R., Singh, S.B., Niven, M.L., Hamei, E. and Schmidt, J.M., 1987. Isolation, structure, and synthesis of combretastatins A-l and B-l, potent new inhibitors of microtubule assembly, derived from *Combretum caffrum. Journal of Natural Products* 50: 119–131. https://doi. org/10.1021/np50049a016.

Reis, R.S., Neves, I., and Lourenço, S.L.S., 2004. Comparison of flow cytometric and alamar blue tests with the proportional method for testing susceptibility of

Mycobacterium tuberculosis to Rifampin and Isoniazid. *Journal of Clinical Microbiology* 42: 2247–2248.

Reller, L.B., Weinstein, M., Jorgensen, J.H., and Ferraro, M.J., 2009. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clinical Infectious Diseases* 49(11): 1749-1755.

Rogers, C.B., and Verotta L., 1996. Chemistry and biological properties of the African Combretaceae. In: Hostettmann, K., Chinyanganya, F., Maillard, M., Wolfender, J.L. (Eds.), *Chemistry, biological and pharmacological properties of African medicinal plants, phytochemistry*. University of Zimbabwe Publications, Harare, 121–142.

Rosengren, Å., Fabricius, A., Guss, B., Sylvén, S. and Lindqvist, R., 2010. Occurrence of foodborne pathogens and characterization of Staphylococcus aureus in cheese produced on farm-dairies. International journal of food microbiology, 144(2), pp.263-269.

Runyoro, D.K., Matee, M.I., and Ngassapa, O.D., 2006. Screening of Tanzanian medicinal plants for anti-Candida activity, BMC Complement. *Alternative Medicine* 6:11.

Schwikkard, S., Zhou, B.N., Glass, T.E., Sharp, J.L., Mattern, M.R., Johnson, R.K., and Kingston, D.G.I., 2000. Bioactive compounds from Combretum erythrophyllum. *Journal of Natural Products* 63: 457–460.

Srivastava, P., Prasad, S.G.M., Mohd Nayeem, A., and Prasad, M., 2015. Analysis of antioxidant activity of herbal yoghurt prepared from different milk. *Journal of Pharmaceutical Innovation* 4:18-20.

Tiwari, B.K., Valdramidi, V.P., O'Donnell, C.P., Muthukumarappan, K., Bourke, P. and Cullen, P.J., 2009. Application of natural antimicrobials for food preservation. *Journal of Agricultural and Food Chemistry* 57: 5987–6000.

Zhang, X.R., Kaunda, J.S., Zhu, H.T., Wang, D., Yang, C.R., and Zhang, Y.J., 2019. The Genus Terminalia (Combretaceae): an ethnopharmacological, phytochemical and pharmacological review, natural products and bioprospecting. Springer Singapore.

CHAPTER 6: CYTOTOXICITY ASSAY OF LEAVES, STEMS AND ASHES OF SOME *COMBRETUM* SPECIES

6.1 Introduction

Cytotoxicity studies are useful initial steps in determining the potential toxicity of a test substance, including plant extracts or biologically active compounds isolated from plants. Minimal to no toxicity is essential for the successful development of a pharmaceutical or cosmetic preparation and in this regard, cellular toxicity studies play a crucial role (McGaw *et al.,* 2014). The cytotoxicity test, one of the biological evaluation and screening tests, uses tissue cells *in vitro* to observe the cell growth, reproduction and morphological effects by the test substances (Soenen *et al.,* 2012). Cytotoxicity assays are based on various cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity. Many workers have established methods such as Colony Formation method, Crystal Violet method, Tritium-Labelled Thymidine Uptake method, MTT, and WST methods, which are used for counting the number of live cells (Riss *et al.,* 2013). There are three types of cytotoxicity test: Extract, direct contact and indirect contact tests (Li *et al.,* 2015).

Direct contact method: It yields direct contact of the solid “test substance” or medical device with cultured mammalian cells *in vitro* (Li *et al*., 2015). The cytotoxic test occurs by observing the morphological changes and detecting the changes in the number of cells; it can directly reflect the impact of testing the “test substance” or medical devices on the cells. Although the method has high sensitivity, it is more demanding for the medical devices, and suitable medical devices are limited (Frei *et al.,* 1987).

Indirect contact method: Molecular filtration detects cytotoxicity by evaluating the activity of the monolayer succinate dehydrogenase effect by the test substance or medical devices. Monolayer cells are cultured on a cellulose ester filter first and the original culture medium is subsequently replaced with medium containing agar, allowing fresh medium gel on cells. Finally, the single-cell membrane gel is separated and reversed to expose the membrane upwards. Following exposure to the sample, the filter is removed and the metabolic activity of cells affected by the sample is measured (Sayes *et al*, 2006). This method can observe the primary and secondary

cytotoxicity of test substances or medical devices, and is simple, rapid, sensitive, reliable, easy to promote and suitable for the evaluation of the short-term and mildly toxic test substances or medical devices (Li *et al.,* 2015).

The extract method: The mitochondrial dehydrogenase performance measurement, also known as the 3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium bromide (methyl thiazolyl tetrazolium; MTT) assay, is a rapid assessment of cell proliferation and cytotoxicity colorimetric assay to measure cell metabolism or function used (Fotakis and Timbrell, 2006). The main principle is as follows: Mitochondrial dehydrogenase in the cytochrome *b* and *c* sites of the living cells can cleave to the tetrazole ring, and the yellow, water-soluble MTT is reduced by living cells to insoluble purple MTT formazan crystals using succinate, and the pyridine nucleotide cofactors, NADH and NADPH as substrates (Berridge and Tan, 1993). This results in a yellow to blue colour change that can be quantified (Hansen *et al.,* 1989). This substance is soluble in dimethyl sulphoxide and other organic solvents but is insoluble in water. The amount of crystals formed has a positive correlation to the number of cells and their activity,and measuring the absorbance colorimetric value reflects the number of surviving cells and metabolic activity (Li *et al*., 2015).

Other methods. Cell growth inhibition tests, the ultraviolet spectrophotometer assay, cell rehabilitation method, the degree of cell proliferation assays, cell morphology observation, dentin barrier and high-pressure liquid chromatography are used for the cytotoxicity analysis. In recent years, evaluation methods have been developed from the whole animal and cellular level to the molecular level using molecular biology techniques, such as the activation of proto-oncogenes and tumour-suppressor gene inactivation studies. Investigators have reported the restoration of the precipitated metal ions on the oral mucosa cells and osteoblast-like cells, DNA damage and induction of apoptosis at the molecular level (Faccioni, 2003; Cortizo and Etcheverry, 1995). Markey *et al.* (2011) reported an estrogen compound (bisphenol propane) leaking from dental medical devices, and plastic products, which can cause changes in DNA synthesis and induce abnormal body morphology, function and behaviour.

Cancer is a complex genetic disease caused by mutation of oncogenes or tumour suppressor genes, has the ability to be developed due to alteration of signalling pathways (Ouyang *et al.,* 2012). Globally, lung cancer is one of the leading causes of,

mostly avoidable mortality and morbidity. According to the World Health Organization (WHO), 16.2 % of the world total of Disability Adjusted Life Years (DALYs) was attributable to cancer in 2000, and 17.1 % in 2015 (Vos *et al.,* 2016). In recent decades, lung cancer has been one of the most frequently diagnosed cancers worldwide and has high mortality. The “Global cancer statistics” data showed that in 2020, there were approximately 2.2 million new lung cancer cases and 1.8 million new deaths worldwide (Wild *et al.,* 2020). The symptoms of lung cancer in the early stage are not obvious and can easily escape diagnosis or be misdiagnosed. Additionally, lung cancer has a high recurrence rate and poor prognosis. After treatment, the 5-year survival rate of lung cancer is approximately 16% in China, which is unsatisfactory (Zeng *et al*., 2015). Treatment options for lung cancer typically include surgical re-section, radiotherapy, chemotherapy, and targeted therapy. Availability of natural products with higher effectiveness and lower side effects when compared with the currently used anticancer drugs are desired (Lachenmayer *et al.,* 2010). Medicinal herbs are important for cancer treatment due to their multiple chemical compound for discovering new active materials against cancer (Newmann and Gregg, 2007). Plants produce a wide range of chemical compounds called secondary metabolites. Alkaloids, terpenoids, flavonoids, pigments, and tannins are important constituents of these compounds. Secondary metabolites have biologic effects such as anti-inflammatory, anticancer, contraceptive, and different effects on hematopoietic cells (Mansourri *et al.,* 2015), lipids (Kooti *et al.,* 2014) and cardiovascular systems (Kooti *et al.,* 2016). Different improvements were reported in common treatments of cancer by finding secondary compounds of natural products and medicinal herbs. It is believed that anticancer effects of plants develop by suppressing cancer’s stimulating enzymes, repairing DNA, stimulating production of antitumor enzymes in cell, increasing body immunity, and inducing antioxidant effects (Sakarkar and Deshmakh, 2011). The positive effect of plants in cancer treatment has been studied extensively and has shown positive results (Asadi-Samani *et al.,* 2016). In addition, studies have proved the positive effect of plants in curing diabetes (Kooti *et al.,* 2016), fertility (Kooti *et al*., 2016) and sterility thyroid disorders, anemia, and psychological disorder (Kooti *et al.,* 2014). Finding plants that replace chemotherapy and cumbersome cures of cancer with cytotoxic effects is necessary (Khalighi-Sigaroodi *et al.*, 2015). The aim of this chapter was to assess the cytotoxicity of the powdered leaves, stems and ashes

on kidney and liver cell lines using the MTT assay. Furthermore, it assess the anticancer properties of the leaves, stems and ashes of some *Combretum* species.

6.2 Methodology

6.2.1 Cytotoxicity and cell viability analysis

The 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay was used to assess the cytotoxic effect of plant extracts on the viability of lung cancer cells (A549). The MTT assay is a colorimetric assay that is used for cellular metabolic activity. It involves the reaction of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)-dependent cellular oxidoreductase enzymes that may, under defined conditions, reflect the number of viable cells present. These enzymes can reduce the tetrazolium dye MTT to its insoluble formazan form, which has a purple colour once solubilised for spectrophotometer measurement. The effect of plant extracts and the negative control (DMSO) on the viability of A459 cells was assessed using the MTT assay. Briefly, lung cancer cells (A549) were seeded in a 96-well microtiter plate at 1×10^5 cells/well and exposed to various concentrations of plant extracts (1.95-1000 µg) and DMSO (1%) for 24 hours after allowing them to attach overnight. After 24 hours, media containing different concentrations of the treated extracts were removed followed by addition of 10 µl of MTT (5 mg/ml) into each well and the plates were incubated for an additional 4 hours at 37°C. After the MTT solution was aspirated off, to achieve solubilisation of the formazan crystal formed in viable cells, 100 µl of DMSO was added into each well before absorbance at 570nM could be measured using a GloMax-Multi+ (Promega, USA). Cell survival rate was calculated with the following formula:

$$Survival\ rate\ (\%) = \frac{Average\ OD\ (experimental\ group)}{Average\ OD\ (Untreated\ group)}\ \mathrm{X}\ 100\%$$

6.2.2 Data analysis

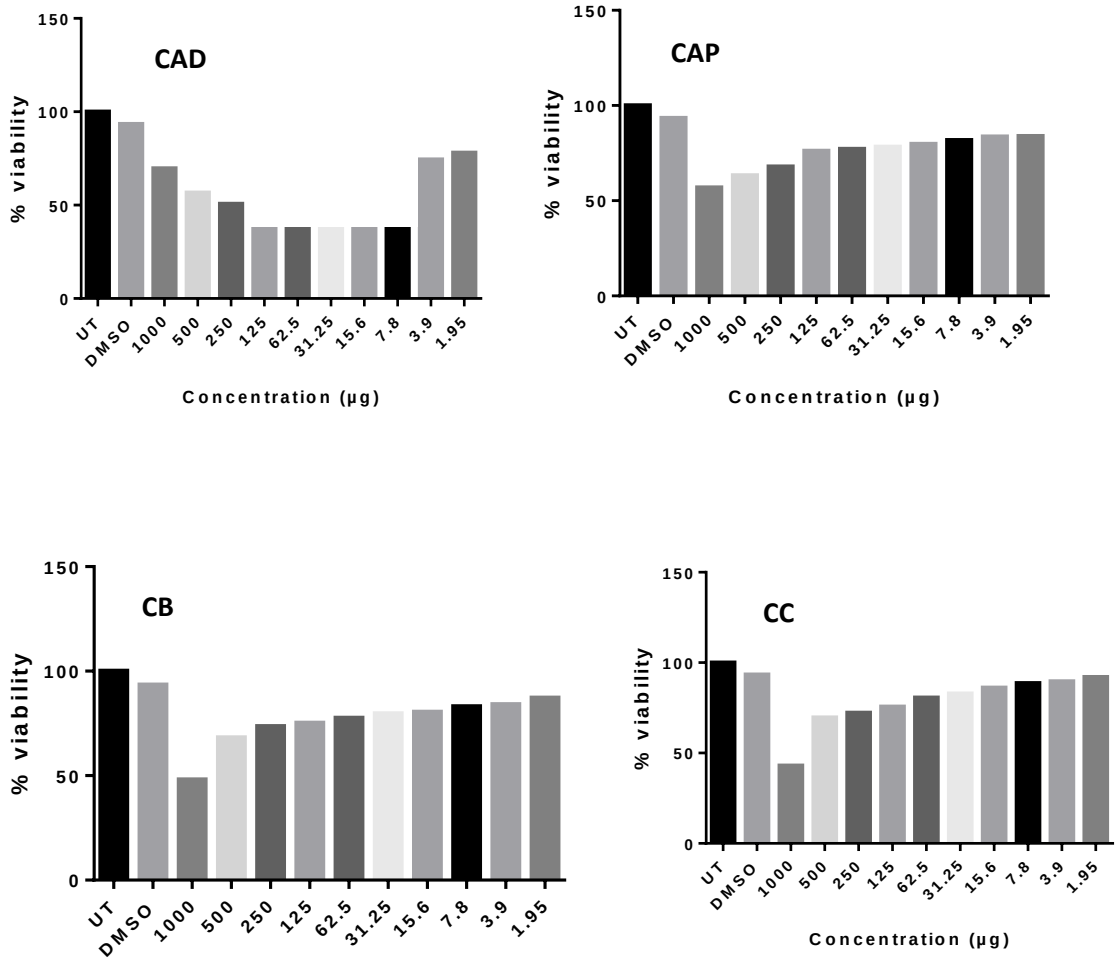
Statistical analysis of results was performed using Statistix 10 data analysis software, a completely randomised test and the Welch's Test was used for comparison of any significant differences between the means. Statistical analysis was performed to determine variation between the cytotoxicity and anticancer activity between a) leaves, b) stems c) ahes. Values were considered significantly different when $p < 0.05$.

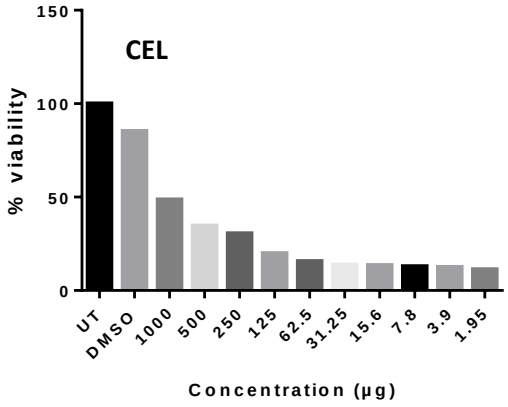
6.3 Results

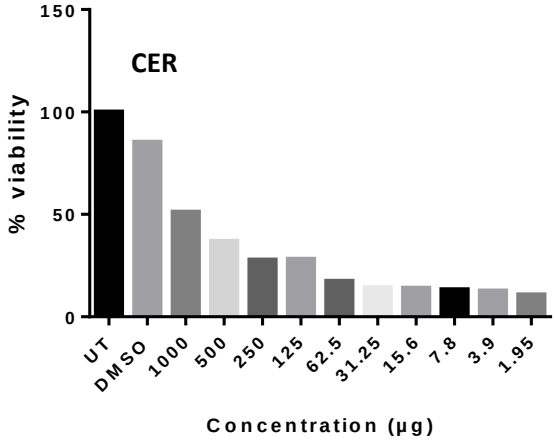
The acetone extracts of the leaves, stems and ashes of the 12 *Combretum* plants were studied for cytotoxicity against A549 lung carcinoma cells using MTT assay. The results showed that *Combretum* plants poseses anticancer activity.

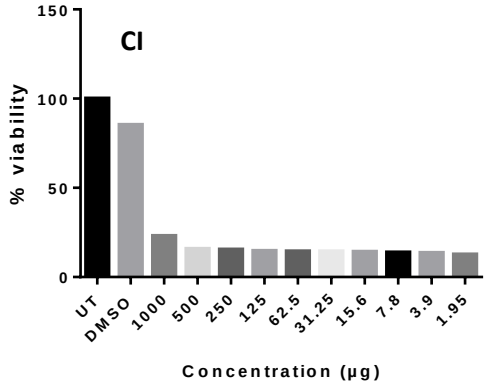
6.3.1 The effect of *Combretum* leaf extracts on A549 cells

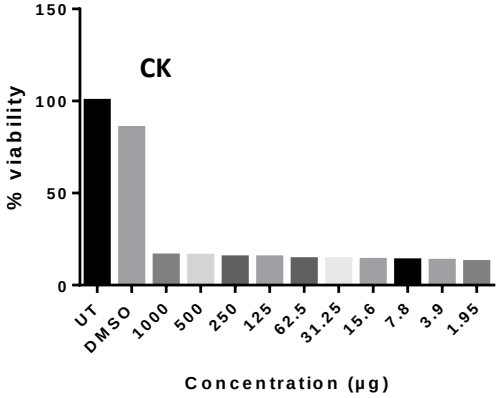
The assays revealed that 50% of the leaf extracts of tested plants showed cytotoxicity and cell proliferation inhibition in lung carcinoma cells A549 in a dose-dependent manner. The A549 cells were more sensitive to the following plants: *(CEl) C. elaegnoides, (CEr) C. erythrophyllum,* (CI), *C. imberbe,* (CK) *C. kraussii* and (CM) *C. mkuzense* as depicted in figure 6.1.

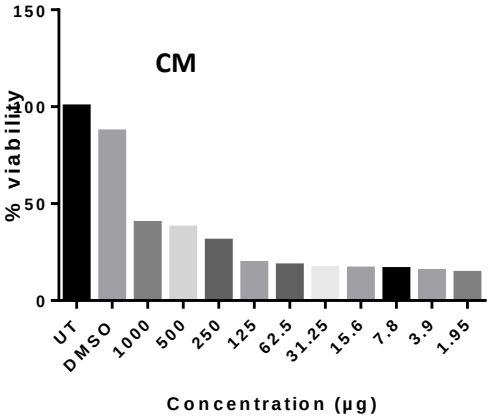


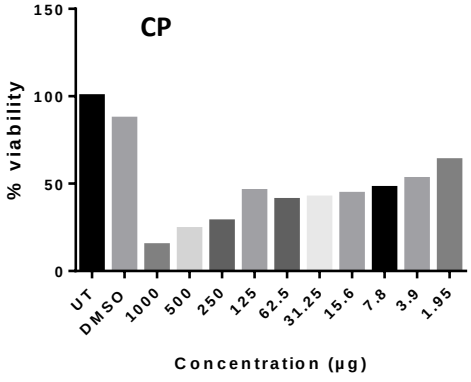

CEL
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)


CER
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)


CI
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)


CK
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)


CM
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)


CP
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)

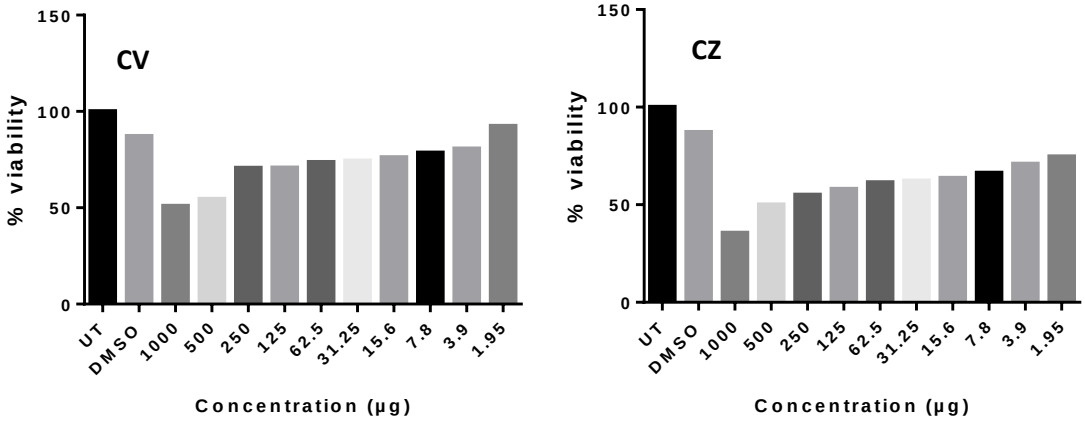


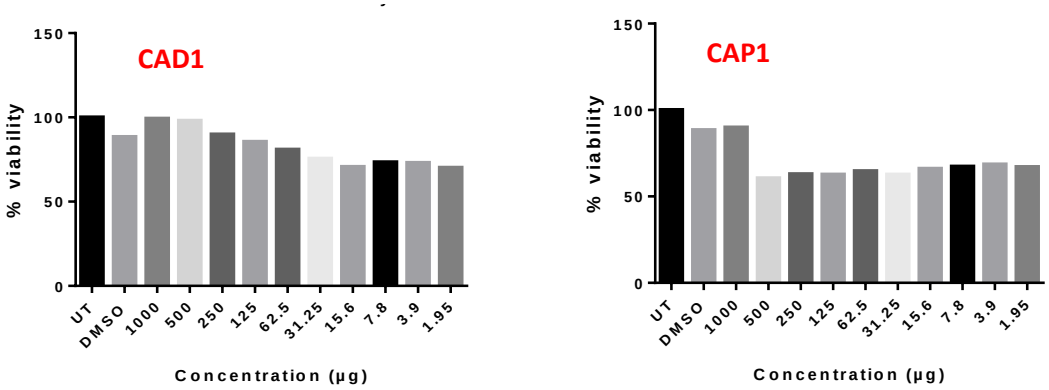
Figure 6.1: Cytotoxicity of twelve *Combretum* acetone leaf extracts against A549 lung cancer cell.

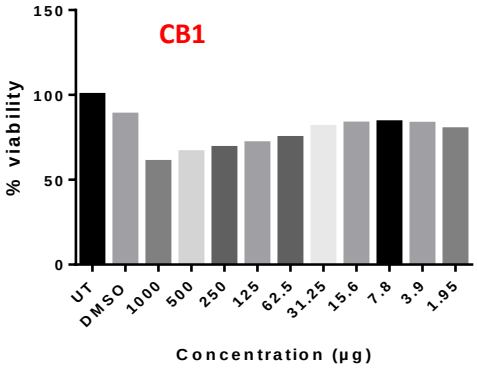
Key:

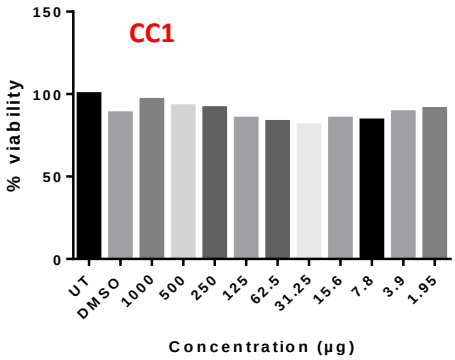
C. adenogdium (CAD), *C. apiculatum* (CAP), *C. bracteosum* (CB), *C. caffrum* (CC), *C. elaegnoides (CEL), C. erythrophyllum (CER),* , *C. imberbe* (CI), *C. kraussii* (CK), *C. mkuzense* (CM), *C. padoides* (CP), *C. vendae* (CV) *and C. zeyherii* (CZ).

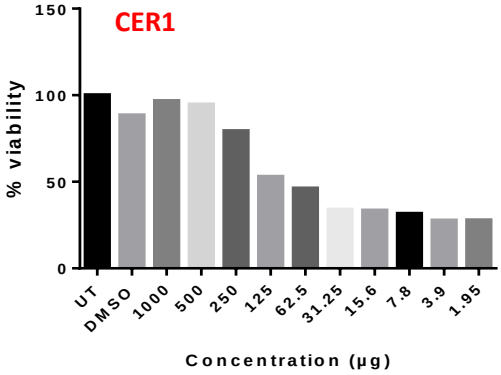
6.3.2 The effect of Combretum stem extracts on A549 cells

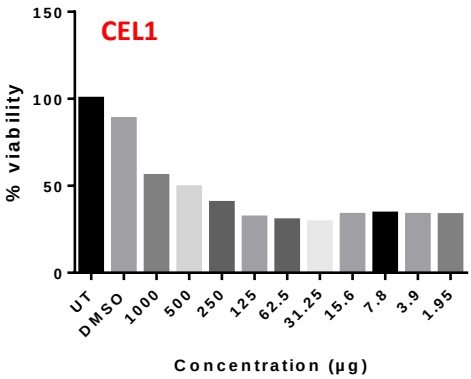
The effects of the stem extracts of Combretum spp. are presented in Figure 6.2. *C. adenogdium* (CAD1) and *C. caffrum* (CC1) did not show anticancer activity, whereas *C. apiculatum* (CAP1) and *C. bracteosum* (CB1) were only able to reduce cell viability to less than 60%. *C. mkuzense* (CM1). *C. padoides* (CP1), *C. vendae (*CV1) and *C. zeyherii* (CZ1) acted in a concentration-depended manner with the highest activity (40%) seen at the 1000 µg/ml.

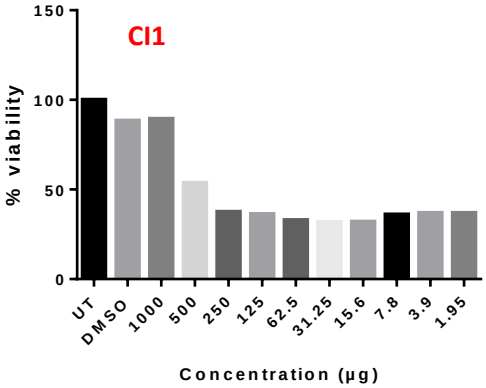


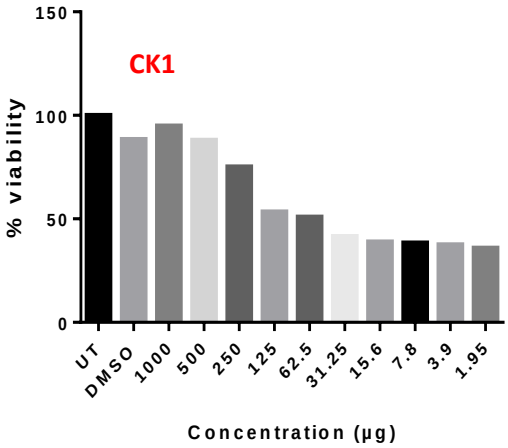

CB1
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)


CC1
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)


CER1
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)


CEL1
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)


CI1
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)


CK1
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)

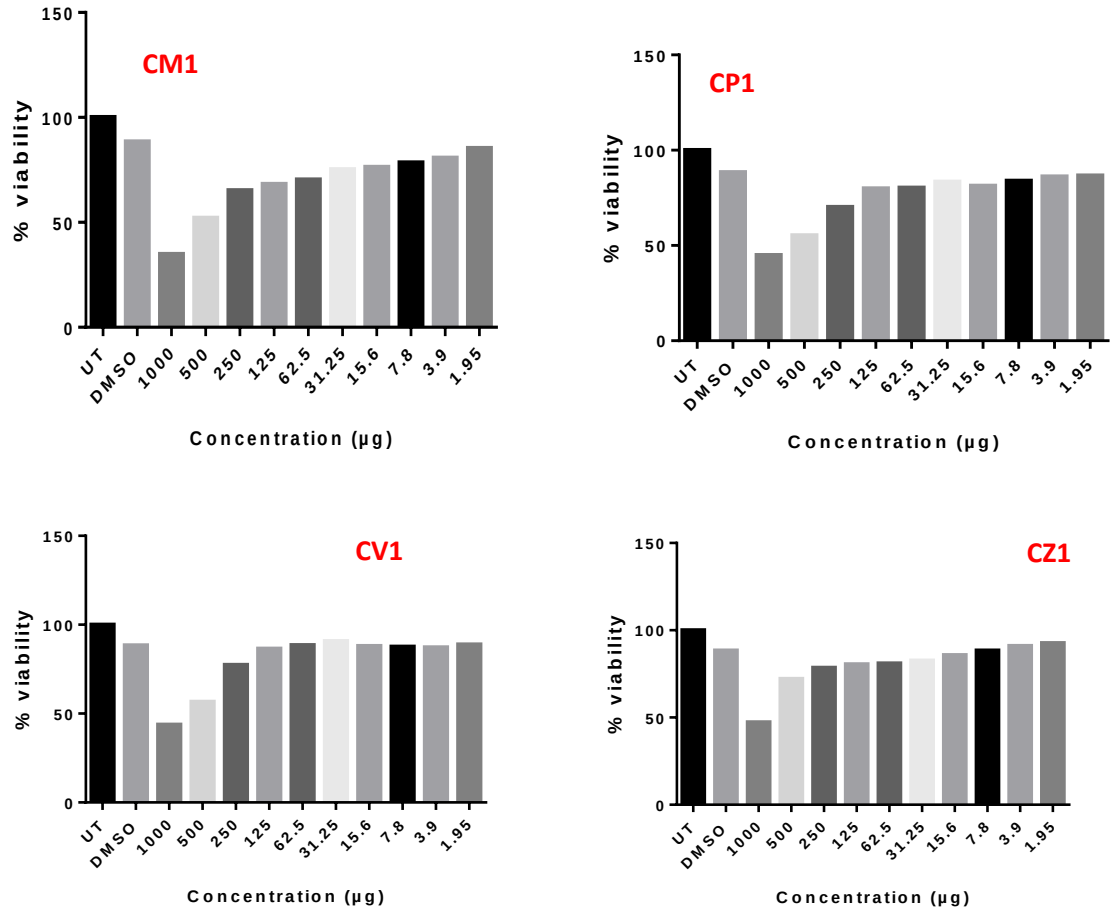


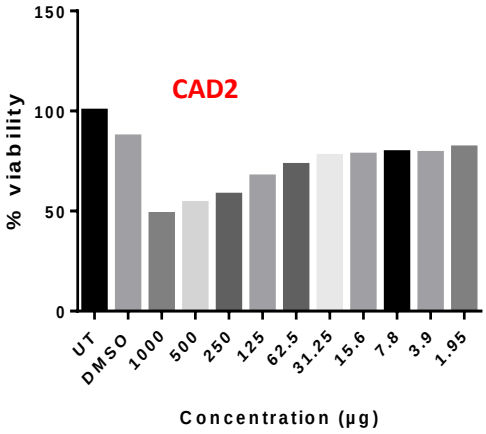
Figure 6.2: Cytotoxicity of twelve *Combretum* acetone stem extracts against A549 lung cancer cell.

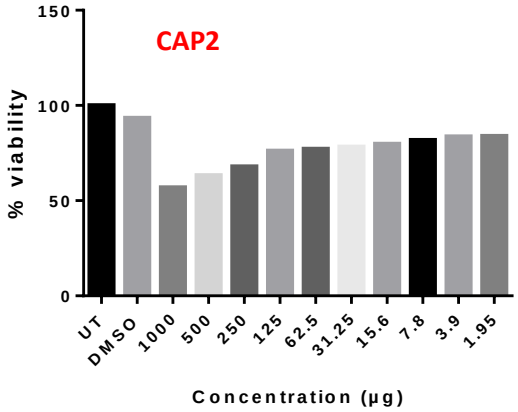
Key:

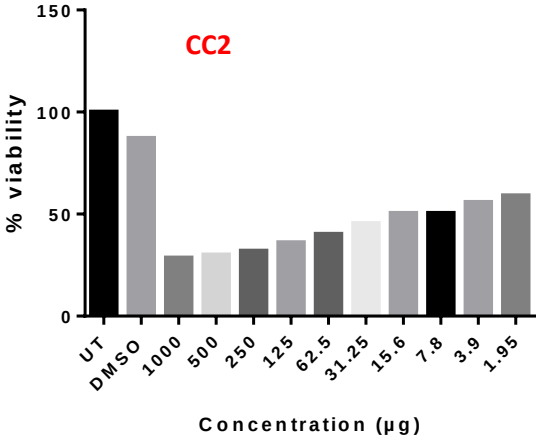
(CAD1) *C. adenogdium*, (CAP1) *C. apiculatum,* (CB1) *C. bracteosum,* (CC1) *C. caffrum, (CEL1) C. elaegnoides, (CER1) C. erythrophyllum,* (CI1), *C. imberbe,* (CK1) *C. kraussii,* (CM1) *C. mkuzense,* (CP1) *C. padoides*, (CV1) *C. vendae and* (CZ1) *C. zeyherii*

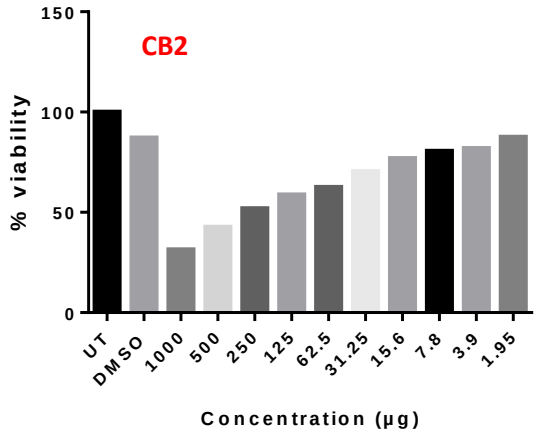
6.3.3 The effect of *Combretum* ash extracts on A549 cells

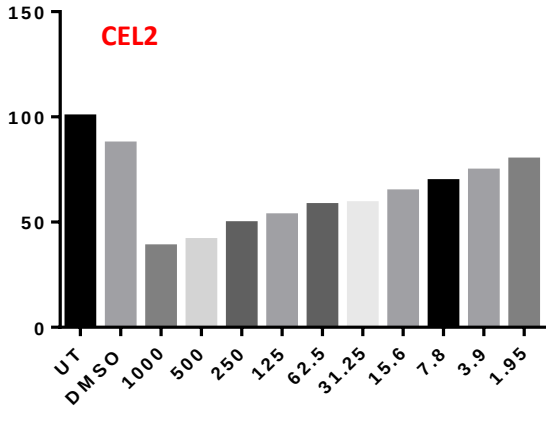
Most of the ashes obtained had shown good activity at concentrations ranging from 31.25-1000 µg/ml with only 20-50 % in cell viability. Ashes from *C. mkusenze* (CM2) showed the greatest anti-cancer activity at the highest concentration (1000 µg/ml) reducing cell viability to around 10% (Figure 6.3).

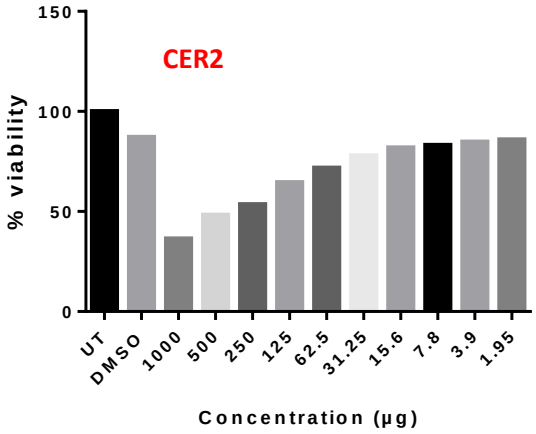

CAD2
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)


CAP2
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)


CC2
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)


CB2
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)


CEL2
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95


CER2
% viability
150
100
50
0
UT
DMSO
1000
500
250
125
62.5
31.25
15.6
7.8
3.9
1.95
Concentration (µg)

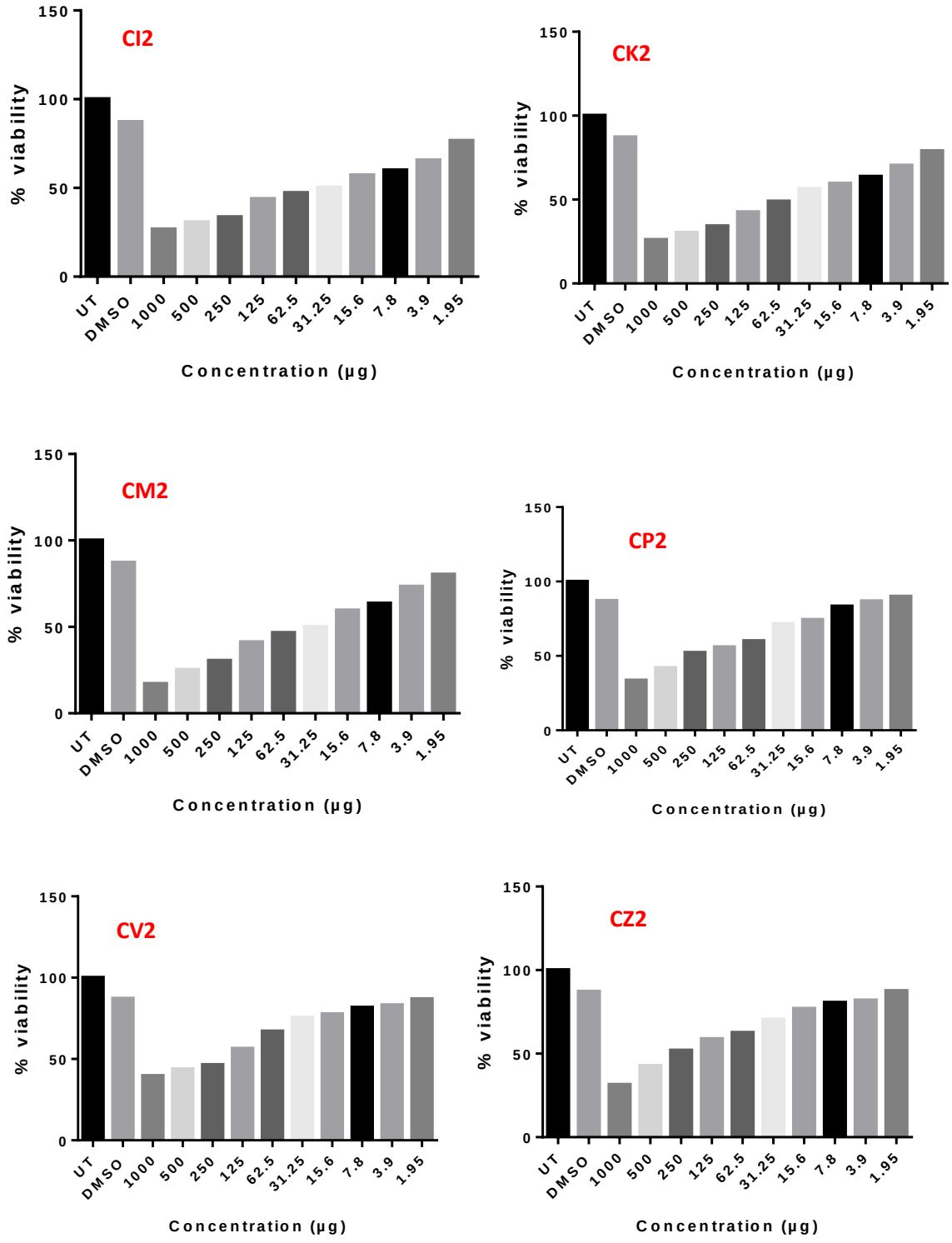


Figure 6.3: Cytotoxicity of twelve *Combretum* acetone ash extracts against A549 lung cancer cell.

Key:

C. adenogdium (CAD2)*, C. apiculatum (CAP2), C. bracteosum (CB2), C. caffrum (CC2), C. elaegnoides (CEL2), C. erythrophyllum (CER2), C. imberbe (CI2), C. kraussii (CK2), C. mkuzens*e *(CM2)*, *C. padoides* (CP2)*, C. vendae (CV2) and C. zeyherii (CZ2).*

6.4 Discussion

Naturally, derived compounds from medicinal plants, microorganisms, and marine organisms may be considered as an important source of novel effective anti-cancer drugs (Cooper, 2008). In 2012, Lancet predicted an increase of 78% in cancer cases in South Africa by the year 2030 (Health 24, 2012). Report given by the National Cancer Registry in 2014 indicated that cancer cases have reached 115,000 per year, which is an increase from the approximate 74,500 reported in 2012 (CANSA, 2019). In South Africa, approximately 27 million individuals rely on traditional medicine as their primary source of health care (Mander, 1998). A few ethnobotanical studies in South Africa have focused specifically on plants traditionally used for the treatment of cancer and have reported that indeed the plants are used for medicinal purposes (Coopoosamy and Naidoo, 2012; Koduru *et al*., 2007a; Thring and Weitz, 2006). An extensive research on *Combretum* plants on normal cells such as Vero kidney cells has been done and the plants were found to be non-toxic (Masoko, 2007; Aderogba *et al.,* 2011; Masoko and Eloff, 2005). In the present study, 50 % of the acetone leaf extracts of *Combretum* plants showed cytotoxicity and cell proliferation inhibition in lung carcinoma cells A549 in a concentration-dependent manner (MTT assay). *C apiculatum, C bracteosum, C caffrum. C pardoides, C mkusenze and C zeyherri* exhibited noticeable cytotoxic effect on the A549 cells . The present study showed that A549 cells were more sensitive to *the C erythrophylum, C elaegnodes, C imberbe, C kraussi and Cmkusenze* since the highest anticancer activity was exhibited at the lowest concentrations of the plant extracts, with less than 15 % cell viability. The anticancer activity of these plants against lung cancer cell line A549 might be due to the presence of remarkable antioxidant components and phytoconstituents such as phenols, flavonoids and tannins reported earlier in this study. The antioxidant activity is well correlated with anticancer activity, since free radicals are one of the major contributing factors in the development of cancer (Singha and Das, 2015; Kalita *et al.,* 2014; Sharma *et al.,* 2015; Krishnamurthy *et al.,* 2015). This antioxidant activity that influences the anticancer activity of these plants could be attributed to the active components like polyphenols and tannins (Widowati *et al.*, 2013). Further, studies

have shown that flavonoids have anticancer activity in inhibiting cell proliferation and angiogenesis through their effect on signal transduction (Islam *et al.*, 2013). Similarly, previous work has shown that polyphenolic compounds possess anticancer activity by instigating apoptosis in cancer cells by acting on a series of signalling pathways linked to apoptosis (Singha and Das, 2015; Yang *et al., 2001*). Other findings also showed that phenolic compounds such as phenolic acids, flavonoids and tannins have antioxidant (Cai *et al.,* 2004; Bouaziz-Ketata *et al.,* 2015; Rajan *et al.,* 2014; How *et al.,* 2010), and anti-tumour properties (Nandi *et al.,* 2007; Koul *et al.,* 2014) through scavenging free radicals. However, further studies are required to isolate the principal active compounds present in the leaves of these *Combretum* species and to study their anticancer activities. *C. adenogdium* and *C. caffrum* did not have any anticancer activity, whereas *C. apiculatum and C. bracteosum* were only able to reduce cell viability to less than 60%. *C mkusenze*, *C. pardoides , C. vendae* and *C. zeyherrii* acted in a concentration-depended manner with the highest activity seen at 1000 µg/ml. This activity may be attributed to the presence of phytoconstituents such as saponins, tannins, terpenoids, steroids, cardiac glycosides and flavonoids. Saponins exhibit cytotoxic effect and growth inhibition against a variety of cell lines making potential anti-inflammatory and anticancer agents (Iniaghe *et al.,* 2009). The presence of the above-mentioned phytoconstituents has been shown to induce a cascade-based apoptosis in cancer cells, thus inducing cytotoxicity (Owen *et al*., 2003). Meanwhile, A549 lung cells were susceptible to the following plants *C erythrophylum, C elaegnoides, C imberbe and C kraussi* which exhibited good anti-cancer effect at very low concentrations of the extract. plants *C erythrophylum, C elaegnoides, and C imberbe* at concentration 125 µg/ml to 1, 95 µg/ml reduced cell viability to less than 25% while *C kraussi*at 31, 25 µg/ml to 1, 95 µg/ml reduced cell viability to less than 35%. All the ashes tested in the study exhibited a concentration-depended effect against the A549 lung cancer cells. Ashes from *C mkusenze* showed the greatest anti-cancer activity at the highest concentration (1000 µg/ml) reducing cell viability to around 10%. Most of the plants had at concentrations between 31.25 and 1000 µg/ml where only 20-50 % of the cells were viable. It was interesting to observe that although the ashes lost many of the phytoconstituents that mainly contribute to the anti-cancer activity, they still showed good activity. This may be due to an increase in the concentration of flavonoids for *C elaegnoides C. mkusenze* - and *C. vendae*. Flavonoids have potential health benefits arising from the antioxidant activities of their

polyphenolic compounds. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions (Kumar and Padey, 2013). As mentioned earlier, flavonoids have anticancer activity in inhibiting cell proliferation and angiogenesis through their effect on signal transduction (Islam *et al.*, 2013). Other reasons for ashes to still retain great anticancer activity may be due to an increase in the concentration of minerals such as calcium, potassium, sodium, iron and proximate composition of their protein. According to Shirwaikar *et al.* (2004), minerals such as calcium, copper, manganese and zinc are well-known antioxidants that have anticancer activity. Moreover, some proteins have biological activities such as antioxidant and antimicrobial characteristics (Jafarirand *et al*., 2016; Doost, *et al.,* 2019). Overal *C imberbe, C kraussi, C mkusense, C imberbe l* had the greatest anti-cancer activity. The leaves and stems of *C imberbe, C kraussii* and *C. mkuzense* together with the ashes of C. *mkuzense* could serve as a potential source of alternative therapeutic agent for treating lung cancer. Further research is required to isolate the active compound(s) and determine their anticancer properties.

Conclusion: The present investigation revealed that the twelve *Combretum* plants studied could act as a potential alternative remedy for lung cancer. The 70 % aqueous extracts of the *Combretum spp* could be used as effective potential anti-cancer drugs.

6.5 References

Aderogba, M.A., Ndhlala, A.R., Rengasamy, K.R. and Van Staden, J., 2013. Antimicrobial and selected in vitro enzyme inhibitory effects of leaf extracts, flavonols and indole alkaloids isolated from Croton menyharthii. Molecules, 18(10), pp.12633-12644.

Asadi-Samani, M., Kooti, W., Aslani, E., and Shirzad, H., 2016. A systematic review of Iran's medicinal plants with anticancer effects. *Journal of Evidence-Based Complementary and Alternative Medicine* 21:143-153.

Berridge, M.V. and Tan, A.S., 1993. Characterization of the cellular reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron

transport in MTT reduction. Archives of biochemistry and biophysics, 303(2), pp.474-482.

Bouaziz-Ketata, H., Zouari, N., Salah, H.B., Rafrafi, M., and Zeghal, N., 2015. Flavonoid profile and antioxidant activities of methanolic extract of *Hyparrhenia hirta* (L.) Stapf. *Indian Journal of Experimental Biology* 53: 208.

Cai, Y., Luo, Q., Sun, M., and Corke, H., 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sciences* 74: 2157.

Cooper, R.G., 2008. Perspective: The stage-gate® idea-to-launch process—update, what's new, and nexgen systems. Journal of product innovation management, 25(3), pp.213-232.

Coopoosamy, R.M. and Naidoo, K.K., 2012. An ethnobotanical study of medicinal plants used by traditional healers in Durban, South Africa. *African Journal of Pharmacy and Pharmacology*, *6*(11), pp.818-823.

Cortizo, A.M., and Etcheverry, S.B., 1995. Vanadium derivatives act as growth factor - mimetic compounds upon differentiation and proliferation of osteoblast-like UMR106 cells. *Molecular and Cellular Biochemistry* 145: 97-102

Doost et al., 2019.???

Faccioni, F., Franceschetti, P., Cerpelloni, M., and Fracasso, M.E., 2003. In vivo study on metal release from fixed orthodontic appliances and DNA damage in oral mucosa cells. *American Journal of Orthodontics and Dentofacial Orthopedics* 124: 687-694.

Frei, K., Siepl, C., Groscurth, P., Bodmer, S., Schwerdel, C., and Fontana, A., 1987. Antigen presentation and tumour cytotoxicity by interferon-γ-treated microglial cells. *European Journal of Immunology* 17: 1271–1278.

Hansen, M.B., Nielsen, S.E. and Berg, K., 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. Journal of immunological methods, 119(2), pp.203-210.

How, Y.L., Yau, Y.L., and Kah, H.K., 2010. *Blechnum orientale* Linn—a fern with potential as antioxidant, anticancer and antibacterial agent. *BMC Complementary Medicine and Therapies* 10:10

Iniaghe, O.M., Malomo S.O., and Adebayo. J. O., 2009. Proximate composition and phytochemical constituents of leaves of some Acalypha species. *Pakistan Journal of Nutrition* 8(3): 256-258.

Islam, S., Nasrin, S., Khan, A., Hossain, A.S.M., Islam, F., Khandokhar, P., Mollah, M.N.H., Rashid, M., Sadik, G., Rahman, M.A.A., and Alam, A.H.M.K., 2013. Evaluation of antioxidant and anticancer properties of the seed extracts of *Syzygium fruticosum Roxb*. Growing in Rajshahi, Bangaladesh. *BMC Complement African Journal of Traditional, Complementary, and Alternative Medicines* 13:142.

Jafarirand et al., 2016.???

Kalita, S, Verma, A.K., and Prasad, S.B., 2014. Chlorambucil and ascorbic acid-mediated anticancer activity and haematological toxicity in Dalton's ascites lymphoma-bearing mice. *Indian Journal of Experimental Biology* 52:112.

Khalighi-Sigaroodi, F., Jeddi-Tehrani, M. and Ahvazi, M., 2014. Cytotoxicity evaluation of Taverniera spartea on human cancer cell lines. *Journal of Medicinal Plants* 2:114-128.

Koduru et al., 2007a???

Kooti, M., Ghasemiboroon, M., and Asadi-Samani, M., 2014a. The effect of alcoholic extract of celery leaves on the delivery rate (fertilization and stillbirths), the number, weight and sex ratio of rat off spring. *Advances in Environmental Biology.* 8:824-830.

Kooti, W., Ghasemiboroon, M., and Ahangarpoor, A., 2014b. The effect of hydro-alcoholic extract of celery on male rats in fertility control and sex ratio of rat offspring. *Journal of Babol University of Medical Sciences* 6(4): 43-49.

Kooti, W., Farokhipour, M, Asadzadeh, Z., Ashtary-Larky, D., and Asadi-Samani, M., 2016a. The role of medicinal plants in the treatment of diabetes: a systematic review. *Electron Physician* 2(8):1832-1842.

Kooti., W, Hasanzadeh-Noohi, Z, Sharafi-Ahvazi, N,. Asadi-Samani, M., and Ashtary-Larky D., 201b6. Phytochemistry, pharmacology, and therapeutic uses of black seed (Nigella sativa). *Chinese Journal of Natural Medicines*14:732-745.

Koul, A., Goyal, R. and Bharati, S., 2014. Protective effect of *Azadirachta indica* A. Juss against doxorubicin-induced cardiac toxicity in tumour bearing mice. *Indian Journal of Experimental Biology* 52: 323

Krishnamurthy, P.T., Vardarajalu. A., Wadhwani, A., and Patel, V., 2015. A identification and characterization of a potent anticancer fraction from the leaf extracts of *Moringa oleifera* L. *Indian Journal of Experimental Biology* 53: 98.

Kumar and Padey, 2013.???

Lachenmayer, A., Alsinet, C., Chang C.Y., and Liovit, J., 2010. Molecular approaches to treatment of hepatocellular carcinoma. *Digestive and Liver Disease* 42:264-272.

Li, W., Zhou, J., and Xu, Y., 2015. Study of the in vitro cytotoxicity testing of medical devices. *Biomedical Reports* 3(5): 617–620. doi:10.3892/br.2015.481.

Mansouri, E., Kooti, W., and Bazvand, M., 2015. The effect of hydro alcoholic extract of Foeniculum vulgare Mill on leukocytes and haematological tests in male rats. *Jundishapur* 10:e18396.

Markey C.M., Wadia P.R., Rubin B.S., Sonnenschein, C., and Soto, A.M., 2011. Long-term effects of fetal exposure to low doses of the xenoestrogen bisphenol-A in the female mouse genital tract. *Biology of Reproduction* 72: 1344-1351.

Masoko, P. and Eloff, J.N., 2005. The diversity of antifungal compounds of six South African Terminalia species (Combretaceae) determined by bioautography. African Journal of Biotechnology, 4(12).

Masoko, P. and Eloff, J.N., 2007. Screening of twenty-four South African Combretum and six Terminalia species (Combretaceae) for antioxidant activities. African Journal of Traditional, Complementary and Alternative Medicines, 4(2), pp.231-239.

McGaw L.J., Elgorashi, E.E., and Eloff J.N., 2014. Cytotoxicity of African medicinal plants against normal animal and human cells toxicological survey of African Medicinal plants: 181–233

Nandi, S., Vracko, M., and Bagchi, M.C., 2007. Anticancer activity of selected phenolic compounds: QSAR studies using ridge regression and neural networks. *Chemical Biology & Drug Design* 70: 424.

Newman, D.J., and Cragg, G.M., 2007. Natural products as sources of new drugs over the last 25 years. *Journal of Natural Products* 70:461-477.

Ouyang, L., Shi, Z., Zhao, S., Wang, F.T., Zhou, T.T., Liu, B. and Bao, J.K., 2012. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell proliferation*, *45*(6), pp.487-498.

Owen, R.W., Haubner, R., Mier, W., Giacosa, A., Hull, W.E., Spiegelhalder, B. and Bartsch, H., 2003. Isolation, structure elucidation and antioxidant potential of the major phenolic and flavonoid compounds in brined olive drupes. *Food and Chemical Toxicology*, *41*(5), pp.703-717.

Rajan, I., Rabindran, R., Jayasree, P.R., and Kumar, P.R.M., 2014. Antioxidant potential and oxidative DNA damage preventive activity of unexplored endemic species of Curcuma. *Indian Journal of Experimental Biology* 52:133.

Riss, T.L., Moravec, R.A., and Niles, A.L., 2013. Cell viability assays. **In:** Sittampalam GS, Grossman A, Brimacombe K, Eds.), *Assay guidance manual* [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences.

Sakarkar, D.M., and Deshmukh, V.N., 2011. Ethno pharmacological review of traditional medicinal plants for anticancer activity. *International Journal of Pharmaceutics* 3:298-308.

Sayes, C.M., Liang, F., Hudson, J.L., Mendez, J., Guo, W., Beach, J.M., Moore, V.C., Doyle, C.D, West, J.L., and Billups, W.E., 2006. Functionalization density dependence of single-walled carbon nanotubes cytotoxicity *in vitro*. *Toxicology Letters* 161: 135–142.

Sharma, D., Rawat. I., and Goel, H.C., 2015. Anticancer and anti-inflammatory activities of some dietary cucurbits. *Indian Journal of Experimental Biology* 53:216.

Shirwaikar, A., Rajendran, K., Kumar, C.D. and Bodla, R., 2004. Antidiabetic activity of aqueous leaf extract of Annona squamosa in streptozotocin–nicotinamide type 2 diabetic rats. Journal of ethnopharmacology, 91(1), pp.171-175.

Singha, I. and Das, S.K., 2015. Grapevine fruit extract protects against radiation-induced oxidative stress and apoptosis in human lymphocyte. *Indian Journal of Experimental Biology* 53: 753.

Soenen, S.J., Manshian, B., Montenegro, J.M., Amin. F., Meermann. B., Thiron, T., Cornelissen, M., Vanhaecke, F., Doak, S., and Parak, W.J., 2012. Cytotoxic effects of gold nanoparticles: a multiparametric study. *ACS Nano* 6: 5767–5783.

Thring and Weitz, 2006.???

Vos, T., Allen C., and Arora, M., 2016. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* 388 (10053): 1545–160.

Widowati, W., Wijaya, L., Wargasetia, T.L, Bachtiar, I., Yelliantty, Y., and Laksmitawati, D.R., 2013. Antioxidant, anticancer, and apoptosis-inducing effects of Piper extracts in HeLa cells. *Journal of Experimental and Integrative Medicine* 3:225.

Wild, C.P., Weiderpass, E., Stewart B.W. 2020. World cancer report: cancer research for cancer prevention. https://publications.iarc.fr/586, (accessed 4 February 2020).

Yang, C.S., Landau, J.M., Huang, M.T., and Newmark, H.L., 2001. Inhibition of carcinogenesis by dietary polyphenolic compounds. *The Annual Review of Nutrition* 21: 381.

Zeng, H. Zheng, R. Guo, Y. Zhang, S. Zou, X. Wang, N. Zhang, L. Tang, J. Chen, J. Wei, K. Huang, J. Wang, L. Yu, D. Zhao, G. Song, Y. Shen, X. Yang, X. Gu, F. Jin, Q. Li, Y. Li, H. Ge, F. Zhu, J. Dong, G. Guo, M. Wu, L. Du, X. Sun, Y. He, M. P. Coleman, P. Baade, W., Chen, W., and Yu, X.Q., 2015. Cancer survival in China, 2003–2005: A population-based study. *International Journal of Cancer* 136 (8): 1921–1930.

CHAPTER 7: MICROBIOLOGICAL AND SHELF-LIFE ANALYSIS OF THE SORGHUM JUICE TREATED WITH EXTRACTS AND ASHES OF *COMBRETUM* SPECIES

7.1 Introduction

Soft drinks represent an important market within the food industry. The increasing variety of these products being released at a bewildering rate has altered the potential for spoilage problems. Relatively few organisms, usually yeasts, and a few acid-tolerant bacteria and fungi generally spoil soft drinks. Many microorganisms are found in soft drinks as environmental or raw material contaminants, but relatively few can grow within the acidic and low oxygen environment. Yeasts are the most significant group of microorganisms associated with the spoilage of soft drinks and fruit juices. As noted above, most spoilage is caused by yeasts and mould species, with yeasts being the most important cause, but some spoilage is caused by acid tolerant bacteria (Hocking and Jensen, 2001; Jay and Anderson, 2001). Presently, a wide variety of chemical preservatives is permitted and used in foods to prevent the growth of food spoilage and disease causing bacteria. The type of preservative, amount allowed to be used and type of foods vary from one country to another, but numerous consumers negatively perceive the use of preservatives. Foodborne disease outbreaks are on the rise even in developed countries, with a shift from challenges posed by foods from animal origin to fresh foods such as produce, shellfish and dry products and ingredients. New risks are being encountered because of changes in food production practices, environment, increase in global trade of food and changes to the genetic characteristics of the relevant pathogenic microorganisms (Havelaar *et al.,* 2010). An increase in the fresh-cut convenient salad market has coincided with an increase in foodborne diseases; pre-cutting of the salad leaves releases nutrients, which support microbial growth. The modified atmosphere within the package reduces spoilage by aerobes but enhances the virulence of pathogens like *E. coli* 0157:H7 (Warriner *et al.,* 2009; Chua, *et al.*, 2008). Traditional food preservation methods are less effective in preventing the growth of these food pathogens in fresh food. New and innovative techniques are needed by the food industry to overcome these challenges. Use of plant antimicrobials is an emerging technology that could be used by the industry to extend the storage life of food and overcome these food safety issues.

Natural products are chemical compounds or substance(s) produced by a living organism or found in nature that have pharmacological or biological activity (Havelaar *et al.,* 2010). Living organisms produce manifold primary and secondary metabolites. Primary metabolites have essential functions in the organism whereas secondary metabolites may have important functions for their producers or could simply be waste products. However, secondary metabolites may also have properties that are beneficial to humans. In many cases, they are used as drugs against human diseases such as cancer, bacterial infections, inflammation, and many other diseases (Havelaar *et al.,* 2010; Warriner *et al.,* 2009; Chua, *et al.*, 2008). However, a number of these secondary metabolites have been noted for their antimicrobial activity. Secondary metabolites with antimicrobial activity are found in most organisms, including: (1) plants such as fruits, vegetables, seeds, herb, and spices, (2) animal sources such as milk, eggs, and tissues, and (3) microorganisms such as bacteria and fungi. Natural antimicrobials are being given more attention due to the increased concerns with chemical preservatives among consumers. Even though chemical preservatives are approved for human consumption by government agencies, many of these preservatives still threaten people's health. Hence, scientific communities are paying more attention to the potential antimicrobial activities of natural products. On the other hand, the increasing antibiotic resistance against chemical preservatives of some pathogens associated with foodborne illness is in increasing rates (Cowan, 1999; Balasundram *et al.,* 2006). Natural antimicrobials seem to be the most promising answer to many of the increasing concerns with antibiotic resistance and could yield better results than antimicrobials from combinatorial chemistry and other synthetic procedures (Manach *et al.,* 2004). Therefore, novel types of effective and healthy antimicrobial compounds that could protect food against contamination and consumers against infection are highly demanded.

In recent years, a large number of studies were conducted in search of the antimicrobial activity of natural products. Plants, especially herbs and spices, are receiving more attention. Nowadays, there are over 1340 plants with defined antimicrobial activities, and over 30,000 antimicrobial compounds have been isolated from plants. Plants produce an array of secondary metabolites that can be found in edible, medicinal, and herbal plants and their derived essential oils (EOs) (Secondary metabolites from plants are extensively studied as a promising healthy ingredient or

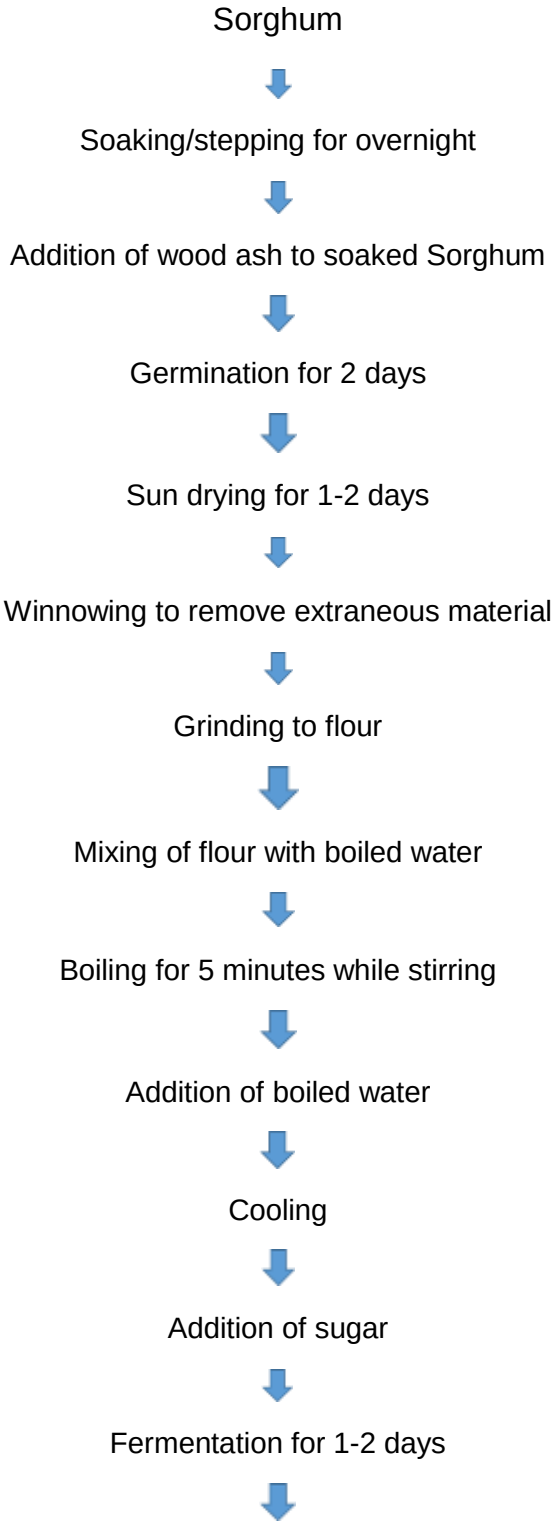
human disease controlling agents). These secondary metabolites possess various benefits, including antimicrobial properties against pathogenic and spoilage microbes (Manach *et al.,* 2004). Natural antimicrobials derived from plants have been recognised for centuries, but only scientifically confirmed in the last 30 years (McMeekin, *et al*., 2010*;* Chua *et al.* 2008). Thus, an increasing interest in finding natural antimicrobials for application in food products to prevent or inhibit microbial growth and extend shelf life is becoming quite notable (McMeekin *et al.,* 2010; Tajkarimi *et al.,* 2010). The presence of both antioxidant and antimicrobial properties in a single molecule makes them more effective and better suited as food preservatives. In general, plants have a much greater inhibition effect against Gram-positive than Gram-negative bacteria. The activity against both types of bacteria may be indicative of the presence of broad-spectrum antibiotic compounds or simply general metabolic toxins (Burt, 2004). The antimicrobial efficacy of components in plants depends on the chemical structure of active components and their concentration. There are various chemical components present in plants with antimicrobial effect, including saponins, flavonoids, thiosulfinates, glucosinolates, phenolics, and organic acids. Plant antimicrobials are phytochemicals, which are important for the proper functioning of the plant. In most cases, these substances act as plant defence agents against microorganisms and other predators. They also regulate growth, pollination and fertilization (Cowan, 1999). These secondary metabolites are among the most widely occurring phytochemicals in plants. They contribute to sensory properties when added to food and have antioxidant and antimicrobial properties (Balasundram *et al.,* 2006), characteristics that are useful in extending the shelf life of food. Antimicrobials are chemical compounds or substances that may delay microbial growth or cause microbial death on entering a food matrix. The current study aimed to assess the microbiological quality and shelf life of the prepared sorghum juice treated with the plants.

7.2. Methodology

7.2.1. Preparation of the juice

Two types of sorghum juices were prepared, one containing powdered leaves, and the other containing ashes. The sorghum juice was prepared by adding germinated sorghum flour to water (2 L) with 1:3 ratio (g/L) used, mixing thoroughly. After boiling

the mixture was left to cool, a cup of household sugar was added for taste; in one juice, ashes (25 g) were added, in the other, powder from dried leaves (25 g) was added and in the other, powder from dried stem (25 g) was added. Untreated juice, which served as a control, was prepared. The mixtures were left for a period of two days to allow the ingredients to blend.



Sweet sorghum juice

Figure 7.1: Flow diagram for production of sweet sorghum juice

7.2.2. Shelf life analysis of the prepared juice

7.2.2.1. Microbial enumeration

Juice samples were collected after preparation; the samples were serially diluted using peptone water. Tempo instrument (Biomereiux) was used to enumerate total coliform, total aerobic count, *E. coli, S. aureus*, lactic acid bacteria, enteric bacteria, yeast and mould using the most probable number following the manufacturers instruction. Ten millilitres of juice were added to 100 ml of buffered peptone water. The mixture was shaken for 20 seconds using a stomacher. After shaking, 0.1 ml of the mixture was added into 3.9ml of liquid media TEMPO AC and TEMPO LAB used for enumeration of aerobic bacteria and lactic acid bacteria. A Tempo card was inserted into the liquid media containing the water sample and was then inserted into the Tempo filler. Inside the Tempo filler, the mixture of the media together with the samples was transferred from the bottle into the card. The cards were removed from the filler and were incubated at 37 ^{o}C for 48 hours. Following incubation, the cards were read using the Tempo reader; the colonies were enumerated as CFU/ml. For enumeration of total coliforms, *S. aureus* and *E. coli*, the same procedure was followed, however, 1ml of the mixture was added into 3ml of the media and the incubation period was 24 hours and for *Enterobacteriaceae* and *B. cereus*; the incubation period was 22 hours at 37 ^{o}C.

7.2.2.2 Isolation and Identification of microorganisms that survived treatment.

Serial Dilution

One millilitre of the juice sample was added into 9 ml of sterile distilled water to prepare stock solution. Thereafter, the test tubes were labelled as ($10^{-1,}$ 10^{-2} and $10^{-3,}$). After that, 1ml from the mixture sample was transferred into the first test tube that was 10^{-1} and shaken well in order to get an equal distribution of microorganisms. Then, 1ml from the first test tube was transferred into the next test tube and again shaken. Finally, the procedure was repeated to complete the serial dilution up to 10^{-3}. Spread plate technique was used to yield the isolation of the bacterial cultures on nutrient agar. Few

well-isolated colonies were picked based on morphological difference and Gram stained and were identified using Vitek 2 Compact.

Gram Stain Procedure

The slide was placed with heat fixed smear on staining tray. A drop of crystal violet safranin (Sigma Aldrich) was added and allowed to stand for 60 seconds and then washed with distilled water. Moreover, one drop of Iodine safranin (Sigma Aldrich) was added, and then allowed to stand for 30 seconds. After this, it was washed with distilled water. Then, 95% ethyl alcohol (VWR) (decolourization) was added and allowed to stand for 30 seconds then washed with distilled water. After that, safranin (Sigma Aldrich) was added and allowed to stand for 60 seconds and then washed with distilled water. Finally, the slide was put under a microscope and then, a purple and pink colour from a single colony of the slide under microscope was observed. A purple colour from a single colony of the slide under microscope indicated that the bacteria were gram positive whereas the pink colour under microscope indicated that the bacteria were gram negative. Microscopic investigation for Gram reaction and morphological features of suspected colony was determined using standard method of Gram's staining.

Identification of the microbial isolates

The VITEK 2 instrument (Biomereiux) was used for identification of the pure cultures of the microbial isolates. The manufacturer's protocol was followed for analysis. The VITEK 2 system is a fully automated microbiology system utilising growth-based technology system and operates *in vitro*. A suspension of a pure culture was prepared by suspending well isolated colonies in 3.0 mL of sterile saline (aqueous 0.45–0.50% NaCl, pH 4.5– 7.0) (Biomereiux) in a 12 × 75 mm clear plastic (polystyrene) (Biomereiux) test tubes using a sterile swab. The test kit card with the transferred suspensions were placed in the VITEK incubator. The VITEK system analyses the card as growth of the organism that occurs and gives an identity of the organism.

7.2.3 Data analysis

Descriptive statistics were computed using ANalysis Of VAriance (ANOVA) programmed into the R statistical software. Tukey and Dunnett's T3 post-hoc tests were run for equal and un-equal variances, respectively. Statistical analysis was

performed to determine variation between the juices in terms of shelf-life evaluation. Values were considered significantly different when $p < 0.05$

7.3. Results

7.3.1 Changes in microbial load of stored juices

The evolution of the following microorganisms (aerobic count, *Enterobacteriaceae*, total coliform, *S. aureus*, *B. cereus, E. coli and* lactic acid bacteria) were examined throughout storage of the prepared sorghum juice.

7.3.1.1 The stability of aerobic bacteria in prepared sorhum juice treated with powdered leaves and ashes of *Combretum* species

The aerobic count for both juices treated with leaves and ashes were found to be above 500 000 CFU/ml during all the 4 weeks of storage. It was interesting to observe that *C. kraussii* was able able to reduce the aerobic count to lower than 40 000 CFU/ml during the four weeks of storage, as depicted in Figure 7.2.

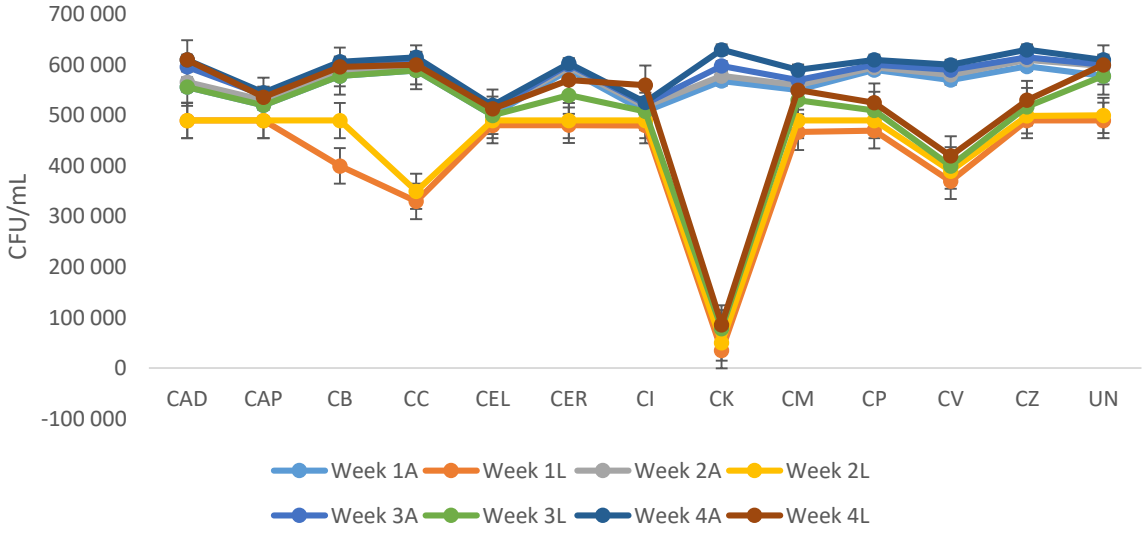


Figure 7.2. Changes in aerobic bacterial count during storage of sorghum juice treated with powdered leaves and ashes of twelve *Combretum* species.

Key:

C. adenogdium (CAD), *C. apiculatum* (CAP), *C. bracteosum* (CB), *C. caffrum* (CC), *C. elaegnoides (CEL), C. erythrophyllum (CER),* , *C. imberbe* (CI), *C. kraussii* (CK), *C. mkuzense* (CM), *C. padoides* (CP), *C. vendae* (CV) *and C. zeyherii* (CZ).

(A = Ashes and L=Leaves).

7.3.1.2 The stability of *Enterobacteriaceae* in the prepared sorghum juices treated with powdered leaves and ashes of *Combretum* species.

The ashes showed more activity against the *Enterobacteriaceae* group when compared with the powdered leaves. From week 1 to 4 of storage, the microbial count was around 50 000 CFU/ml (figure 7.3.)

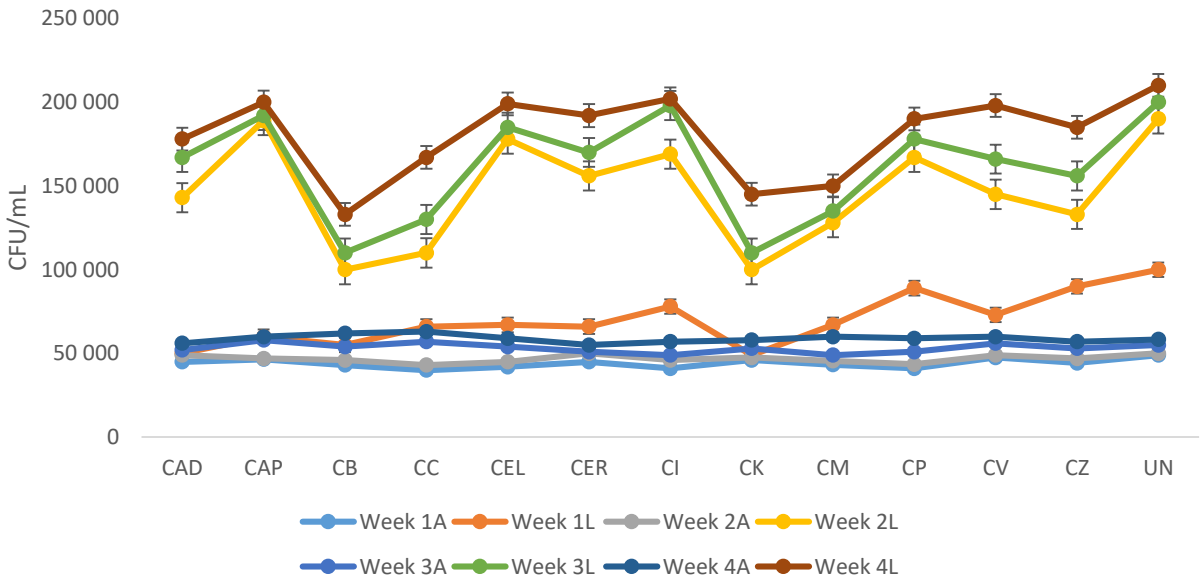


Figure 7.3 Changes in *Enterobacteriaceae* during the storage of sorghum juice treated with powdered leaves and ashes of *Combretum* species.

Key:

C. adenogdium (CAD), *C. apiculatum* (CAP), *C. bracteosum* (CB), *C. caffrum* (CC), *C. elaegnoides (CEL), C. erythrophyllum (CER), C. imberbe* (CI), *C. kraussii* (CK), *C. mkuzense* (CM), *C. padoides* (CP), *C. vendae* (CV) *and C. zeyherii* (CZ).

(A = Ashes and L=Leaves).

7.3.1.3 The stability of total coliform bacterial count inprepared sorghum juices treated with powdered leaves and ashes of *Combretum* species..

The leaves showed more activity against the bacteria reducing total coliforms during storage compared to the ashes. Total coliforms were more susceptible to the leaves of *C. apiculatum, C. bracteseoum*, *C. erythrophyllum, C. kraussii and C. vendae* especially in first week of storage. The leaves of *C. caffrum, C. mkuzense* showed lower antibacterial activity (Figure 7.4).

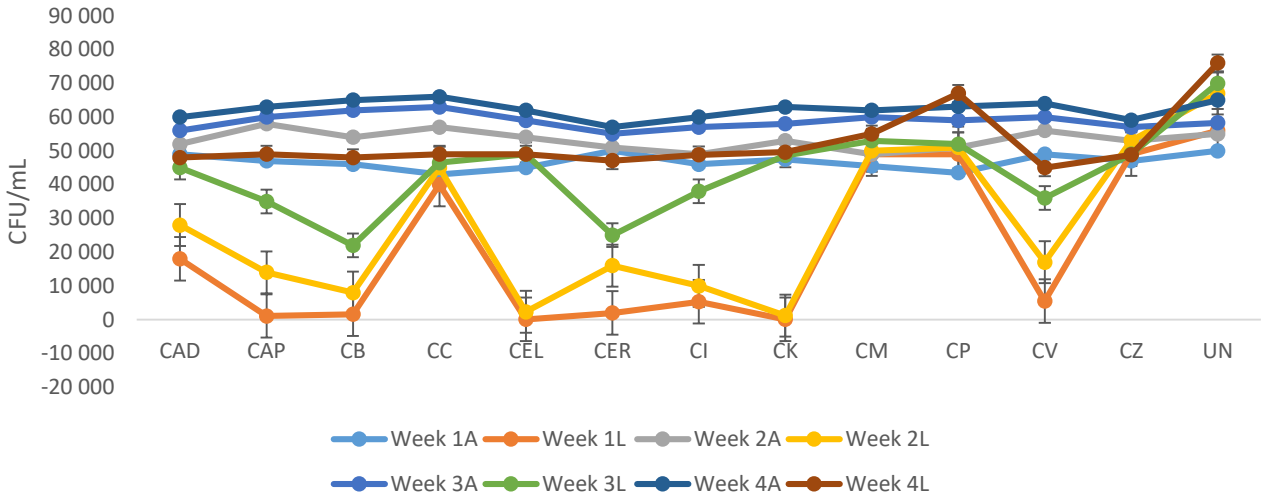


Figure 7.4 Changes in total coliform bacterial count during the storage of sorghum juice treated with leaves and ashes of *Combretum* species.

Key:

C. adenogdium (CAD), *C. apiculatum* (CAP), *C. bracteosum* (CB), *C. caffrum* (CC), *C. elaegnoides (CEL), C. erythrophyllum (CER), C. imberbe* (CI), *C. kraussii* (CK), *C. mkuzense* (CM), *C. padoides* (CP), *C. vendae* (CV) *and C. zeyherii* (CZ).

7.3.1.4 The stability of *Staphyloccocus aureus* in prepared sorhum juices treated with powdered leaves and ashes of *Combretum* species.

The powdered leaves and ashes of *Combretum* spp. showed antibacterial activity against *S. aureus* during the 4-week storage period with bacterial counts below 10 000 in the treated juices, while the control (untreated juice) has around 50 000 bacterial count, as shown in Figure 7.5

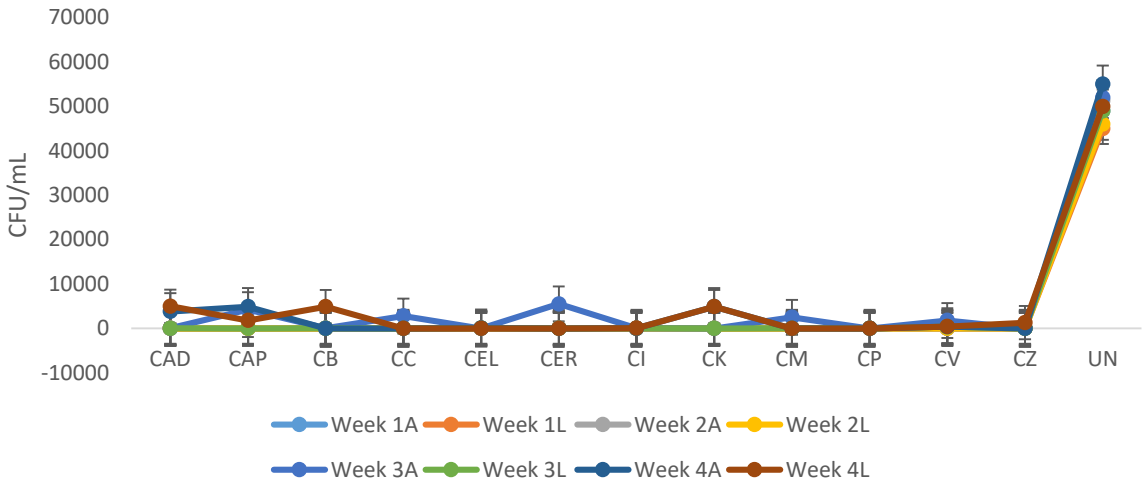


Figure 7.5 Changes in *Staphyloccocus aureus* bacterial during storage of sorghum juice treated with powdered leaves and ashes of *Combretum* species.

Key:

C. adenogdium (CAD), *C. apiculatum* (CAP), *C. bracteosum* (CB), *C. caffrum* (CC), *C. elaegnoides (CEL), C. erythrophyllum (CER), C. imberbe* (CI), *C. kraussii* (CK), *C. mkuzense* (CM), *C. padoides* (CP), *C. vendae* (CV) *and C. zeyherii* (CZ).

7.3.1.5 The stability of the *Bacillus cereus* in the prepared sorhum juices treated with powdered leaves and ashes of *Combretum* species.

The leaves of *C. caffrum, C. elaegnoides and C. erythrophyllum* inhibited the growth *of B. cereus* in the first three weeks of storage. After the fourth week, the bacterial growth was enhanced. *B. cereus* was found to be resistant to the tested powdered leaves and ashes of *Combretum* species as depicted in figure 7.6.

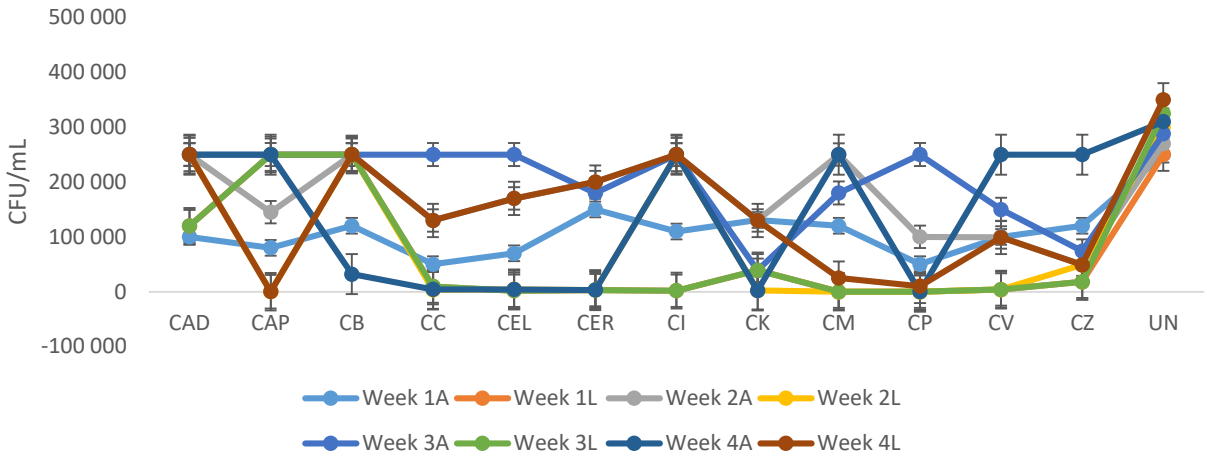


Figure 7.6 Changes in *Bacillus cereus* during the storage storage of sorghum juice treated with powdered leaves and ashes of *Combretum* species.

Key:

C. adenogdium (CAD), *C. apiculatum* (CAP), *C. bracteosum* (CB), *C. caffrum* (CC), *C. elaegnoides (CEL), C. erythrophyllum (CER), C. imberbe* (CI), *C. kraussii* (CK), *C. mkuzense* (CM), *C. padoides* (CP), *C. vendae* (CV) *and C. zeyherii* (CZ).

7.3.1.6 The stability of *Lactic acid bacteria* in prepared sorghum juices treated with powdered leaves and ashes of *Combretum* species.

The leaves were able to totally inhibit the growth of lactic acid bacteria for the first three weeks of storage; growth was later detected in the fourth week of storage. However, the bacteria resisted the ashes-treated juice as depicted in Figure 7.7.

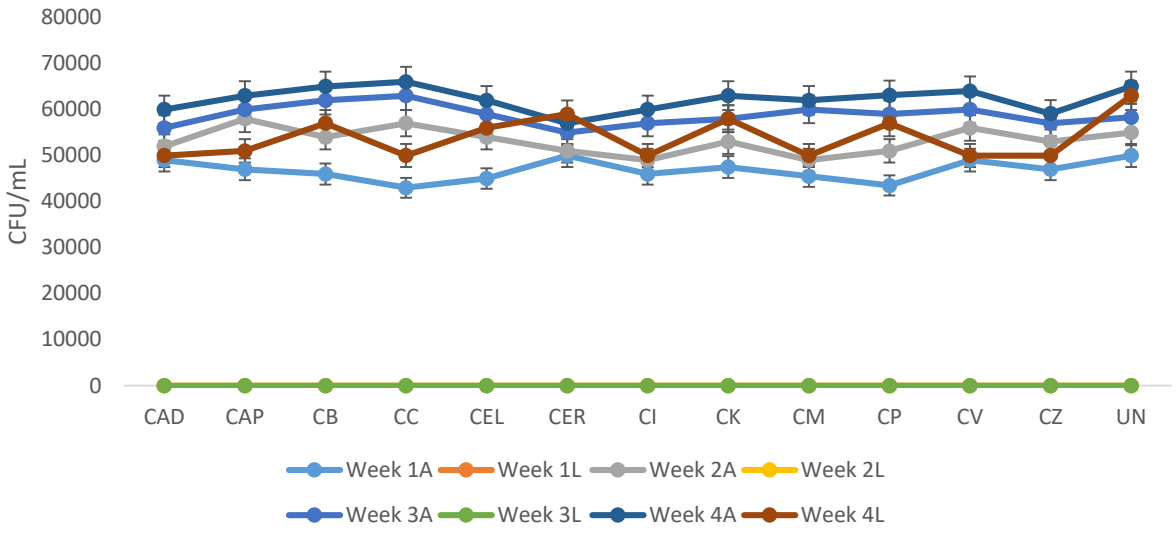


Figure 7.7 Changes in lactic acid bacteria during storage of sorghum juice treated with powdered leaves and ashes of *Combretum* species.

Key:

C. adenogdium (CAD), *C. apiculatum* (CAP), *C. bracteosum* (CB), *C. caffrum* (CC), *C. elaegnoides (CEL), C. erythrophyllum (CER), C. imberbe* (CI), *C. kraussii* (CK), *C. mkuzense* (CM), *C. padoides* (CP), *C. vendae* (CV) *and C. zeyherii* (CZ).

7.3.1.7 The stability of the *Escherichia coli* in prepared sorghum juices treated with powdered leaves and ashes of *Combretum* species.

E. coli was not detected in any of the juicies, including that of the control, as depicted in figure 7.8.

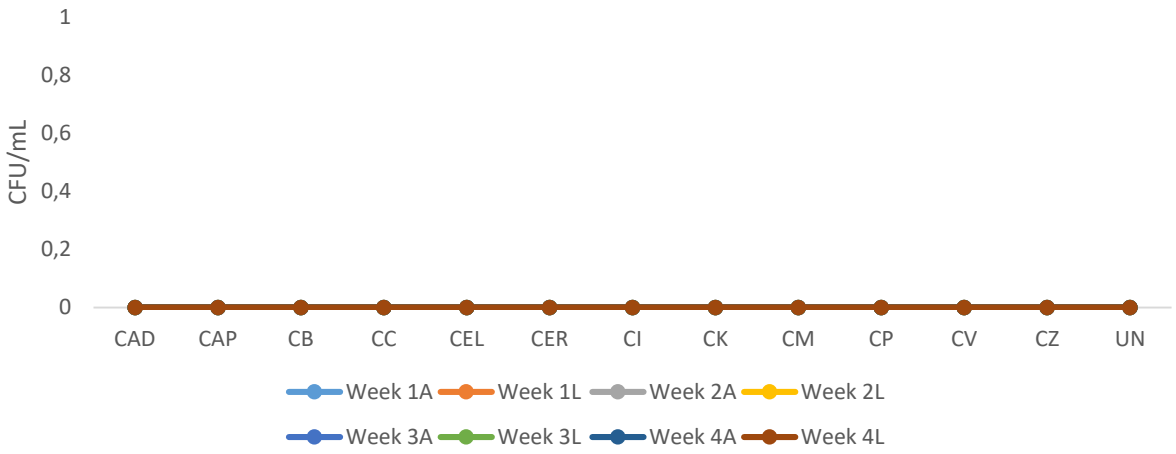


Figure 7.8 Changes in *Escherichia coli* during storage of sorghum juice treated with powdered leaves and ashes of *Combretum* species.

Key:

C. adenogdium (CAD), *C. apiculatum* (CAP), *C. bracteosum* (CB), *C. caffrum* (CC), *C. elaegnoides (CEL), C. erythrophyllum (CER), C. imberbe* (CI), *C. kraussii* (CK), *C. mkuzense* (CM), *C. padoides* (CP), *C. vendae* (CV) *and C. zeyherii* (CZ).

7.3.2. Microbial Identification of the Gram-stained isolates

Spread plate technique was used for the isolation of the bacterial cultures on nutrient agar. Few well-isolated colonies, as depicted in Figure 7.9, were picked based on morphological differences for identification purposes.

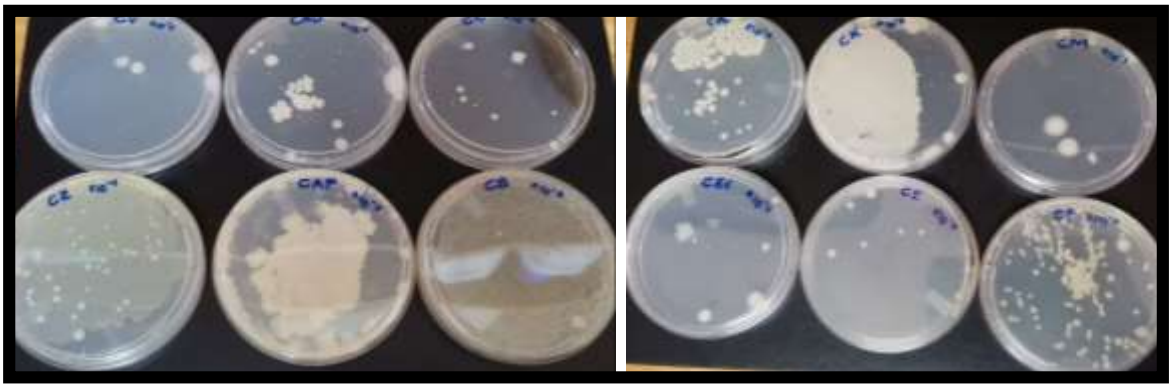


Figure 7.9 Bacterial isolates from the Sorghum juice treated with the leaves and ashes of the twelve *Combretum* plants

Vitek 2 Compact was used for the characterisation and identification of the dominant bacterial isolates using biochemical reactions highlighted in Appendix V and VI. The identities of bacterial isolates are shown in Table 7.1. *Enterobacter* spp. were predominant in the juice treated with ashes.

Table 7.1 Identities of bacterial isolates from the sorghum juice based on biochemical reactions.

Bacterial Isolate	**Grams reaction**	**Bacterial Identity**	**Identification confidence**
CI*	Negative Positive Negative	*Stenotrophomonas maltophilia* *Staphylococcus intermedius* *Aeromonas sobria*	95% 87 % 86 %
CI	Negative	*Enterobacter cloacae* *Enterobacter kobei* *Enterobacter hormaechei*	87% 90% 89%
CEL*	Positive	*Staphylococcus intermedius*	87%
CV, CZ	Positive	*Staphylococcus intermedius*	86%
CC, CB, CAD*	Negative	*Enterobacter cloacae* *Enterobacter kobei* *Enterobacter hormaechei*	50% 50% 86%
CAP*	Negative	unidentified	0
CER*	Negative	unidentified	0

Key: * Microorganisms isolated form the juice infused with ashes. CAD) *C. adenogdium*, (CAP) *C. apiculatum,* (CB) *C. bracteosum,* (CC) *C. caffrum, (CEL) C. elaegnoides, (CER) C. erythrophyllum,* (CI), *C. imberbe,* (CK) *C. kraussii,* (CM) *C. mkuzense,* (CP) *C. padoides*, (CV) *C. vendae and* (CZ) *C. zeyherii*

7.4 Discussion

Most traditional food preservation methods depend on the application of high temperature and pressure. The mild heat processing and modified-atmosphere packaging, adopted for preserving food products, are not sufficiently reliable for effective control of spoilage by pathogenic microorganisms. Moreover, low temperature storage of perishable foods cannot assure the safety and quality of food products (Negi, 2012; Tajkarimi, et al., 2010), and it costs a lot of energy. Finally, chemical preservatives alone cannot destroy all food pathogens or restrict microbial spoilage and are increasingly frowned upon by (at least some) consumers. Many consumers prefer to avoid chemical preservatives and are concerned about negative

side effects. Chemical preservatives like nitrates, benzoates, sulphites, sorbates, parabens, formaldehyde, butylated hydroxytoluene or–hydroxyanisole, can cause serious health hazards such as hypersensitivity, allergy, asthma, hyperactivity, neurological damage or cancer (Anand and Sati, 2013). Therefore, serious attention should be devoted to the safety and necessity of using chemical preservatives in the food industry (Shakiba *et al.,* 2011; Tajkarimi *et al.,* 2010). Elimination of microorganisms from food without compromising the desirable properties of the product is still a challenge for the food industry. The current study aimed to assess the effect of the addition of ashes and leaves to the microbiological quality and shelf life of sorghum juice. All the juices prepared had a noticeable number of aerobic bacteria with the juices treated with ashes showing higher colony forming units (CFU) counts. As the storage time increased, the bacterial counts also increased, which ranged from 400 000-600 000 CFU/ml for all the samples. It was interesting to see that the juice treated with the leaves of *C. kraussii*, the CFU was exceptionally low (>40 000) CFU count during the storage time. The juices treated with leaves and ashes had high levels of *Enterobacteriaceae*, with CFU counts ranging from 40 000-60 00. It was interesting to see that this group of bacteria were more resistant to leaves than ashes. *Enterobacteriaceae* are useful indicators of hygiene and of post-processing contamination of ready-to-eat foods. Their presence in high numbers (>105 per gram) in ready-to-eat foods indicates that an unacceptable level of contamination has occurred or there has been under processing. The juice treated with the leaves of *C. kraussii*, was found to be within the permissible limits. The group includes both pathogenic and non-pathogenic bacteria. In ready-to-eat foods that are fully cooked, detection of *Enterobacteriaceae* is used as an indication of either post-processing contamination or inadequate cooking. Total coliforms detected in the juices increased with storage time, with CFU's ranging from 0- 40 000 CFU/ml for leaves and 45 000-60 000 CFU/ml . The leaves of *C. apiculatum, C. bracteosum, C. kraussii and C. vendae* were able to inhibit the coliforms during the first week one of storage; however, as the storage time increased, coliforms also increased. *S. aureus* was typically detected in low amounts for in both ashe- and lea-treated juices. In comparison to the control (untreated juice), the treated juices were able to inhibit *S. aureus*, which confirms our earlier report where the leaves and ashes showed great antibacterial activity against *S. aureus* with MIC value ranging from 0.04 mg/ml to 0.16 ml/ml as reported earlier. The antimicrobial activity may be due to the presence of several

classes of secondary metabolites, including triterpenoids, flavonoids, stilbenes, tannins and lignans, (Lima De Morais *et al., 2012*; Zhang *et al.,* 2019). The following leaves *C. caffrum, C. elaegnoides, C. erythrophyllum, C. imberbe, C. kraussii C. mkuzense and C padoides i*nhibited the growth *of B. cereus* in the first 3 weeks. The untreated juice had a high count of *B. cereus* from the first week of treatment to the fourth. *B cereus* was found to be resistant to *C. apiculatum and C. bracteseum.* The following ashes; *C. elaegnoides, C. erythrophyllum, C. kraussii* and *C. padoides* were effective in reducing the levels of *B. cereus* when compared to the untreated, however, when the storage time increased, the bacterial count also increased. *B. cereus* produces two types of toxins – emetic (vomiting) and diarrhoeal – causing two types of illness. The emetic syndrome is caused by emetic toxin produced by the bacteria during the growth phase in the food. The diarrhoeal syndrome is caused by diarrhoeal toxins produced during the growth of the bacteria in the small intestine (Ehling-Schulz *et al.,* 2006). The ability of the *C. caffrum, C. elaegnoides, C. erythrophyllum, C. imberbe, C. kraussii C. mkuzense and C. padoides* plants to inhibit the growth of *B. cereus* is of importance, as this will help alleviate some of the health issues associated with presence of this microorganism. Lactic acid bacteria were not detected in throughout the four weeks of storage for juices treated with powdered leaves of *Combretum* spp. and the control. However, the juices treated with ashes had high bacterial counts ranging from 40 000 CFU/ml to 60 000 CFU/ml. The increase in lactic acid bacteria followed the same trend as reported on other natural fermented foods (Sulma *et al.,* 1991; Choi *et al.,* 1994; Dziedzoaze *et al.,* 1996). This increase may be because, after a couple weeks, the beverage started to ferment. Other workers have reported the dominance of lactic acid bacteria in various fermentation products, including *Obushera* (Muyanja *et al*., 2003), *Togwa* (Mugula *et al.,* 2003), *Poto poto* (Abriouel *et al*., 2006) and *Ting* (Sekwati-Monang and Gänzle, 2011). *E. coli* was not present throughout the storage time. The presence of lactic acid bacteria more frequently occurs in unpasteurised juices (Oliveira *et al.*, 2006). These microorganisms produce acetic and formic acids along with ethanol and carbon dioxide that can alter the flavour of juice (Jay and Anderson, 2001). *Escherichia coli (E. coli)* naturally form part of the normal fora in the gut of humans and other animals. In fact, most *E. coli* are considered harmless to humans (Croxen and Finlay, 2010). However, certain pathogenic *E. coli strains c*an infect the gut area and cause severe illness (Croxen *et al.,* 2013). The presence of *E. coli* in a food product is often an indicator of faecal

contamination. The fact that it was not detected in any of the juices, it implies that proper hygiene was followed in the preparation. Isolation of the most common bacteria were done and identified using Vitek 2 compact. The isolates were characterised by morphological differences. Most of the isolates were in the *Enterobacter genus*. *Enterobacter* spp. are named thus for their enteric recovery as gram-negative bacteria (Richard, 1984). They are commonly found in soil, water, and sewage. They also are causes of botanical disease. These organisms are facultative anaerobic and motile by peritrichous flagella, with the exception of *Enterobacter asburiae*. These organisms may be introduced to the juices during the preparation process (sun-drying)It will be advisable to use an alternative method for drying such as oven or microwave to avoid to introducing such microorganisms. *E. cloacae* has been implicated in a broad range of clinical syndromes and was detected in six types of street foods obtained from five locations in Malaysia, revealing a prevalence of 100% in *kuah* chutney, curry *samosa*, *surimi* lobster, *kuih lapis* and *kuih koci*; and 66.7% in *tauhu sumbat* (Haryani *et al.,* 2008). Based on European recommendations for antimicrobial resistance surveillance, *E. cloacae* is one of the few bacteria that has to be monitored in healthcare facilities (Cornaglia *et al.,* 2004). Antibiotic susceptibility testing results showed that all of *E. cloacae* studied were resistant to ampicillin, erythromycin, rifampicin, and sulphamethoxazole. They demonstrated various degrees of resistance to streptomycin (85.71%), ciprofloxacin and tetracycline (42.86%), trimethoprim and cefuroxime (28.57%); but they were susceptible to chloramphenicol and gentamicin. This might be the reason why this bacterium was prevalent in the drink because of its resistance. *S. intermedius* was also found to be prevalent in the juices. *S. intermedius* is a very rare human pathogen. There are only 16 reported cases that have described *S. intermedius* as a cause of infection in human (Kelesidis and Tsiodras, 2010). Most of these cases have been described in association with exposure to animals, mostly dogs. This emphasise the need to change the drying method as indicated earlier in this study. *Aeromonas sobria* was isolated in one of the juices. Species of *Aeromonas* are Gram-negative, non-spore-forming, rod-shaped, facultative anaerobic bacteria that occur in aquatic environments. Although historically, *Aeromonas* genus has been placed in the family Vibrionaceae (Popoff, 1984), there have been proposals to place it under the family *Aeromonadaceae* (Colwell *et al.,* 1986). The *aeromonads* share many biochemical characteristics with members of the *Enterobacteriaceae*, from which they are primarily differentiated by being oxidase-positive (Appendix V, Vitek 2

results). The mesophilic *aeromonads* have been commonly isolated from patients with gastroenteritis although their role in disease causation remains unclear. They are also associated with sepsis and wounds, and with eye, respiratory tract, and other systemic infections (Janda and Duffey, 1988; Janda and Abbott, 1996; Nichols *et al.,* 1996). The presence of *Aeromonas sobria* in one of the juices was a cause for concern about its safety. *Stenotrophomonas maltophilia* as an environmental bacterium was also isolated in this study. It is found in aqueous habitats, including plant rhizospheres, animals, foods, and water sources. Studies have shown that this bacterium is associated with plant-based food (Qureshi *et al*., 2005).

Conclusion: Additions of the powdered leaves and ashes of *Combretum* plants were able to enhance the shelf life of the sorghum juice by reducing *S. aureus, B. cereus* and lactic acid bacteria. However, additional preservation methods may be required to eliminate other food pathogens.

7.5. References

Abriouel, H., Omar, N.B., López, R.L., Martínez-Cañamero, M., Keleke, S., and Gálvez, A., 2006. Culture-independent analysis of the microbial composition of the African traditional fermented foods poto poto and dégué by using three different DNA extraction methods. *International Journal of Food Microbiology* 111: 228–233.

Anand, S. P., and Sati, N., 2013. Artificial preservatives and their harmful effects: Looking toward nature for safer alternatives. *International Journal of Pharmaceutical Sciences and Research* 4(7): 2496–2501.

Balasundram, N.K., Sundram K., and Samman, S., 2006. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry* 99(1): 191-203.

Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods--a review. *International Journal of Food Microbiology* 94(3): 223-253.

Choi, S., Beuchat, L.R., Perkins, L.M., and Nakayama, T., 1994. Fermentation and sensory characteristics of kimichi containing potassium chloride as partial replacement of sodium chloride. *International Journal of Food Microbiology* 21:335– 340.

Chua, D., Goh, J.K., Saftner, R.A., and Bhagwat, A.A., 2008. Fresh-cut lettuce in modified atmosphere packages stored at improper temperatures supports Enterohemorrhagic E. coli isolates to survive Gastric acid challenge. Journal of Food Science 73(3): M148-M153.

Colwell, R.R., MacDonell, M.R., and De Ley, J., 1986. Proposal to recognize the family Aeromonadaceae fam. *International Journal of Systematic Bacteriology* 36:473–477.

Cooper, E.L., 2008. eCAM: an emerging linkage with ethno pharmacology? *Evidence-Based Complementary and Alternative Medicine* 5: 365–366.

Cornaglia, G., Hryniewicz, W., Jarlier, V., Kahlmeter, G., Mittermayer, H., Stratchounski, L., Baquero, F. and ESCMID Study Group for Antimicrobial Resistance Surveillance (ESGARS, 2004. European recommendations for antimicrobial resistance surveillance. Clinical Microbiology and Infection, 10(4), pp.349-383.

Cowan, M.M., 1999. Plant products as antimicrobial agents. *Clinical Microbiology and Infection* (4): 564-582.

Croxen, M.A. and Finlay, B.B., 2010. Molecular mechanisms of Escherichia coli pathogenicity. Nature Reviews Microbiology, 8(1), pp.26-38.

Croxen, M.A., Law, R.J., Scholz, R., Keeney, K.M., Wlodarska, M. and Finlay, B.B., 2013. Recent advances in understanding enteric pathogenic Escherichia coli. Clinical microbiology reviews, 26(4), pp.822-880.

Dziedzoaze, T.N., Ellis, W.O., and Oldham, J.H., 1996. Effect of cassava varietal differences and fermentation time on the quality of agbelina. In: Halm, M., Jakobsen, M. (Eds.), *Traditional fermented food processing in Africa*. Proceedings of the Third Biennial Seminar on African Fermented Food, FRI, DANIDA, KVL, July. Accra, Ghana, 17–25.

Ehling-Schulz, M., Guinebretière, M., Monthan, A., Berge, O., Fricker, M., and Svensson, B., 2006. Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. *FEMS Microbiology Letters* 260(2):232–240.

Haryani, Y., Tunung, R., Chai, L.C., Lee, H.Y., Tang, S.Y. and Son, R., 2008. Characterization of Enterobacter cloacae isolated from street foods. *International Food Research Journal*, *15*(1).

Havelaar, A.H, Brul, S., de Jong, A., de Jonge, R., Zwietering, M.H., and Ter Kuile, B.H. 2010. Future challenges to microbial food safety. *International Journal of Food Microbiology* 139 Suppl 1:S79-94. doi: 10.1016/j.ijfoodmicro.2009.10.015. Epub 2009 Oct 23. PMID: 19913933.

Hocking, A.D. and Jensen, N., 2001. Soft drinks, cordials, juices, bottled water and related products. Spoilage of processed foods: causes and diagnosis,.84-100.

Janda, J.M., and Abbott, S.L., 1996. Human pathogens. In: Austin B (eds.), *the genus Aeromonas*. London, Wiley: 151–173.

Janda, J.M., and Duffey, P.S., 1988. Mesophilic aeromonads in human disease: current taxonomy, laboratory identification, and infectious disease spectrum. *Reviews in Infectious Diseases* 10:980–987.

Jay, S., and Anderson, J. 2001. In Spoilage of Processed Foods. Causes and Diagnosis; Southwood Press: Marrickville, Australia, 187–198.

Koduru, S., Grierson, D.S., and Afolayan, A.J., 2007. Ethnobotanical information of medicinal plants used for treatment of cancer in the Eastern Cape Province, South Africa. *Current Science* 92: 906–908.

Kumar, S., and Pandey, A.K., 2013. Chemistry and biological activities of flavonoids: an overview. *The Scientific World Journal* Article ID: 16275.

Lima De Morais, G.R., De Sales, I.R.P., Filho, M.R.D.C., De Jesus, N.Z.T., De Sousa Falcao, H., Barbosa-Filho, J.M., Cabral, A.G.S., Souto, A.L., Tavares, J.F., and Batista, L.M., 2012. Bioactivities of the genus *Combretum* (Combretaceae): a review. *Molecules* 17. https://doi.org/10.3390/molecules17089142, 9142–7206

Manach, C., Scalbert, A., Morand, C., Rémésy, C., and Jiménez, L., 2004. Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition* 79(5):727-747.

McMeekin, T.A., Hill, C., Wagner, M., Dahl, A., and Toss, T., 2010. Ecophysiology of food-borne pathogens: Essential knowledge to improve food safety. *International Journal of Food Microbiology* 139(Supplement 1): S64-S78.

Mugula, J.K., Nnko, S.A.M., Narvhus, J.A., Sørhaug, T., 2003. Microbiological and fermentation characteristics of togwa, a Tanzanian fermented food. *International Journal of Food Microbiology* 80: 187–199.

Muyanja, C.M.B.K., Narvhus, J.A., Treimo, J., and Langsrud, T., 2003. Isolation, characterisation and identification of lactic acid bacteria from bushera: a Ugandan traditional fermented beverage. *International Journal of Food Microbiology* 80: 201–210.

Negi, P., 2012. Plant extracts for the control of bacterial growth: efficacy stability and safety issues for food application. *International Journal of Food Microbiology* 156: 7–17.

Nichols, G.L., Lightfoot, N.F. and de Louvois, J., 1996. Health significance of bacteria in distribution systems— review of Aeromonas. London, UK Water Industry Research Ltd (Report DW-02/A).

Oliveira, J.C., Setti-Perdigao, P., Siqueira, K.A.G., Santos, A.C., and Miguel M. A. L. 2006. Microbiological characteristics of orange juices. *Ciencia e Tecnologia de Alimentos* 26 (2):241– 245.

Qureshi, A., Mooney, L., Denton, M., and Kerr, K.G., 2005. *Stenotrophomonas maltophilia* in salad *Emerging infectious diseases* 11:1157 –1158.

Richard, C., 1984. Enterobacter. In Garrity, G.M. (ed.): *Bergey's Manual of Systematic Bacteriology*. New York, Springer. 465-467

Sekwati-Monang, B., 2011. Microbiological and chemical characterization of *ting*, a sorghum-based gluten-free fermented cereal product from Botswana. Ph.D. Thesis. University of Alberta, Edmonton, Canada.

Sekwati-Monang, B., and Gänzle, M.G., 2011. Microbiological and chemical characterisation of ting, a sorghum-based sourdough product from Botswana. *International Journal of Food Microbiology* 150: 115–121.

Shakiba, M., Kariminik, A., and Parsia, P. 2011. Antimicrobial activity of different parts of Phoenix dactylifera. *International Journal of Molecular and Clinical Microbiology* 1: 107–111.

Sulma, I., Larry, R.S., and Kirleis, A., 1991. Isolation and characterisation of microorganisms associated with the traditional sorghum fermentation for production of Sudanese kisra. *Applied and Environmental Microbiology Journal* 57: 2529– 2533.

Tajkarimi, M.M., Ibrahim, S.A., and Cliver, D.O., 2010. Antimicrobial herb and spice compounds in food. *Food Contro*l 21(9): 1199-1218.

Warriner, K., Huber, A., Namvar, A., Fan, W., and Dunfield, K., 2009. Recent advances in the microbial safety of fresh fruits and vegetables. **In:** L.T. Steve, *Advances in food and nutrition research*. Academic Press. 155-208.

Zhang, X.R., Kaunda, J.S., Zhu, H.T., Wang, D., Yang, C.R., and Zhang, Y.J., 2019. The Genus *Terminalia* (Combretaceae): an ethnopharmacological, phytochemical and pharmacological review, natural products and bioprospecting. Springer Singapore.

CHAPTER 8: SENSORY EVALUATION AND NUTRIONAL COMPOSITION OF PREPARED SORGHUM JUICES TREATED WITH EXTRACTS AND ASHES OF *COMBRETUM* SPECIES

8.1 Introduction

Sensory food science is a discipline dealing with human sensory perceptions of and affective responses to foods, beverages and their components. It is multidisciplinary in nature, deriving research questions from food science and applying behavioural research methods to solve these questions. Sensory food science uses sensory evaluation as its central method of analysis. Sensory evaluation is defined as a scientific method used to evoke, measure, analyse, and interpret those products as perceived through the senses of sight, smell, touch, taste, and hearing (Lawless and Heymann, 1999; Stone and Sidel, 1993). Depending upon the research question, sensory food science also utilises physicochemical, physiological, and consumer-based research methods. When a consumer buys a food product, they often buy nutrition, convenience, and image. Nevertheless, most importantly consumers are buying sensory properties/performance and sensory consistency. Therefore, sensory evaluation is an integral part in defining and controlling product quality. Sensory evaluation comprises a set of techniques for accurate measurements of human responses to foods and minimises the potentially biasing effects of brand identity (Lawless and Heymann, 2010). Sensory characteristics comprising appearance, odour, flavour and texture are included within the quality of food products. There are a limited number of internationally accepted standards on general methods in sensory analysis, such as general guidance (ISO 6658, 1985), assessor's selection and panel training procedures (ISO 8586, 1993) and sensory tests (ISO 5495, 1983; ISO 4120, 1983; ISO 6564, 1985; ISO 10399, 1991; ISO 1036, 1994). These standards permit the selection, basic training of panellists and general application of discriminative and descriptive sensory methods. In the context of food industry, sensory evaluation is one of the tools that marketing management can use in order to understand the target market, identify the most important features of a product, eliminate wasted effort during product development, deal with quality issues, compare their brands to others and try to ensure long shelf life.

There are many types of sensory analysis methods, the most popular being difference tests, descriptive analysis and consumer acceptance testing (Lawless and Heymann, 1998). Difference tests include the triangle test, where the panel member attempts to detect which one of three samples is different from the other two, and duo-trio tests, where the panel member selects which one of two samples is different from the identified standard. Difference tests estimate the magnitude of sensory differences between samples, but one limitation of these tests is that the nature of the differences is not defined. It is usually a common practice to use a combination of difference tests and descriptive sensory analysis for problem solving. Descriptive sensory analysis uses several techniques that seek to discriminate between a range of products based on their sensory characteristics and also to determine a quantitative description of the sensory differences that can be identified, not just the defects. No judgment of “good” or “bad” is made as in traditional quality judging methods because this is not the purpose of the evaluation. Here, the panel is a powerful instrument that identifies and quantifies a product’s sensory properties. The current chapter focused on assessing the nutritional composition and sensory evaluation of the prepared sorghum juices.

8.2 Methods and materials

8.2.1 Proximate and nutritional analysis

The proximate and mineral analysis of the prepared juices were performed as described in Chapter 4, section 4.2.

8.2.2 Sensory analysis of wine using a hedonic scale

An affective test that involved 20 untrained cosumers/panellists aged 18 and above was used to evaluate the acceptance of the samples by using an intensity scale from 1 to 9 for the attributes that best represent the product and are more susceptible to changes during storage, i.e. flavour, acidity, off odours and pasta friability (ISO 8589, 1988). The scale comprised nine verbal categories ranging from “dislike extremely” to “like extremely” (Appendix IX). The participants who consented to be part of the study were given coded samples. After tasting, they selected the category on the scale that best described each sample. The consent form is attached in (Appendix X). The samples were presented following a completely randomised design. Mineral water and cream cracker biscuits were available as neutralisers between samples in order to avoid carryover effects. Taste preference was evaluated using the ranking test

according to the subjects' degree of liking. Prior to each assessment, the panellists were informed about the task. In addition, to the oral information, a detailed set of written instructions on the testing methods was available in the testing booth. A total of 15 ml of each beverage at 7 °C was served to each panellist in coded opaque plastic tumblers. The tests were performed under conditions of standard light and temperature (20 °C) with groups of five panellists.

8.2.3 Data analysis

Statistical analysis of results was performed using Statistix 10 data analysis software, a completely randomised test and the Welch's Test was used for comparison of any significant differences between the means. Statistical analysis was performed to determine variation between the juices in terms of proximate, nutritional and sensory evaluation. Values were considered significantly different when $p < 0.05$.

8.3 Results

8.3.1 Mineral and proximate analysis

8.3.1.1 Mineral analysis of the juice infused with leaves of *Combretum* species

The juices prepared in the study possessed a substantial amount of trace element or minerals. It was interesting to observe that potassium has the highest concentrations ranging from 3, 55–104 mg/l followed by calcium (3.2-148 mg/l), as shown in Table 8.1. Minerals such as cobalt, copper, iron, magnesium, sodium, nickel and zinc were present in low concentrations.

Table 8.1: Trace elements in prepared sorghum juices infused with powdered leaves of *Combretum* species.

Plant	**Trace elements/minerals mg/l**									
	Calcium	**Cobalt**	**Copper**	**Iron**	**Potassium**	**Magnesium**	**Manganese**	**Sodium**	**Nikel**	**Zinc**
C. apiculatum	**2**	-1,22	-1,11	-1,15	**59,6**	-0,467	-0,222	0,202	-3,1	-1,51
C. bracteosum	**9,27**	-1,11	-0,978	-1,02	**57,3**	1,7	-0,143	0,289	-2,77	-1,06

C. caffrum	**1,36**	-1,23	-0,999	-1,14	**3,55**	0,507	-0,231	-0,142	-3,11	-1,4
C. elaegnoides	**1,72**	-1,2	-1,09	-1,14	**93,2**	-0,0043	-0,179	0,426	-3,06	-1,25
C. erythrophyllum	**14,5**	-1,09	-0,974	-0,962	**97,5**	3,04	-0,128	0,689	-2,7	-1,48
C. imberbe	**11,6**	-1,13	-1,03	-1,04	**96,2**	0,441	-0,174	0,86	-2,82	-1,37
C. kraussii	**9,05**	-1,13	-1,04	-1,05	**82,3**	-0,411	-0,183	0,518	-2,82	-1,32
C. mkuzense	**2,71**	-1,21	-1,08	-1,14	**28,4**	-0,142	-0,225	0,0204	-3,07	-1,35
C. padoides	**4,37**	-1,26	-0,991	-1,18	**58,4**	-1,2	-0,242	-0,169	-3,16	-1,43
C. vendae	**3,34**	-1,19	-1,1	-1,12	**104**	0,301	-0,209	0,817	-3,04	-1,38
C. zeyherii	**1,16**	1,19	-1,14	-1,18	**11,5**	-3,15	-0,261	-0,646	-3,08	-1,2

The juices infused with ashes had different levels of minerals. It is important to note that there was a substantial increase in the concentratiions of minerals such as calcium, potassium, magnesium and sodium. Minerals such as cobalt, nickel, iron and zinc were detected in the lowest concentrations, as indicated in Table 8.2

Table 8.2: Mineral content in prepared sorghum juices infused with ashes of *Combretum* species.

Plant	**Trace elements/minerals**									
	Calcium	**Cobalt**	**Copper**	**Iron**	**Potassium**	**Magnesium**	**Manganese**	**Sodium**	**Nikel**	**Zinc**
C. adenogonium	**6**	-0,964	-0,83	-0,743	**165**	**2,62**	-0,063	**3,51**	-3,1	-1,53

C. apiculatum	**61,2**	-0,789	-0,605	-0,554	**66,1**	**15,2**	0,212	**0,678**	-2,06	-0,992
C. bracteosum	**25,4**	-0,991	-0,813	-0,815	**145**	**5,43**	0,0469	**2,17**	-2,09	-0,979
C. caffrum	**40,7**	-0,876	-0,662	-0,317	**136**	**12**	0,267	**4,7**	-2,95	-1,44
C. elaegnoides	**53,3**	-0,837	-0,744	-0,633	**336**	**9,53**	0,126	**4,05**	-2,48	-1,24
C. erythrophyllum	**148**	-0,555	-0,188	0,219	**922**	**23,7**	0,38	**-12,5**	-2,97	-1,43
C. imberbe	**23,7**	-1,01	-0,85	-0,848	**52,4**	**2,83**	0,0786	**1,72**	-3,05	-1,46
C. kraussii	**3,2**	-1,16	-1,01	-1,1	**12,1**	**0,0515**	-0,187	**0,311**	-3,12	-1,48
C. mkuzense	**43,2**	-0,871	-0,635	-0,481	**43,9**	**11,5**	0,0716	**1,37**	-1,18	-0,588
C. padoides	**7,36**	-1,13	-1,04	-1,05	**130**	**0,764**	-0,166	**0,73**	-2,95	-1,42
C. vendae	**21,1**	-1,03	-0,921	-0,88	**167**	**2,95**	0,0557	**2,75**	-2,74	-1,34
C. zeyherii	**76,3**	-0,809	-0,432	0,0669	**105**	**23,2**	0,307	**3,49**	-1,85	-0,899

8.3.1.2 Proximate analysis

Proximate analysis refers to the quantitative analysis of macromolecules in food. A combination of different techniques, such as extraction, ICP were used to determine protein, moisture, ash and energy levels.

Food energy is defined as the energy released from carbohydrates, fats, proteins, and other organic compounds. Food energy is usually measured by a bomb calorimeter based on the heat of combustion (Insel *et al.,* 2012). Energy released by a particular food is a critical parameter in nutrition. All the juice prepared both infused with leaves

and ashes had appreciable amount of energy, ranging from 12-17 KJ/g, as indicated in Figure 8.1.

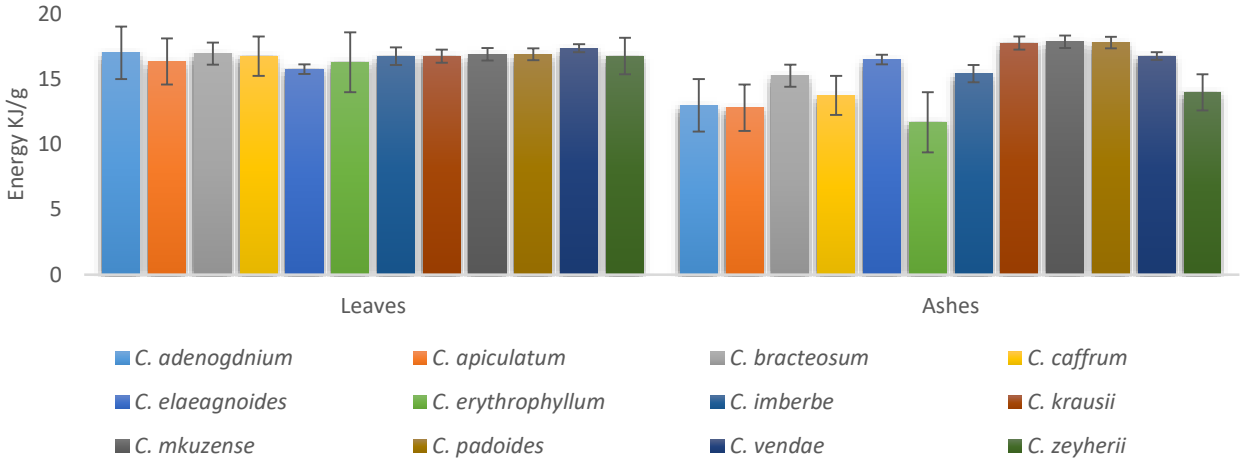


Figure 8.1: Energy content of prepared sorghum juices infused with leaves and ashes of *Combretum* species.

Proteins consist of one or more chains of amino acids, and differ from one another primarily in the sequence of amino acids. Proteins can be hydrolysed into polypeptides oramino acids by proteases. Amino acids are essential nutrients, and some are supplemented in foods in both natural and synthetic forms (Ikeda, 2003). All the juices had appreciable amounts of protein. Interestingly juices infused with ashes from *C. caffrum, C. erythrophyllum* and *C. kraussii* had the highest concentrations of proteins compared to the rest of the juices (Figure 8.2).

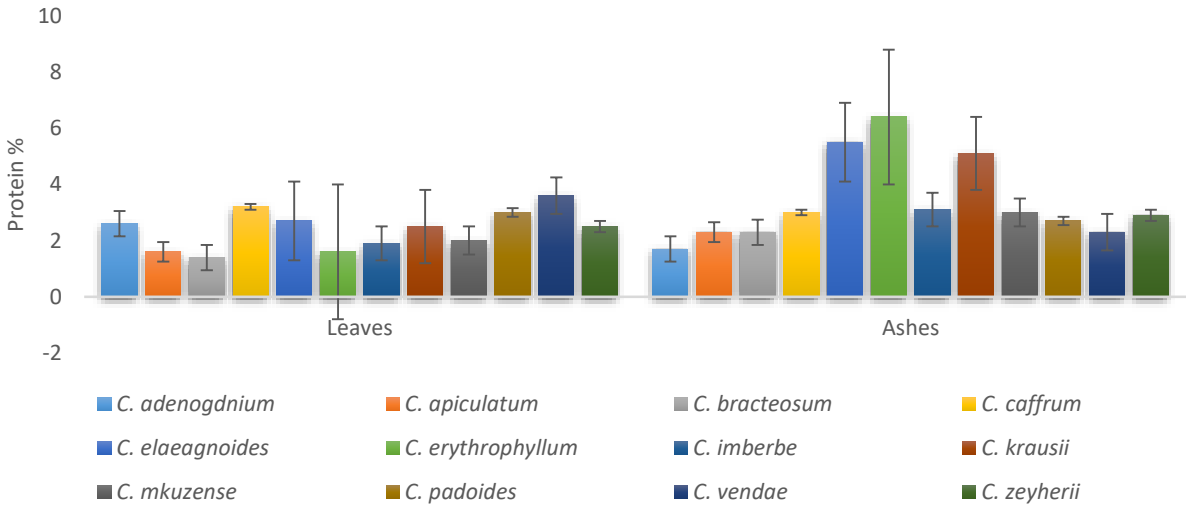


Figure 8.2 Protein content of the prepared juices infused with leaves and ashes

Determining the ash content of food is part of proximate analysis for nutritional evaluation and it is an important quality attribute for some food ingredients (Harris and Marshall, 2017). Ash content for all the juices was found to be below 2 % with the highest concentrations found in the juice infused with ashes of *C. adenognium*, as shown in figure 8.3.

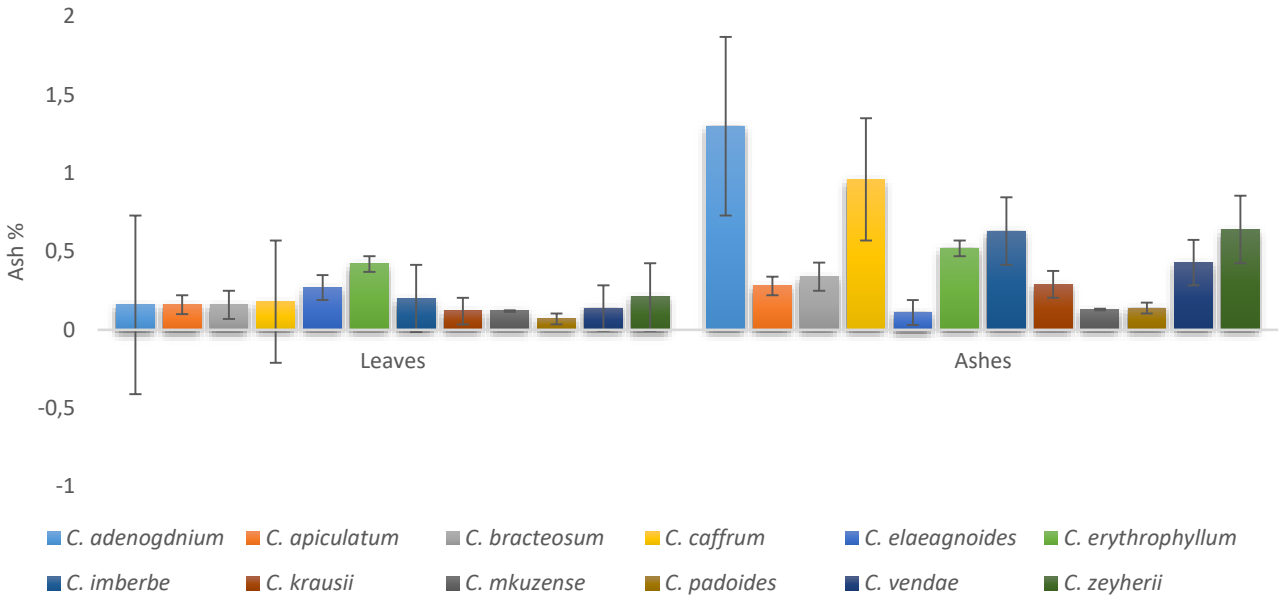
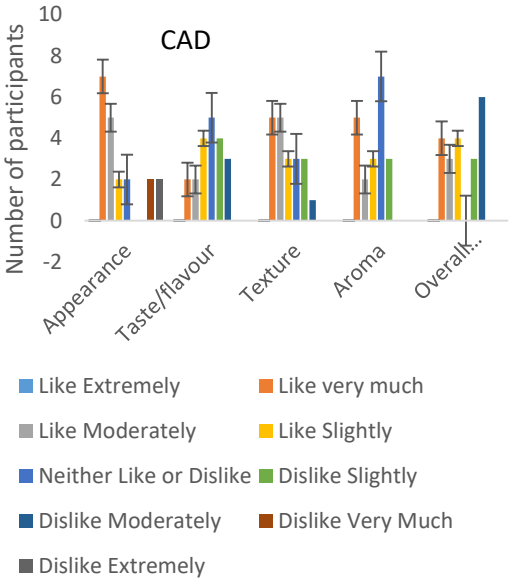
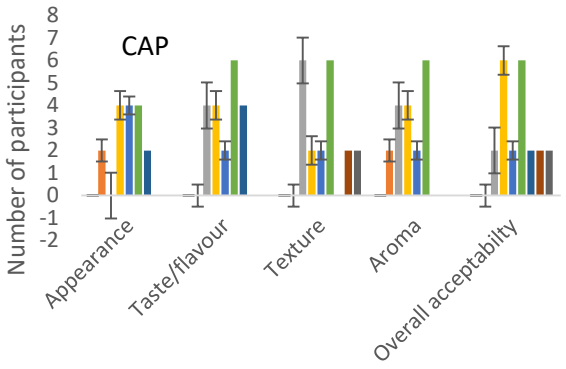


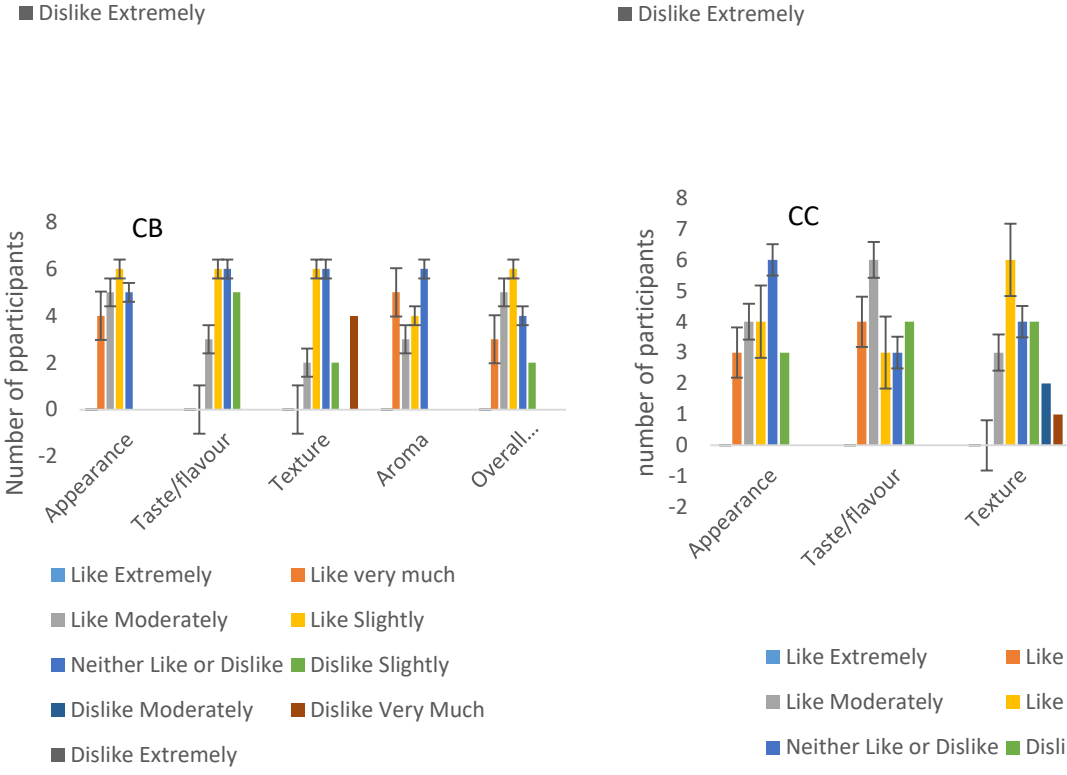
Figure 8.3: The composition of ash in prepared sorghum juices infused with leaves and ashes of *Combretum* species.

8.3.2 Sensory analysis

Sensory evaluation is necessary to build a relationship between the product characteristics and the consumer. Appearance, taste, colour and texture are the important sensory attributes (Escribano *et al.,* 2010). Addition of ashes into the prepared juices of sorghum impart better sensory and organoleptic properties than addition of the powdered leaves of the plants. The juice containing ashes of *C. caffrum* and *C. bracteseuom* (Appendix X) were well accepted by most of the panellists compared to the rest of the additives (Figure 8.4 and Figure 8.5).


CAD
Number of participants
10
8
6
4
2
0
-2
Appearance
Taste/flavour
Texture
Aroma
Overallk..

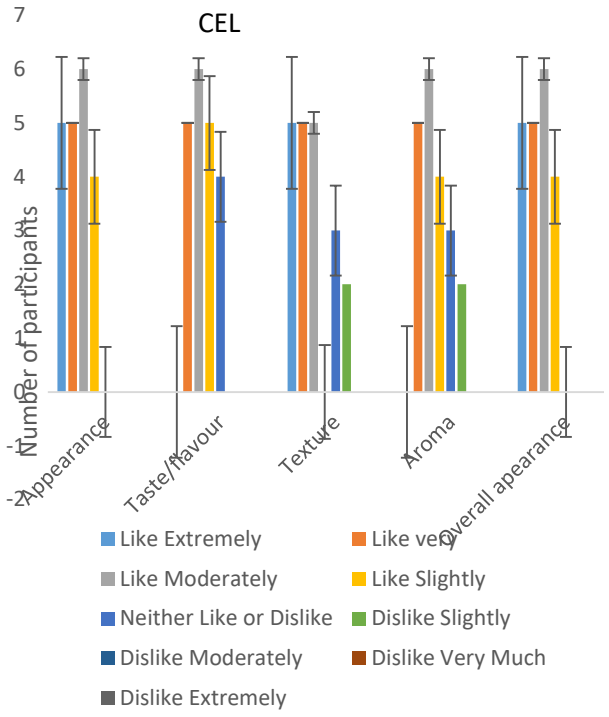

CAP
Number of participants
8
7
6
5
4
3
2
1
0
-1
-2
Appearance
Taste/flavour
Texture
Aroma
Overall acceptabilty

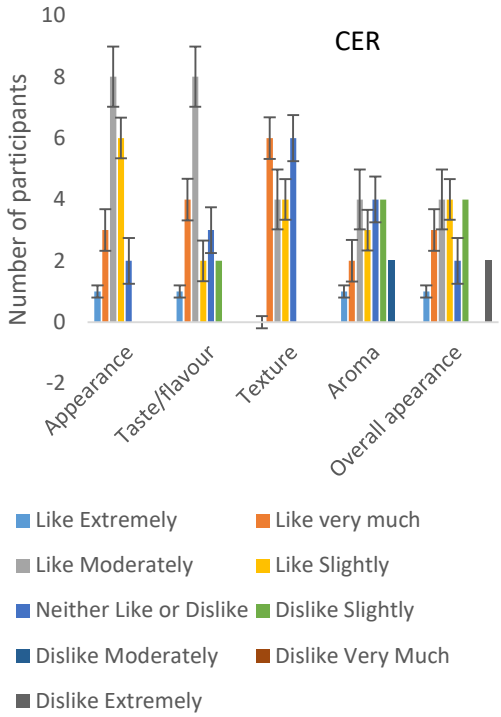

Like Extremely
Like very much
Like Moderately
Like Slightly
Neither Like or Dislike
Dislike Slightly
Dislike Moderately
Dislike Very Much
Dislike Extremely

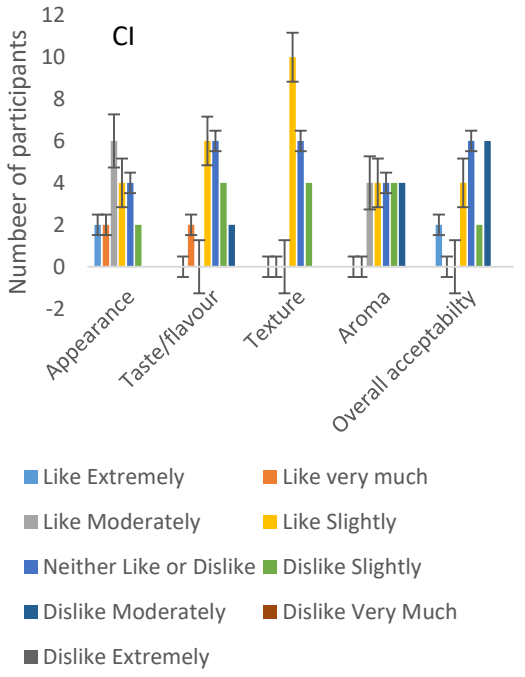
CB
Number of pparticipants
8
6
4
2
0
-2
Appearance
Taste/flavour
Texture
Aroma
Overall...

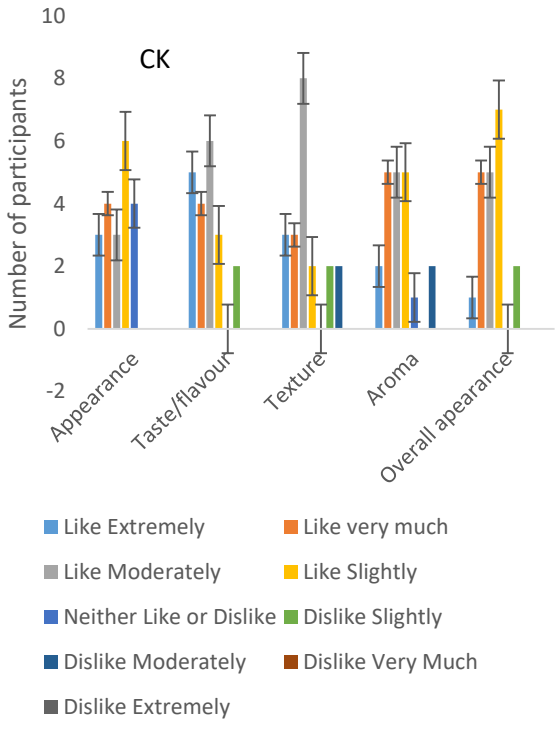
CC
number of participants
8
7
6
5
4
3
2
1
0
-1
-2
Appearance
Taste/flavour
Texture
Aroma
Overall acceptability


Like Extremely
Like very much
Like Moderately
Like Slightly
Neither Like or Dislike
Dislike Slightly
Dislike Moderately
Dislike Very Much
Dislike Extremely


CEL
Number of participants
7
6
5
4
3
2
1
0
-1
-2
Appearance
Taste/flavour
Texture
Aroma
Overall apearance
Like Extremely
Like very much
Like Moderately
Like Slightly
Neither Like or Dislike
Dislike Slightly
Dislike Moderately
Dislike Very Much
Dislike Extremely


CER
Number of participants
10
8
6
4
2
0
-2
Appearance
Taste/flavour
Texture
Aroma
Overall apearance
Like Extremely
Like very much
Like Moderately
Like Slightly
Neither Like or Dislike
Dislike Slightly
Dislike Moderately
Dislike Very Much
Dislike Extremely


CI
Numbeer of participants
12
10
8
6
4
2
0
-2
Appearance
Taste/flavour
Texture
Aroma
Overall acceptabilty
Like Extremely
Like very much
Like Moderately
Like Slightly
Neither Like or Dislike
Dislike Slightly
Dislike Moderately
Dislike Very Much
Dislike Extremely


CK
Number of participants
10
8
6
4
2
0
-2
Appearance
Taste/flavour
Texture
Aroma
Overall apearance
Like Extremely
Like very much
Like Moderately
Like Slightly
Neither Like or Dislike
Dislike Slightly
Dislike Moderately
Dislike Very Much
Dislike Extremely

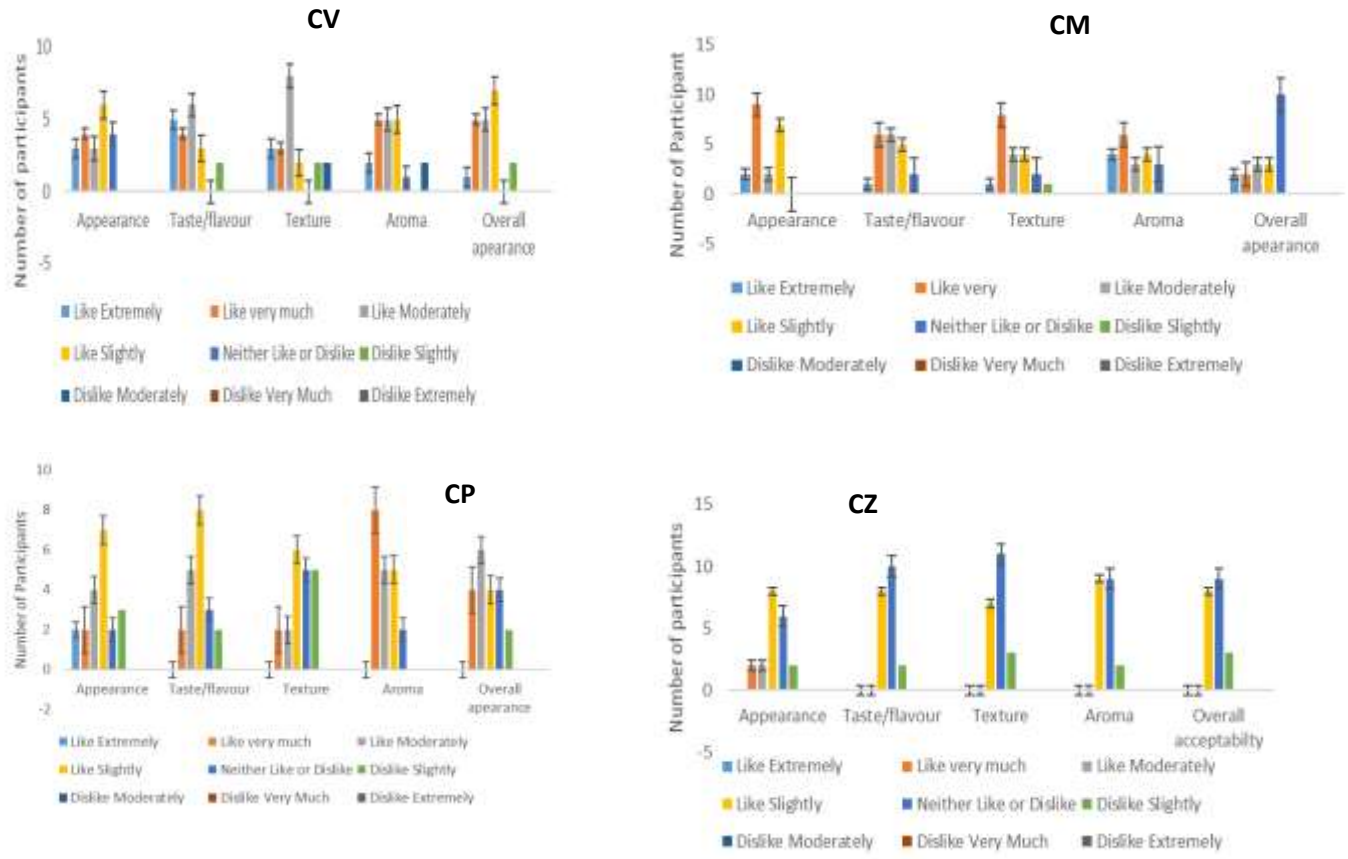
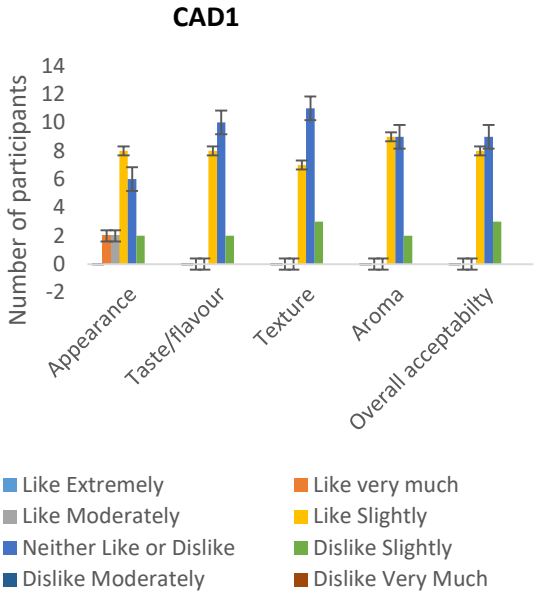
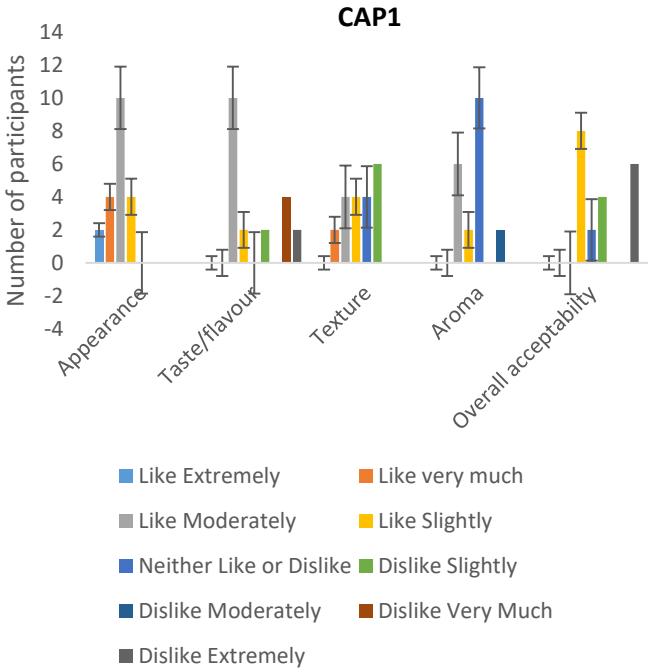


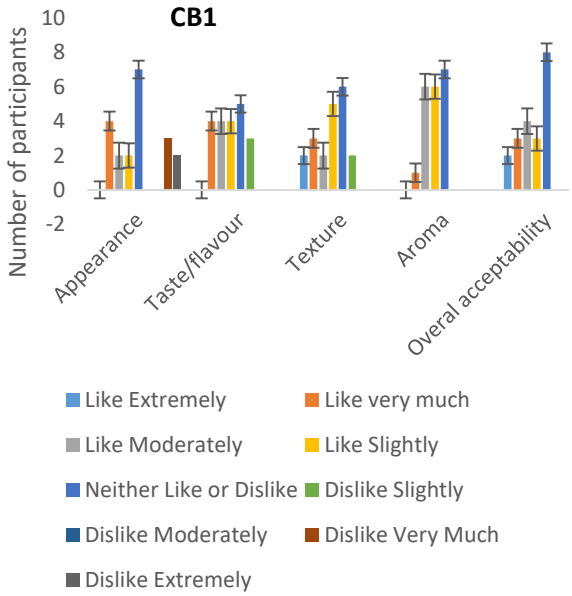
Figure 8.4 Sensory evaluation of prepared sorghum beverages/juices treated with the powdered leaves of twelve *Combretum* species.

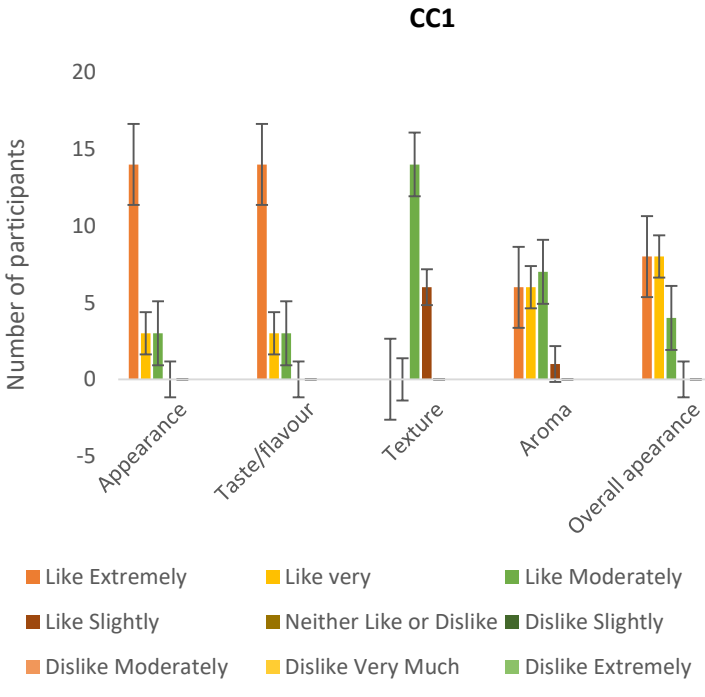
Key:*C. adenogdium* (CAD), *C. apiculatum* (CAP), *C. bracteosum* (CB), *C. caffrum* (CC), *C. elaegnoides (CEL), C. erythrophyllum (CER), C. imberbe* (CI), *C. kraussii* (CK), *C. mkuzense* (CM), *C. padoides* (CP), *C. vendae* (CV) *and C. zeyherii* (CZ).

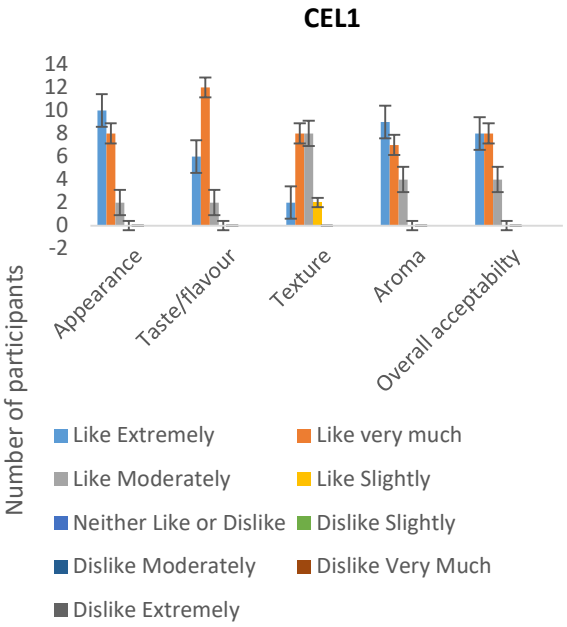
The juices infused with ashes were received better in comparison to the juices infused with powdered leaves. *C. bractesoum, C. caffrum* and *C. mkuzense* perfomed well as far as taste was concerned. *C. caffrum* received the highest score in all the parameters (aroma, texture, appearance and overall acceptability) tested in the study (p=001) (Appendix X).

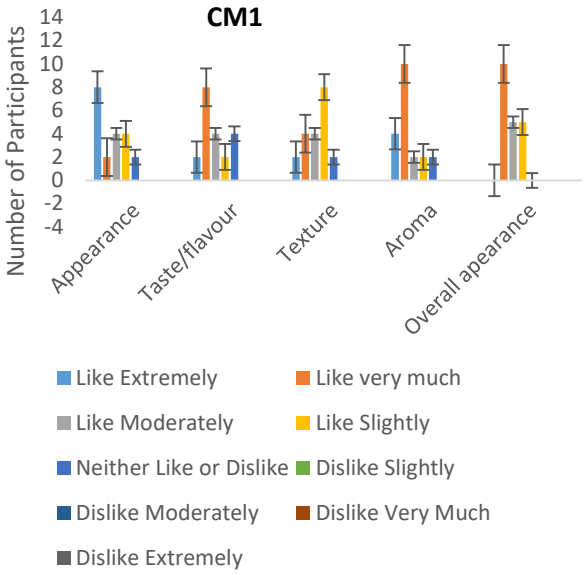


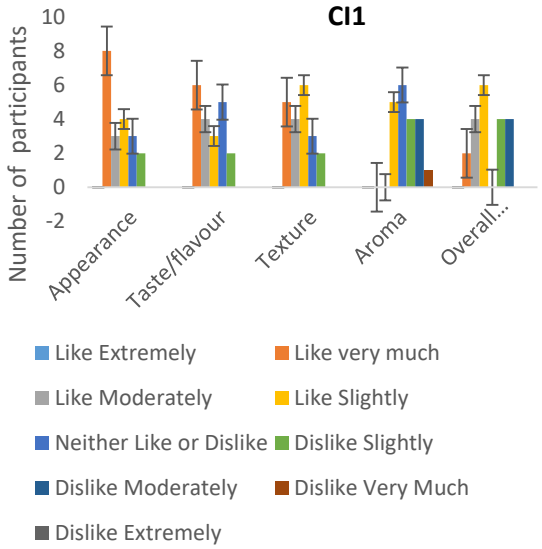


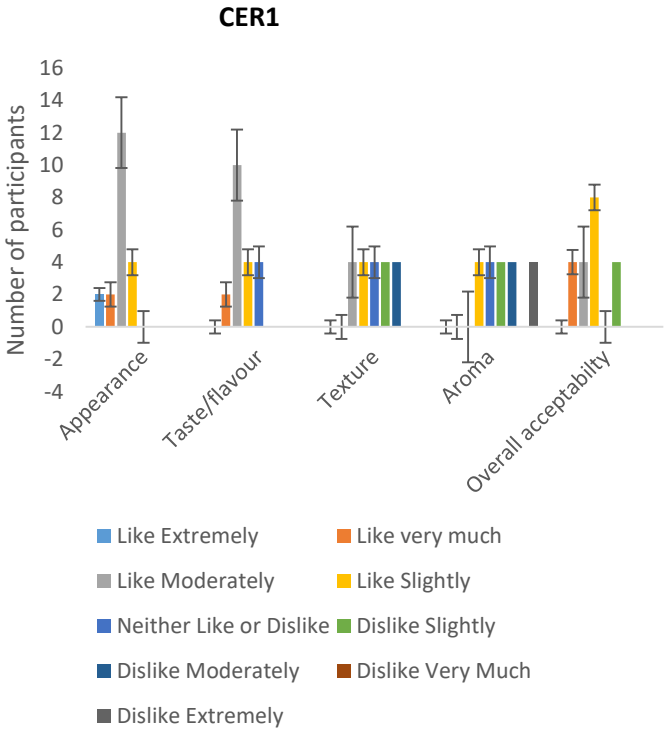


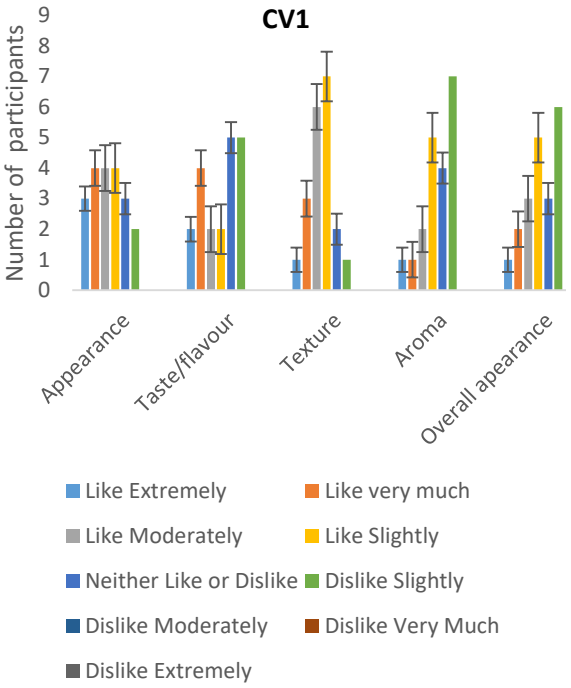



CEL1
Number of participants
14
12
10
8
6
4
2
0
-2
Appearance
Taste/flavour
Texture
Aroma
Overall acceptabilty
Like Extremely
Like very much
Like Moderately
Like Slightly
Neither Like or Dislike
Dislike Slightly
Dislike Moderately
Dislike Very Much
Dislike Extremely

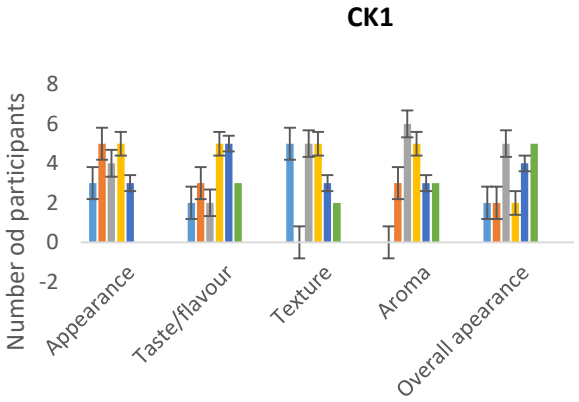

CM1
Number of Participants
14
12
10
8
6
4
2
0
-2
-4
Appearance
Taste/flavour
Texture
Aroma
Overall apearance
Like Extremely
Like very much
Like Moderately
Like Slightly
Neither Like or Dislike
Dislike Slightly
Dislike Moderately
Dislike Very Much
Dislike Extremely


CI1
Number of participants
10
8
6
4
2
0
-2
Appearance
Taste/flavour
Texture
Aroma
Overall...
Like Extremely
Like very much
Like Moderately
Like Slightly
Neither Like or Dislike
Dislike Slightly
Dislike Moderately
Dislike Very Much
Dislike Extremely

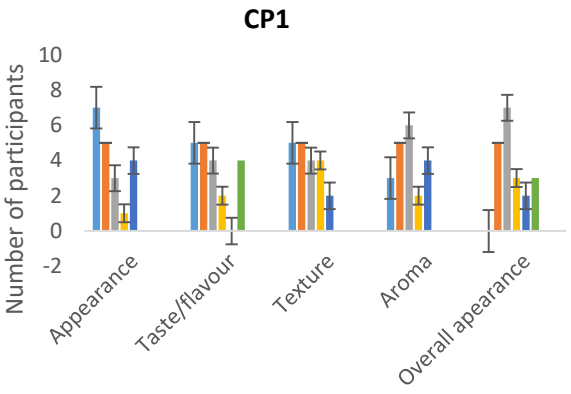

CER1
Number of participants
16
14
12
10
8
6
4
2
0
-2
-4
Appearance
Taste/flavour
Texture
Aroma
Overall acceptabilty
Like Extremely
Like very much
Like Moderately
Like Slightly
Neither Like or Dislike
Dislike Slightly
Dislike Moderately
Dislike Very Much
Dislike Extremely


CV1
Number of participants
9
8
7
6
5
4
3
2
1
0
Appearance
Taste/flavour
Texture
Aroma
Overall apearance

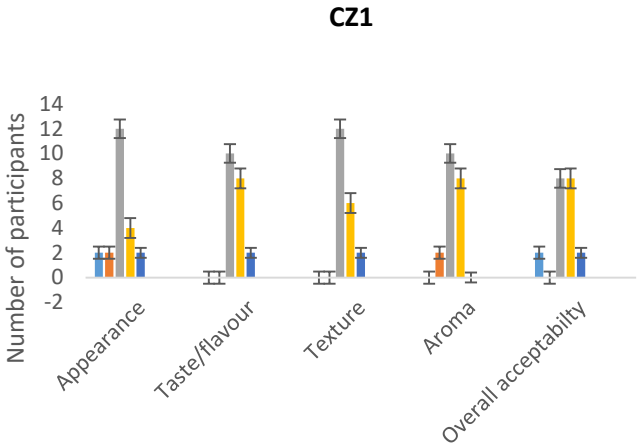
Like Extremely
Like very much
Like Moderately
Like Slightly
Neither Like or Dislike
Dislike Slightly
Dislike Moderately
Dislike Very Much
Dislike Extremely


CK1
Number od participants
8
6
4
2
0
-2
Appearance
Taste/flavour
Texture
Aroma
Overall apearance


Like Extremely
Like very much
Like Moderately
Like Slightly
Neither Like or Dislike
Dislike Slightly
Dislike Moderately
Dislike Very Much
Dislike Extremely


CP1
Number of participants
10
8
6
4
2
0
-2
Appearance
Taste/flavour
Texture
Aroma
Overall apearance


Like Extremely
Like very much
Like Moderately
Like Slightly
Neither Like or Dislike
Dislike Slightly
Dislike Moderately
Dislike Very Much
Dislike Extremely


CZ1
Number of participants
14
12
10
8
6
4
2
0
-2
Appearance
Taste/flavour
Texture
Aroma
Overall acceptablity


Like Extremely
Like very much
Like Moderately
Like Slightly
Neither Like or Dislike
Dislike Slightly
Dislike Moderately
Dislike Very Much
Dislike Extremely

Figure 8.5 Sensory evaluation of prepared sorghum beverages/juices treated with the powdered leaves of twelve *Combretum* species.

Key:

C. adenogdium (CAD), *C. apiculatum* (CAP)*, C. bracteosum* (CB)*, C. caffrum* (CC)*, C. elaegnoides (CEL), C. erythrophyllum (CER), C. imberbe* (CI)*, C. kraussii* (CK)*, C. mkuzense* (CM)*, C. padoides* (CP), *C. vendae* (CV) *and C. zeyherii* (CZ)*.*

8.4 Discussion

Product developers make use of many tools in the development of a product. These tools include, for example, chemical tests, microbiological procedures and the use of physical equipment to determine elasticity, hardness, viscosity, colour intensity and more. It is possible for food products to reflect similar measurements or results when these tools are applied individually, yet still result in different perceptions, acceptability or preferences on consumption of the product. Grading methods for food and beverage products, traditionally involved one or two trained “experts” assigning quality scores on the appearance, flavour and texture of the products based on the presence or absence of predetermined defects. These traditional judging methods have several shortcomings: they cannot predict consumer acceptance; their quality assessments are subjective; assigning quantitative scores is difficult; and they do not combine analytically oriented attribute ratings with affectively oriented quality scores (Claassen and Lawless, 1992). Thus, by using traditional methods of evaluation, some products with very different sensory characteristics, such as those identified by a product flavour profile, but with no product defect will obtain the same quality score. The focus of this chapter was to assess the nutritional composition and sensory properties of the prepared sorghum juices infused with leaves and ashes obtained from the twelve *Combretum* plants using a 9-point hedonic scale. The mineral analysis revealed that potassium was detected in the highest concentrations in both the juices infused with powdered leaves and ashes, with the ashes possessing more potassium. The presence of potassium in juices provided great health promoting benefits, as highlighted earlier. Some of the benefits include maintaining the ionic balance of the human body and maintaining tissue excitability. Sodium plays an important role in the transportation of metabolites (Sinha *et al.*, 2019). The ratio of potassium/sodium in any food is an important factor associated with hypertension and arteriosclerosis. Sodium enhances and potassium depresses blood pressure (Saupi *et al.,* 2009).. It was interesting to observe that there was a serious decline in the

concentatrion of calcium in the juices in comparison to the 'stand-alone' ashes and powdered leaves (Chapter 4,). These minerals might be lost during cooking (sensitive to heat) and fermentation process (due to the presence of microorganisms). For the juice infused with the leaves, the concentration of potassium followed this trend: *C. vendae >C. erythrophyllum>C. elaegnoides while for C. erythrophyllum>C. elaegnoides> C. adenogonium > C. vendae.* Sodium, magnesium, nickel, iron, zinc and calcium were lost during the preparation of the drink. All the juices infused with leaves and ashes had an appreciable amount of energy, protein and ash content; ranging from 12-17 KJ/g, 2-8% and 0.2- 1,5 %, respectively. The sensory evaluation revealed that for the juice infused with *C. adenogonium* leaves, 35 % of the panellists liked the appearance very much, while 25% neither liked nor disliked the texture and aroma. The overall acceptability was ranked in the following order: 15% was 'liked very much'; 10% 'liked moderately', 10% 'liked slightly', 20 % 'disliked slightly' while the remaining were 'disliked moderately'. The drink that was added with leaves, that is, *C. apiculatum,* was 'fairly liked' by the panellists; 30 % liked the taste, texture, aroma and overall acceptability. Only a few panellists rated the drink in the 'like very much' score. None of the panellists disliked any of the organoleptic properties of the drink. *C. bracteosum* was neither liked nor disliked by the majority of the panellists. Taste, texture, aroma and overall acceptability was marked in the neutral score. With that said, some of the panellists appreciated its appearance; 35% gave it a 'like very much' score; 30% gave it a 'like slightly' and 20% gave it for 'like moderately' (Figure 8.5). *C. caffrum* was accepted moderately as far as taste and aroma were concerned (30%) while texture was liked slightly. Thirty percent of the panellists neither liked nor disliked the texture of the juice. It was interesting to see that 25 % of the panellists was pleased with the overall acceptability of the juice (it was scored 'like very much'), 30% neither liked nor disliked it while the remaining did not like it very much. *C. elaegnoides* was fairly appreciated. Twenty five percent of the panellists scored the following organoleptic parameters (appearance, and texture) in the 'like very much' rank while 25% scored it in the 'like moderately' rank. Majority of the panellists scored the taste and aroma in the like moderately' rank, which led to the overall acceptability falling into the 'like moderately' rank. Appearance and taste of *C. erythrophyllum* were appreciated by the majority of the panellists; 40% scored it in the 'like moderately' rating while 20% scored in the 'like very much'. Texture was also appreciated; however, the aroma was largely disliked, which affected the overall acceptability. *C. imberbe* was largely liked as far as appearance and texture were concerned, however, taste and aroma were disliked, which affected the overall acceptability of the juice. Appearance, taste, texture and aroma

of *C. kraussii* were fairly appreciated by the panel. Overall acceptability of *C. kraussii* was rated better than the other juices, as 35% of the panellists gave it 'Like slightly' *score C. mkusenze* was received well by a majority of the panellists, where 40% scored it the 'like very much' based on the appearance, taste, texture and aroma properties, which led to the overall acceptability being fairly appreciated. *C. vendae* was slightly liked by the panellists. Forty percent of the participants appreciated the appearance, taste and texture while the aroma was liked very much. The *C. zeyherii* juice was neither liked nor disliked by 50% of the participants as far as taste, texture, aroma and overall acceptability were concerned. There was a noticeable change in trends with the juices that were infused with the ashes from the ones treated with leaves. The *C. adenogonium* juice was neither liked nor disliked by 50% of the participants as far as taste, texture, aroma and overall acceptability were concerned. With that said, 40% of the panellists slightly appreciated the product. The *C. apiculatum* juice was well received. 50% of the participants moderately liked the taste and appearance while overall acceptability was liked slightly. *C. bracteosum* was neither liked nor disliked by a majority of the panel. Taste, texture, aroma and overall acceptability was rated in the neutral score, however, a few of the panellists appreciated the juice *C. caffrum* was liked more than any other juice. 70% of the panellists scored it in the 'like extremely' rank as far as appearance and taste were concerned; however, texture was not well received. Aroma and overall acceptability was scored by the majority in the 'like very much' and 'like moderately' scores, respectively. *C. elaegnoides* was also appreciated. 50% of the panellists scored appearance, taste and texture in the 'like extremely' rank while 40% scored overall acceptability as well. *C erythrophyllum* and *C. imberbe* were found to have a similar trend; they were both well received. 40% of the panellists scored appearance in the 'like extremely' rank while taste, aroma texture and overall acceptability were liked very much. A few panellists neither liked nor disliked the juice. The appearance and taste of *C krauss*i was 'liked moderately' by 60% of the panel while the rest of the panel did not like the aroma and taste, which affected the overall acceptability of the juice. *C. mkusense* and C. *pardoides h*ad similar properties; a few panellists slightly liked it while others did not like it. *C. vendae* was fairly appreciated. Thirty five percent of the panellists scored the following organoleptic parameters (appearance, taste, texture and overall acceptability) in the 'like extremely' rank while 30% scored it in the 'like very much' rank. *C zeyherri* was fairly appreciated. 60% scored it in the 'like moderately' while 40% scored it in the 'like slightly' in the majority of organoleptic properties. Summing up the evaluation, the juices that were liked the most as far as taste was concerned, were *C bracteosum>C. caffrum>C. mkusenze*

{p=0001(Appendix X)} with *C. apiculatum* being liked the least. As far as texture was concerned, the juice that was liked the most was *C. caffrum> C. bracteosum* > with *C. pardoides* being liked the least. For aroma and appearance, *C. caffrum >C. erythrophyllum > C. bracteosum>* were liked the most compared to rest of the juices. The overall acceptability was rated in the C *erythrophyllum, C. caffrum* (Ashes) with *C. elaegnoides* receiving the lowest score ({p=0001(Appendix X)}. Overall, an addition of the ashes had better sensory and organoleptic properties than an addition of the leaves. The juice containing ashes of *C. caffrum* and *C. bractesuom* was well received by majority of the panellists compared to the rest of the additives. On the other hand, sensory profiling has received some criticism. Some authors suggest that the grouping of individual sensory elements (sensory attributes) does not necessarily convey what is really being perceived, that is to say, that words cannot clearly describe what is being felt (Chauhan and Harper,1986). In order to avoid this negative point, other techniques such as DSM (Carroll and Chang, 1970) and hedonic measurements (Barcenas *et al.,* 1998; Barcenas *et al.,* 2000 are recommended. A number of studies dealing with different sensory comparison methodologies have pointed out that a multidimensional map sample score correlation analysis can be considered a useful tool in this approach (Heymann, 1994; Gilbert and Heymann, 1995; Risvik *et al.,* 1997).

Conclusion: Addition of the *Combretum* ashes to the prepared juices better improved the organoleptic properties such as taste, tecture, smell and appearance compared to the addition of powdered leaves. This explains why wood ash is frequently used in the preparation of traditional sorghum juice in many African countries.

8.5. References

Barcenas, P., Perez Elortondo, F. J., and Albisu, M., 2000. Selection and screening of a descriptive panel for ewe's milk cheese sensory profiling. *Journal of Sensory Studies* 15: 79–99.

Barcenas, P., Perez Elortondo, F.J., Salmeron, J., and Albisu, M., 1998. Recalled preference of Spanish consumers for smoked food. *Nutrition and Food Science* 6,338–342.

Carroll, J.D., and Chang, J.J., 1970. Analysis of individual differences in multidimensional scaling via an N-way generalisation of 'EckartYoung' decomposition. *Psychometrika* 35: 283–319.

Chauhan, J., and Harper, R., 1986. Descriptive profiling versus direct similarity assessments of soft drinks. *Journal of Food Technology* 21: 175–187.

Claassen, M.R., and Lawless, H.T., 1992. A comparison of descriptive terminology systems for the sensory analysis of flavour defects in milk. *Journal of Food Science* 57: 596-621. http://dx.doi.org/10.1111/j.1365-2621.1992.tb08051.

Gilbert, J.M., and Heymann, H. 1995. Comparison of four sensory methodologies as alternatives to descriptive analysis for the evaluation of apple essence aroma. *The Food Technologist (NZIFST)* 24: 28–32.

Harris, G.K., and Marshall, M.R., 2017. Ash analysis. In: Nielsen, S.S. (ed.), *Food analysis*. 5th edition. Springer, New York.

Heymann, H., 1994. A comparison of free choice profiling and multidimensional scaling of vanilla samples. J*ournal of Sensory Studies* 9: 445–453

Ikeda, M., 2003. Amino acid production processes. *Advances in Biochemical Engineering/Biotechnology* 79: 1-35.

Insel, P.M., Ross, D., and McMahon, K., 2012. Nutrition: myplate update. Jones and Bartlett Publishers, Massachusetts,

ISO 1036. 1994. Sensory analysis. Methodology. Texture profile. International Organization for Standardization, Geneva, Switzerland.

ISO 4120 .1983. Sensory analysis. Methodology. Triangular test. International Organization for Standardization, Geneva, Switzerland.

ISO 5495. 1983. Sensory analysis. Methodology. Paired comparison test. International Organization for Standardization, Geneva, Switzerland.

ISO 6564. 1985. Sensory analysis. Methodology. Flavour profile methods. International Organization for Standardization, Geneva, Switzerland.

ISO 6658 .1985. Sensory analysis. Methodology. General guidance. International Organization for Standardization, Geneva, Switzerland.

ISO 8586. 1993. Sensory analysis. General guidance for the selection, training and monitoring of assessors. Part 1: Selected assessors. International Organization for Standardization, Geneva, Switzerland.

ISO. 10399. 1991. Sensory analysis. Methodology. Duo-trio test. International Organization for Standardization, Gene`ve, Switzerland.

Lawless, H. T., and Heymann, H., 1998. Sensory evaluation of food: principles and practices. New York: Chapman and Hall.

Lawless, H.T., and Heymann, H., 2010. Sensory evaluation of food – principles and practices. 2nd edition. Springer, New York. p.587.

Risvik, E., McEwan. A., and Rodbotten, M., 1997. Evaluation of sensory profiling and projective mapping data. *Food Quality and Preference* 8: 63–71.

Saupi, N., Zakaria, M.H., and Bujang, J.S., 2009. Analytic chemical composition and mineral content of yellow velvet leaf (*Limnocharis flava L. Buchenau*)'s edible parts. *Journal of Applied Science* 9 (16): 2969e2974.

Sinha, B.K., Bhattacharjee, S., and Tapan, S., 2019. Nutritional composition, mineral content, antioxidant activity and quantitative estimation of water-soluble vitamins and phenolics by RP-HPLC in some lesser-used wild edible plants. Heliyon e01431.

Sidel, J.L. and Stone, H., 1993. The role of sensory evaluation in the food industry. Food Quality and Preference, 4(1-2), pp.65-73

CHAPTER 9: GENERAL DISCUSSION

9.1 Discussion

Food spoilage due to microbial contamination is a major problem for consumers, industries and regulatory agencies (Penha *et al.,* 2017). To ensure microbial elimination and safety, the industry relies on the use of chemical preservatives, pasteurisation, and other methods such as dehydration, irradiation, and high hydrostatic pressure (Gonzalez and Barret, 2010). Most traditional food preservation methods depend on the application of high temperature and pressure. The mild heat processing and modified-atmosphere packaging methods, adopted for preserving food products, are not sufficiently reliable for effective control of spoilage by pathogenic microorganisms. Moreover, low temperature storage of perishable foods cannot assure the safety and quality of food products (Negi, 2012; Tajkarimi *et al.,* 2010), and it involves a lot of energy. Certainly, chemical preservatives alone cannot destroy all food pathogens or restrict microbial spoilage, which is why they are increasingly being frowned upon by (at least some) consumers. Many consumers prefer to avoid chemical preservatives and are concerned about negative side effects. Chemical preservatives like nitrates, benzoates, sulphites, sorbates, parabens, formaldehyde, butylated hydroxytoluene or – hydroxyanisole can cause serious health hazards such as hypersensitivity, allergy, asthma, hyperactivity, neurological damage or cancer (Anand and Sati, 2013). Therefore, serious attention should be devoted to the safety of using chemical preservatives in the food industry (Shakiba *et al.,* 2011; Tajkarimi *et al.,* 2010). The elimination of microbes from food without compromising the desirable properties of the product is still a challenge for the food industry. Studies on controlling the spoilage and increasing the shelf life as well as the quality of food products reveals the need for specific measures and technologies at each production step. Natural plant antimicrobials such as plant extracts, essential oils and organic acids may offer considerable advantages as potential substitutes to safeguard food safety (Hsouna *et al.,* 2011; Negi, 2012; Takahashi *et al.,* 2013). For centuries, based on ethno medicinal knowledge, edible plant extracts and essential oils have been traditionally used as folk medicine as additives in food to extend the shelf life of products (Al-zoreky and Al-Taher, 2015). Moreover, the lower price of herbal medicine compared to conventional pharmaceuticals and some synthetic chemicals, encourages their cost-effective use for large-scale food preparation, which ultimately also benefits consumers (Shakiba *et al.,* 2011). The major advantages of using natural plant-based preservatives over synthetic chemicals in food is to control microbial contamination, growth, eliminating food pathogens and to extend shelf life, acting as better antioxidants

to inhibit oxidation and strengthening the immune system of the consumer by increasing immunoglobulin synthesis (Kim *et al.,* 2013; Tajkarimi *et al.,* 2010). In this study, we tested twelve different *Combretum* plants (using different plant parts, i.e. leaves, stems and ashes) with the aim of improving the microbiological quality and shelf life of the traditionally produced sorghum juice. The findings of the current study could pave way for further detailed studies on traditional medicinal plants as edible resources that are suitable as natural preservatives for the food-processing industry. Furthermore, we tested the plant extracts for their antimicrobial activity against four food pathogens, antioxidant activity, phytoconstituents and nutritional composition. We also briefly considered their broader potenti al as anticancer therapeutics. All the plants (*C. caffrum, C. vendae, C. erythrophyllum, C. elaegnoides, C. apiculatum, C. imberbe, C. adenogdium, C. padoides, C. bracteosum, C. kraussii, C. mkuzens*e and *C. zeyherii)* investigated are traditionally used for medicinal purposes in South Africa and other African countries, with the majority of the plants being indigenous to South Africa. Phytochemicals such as saponins, tannins, terpenoids, steroids, cardiac glycosides and flavonoids were found in all the leaves and stems tested in the study. Phytochemicals such as saponins, tannins, terpenoids, steroids, cardiac glycosides and flavonoids were found in all the leaves tested in this study. The ashes had a different profile of those phytoconstituents in that, when they were compared to the stems and leaves, the following phytoconstituents were lost; tannins, with the exception of *C. mkuzense* and *C. padoides*; cardiac glycosides and flavonoids. These phytoconstituents may be sensitive to high temperature. The quantitative phytochemical analyses revealed that both the leaves, stems and some ashes such as *C. apiculatum* and *C. vendae* contained appreciable levels of phenolic compounds, tannins and flavonoids. These secondary metabolites detected in this study have been associated with antimicrobial activities and numerous physiological activities in mammalian cells in various studies (Sofowora, 1993; Abo *et al.,* 1999; Nweze *et al.,* 2004; Mishra *et al*., 2015). Phenolic compounds, the most abundant and largest groups of secondary metabolites (Singh *et al.*, 2007), possess antimicrobial activity against microorganisms *in vitro*; due to their ability to compete with the soluble and extracellular protein, and their ability to compete with the cell wall of bacteria (Marjorie, 1996). Flavonoids were reported to inhibit bacterial virulence factors, for instance, haemolysis activity of *S. aureus* (Qiu *et al.*, 2010). Other compounds with antibacterial activity include steroids (Raquel, 2007) and alkaloids (Okwu and Okwu, 2004). Generally, the leaves showed higher concentrations of the phytoconstituents when compared to the stems. The results in the current study revealed that the leaves of *C. apiculatum* could be a good

source of phenolic compounds when compared to other plant leaves in the study. These results are in line with those of Masoko *et al. (*2007) who investigated the qualitative antioxidant activity and phytochemical properties of 30 members of the *Combretaceae*. A study by Aderogba *et al.* (2012) also supports these findings. Qualitative and quantitative analyses of phytochemicals help in the understanding the diverse variety of compounds that are produced by plants, and help in extracting, identifying and purifying the bioactive compounds for their medicinal values (Santhi and Sengottuvel, 2016). The results presented in this study have established systematic scientific evidence of the phytochemical constituents of the 12 *Combretum* traditional medicinal plants. These plants contain compounds such as tannins, flavonoids and alkaloids that are of pharmacological importance. Plant phytochemicals have natural defence bioactive constituents that can be used to treat diseases caused by pathogenic species of bacteria. Antioxidant activities of many plants are of great interest in the food, cosmetics and pharmaceutical industries, since their possible use as natural additives emerged from a growing tendency to replace synthetic preservatives with natural ones. The antioxidant activities of the different extracts were evaluated by measuring the scavenging activity of these extracts toward the stable 2,2-diphenyl-1-picrylhydrazyl-hydrate (DPPH) free radicals. Among the plants examined, the leaves of *C. kraussii* and *C. mkuzense* exerted the best antioxidative activities. Statistical analysis revealed the highest antiradical power for *C. mkuzense* at concentrations of 0.125, 0.25, 0.0625 and 0.5 mg/ml; *C. zeyherii*; 0.5 and 1mg/ml; *C. kraussii*; 0.25 and 0.125 mg/ml and *C. padoides* at 1 mg/ml. The overall results obtained from this study indicate that the majority of the plants species investigated have the potential to be used as antioxidants. These findings are consistent with those found in the literature, since plants are well known for their potent antioxidant activities (Maxwell, 1995; Ruberto *et al.,* 2000). Plant phytochemicals are natural defence bioactive constituent that can be used for treating diseases caused by pathogenic species of bacteria. Therefore, further exploration and investigation was done to validate the traditional medicinal value claimed for these plants. Trace elements, which are also known as trace minerals, are dietary minerals that are useful for proper growth, development, maintaining and recovering the health of the organism (Aliasgharpour and Marjan, 2013). Most of the plants had appreciable levels of trace elements such as Ca, Co, Cu, Fe, K, Mg, Mn and Ni. These trace elements control important biological processes through such actions as facilitating the binding of molecules to receptor sites on cell membranes, altering the structure or ionic nature of membranes to prevent or allow specific molecules to enter or leave a cell, and inducing gene expression resulting

in the formation of proteins involved in life processes. It was interesting to observe that the concentration of calcium in all the ashes increased significantly, approximately four times more than the leaves and stems. Based on this findings, it can be concluded that the leaves and ashes of *C. adenogonium* and *C. apiculatum* could provide a good source of Ca to the human diet. Calcium is important for blood coagulation and the normal functioning of the cardiac muscles (Sundriyal and Sundriyal, 2004). It also noteworthy to highlight that all the *C. zeyherii* minerals increased drastically. These minerals are necessary for the human body to maintain good health and the mineral elements affect biochemical processes and play crucial roles in living organisms, specifically the biological, metabolic and enzymatic reactions leading to the development of active organic components (Serfor-Armah *et al.,* 2002). Based on the findings in thiistudy, it can be established that these plants can be exploited as a source of natural nutrients and minerals. The minimal inhibitory concentration (MIC) of the *Combretum* leaves, stems and their ash extracts were determined in order to assess their antimicrobial activity. The leaves tested in the study showed great antibacterial properties with the lowest MIC value being 0.04 mg/ml against *E. coli* and *S. aureus. E. faecalis* was found to be resistant against all the leaves with the exception of *C. Imberbe.* Majority of the stems tested in the study showed antimicrobial properties with the lowest MIC value being 0.04 mg/ml against *E coli.* However, *E. faecalis* showed resistance against all the 12 plants tested. It was interesting to observe that the all test microorganisms showed resistance to the ash extracts, with the exception of *S. aureus*, which was found to be susceptible to 75% of the test ashes with MIC value of 0.16 mg/ml. The activity may be due to the presence of several classes of secondary metabolites, including triterpenoids, flavonoids, tannins and lignans, (Lima De Morais *et al.,* 2012; Zhang *et al.,* 2019). The same compounds were isolated from different species of the genus *Combretum*. For example, a series of unique stilbenes (combretstatins) were isolated from *C. kraussii, C. caffrum,* and *C. erythrophyllum* (Pettit *et al.,* 1987; Rogers and Verotta, 1996; Brookes *et al.,* 1999; Schwikkard *et al.,* 2000; Eloff *et al.,* 2005; Famakin *et al.,* 2005). Several pharmacological activities of *Combretum* species and some of the isolated compounds have been reported from South Africa, Democratic Republic of Congo and Burkina Faso (Martini and Eloff, 1998; Eloff, 1999; McGaw *et al.,* 2001; Atindehou *et al.,* 2004; Masoko and Eloff, 2005; Eloff and McGaw, 2006; Manga *et al*., 2012). Overall, majority of the plant extracts (leaves, stems and ashes) were found to be active against *E. coli and S. aureus. C. bracteosum* was found to be the most active extract against *E. coli* and *S. aureus* while *C. vendae* was active against *P. aeruginosa*. Several workers investigated the efficiency

of plant extracts and their effective compounds as antimicrobial agents to control the growth of food borne and spoilage bacteria. They suggested that antimicrobial components of the plant extracts (terpenoids, alkaloids and phenolic compounds) interact with enzymes and proteins of the microbial cell membrane causing its disruption to disperse a flux of protons towards cell exterior, which induces cell death or may inhibit enzymes necessary for amino acids biosynthesis (Burt, 2004; Gill and Holley, 2006). The present study suggested that plant extracts, which proved to be potentially effective, could be used as natural preservatives to control food poisoning diseases and preserve food, avoiding application of health hazards of chemical preservatives. Most traditional food preservation methods depend on the application of high temperature and pressure. The mild heat processing and modified-atmosphere packaging, adopted for preserving food products, are not sufficiently reliable for effective control of spoilage by pathogenic microorganisms. Chemical preservatives alone cannot destroy all food pathogens or restrict microbial spoilage and are increasingly frowned upon by (at least some) consumers. Many consumers prefer to avoid chemical preservatives and are concerned about negative side effects. The current study further assessed the effect of the addition of ashes and leaves on the microbiological quality and shelf life of sorghum juice. As the storage time of the juice increased, the bacterial counts also increased, ranging from 400 000-600 000 CFU/ml for all the samples. It was interesting to see that when the juice was treated with the leaves of *C kraussii*, the CFU was exceptionally low (>40 000 CFU/ml) throughout the storage time. Their presence in high numbers (>105 per gram) in ready-to-eat foods indicates that an unacceptable level of contamination has occurred or there has been under processing. The juice treated with the leaves of *C. kraussii* was found to be within the permissible limits. Total coliforms detected in the juices increased with storage time, with CFU's ranging from 0- 40 000 CFU/ml for leaves and 45 000-60 000 CFU/ml. The leaves of *C. apiculatum, C. bracteosum, C. kraussii and C. vendae* were able to inhibit the coliforms in the first week one of treatment; however, as the storage time increased, coliforms also increased. *S. aureus* was typically detected in low amounts for both the ashes and leaves. The leaves of *C. caffrum, C. elaegnoides, C. erythrophyllum, C. imberbe, C. kraussii C. mkuzense and C. padoides* inhibited the growth *of B. cereus* in the first three weeks of treatment. The following ashes; *C. elaegnoides, C. erythrophyllum, C. kraussii and C. padoides* were effective in reducing the levels of *B. cereus* when compared with the untreated; however, when the storage time increased, the bacterial count also increased. The ability of the *C. caffrum, C. elaegnoides, C. erythrophyllum, C. imberbe, C. kraussii C. mkuzense* and *C. padoides*

plants to inhibit the growth of *B. cereus* is great, as this will help alleviate some of the health issues associated with presence of this microorganism. Vitek 2 Compact was used for the characterisation and identification of the dominant bacterial isolates using biochemical reactions. *Enterobacter* spp. were predominant in the juice treated with ashes. Additions of the leaves and ashes of *Combretum* was able to enhance the shelf life of the sorghum juice by reducing *S. aureus, B. cereus* and lactic acid bacteria. In South Africa, approximately 27 million individuals rely on traditional medicine as their primary source of health care (Mander, 1998). A few ethnobotanical studies in South Africa have been reported which focus specifically on plants traditionally used for the treatment of cancer (Coopoosamy and Naidoo, 2012; Koduru *et al*., 2007; Thring and Weitz, 2006). In the present study, 50 % of the acetone leaf extracts of *Combretum* plants showed cytotoxicity and cell proliferation inhibition in lung carcinoma cells A549 in a dose-dependent manner (MTT assay). *C. apiculatum, C. bracteseum, C. caffrum*, *C. padoides*, C. *mkuzense* and *C. zeyherii* exhibited a noticeable cytotoxic effect on the A549 cells. The present study showed that A549 cells were more sensitive to the *C. elaegnoides, C. erythrophyllum, C. imberbe, C. kraussii* and *C. mkuzense* since a higher anticancer activity was exhibited at the lowest concentration of the plant extracts, with the cell viability that is lower than 15%. The anticancer activity of these plants against lung cancer cell line A549 might be due to the presence of the remarkable antioxidant components and phytoconstituents such as phenols, flavonoids and tannins. Most of the plants had good activity at concentrations between 31.25 and1000 µg/ml with only 20 and 50 %, respectively, of viable cells. It was interesting to observe that although the ashes lost most of the phytoconstituents that are mainly attributed to anti-cancer activity, majority of the ashes had great activity. This may be due to an increase in the concentration of flavonoids for *C. erythrophyllum, C. mkuzense* and *C. vendae*. Flavonoids have the potential health benefits arising from the antioxidant activities of polyphenolic compounds. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions (Kumar *et al.,* 2013; Kumar and Padey, 2013). As already highlighted above, flavonoids have anticancer activity in inhibiting cell proliferation and angiogenesis through their effect on signal transduction (Islam *et al.*, 2013). Other reasons for ashes to still retain great anticancer activity may be due to increase in the concentration of minerals such as calcium, potassium, sodium, iron and their protein content. According to Shirwaikar *et al.* (2004), minerals such as calcium, copper, manganese and zinc are well-known antioxidants that have anticancer activity. The leaves and stems of *C. imberbe, C. kraussii* and *C. mkuzense* together with

the ashes of C. *mkuzense* could serve as a potential source of alternative therapeutic agents for treating lung cancer. Further studies are required to isolate the active compound(s) in the plants The present investigation revealed that the 12 *Combretum* plants studied could act as a potential alternative remedy for lung cancer. The 70% aqueous extracts of *the Combretum* spp. have potential anti-cancer activity. The study also revealed that addition of the *Combretum* ashes to the prepared juices better improved the organoleptic properties such as taste, tecture, smell and appearance of the sorghum juices when compared with that of the powdered leaves.

Conclusion: The results of the present study demonstrated that leaves and stems of *Combretum* plants and extracts ashes possess good antioxidant and free radical scavenging activities. The 70% acetone extracts possessed many functional phytoconstituents. Furthermore, *Combretum* extracts also exhibited appreciable antimicrobial and great anticancer activity. The use of these plants and bioactive components from indigenous resources and their utilisation as potential natural food preservatives could be of economic value. However, further investigations involving more detailed *in vitro* and *in vivo* studies to establish which components of the plants or extracts offer the best antioxidant, antimicrobial and anticancer activity are recommended. Additions of these plants showed to have a positive effect on the microbiological, nutritional and sensory properties of the juice. Overall, this study presents valuable information on the phytochemical composition, nutritional composition and antioxidant attributes of the *Combretum* plants in South Africa and advocates their use as food and pharmaceutical preparations for the local industries. In addition, *Combretum* plants showing antioxidant activity and anticancer might be explored for functional food and nutraceutical applications, besides their traditional uses.

Recommendations: Several plants are currently being investigated to for their antimicrobial and medicinal properties. The present study revealed that the selected plants, including ashes, contained appreciable amounts of nutrients, mineral elements and phytochemical constituents that are supported by their antimicrobial activity [minimum inhibitory concentration (MIC)], anticancer and shelf-life enhancing activity. Thus, the outcome of this work supports the use of these plants as medicine, as was the case in ancient medicinal traditions as well as the traditional usage of the studied plants. It is very clear that these extracts possess compounds with antimicrobial properties that can be used as antimicrobial agents in new drugs for the therapy against pathogenic microorganisms. It is, therefore, recommended that:

- The most active extracts of these plants should be subjected for further analysis to isolate and characterise the active compound(s)for possible development into therapeutic agents.
- The potentially useful active phytochemicals isolated from the plants should be synthesised chemically to enhance the sustainable use of the agents.

9.2 References

Abo, K.A., Ogunleye V.O., and Ashidi, J.S., 1999. Antimicrobial potential of *Spondiasmonbin, croton zambesicus and zygotritonia crocea*. *Phyto-therapy Research* 13: 494-497.

Aderogba, M.A., Kgatle, D.T., McGaw, L.J., Eloff, J.N., 2012. Isolation of antioxidant constituents from *Combretum apiculatum subsp. apiculatum*. *South African Journal of Botany* 79(1): 125-131.

Aliasgharpour and Marjan, 2013.???

Al-zoreky, N.S., and Al-Taher, A.Y. 2015. Antibacterial activity of spathe from Phoenix dactylifera L. against some food-borne pathogens. *Industrial Crops and Products* 65: 241–246. https://doi.org/10.1016/j.indcrop.2014.12.014.

Anand, S.P., and Sati, N., 2013. Artificial preservatives and their harmful effects: looking toward nature for safer alternatives. *International Journal of Pharmaceutical Sciences and Research* 4(7): 2496–2501. https://doi.org/10.13040/IJPSR.0975-8232.4(7). 24960-01.

Atindehou, K.K., Schmid, C., Brun, R., Koné, M.W., and Traore, D. 2004. Antitrypanosomal anti-plasmodial activity of medicinal plants from Côte d'Ivoire. *Journal of Ethno Pharmacology* 90: 221–227.

Brookes, K.B., Doudoukina, O.V., Katsoulis, L.C., Veale, D.J.H., 1999. Uteroactive constituents from Combretum kraussii. *South Africa Journal of Chemistry* 52: 127–132

Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods--a review. *International Journal of Food Microbiology* 94(3): 223-253.

Coopoosamy, R.M., and Naidoo, K.K., 2012. An ethnobotanical study of medicinal plants used by traditional healers in Durban, South Africa. *African Journal of Pharmacy and Pharmacology* 6: 818–823. https://doi.org/10.5897/AJPP11.700.

Eloff, J.N., 1999. It is possible to use herbarium specimens to screen for antibacterial components in some plants. *Journal of Ethnopharmacology* 67: 355–360.

Eloff, J.N., Famakin, J.O., and Katerere, D.R.P., 2005b. Isolation of an antibacterial compounds from *Combretum erythrophyllum* (Combretaceae). *Journal of Ethnopharmacology* 62(3): 255 -263.

Eloff, J.N., and McGaw, L.J., 2006. Plant extracts use to manage bacterial, fungal and parasitic infection in South Africa. In: Ahmad, 1, Aqil, F., Owais, M. (Eds.), *Modern phytomedicine*. Wiley- VCH Verlag GmbH and Co., KGaA, 71–121.

Famakin, J.O. and Katerere, D.R.P., 2005. Isolation of an antibacterial stilbene from Combretum woodii (Combretaceae) leaves. African Journal of Biotechnology, 4(10).

Gill, A.O., and Holley, R.A., 2006. Disruption of Escherichia coli, Listeria monocytogenes and Lactobacillus sakei cellular membranes by plant oil aromatics. International Journal of Food Microbiology 108: 1–9.

Gonzalez, M., and Barret, D.M., 2010. Thermal, high pressure and electric field processing effects on plant cell membrane integrity and relevance to fruit and vegetable quality. *Journal of Food Science* 75: 121–130. https://doi.org/10.1111/j.1750-3841.2010. 01763.

Kim, S., Cho, A., and Han, J. 2013. Antioxidant and antimicrobial activity of leafy green vegetable extracts and their applications to meat product preservation. *Food Control* 29: 112–120. https://doi.org/10.1016/j.foodcont.2012.05.060.

Koduru, S., Grierson, D.S., and Afolayan, A.J., 2007. Ethnobotanical information of medicinal plants used for treatment of cancer in the Eastern Cape Province, South Africa. *Current Science* 92: 906–908.

Kumar, A., Kumar, P. and Nadendla, R., 2013. A review on: Abelmoschus esculentus (Okra). International Research Journal of Pharmaceutical and Applied Sciences, 3(4), pp.129-132.

Kumar, S. and Pandey, A.K., 2013. Chemistry and biological activities of flavonoids: an overview. The scientific world journal, 2013.

Kumar, D., Sukapaka, M., Babu, G.D., and Padwad, Y., 2015. Chemical composition and in vitro cytotoxicity of essential oils from leaves and flowers of Callistemon citrinus from WesternHimalayas. PLoSOne10,e0133823.

Lima De Morais, G.R., De Sales, I.R.P., Filho, M.R.D.C., De Jesus, N.Z.T., De Sousa Falcao, H., Barbosa-Filho, J.M., Cabral, A.G.S., Souto, A.L., Tavares, J.F., and Batista, L.M., 2012. Bioactivities of the genus Combretum (Combretaceae): a review. Molecules 17. https://doi.org/10.3390/molecules17089142, 9142–7206

Mander, M., 1998. Marketing of indigenous medicinal plants in South Africa – A case in KwaZulu-Natal. Report, FAO.

Manga, F.N., Khattabi, C.E.I., Fontaine, J., Berkenboom, G., Duez, P., Nzunzu, J.L., Pochet, S., 2012. Vascular effects and antioxidant activity of two Combretum species from Democratic Republic of Congo. *Journal of Ethno Pharmacology* 142: 194–200.

Marjorie, C., 1996. Plant products as antimicrobial agents. *Clinical Microbiology Review* 1996; 12: 564-582.

Martini, N., and Eloff, J.N., 1998. The preliminary isolation and of several antibacterial compounds from Combretum erythrophyllum (Combretaceae). *Journal of Ethno Pharmacology* 62: 255–263.

Masoko, P., and Eloff J.N., 2005. The diversity of antifungal compounds in six South African *Terminalia* species (Combretaceae) determined by bio-autography. *African Journal of Biotechnology* 14: 1425–1431.

Masoko, P., Picard, J., and Eloff, J.N., 2007. The antifungal activity of twenty-four southern African *Combretum* species (*Combretaceae)*. *South African Journal of Botany* 73: 173–183.

Maxwell, S.R.J. 1995. Prospects for the use of antioxidant therapies. *Drugs*, 49:3, 345-361.

McGaw, L.J., Rabe, T., Sparg, S.G., Jäger, A.K., Eloff, J.N., Van Staden, J., 2001. An investigation of the biological activity of Combretum species. *Journal of Ethno Pharmacology* 75: 45–50.

Mishra, M.P., Rath, S, Swain, S.S., Ghosh, G., Das, D., and Padhy R, N., 2009. In vitro antibacterial activity of crude extracts of selected medicinal plants against UTI causing MDR bacteria. *Journal of King Saud University – Science*. http://dx.doi.org/10.1016/j.jksus.2015.05.007. 29: 84-95.

Negi, P. 2012. Plant extracts for the control of bacterial growth: efficacy stability and safety issues for food application. *International Journal of Food Microbiology* 156: 7–17. https://doi.org/10.1016/j.ijfoodmicro.2012.03.006.

Nweze, E.I., Okafor, J.I. and Njoku, O., 2004. Antimicrobial activities of methanolic extracts of Trema guineensis (Schumm and Thorn) and Morinda lucida benth used in Nigerian. Bio-research, 2(1), pp.39-46.

Okwu, D.E., and Okwu M.E., 2004. Chemical composition of Spondias mombin linn. Plant parts. *Journal of Sustainability. Agricultural Environment* 6(2): 140-147.

Penha, C. B., Bonin, E., da Silva, A.F., Hioka, N., Zanqueta, É. B., and Nakamura, T.U. 2017. Photodynamic inactivation of foodborne and food spoilage bacteria by curcumin. *LWT-Food Science and Technology* 76: 198–202. https://doi.org/10.1016/j. lwt.2016.07.037.

Pettit, G.R., Smith, C.R., and Singh, S.B.1987. Recent advances in the chemistry of plant antineoplastic constituents. In: Hostettmann K, Lea PJ (eds.), *Biologically active natural products*. Proceedings of the Phytochemical Society of Europe. Oxford Science Publications, UK.

Qiu J., Jiang, Y., Xia, L., Xiang, H., Feng, H., Pu, S., and Deng, X. 2010. Sub inhibitory concentrations of licochalcone A decrease alpha - toxin production in both methicillin - sensitive and methicillin - resistant *Staphylococcus aureus* isolates. *Applied Microbiology* 50(2): 223-229.

Raquel, F.E., 2007. Bacterial lipid composition and antimicrobial efficacy of cationic steroid compounds. *Biochemica et Biophysica Acta* 1768(10): 2500-2509.

Rogers, C.B., and Verotta, L., 1996. Chemistry and biological properties of the African Combretaceae. *Chemistry, Biological and Pharmacological Properties of African Medicinal Plants*: 121–141.

Ruberto, G., Baratta, M.T., Deans, S., and Dorman, H.J.D., 2000. Antioxidant and antimicrobial activity of *Foeniculum vulgare* and *Crithmum maritimum* essential oils. *Planta Medica* 66: 687-693.

Santhi, K., and Sengottuvel, R., 2016. Qualitative and quantitative phytochemical analysis of Moringa concanensis Nimmo. *International Journal of Current Microbiology and Applied Sciences* 5(1): 633-640.

Schwikkard, S., Zhou, B.N., Glass, T.E., Sharp, J.L., Mattern, M.R., Johnson, R.K., and Kingston, D.G.I., 2000. Bioactive compounds from *Combretum erythrophyllum. Journal of Natural Products* 63: 457–460.

Serfur-Armah et al., 2002.???

Shakiba, M., Kariminik, A., and Parsia, P., 2011. Antimicrobial activity of different parts of Phoenix dactylifera. *International Journal of Molecular and Clinical Microbiology* 1: 107–111. https://doi.org/10.4314/bajopas.v10i1.7S.

Shirwaikar, A., Rajendran, K. and Kumar, C.D., 2004. In vitro antioxidant studies of *Annona squamosa* Linn. Leaves. *Indian Journal of Experimental Biology* 42: 803-807.

Singh, R., Singh, S.K., and Arora, S., 2007. Evaluation of antioxidant potential of ethyl acetate extract/fractions of *Acacia auriculiformis* A. Cunn. *Food Chemistry Toxicology* 45: 1216-1223.

Sofowora, L.A., 1993. Medicinal plants and traditional medicine in Africa. Spectrum Books Ltd, Ibaban. 55–71.

Sundriyal, M., and Sundriyal, R.C., 2004. Wild edible plants of the Sikkim Himalaya: nutritive values of selected species. *The Society for Economic Botany* 58 (2): 286-299.

Tajkarimi, M.M., Ibrahim, S.A., and Cliver, D.O. 2010. Antimicrobial herb and spice compounds in food. *Food Control* 21(9): 1199–1218. https://doi.org/10.1016/j.foodcont.2010.02.003.

Takahashi et al., 2013.???

Thring, T.S.A., and Weitz, F.M., 2006. Medicinal plant use in the bredasdorp/elim region of the southern Overberg in the Western Cape province of South Africa. *Journal of Ethnopharmacology* 103: 261–275. https://doi.org/10.1016/j.jep.2005.08.013.

Zhang, X.R., Kaunda, J.S., Zhu, H.T., Wang, D., Yang, C.R., Zhang, Y.J., 2019. The Genus Terminalia (Combretaceae): an ethnopharmacological, phytochemical and pharmacological review, natural products and bioprospecting. Springer Singapore. https://doi.org/10.1007/s13659-019-00222-3

APPENDIX I: CHAPTER 3 STATS RESULTS

Statistix 10.0 Morongoa_Conc2020_In..., 7/22/2020, 2:27:03 PM

Factorial AOV Table for One

Source	DF	SS	MS	F	P
Rep	2	43.7	21.847		
Plant	11	8744.8	794.984	58.70	0.0000
PParts	1	517.3	517.347	38.20	0.0000
Plant*PParts	11	6859.2	623.559	46.04	0.0000
Error	46	623.0	13.543		
Total	71	16788.0			

Grand Mean 71.736
CV 5.13

Factorial AOV Table for half

Source	DF	SS	MS	F	P
Rep	2	36.8	18.39		
Plant	11	11883.5	1080.32	74.26	0.0000
PParts	1	55.1	55.13	3.79	0.0577
Plant*PParts	11	26298.7	2390.79	164.33	0.0000
Error	46	669.2	14.55		
Total	71	38943.3			

Grand Mean 63.597
CV 6.00

Factorial AOV Table for Qaurter

Source	DF	SS	MS	F	P
Rep	2	5.6	2.79		
Plant	11	32575.0	2961.36	192.29	0.0000
PParts	1	18.0	18.00	1.17	0.2853
Plant*PParts	11	22137.0	2012.45	130.68	0.0000
Error	46	708.4	15.40		
Total	71	55444.0			

Grand Mean 54.500
CV 7.20

Factorial AOV Table for QofQ

Source	DF	SS	MS	F	P
Rep	2	4.1	2.06		
Plant	11	30640.8	2785.53	299.92	0.0000

PParts	1	171.1	171.13	18.43	0.0001
Plant*PParts	11	24902.7	2263.88	243.76	0.0000
Error	46	427.2	9.29		
Total	71	56146.0			

Grand Mean	46.486
CV	6.56

Factorial AOV Table for QofQofQ

Source	**DF**	**SS**	**MS**	**F**	**P**
Rep	2	15.1	7.54		
Plant	11	23909.0	2173.55	272.99	0.0000
PParts	1	115.0	115.01	14.45	0.0004
Plant*PParts	11	24955.5	2268.68	284.94	0.0000
Error	46	366.3	7.96		
Total	71	49360.9			

Grand Mean	38.042
CV	7.42

Tukey HSD All-Pairwise Comparisons Test of One for Plant

Plant	Mean	Homogeneous Groups
zeyherii	*88.000*	*A*
imberbe	*84.833*	*AB*
bracteosum	*81.167*	*ABC*
erythrophyllum	*77.667*	*BCD*
vendae	*76.833*	*CDE*
pardoides	*75.333*	*CDEF*
elaegnoides	*72.167*	*DEF*
krausii	*69.833*	*EF*
Caffrum	*68.833*	*F*
adenogdnium	*59.333*	*G*
mkuzense	*56.667*	*GH*
apiculum	*50.167*	*H*

Alpha	0.05	Standard Error for Comparison	2.1247
Critical Q Value	4.865	Critical Value for Comparison	7.3084

There are 8 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of One for PParts

PParts	Mean	Homogeneous Groups
Stem	74.417	A
Leaves	69.056	B

Alpha	0.05	Standard Error for Comparison	0.8674
Critical Q Value	2.845	Critical Value for Comparison	1.7448

All 2 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of One for Plant*PParts

Plant	PParts	Mean	Homogeneous Groups
zeyherii	Leaves	93.000	A
bracteosum	Stem	91.333	AB
pardoides	Leaves	89.333	AB
imberbe	Leaves	86.667	ABC
krausii	Stem	85.333	ABC
imberbe	Stem	83.000	ABCD
zeyherii	Stem	83.000	ABCD
erythrophyllum	Leaves	82.667	ABCD
elaegnoides	Leaves	81.000	BCDE
vendae	Leaves	77.333	CDE
Caffrum	Stem	76.667	CDE
adenogdnium	Stem	76.333	CDE
vendae	Stem	76.333	CDE
erythrophyllum	Stem	72.667	DEF
bracteosum	Leaves	71.000	EFG

apiculum	Stem	64.333	FGH
elaegnoides	Stem	63.333	FGH
pardoides	Stem	61.333	FGH
Caffrum	Leaves	61.000	GH
mkuzense	Stem	59.333	H
krausii	Leaves	54.333	H
mkuzense	Leaves	54.000	H
adenogdnium	Leaves	42.333	I
apiculum	Leaves	36.000	I

Alpha	0.05	Standard Error for Comparison	3.0048
Critical Q Value	5.458	Critical Value for Comparison	11.597

There are 9 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of half for Plant

Plant	Mean	Homogeneous Groups
vendae	87.333	A
zeyherii	79.167	B
bracteosum	77.333	B
pardoides	67.333	C
imberbe	65.167	CD
mkuzense	65.000	CD
erythrophyllum	62.000	CD
Caffrum	61.833	CD
apiculum	58.833	DE
krausii	52.167	E
adenogdnium	44.167	F
elaegnoides	42.833	F

Alpha	0.05	Standard Error for Comparison	2.2021
Critical Q Value	4.865	Critical Value for Comparison	7.5749

There are 6 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of half for PParts

PParts	Mean	Homogeneous Groups
Leaves	64.472	A
Stem	62.722	A

Alpha	0.05	Standard Error for Comparison	0.8990
Critical Q Value	2.845	Critical Value for Comparison	1.8084

There are no significant pairwise differences among the means.

Tukey HSD All-Pairwise Comparisons Test of half for Plant*PParts

Plant	PParts	Mean	Homogeneous Groups
zeyherii	Leaves	94.000	A
mkuzense	Leaves	93.000	A
krausii	Stem	92.333	A

bracteosum	Stem	90.333	A
erythrophyllum	Leaves	88.000	A
pardoides	Leaves	87.333	A
vendae	Leaves	87.333	A
vendae	Stem	87.333	A
Caffrum	Leaves	68.667	B
imberbe	Stem	68.000	B
bracteosum	Leaves	64.333	BC
zeyherii	Stem	64.333	BC
imberbe	Leaves	62.333	BC
apiculum	Leaves	60.333	BC
elaegnoides	Stem	59.333	BCD
adenogdnium	Stem	58.333	BCD
apiculum	Stem	57.333	BCD
Caffrum	Stem	55.000	CD
pardoides	Stem	47.333	DE
mkuzense	Stem	37.000	EF
erythrophyllum	Stem	36.000	EF
adenogdnium	Leaves	30.000	F
elaegnoides	Leaves	26.333	F
krausii	Leaves	12.000	G

Alpha	0.05	Standard Error for Comparison	3.1143
Critical Q Value	5.458	Critical Value for Comparison	12.020

There are 7 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Qaurter for Plant

Plant	Mean	Homogeneous Groups
krausii	92.667	A
vendae	78.667	B
zeyherii	72.333	BC
mkuzense	68.500	CD
pardoides	61.333	DE
Caffrum	56.833	EF
apiculum	50.000	FG
bracteosum	49.500	FG
imberbe	44.167	G
elaegnoides	35.000	H
adenogdnium	33.500	H
erythrophyllum	11.500	I

Alpha	0.05	Standard Error for Comparison	2.2657
Critical Q Value	4.865	Critical Value for Comparison	7.7935

There are 9 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Qaurter for PParts

PParts	Mean	Homogeneous Groups
Leaves	55.000	A

Stem 54.000 A

Alpha	0.05	Standard Error for Comparison	0.9250
Critical Q Value	2.845	Critical Value for Comparison	1.8606

There are no significant pairwise differences among the means.

Tukey HSD All-Pairwise Comparisons Test of Qaurter for Plant*PParts

Plant	PParts	Mean	Homogeneous Groups
mkuzense	Leaves	99.000	A
zeyherii	Leaves	94.667	AB
krausii	Stem	93.000	AB
krausii	Leaves	92.333	AB
vendae	Stem	87.333	ABC
pardoides	Leaves	85.333	BC
Caffrum	Stem	76.667	CD
bracteosum	Stem	71.667	DE
vendae	Leaves	70.000	DE
apiculum	Leaves	64.333	DE
imberbe	Stem	61.333	EF
zeyherii	Stem	50.000	FG
adenogdnium	Stem	45.333	GH
elaegnoides	Stem	43.667	GH
mkuzense	Stem	38.000	GHI
pardoides	Stem	37.333	HI
Caffrum	Leaves	37.000	HI
apiculum	Stem	35.667	HI
bracteosum	Leaves	27.333	IJ
imberbe	Leaves	27.000	IJ
elaegnoides	Leaves	26.333	IJ
adenogdnium	Leaves	21.667	J
erythrophyllum	Leaves	15.000	JK
erythrophyllum	Stem	8.000	K

Alpha	0.05	Standard Error for Comparison	3.2042
Critical Q Value	5.458	Critical Value for Comparison	12.367

There are 11 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of QofQ for Plant

Plant	Mean	Homogeneous Groups
krausii	92.333	A
Caffrum	63.167	B
zeyherii	62.667	B
mkuzense	59.833	B
vendae	52.833	C
pardoides	52.333	C
imberbe	38.667	D
bracteosum	37.667	D
apiculum	30.500	E
adenogdnium	29.833	E

erythrophyllum	22.833	F
elaegnoides	15.167	G

Alpha	0.05	Standard Error for Comparison	1.7595
Critical Q Value	4.865	Critical Value for Comparison	6.0522

There are 7 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of QofQ for PParts

PParts	Mean	Homogeneous Groups
Leaves	48.028	A
Stem	44.944	B

Alpha	0.05	Standard Error for Comparison	0.7183
Critical Q Value	2.845	Critical Value for Comparison	1.4449

All 2 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of QofQ for Plant*PParts

Plant	PParts	Mean	Homogeneous Groups
mkuzense	Leaves	99.000	A
krausii	Stem	94.000	A
krausii	Leaves	90.667	A
zeyherii	Leaves	81.000	B
Caffrum	Stem	76.333	B
pardoides	Leaves	76.333	B
bracteosum	Stem	60.333	C
imberbe	Stem	60.333	C
vendae	Leaves	57.333	CD
Caffrum	Leaves	50.000	DE
vendae	Stem	48.333	DE
zeyherii	Stem	44.333	EF
erythrophyllum	Leaves	38.667	FG
adenogdnium	Stem	38.333	FG
apiculum	Stem	31.000	GH
elaegnoides	Stem	30.333	GHI
apiculum	Leaves	30.000	GHIJ
pardoides	Stem	28.333	HIJ
adenogdnium	Leaves	21.333	IJK
mkuzense	Stem	20.667	JK
imberbe	Leaves	17.000	K
bracteosum	Leaves	15.000	KL
erythrophyllum	Stem	7.0000	LM
elaegnoides	Leaves	0.0000	M

Alpha	0.05	Standard Error for Comparison	2.4883
Critical Q Value	5.458	Critical Value for Comparison	9.6041

There are 13 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of QofQofQ for Plant

Plant	Mean	Homogeneous Groups
krausii	70.167	A
mkuzense	65.333	A
Caffrum	49.000	B
bracteosum	48.667	B
vendae	41.500	C
zeyherii	40.167	CD
apiculum	35.500	DE
imberbe	33.167	E
pardoides	32.167	E
adenogdnium	20.000	F
elaegnoides	15.000	F
erythrophyllum	5.833	G

Alpha	0.05	Standard Error for Comparison	1.6291
Critical Q Value	4.865	Critical Value for Comparison	5.6037

There are 7 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of QofQofQ for PParts

PParts	Mean	Homogeneous Groups
Stem	39.306	A
Leaves	36.778	B

Alpha	0.05	Standard Error for Comparison	0.6651
Critical Q Value	2.845	Critical Value for Comparison	1.3378

All 2 means are significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of QofQofQ for Plant*PParts

Plant	PParts	Mean	Homogeneous Groups
mkuzense	Leaves	100.00	A
krausii	Leaves	81.000	B
Caffrum	Stem	79.667	B
bracteosum	Stem	76.333	B
krausii	Stem	59.333	C
vendae	Stem	51.667	CD
imberbe	Stem	50.333	D
zeyherii	Leaves	46.000	DE
pardoides	Leaves	40.667	EF
apiculum	Leaves	40.333	EF
adenogdnium	Leaves	40.000	EFG
zeyherii	Stem	34.333	FGH
vendae	Leaves	31.333	GHI
apiculum	Stem	30.667	HI
mkuzense	Stem	30.667	HI
elaegnoides	Stem	30.000	HI
pardoides	Stem	23.667	IJ
bracteosum	Leaves	21.000	J
Caffrum	Leaves	18.333	J

imberbe	Leaves	16.000	J
erythrophyllum	Leaves	6.6667	K
erythrophyllum	Stem	5.0000	K
adenogdnium	Stem	0.0000	K
elaegnoides	Leaves	0.0000	K

Alpha	0.05	Standard Error for Comparison	2.3039
Critical Q Value	5.458	Critical Value for Comparison	8.8924

There are 11 groups (A, B, etc.) in which the means

Conce1mg

Test for equal means			
	Sum of sqrs	df	Mean square
Between groups:	2247,3	11	204,3
Within groups:	28949,1	24	1206,21
Total:	31196,4	35	0,9979
Components of variance (only for random			
Var(group):	-333,971	Var(error):	1206,21
omega2:	0		
Levene´s test for	p (same):	0,5291	
Levene´s test, from medians	p (same):	0,9979	
Welch F test in the case of unequal variances: F=0.1419, df=9.315,			

	C. adenogdnium	apiculatum	bracteosum
C. adenogdnium		1	0,9992
C. apiculatum	0,3823		1
C. bracteosum	1,18	0,7979	
C. caffrum	0,6201	0,2377	0,5602
C. elaegnoides	0,3823	0	0,7979
C. erythrophyllum	0,5768	0,1945	0,6034
C. imberbe	1,33	0,9476	0,1496
C. krausii	0,6018	0,2194	0,5785
C. mkuzense	0,266	0,1164	0,9143
C. pardoides	1,047	0,6649	0,133
C. vendae	1,097	0,7148	0,08312
C. zeyherii	0,9143	0,532	0,266

Concentration0.5mg/ml

Test for equal means			
	Sum of sqrs	df	Mean square
Between groups:	3871,43	11	351,949
Within groups:	32725,8	24	1363,57

Total:	36597,2	35	0,987
Components of variance (only for random			
Var(group):	-337,209	Var(error):	1363,57
omega2:	0		
Levene´s test for	p (same):	0,7758	
Levene´s test, from medians	p (same):	0,9964	

are not significantly different from one another.

	C. adenogdnium	*C. caffrum*	*C. imberbe*
C. adenogdnium		1	0,9989
C. apiculatum	0,8287		1
C. bracteosum	1,22	0,3909	
C. caffrum	0,5441	0,2846	0,6754
C. elaegnoides	0,2033	1,032	1,423
C. erythrophyllum	0,3596	0,4691	0,8599
C. imberbe	0,7974	0,03127	0,4221

C. krausii	0,2251	0,6035	0,9944
C. mkuzense	0,6301	0,1986	0,5894
C. pardoides	1,063	0,2345	0,1564
C. vendae	1,548	0,7192	0,3283
C. zeyherii	0,8912	0,06254	0,3283

Concentration0.25mg/ml

Test for equal means			
	Sum of sqrs	df	Mean square
Between groups:	8337,64	11	757,967
Within groups:	29113,8	24	1213,07
Total:	37451,4	35	0,7888
Components of variance (only for random			
Var(group):	-151,702	Var(error):	1213,07
omega2:	0		
Levene´s test for	p (same):	0,2903	
Levene´s test, from medians	p (same):	0,9308	
Welch F test in the case of unequal variances: F=1.513, df=9.231,			

	C. adenogdnium	C.	C.
C. adenogdnium		1	0,9999
C. apiculatum	0,7957		1
C. bracteosum	0,9117	0,116	
C. caffrum	0,7625	0,03315	0,1492
C. elaegnoides	0,04973	0,8454	0,9614
C. erythrophyllum	0,7791	1,575	1,691
C. imberbe	1,011	0,2155	0,09946
C. krausii	1,986	1,19	1,074
C. mkuzense	1,094	0,2984	0,1823
C. pardoides	1,011	0,2155	0,09946

C. vendae	1,89	1,094	0,978
C. zeyherii	1,293	0,4973	0,3813

Concentration 0.125mg/ml

Test for equal means			
	Sum of sqrs	df	Mean square
Between groups:	6895,22	11	626,838
Within groups:	27471,3	24	1144,64
Total:	34366,6	35	0,8498
Components of variance (only for random			
Var(group):	-172,6	Var(error):	1144,64
omega2:	0		
Levene´s test for	p (same):	0,215	
Levene´s test, from medians	p (same):	0,9777	
Welch F test in the case of unequal variances: F=0.4465, df=9.421,			

	C. adenogdnium	*C. apiculatum*	*C. bracteosum*
C. adenogdnium		1	1
C. apiculatum	0,1365		1
C. bracteosum	0,6485	0,785	
C. caffrum	1,007	1,143	0,3584
C. elaegnoides	0,5631	0,4266	1,212
C. erythrophyllum	0,3584	0,2218	1,007
C. imberbe	1,007	1,143	0,3584
C. krausii	2,099	2,236	1,451
C. mkuzense	0,9215	1,058	0,273
C. pardoides	0,6655	0,8021	0,01706
C. vendae	0,7167	0,8532	0,06826
C. zeyherii	0,9898	1,126	0,3413

Concentration 0.0625 mg/ml

Test for equal means			
	Sum of sqrs	df	Mean square
Between groups:	6014,31	11	546,755
Within groups:	21440	24	893,333
Total:	27454,3	35	0,8021
Components of variance (only for random			
Var(group):	-115,526	Var(error):	893,333

omega2:	0		
Levene´s test for	p (same):	0,154	
Levene´s test, from medians	p (same):	0,913	
Welch F test in the case of unequal variances: F=1.215, df=8.956,			

	C. adenogdnium	apiculatum	bracteosum
C. adenogdnium		1	0,9999
C. apiculatum	0,5409		1
C. bracteosum	0,9465	0,4057	
C. caffrum	1,178	0,6375	0,2318
C. elaegnoides	0,2511	0,792	1,198
C. erythrophyllum	0,5602	1,101	1,507
C. imberbe	1,7	1,159	0,7534
C. krausii	1,874	1,333	0,9272
C. mkuzense	1,719	1,178	0,7727
C. pardoides	0,4636	0,07727	0,4829
C. vendae	0,7534	0,2125	0,1932
C. zeyherii	0,7727	0,2318	0,1739

F	p (same)
0,1694	0,998
Permutation p (n=99999)	
ICC:	-0,38289

C. caffrum	elaegno	erythro	imberbe	krausii	mkuzen	pardoid	vendae
1	1	1	0,9977	1	1	0,9997	0,9996
1	1	1	0,9999	1	1	1	1
1	1	1	1	1	0,9999	1	1
	1	1	1	1	1	1	1
0,2377		1	0,9999	1	1	1	1
0,04322	0,1945		1	1	1	1	1
0,7098	0,9476	0,7531		1	0,9997	1	1
0,01829	0,2194	0,02494	0,7281		1	1	1
0,3541	0,1164	0,3109	1,064	0,3358		1	1
0,4272	0,6649	0,4705	0,2826	0,4455	0,7813		1
0,4771	0,7148	0,5203	0,2327	0,4954	0,8312	0,04987	
0,2942	0,532	0,3375	0,4156	0,3125	0,6483	0,133	0,1829

F	p (same)
0,2581	0,9884
Permutation p (n=99999)	
ICC:	-0,32855

C. caffrum	adeadeelae	erythr	imberbe	krausii	mkuzen	pardoid	vendae
0,9992	1	1	0,9836	0,9671	0,9822	1	1
1	1	0,999	0,9993	0,9977	0,9992	1	1
1	0,9991	0,993	1	0,9999	1	1	1
	0,9958	0,980	1	1	1	1	1
1,429		1	0,9568	0,9258	0,9539	1	0,9998
1,739	0,3091		0,8936	0,8419	0,8884	0,9998	0,998
0,5216	1,951	2,26		1	1	0,9988	0,9999
0,6954	2,125	2,434	0,1739		1	0,9963	0,9995
0,5409	1,97	2,279	0,01932	0,1545		0,9986	0,9999
0,7147	0,7147	1,024	1,236	1,41	1,256		1
0,425	1,004	1,314	0,9465	1,12	0,9658	0,2898	
0,4057	1,024	1,333	0,9272	1,101	0,9465	0,3091	0,01932

C. caffrum	elaegno	erythro	imberbe	krausii	mkuzen	pardoid	vendae
1	1	1	1	1	1	0,9997	0,992
1	0,9998	1	1	1	1	1	1
1	0,996	1	1	0,9998	1	1	1
	1	1	1	1	1	1	0,9998
0,7474		1	0,9998	1	1	0,9985	0,9796
0,1845	0,5629		1	1	1	1	0,9992
0,2533	1,001	0,4378		1	1	1	1
0,319	0,4284	0,1345	0,5722		1	1	0,9978
0,08599	0,8333	0,2705	0,1673	0,4049		1	0,9999
0,5191	1,266	0,7036	0,2658	0,838	0,4331		1
1,004	1,751	1,188	0,7505	1,323	0,9178	0,4847	
0,3471	1,094	0,5316	0,09381	0,6661	0,2611	0,172	0,6567

F	p (same)
0,6248	0,7901
Permutation p	
ICC:	-0,14293

C. caffrum	C.	C.	C.	C.	C.	C.	C.
1	1	1	0,9998	0,9515	0,9996	0,9998	0,9651
1	1	0,9909	1	0,9992	1	1	0,9996
1	0,9999	0,9843	1	0,9997	1	1	0,9999
	1	0,9923	1	0,9989	1	1	0,9995
0,8123		1	0,9997	0,9431	0,9994	0,9997	0,9585
1,542	0,7294		0,9761	0,7163	0,9672	0,9761	0,7557
0,2486	1,061	1,79		0,9999	1	1	1
1,223	2,036	2,765	0,9747		0,9999	0,9999	1
0,3315	1,144	1,873	0,08288	0,8918		1	1
0,2486	1,061	1,79	0	0,9747	0,08288		1

1,127	1,939	2,669	0,8786	0,09614	0,7957	0,8786	
0,5305	1,343	2,072	0,2818	0,6929	0,1989	0,2818	0,5968

F	p (same)
0,5476	0,8508
Permutation p	
ICC:	-0,17757

C. caffrum	C.	C.	C.	C.	C.	C.	C.
0,9998	1	1	0,9998	0,9311	0,9999	1	1
0,9994	1	1	0,9994	0,9	0,9997	1	1
1	0,999	0,9998	1	0,9953	1	1	1
	0,9911	0,9972	1	0,9996	1	1	1
1,57		1	0,9911	0,7584	0,9943	0,9989	0,9984
1,365	0,2048		0,9972	0,834	0,9984	0,9998	0,9997
0	1,57	1,365		0,9996	1	1	1
1,092	2,662	2,457	1,092		0,9992	0,9957	0,9969
0,08532	1,485	1,28	0,08532	1,177		1	1
0,3413	1,229	1,024	0,3413	1,433	0,256		1
0,2901	1,28	1,075	0,2901	1,382	0,2048	0,05119	
0,01706	1,553	1,348	0,01706	1,109	0,06826	0,3242	0,273

F	p (same)
0,612	0,8006
Permutation p	
ICC:	-0,14853

Within groups:	5157,54	24	214,898	Permutation p	
Total:	7433,93	35	0,498		
Components of variance (only for random					
Var(group):	-2,65113	Var(error	214,898	ICC:	-
omega2:	0				
Levene´s test for	p (same):	0,3403			
Levene´s test, from	p (same):	0,971			
Welch F test in the case of unequal variances: F=0.7653,					

	C.	*C.*	*C. bracteosum*	*C. caffrum*	*C.*
C. adenogdnium		0,8913	1	1	1
C. apiculatum	2,269		0,7014	0,9409	0,8139
C. bracteosum	0,5317	2,8		1	1
C. caffrum	0,2206	2,048	0,7522		1
C. elaegnoides	0,2469	2,515	0,2847	0,4675	
C. erythrophyllum	0,445	1,823	0,9767	0,2245	0,692
C. imberbe	0,4884	2,757	0,04332	0,7089	0,2414
C. krausii	1,205	3,473	0,6731	1,425	0,9578
C. mkuzense	0,8349	3,103	0,3033	1,056	0,588
C. pardoides	0,2363	2,505	0,2954	0,4569	0,01063
C. vendae	1,049	3,317	0,5171	1,269	0,8019
C. zeyherii	1,155	1,113	1,687	0,9346	1,402

Mn

Test for equal means					
	Sum of sqrs	df	Mean square	F	p
Between groups:	15,347	11	1,39518	1,565	0,173
Within groups:	21,3919	24	0,891329	Permutation p	
Total:	36,7389	35	0,1688		
Components of variance (only for random					
Var(group):	0,167951	Var(error	0,891329	ICC:	0,15855
omega2:	0,1473				
Levene´s test for	p (same):	0,243			
Levene´s test, from	p (same):	0,972			
Welch F test in the case of unequal variances: F=0.9077,					

	C.	*C.*	*C. bracteosum*	*C. caffrum*	*C.*

C. adenogdnium		0,6615	1	0,983	1
C. apiculatum	2,893		0,366	0,9992	0,3543
C. bracteosum	0,6923	3,585		0,8524	1
C. caffrum	1,709	1,183	2,401		0,8423
C. elaegnoides	0,7234	3,616	0,03119	2,433	
C. erythrophyllum	0,002446	2,895	0,6898	1,712	0,721
C. imberbe	1,027	3,92	0,3351	2,737	0,3039
C. krausii	1,237	4,13	0,5449	2,946	0,5137
C. mkuzense	0,07033	2,963	0,6219	1,78	0,6531
C. pardoides	0,2997	2,593	0,9919	1,41	1,023
C. vendae	0,595	3,488	0,09723	2,304	0,1284
C. zeyherii	1,498	1,395	2,19	0,2116	2,221

Na

Test for equal means					
	Sum of sqrs	df	Mean square	F	p (same)
Between groups:	359,094	11	32,6449	0,893	0,5599
Within groups:	877,402	24	36,5584	Permutation p	
Total:	1236,5	35	0,558		
Components of variance (only for random					
Var(group):	-1,30451	Var(error	36,5584	ICC:	-0,037
omega2:	0				
Levene´s test for	p (same):	0,1061			
Levene´s test, from	p (same):	0,9618			
Welch F test in the case of unequal variances: F=1.1, df=9.398,					

	C.	C.	C. bracteosum	C. caffrum	C.
C. adenogdnium		0,9999	1	0,9998	0,9995
C. apiculatum	0,9558		0,9968	0,9522	0,9343
C. bracteosum	0,4297	1,386		1	1
C. caffrum	1,026	1,981	0,5958		1
C. elaegnoides	1,127	2,083	0,6971	0,1012	
C. erythrophyllum	0,401	0,5548	0,8307	1,427	1,528
C. imberbe	1,776	2,732	1,346	0,7505	0,6493
C. krausii	1,32	2,275	0,8899	0,2941	0,1929
C. mkuzense	1,146	2,102	0,7162	0,1203	0,0191
C. pardoides	1,48	2,436	1,05	0,4545	0,3533
C. vendae	1,782	2,738	1,352	0,7563	0,655
C. zeyherii	0,5252	0,4306	0,9549	1,551	1,652

Test for equal means					
	Sum of sqrs	df	Mean square	F	p
Between groups:	6,68212	11	0,607465	0,3299	0,9704
Within groups:	44,1875	24	1,84115	Permutation p	
Total:	50,8696	35	0,9681		
Components of variance (only for random					
Var(group):	-0,411227	Var(error	1,84115	ICC:	-
omega2:	0				
Levene´s test for	p (same):	0,9483			
Levene´s test, from	p (same):	0,9996			
Welch F test in the case of unequal variances: F=0.3372,					

	adenogdnium	*apiculat*	*C. bracteosum*	*C. caffrum*	*elaegno*
C. adenogdnium		1	0,9988	0,999	0,9991
C. apiculatum	0,3489		0,9999	1	1
C. bracteosum	1,234	0,885		1	1
C. caffrum	1,214	0,8646	0,02042		1
C. elaegnoides	1,198	0,8493	0,0357	0,01528	
C. erythrophyllum	0,7331	0,3842	0,5008	0,4804	0,4651
C. imberbe	1,31	0,9612	0,07616	0,09659	0,1119
C. krausii	1,926	1,577	0,6919	0,7123	0,7276
C. mkuzense	1,425	1,077	0,1915	0,2119	0,2272
C. pardoides	1,408	1,059	0,1745	0,1949	0,2102
C. vendae	1,925	1,576	0,6906	0,711	0,7263
C. zeyherii	0,8127	0,4638	0,4212	0,4008	0,3855

Pb

Test for equal means					
	Sum of sqrs	df	Mean square	F	p
Between groups:	125,831	11	11,4392	0,3515	0,9629
Within groups:	780,949	24	32,5396	Permutation p	
Total:	906,78	35	0,971		
Components of variance (only for random					
Var(group):	-7,03346	Var(error	32,5396	ICC:	-

omega2:	0				
Levene´s test for	p (same):	0,01088			
Levene´s test, from	p (same):	0,9631			

Welch F test in the case of unequal variances: F=0.7824,					

	adenogdnium	*apiculat*	*bracteosum*	*C. caffrum*	*elaegno*
C. adenogdnium		*0,9963*	*1*	*1*	*1*
C. apiculatum	*1,408*		*1*	*1*	*0,9913*
C. bracteosum	*0,784*	*0,6245*		*1*	*0,9999*
C. caffrum	*0,7389*	*0,6696*	*0,04514*		*0,9999*
C. elaegnoides	*0,1574*	*1,566*	*0,9414*	*0,8962*	
C. erythrophyllum	*1,168*	*0,2409*	*0,3836*	*0,4287*	*1,325*
C. imberbe	*0,2233*	*1,185*	*0,5607*	*0,5156*	*0,3807*
C. krausii	*0,6411*	*2,05*	*1,425*	*1,38*	*0,4837*
C. mkuzense	*1,103*	*0,3057*	*0,3188*	*0,364*	*1,26*
C. pardoides	*0,4692*	*0,9393*	*0,3148*	*0,2696*	*0,6266*
C. vendae	*0,3588*	*1,05*	*0,4252*	*0,3801*	*0,5162*
C. zeyherii	*0,617*	*0,7915*	*0,167*	*0,1219*	*0,7744*

Zn

Test for equal means					
	Sum of sqrs	df	Mean square	F	p
Between groups:	93,6713	11	8,51558	0,7746	0,6613
Within groups:	263,837	24	10,9932	Permutation p	
Total:	357,508	35	0,9189		
Components of variance (only for random					
Var(group):	-0,825872	Var(error	10,9932	ICC:	-
omega2:	0				
Levene´s test for	p (same):	2,15E-			
Levene´s test, from	p (same):	0,59			
Welch F test in the case of unequal variances: F=0.5728,					

	adenogdnium	*apiculat*	*bracteosum*	*caffrum*	*elaegno*
C. adenogdnium		1	1	1	1
C. apiculatum	0,2027		1	1	1
C. bracteosum	0,4374	0,6401		1	1
C. caffrum	0,4139	0,6166	0,02351		1
C. elaegnoides	0,2568	0,4595	0,1806	0,1571	

C. erythrophyllum	0,2187	0,01602	0,6561	0,6326	0,4756
C. imberbe	0,4977	0,7004	0,06025	0,08376	0,2408
C. krausii	0,4172	0,2145	0,8546	0,8311	0,6741
C. mkuzense	0,494	0,6967	0,05659	0,0801	0,2372
C. pardoides	0,5259	0,7286	0,08846	0,112	0,269

C. vendae	0,4675	0,6702	0,03012	0,05363	0,2107
C. zeyherii	2,618	2,415	3,055	3,032	2,875

C. erythro	C.	C.	C.	C.	C.	C. zeyherii
1	1	0,9991	0,9999	0,00039	1	1
1	1	0,7976	0,8865	0,00395	0,9547	0,9881
1	1	0,96	0,9866	0,00135	0,9977	0,9998
1	1	0,9859	0,9967	0,00086	0,9997	1
0,9995	0,9997	0,9999	1	0,00025	1	1
	1	0,9348	0,9745	0,00175	0,9943	0,9994
0,05084		0,9442	0,9793	0,00160	0,9957	0,9996
2,08	2,029		1	5,16E-05	1	0,9999
1,806	1,756	0,274		8,15E-05	1	1
7,139	7,19	9,219	8,945			0,000247
1,485	1,434	0,5952	0,3212	8,624		1
1,15	1,099	0,9305	0,6565	8,289	0,3353	

erythro	imberbe	krausii	mkuzen	pardoid	vendae	zeyherii
1	0,9471	0,9264	0,9396	0,928	0,8437	1
1	0,9033	0,8736	0,8922	0,8758	0,7677	1
0,9981	1	1	1	1	1	0,9831
0,9918	1	1	1	1	1	0,9561
1	0,9996	0,9991	0,9995	0,9991	0,9935	1
	0,9941	0,9896	0,9926	0,99	0,9628	1
1,492		1	1	1	1	0,9647
1,601	0,1094		1	1	1	0,9488
1,535	0,0427	0,06674		1	1	0,959
1,594	0,1018	0,00760	0,05913		1	0,9501
1,908	0,416	0,3066	0,3733	0,3142		0,8802
0,4009	1,893	2,002	1,935	1,995	2,309	

APPENDIX II: CHAPTER 4 STATS RESULTS

As

Test for equal means					
	Sum of sqrs	df	Mean square	F	p (same)
Between groups:	591,707	11	53,7916	5,85	0,00015
Within groups:	220,673	24	9,1947	Permutation p	
Total:	812,38	35	0,00142		
Components of variance (only for random					
Var(group):	14,8656	Var(error	9,1947	ICC:	0,61785
omega2:	0,5971				
Levene´s test for	p (same):	0,3022			
Levene´s test, from	p (same):	0,9743			
Welch F test in the case of unequal variances: F=19.58, df=9.365, p=4.377E-					

	adenogdnium	apiculat	bracteosum	caffrum	elaegno
C. adenogdnium		0,9973	1	1	1
C. apiculatum	1,359		1	0,9999	0,9894
C. bracteosum	0,7277	0,6318		1	0,9999
C. caffrum	0,4652	0,8943	0,2626		1
C. elaegnoides	0,2456	1,605	0,9733	0,7108	
C. erythrophyllum	0,8795	0,48	0,1518	0,4143	1,125
C. imberbe	0,8286	0,5308	0,1009	0,3635	1,074
C. krausii	1,201	2,56	1,929	1,666	0,9552
C. mkuzense	0,9269	2,286	1,655	1,392	0,6813
C. pardoides	8,018	6,659	7,29	7,553	8,264
C. vendae	0,6057	1,965	1,333	1,071	0,36
C. zeyherii	0,2704	1,63	0,9981	0,7355	0,02475

Ca

Test for equal means					
	Sum of sqrs	df	Mean square	F	p (same)
Between groups:	682302	11	62027,5	0,97	0,4975
Within groups:	1,53E+06	24	63944,1	Permutation p	
Total:	2,22E+06	35	0,4579		
Components of variance (only for random					
Var(group):	-638,877	Var(error	63944,1	ICC:	-0,01009

omega2:	0				
Levene´s test for	p (same):	0,00051			
Levene´s test, from	p (same):	0,7824			
Welch F test in the case of unequal variances: F=0.9152,					

	C.	*C.*	*C. bracteosum*	*C. caffrum*	*C.*
C. adenogdnium		1	0,9725	0,9358	0,9999
C. apiculatum	0,2101		0,9429	0,8868	0,9995
C. bracteosum	1,827	2,037		1	0,9999
C. caffrum	2,075	2,285	0,2486		0,9994
C. elaegnoides	0,9204	1,13	0,9061	1,155	
C. erythrophyllum	0,5206	0,7306	1,306	1,555	0,3999
C. imberbe	2,012	2,222	0,1859	0,06279	1,092
C. krausii	2,122	2,332	0,2953	0,04665	1,201
C. mkuzense	2,055	2,265	0,2285	0,02009	1,135
C. pardoides	2,114	2,324	0,2877	0,03904	1,194
C. vendae	2,428	2,638	0,6019	0,3533	1,508
C. zeyherii	0,1196	0,3297	1,707	1,956	0,8008

Cd

Test for equal means					
	Sum of sqrs	df	Mean square	F	p
Between groups:	59,8054	11	5,43685	0,4816	0,8966
Within groups:	270,959	24	11,29	Permutation p	
Total:	330,764	35	0,8933		
Components of variance (only for random					
Var(group):	-1,95103	Var(error	11,29	ICC:	-
omega2:	0				
Levene´s test for	p (same):	0,6328			
Levene´s test, from	p (same):	0,95			
Welch F test in the case of unequal variances: F=0.976,					

	adenogdnium	apiculat	bracteosum	caffrum	elaegno
C. adenogdnium		0,9999	1	1	1
C. apiculatum	0,9743		1	0,9999	0,9985
C. bracteosum	0,39	0,5842		1	1
C. caffrum	0,006873	0,9674	0,3832		1
C. elaegnoides	0,29	1,264	0,6801	0,2969	
C. erythrophyllum	0,6409	0,3333	0,2509	0,634	0,931

C. imberbe	0,244	0,7303	0,1461	0,2371	0,534
C. krausii	1,538	2,512	1,928	1,545	1,248
C. mkuzense	0,2096	1,184	0,5997	0,2165	0,08042
C. pardoides	0,2285	1,203	0,6186	0,2354	0,06151

C. vendae	1,119	2,093	1,509	1,125	0,8286
C. zeyherii	0,3248	1,299	0,7148	0,3316	0,03471

Co

Test for equal means					
	Sum of sqrs	df	Mean square	F	p (same)
Between groups:	2,55839	11	0,232581	0,8219	0,6203
Within groups:	6,79158	24	0,282982	Permutation p	
Total:	9,34996	35	0,6149		
Components of variance (only for random					
Var(group):	-0,0168006	Var(error	0,282982	ICC:	-0,06312
omega2:	0				
Levene´s test for	p (same):	0,3142			
Levene´s test, from	p (same):	0,9463			
Welch F test in the case of unequal variances: F=1.783,					

	C.	*C.*	*C. bracteosum*	*C. caffrum*	*C.*
C. adenogdnium		0,9792	1	1	1
C. apiculatum	1,756		0,9434	0,9921	0,8902
C. bracteosum	0,2778	2,034		1	1
C. caffrum	0,2084	1,548	0,4862		1
C. elaegnoides	0,5166	2,273	0,2388	0,725	
C. erythrophyllum	0,7087	1,047	0,9866	0,5003	1,225
C. imberbe	0,4623	2,218	0,1845	0,6707	0,05427
C. krausii	1,653	3,409	1,375	1,861	1,136
C. mkuzense	0,4851	2,241	0,2073	0,6935	0,03147
C. pardoides	0,4146	2,171	0,1368	0,623	0,102
C. vendae	1,321	3,077	1,043	1,529	0,8042
C. zeyherii	0,5286	1,228	0,8064	0,3202	1,045

Cu

Test for equal means					
	Sum of sqrs	df	Mean square	F	p (same)
Between groups:	0,785603	11	0,0714185	1,196	0,3405
Within groups:	1,43257	24	0,0596906	Permutation p	
Total:	2,21818	35	0,3436		
Components of variance (only for random					

Var(group):	0,00390931	Var(error	0,0596906	ICC:	0,06147
omega2:	0,05664				
Levene´s test for	p (same):	0,4665			
Levene´s test, from	p (same):	0,9703			
Welch F test in the case of unequal variances: F=1.297,					

	C.	*C.*	*C. bracteosum*	*C. caffrum*	*C.*
C. adenogdnium		1	0,9998	1	0,9999
C. apiculatum	0,7538		0,9767	0,9949	0,9835
C. bracteosum	1,03	1,784		1	1
C. caffrum	0,7113	1,465	0,319		1
C. elaegnoides	0,9476	1,701	0,08271	0,2363	
C. erythrophyllum	0,612	0,1418	1,642	1,323	1,56
C. imberbe	1,096	1,85	0,06617	0,3852	0,1489
C. krausii	2,546	3,3	1,515	1,834	1,598
C. mkuzense	1,371	2,124	0,3403	0,6593	0,423
C. pardoides	1,338	2,091	0,3072	0,6262	0,3899
C. vendae	2,002	2,756	0,972	1,291	1,055
C. zeyherii	0,9004	0,1465	1,931	1,612	1,848

Fe

Test for equal means					
	Sum of sqrs	df	Mean square	F	p
Between groups:	178,498	11	16,2271	0,9577	0,5072
Within groups:	406,642	24	16,9434	Permutation p	
Total:	585,141	35	0,5705		
Components of variance (only for random					
Var(group):	-0,238767	Var(error	16,9434	ICC:	-
omega2:	0				
Levene´s test for	p (same):	3,21E-			
Levene´s test, from	p (same):	0,5613			
Welch F test in the case of unequal variances: F=0.6944,					

	C.	*C.*	*C. bracteosum*	*C. caffrum*	*C.*
C. adenogdnium		1	1	1	1
C. apiculatum	0,07855		1	1	1
C. bracteosum	0,06312	0,1417		1	1
C. caffrum	0,004488	0,07406	0,06761		1

C. elaegnoides	0,3891	0,4676	0,326	0,3936	

C. erythrophyllum	0,2567	0,1781	0,3198	0,2522	0,6458
C. imberbe	0,347	0,4256	0,2839	0,3515	0,04208
C. krausii	0,2588	0,3373	0,1957	0,2633	0,1303
C. mkuzense	0,009818	0,08836	0,0533	0,01431	0,3793
C. pardoides	0,2791	0,3577	0,216	0,2836	0,11
C. vendae	0,3111	0,3896	0,248	0,3156	0,07799
C. zeyherii	3,2	3,122	3,263	3,196	3,589

K

Test for equal means					
	Sum of sqrs	df	Mean square	F	p (same)
Between groups:	14019,8	11	1274,53	0,8347	0,6092
Within groups:	36647,5	24	1526,98	Permutation p	
Total:	50667,3	35	0,6202		
Components of variance (only for random					
Var(group):	-84,1497	Var(error	1526,98	ICC:	-0,05832
omega2:	0				
Levene´s test for	p (same):	0,00379			
Levene´s test, from	p (same):	0,9375			
Welch F test in the case of unequal variances: F=0.7412,					

	C.	*C.*	*C. bracteosum*	*C. caffrum*	*C.*
C. adenogdnium		0,9999	1	0,9999	1
C. apiculatum	0,9146		0,9968	1	0,9984
C. bracteosum	0,4728	1,387		0,9956	1
C. caffrum	0,9663	0,05171	1,439		0,9977
C. elaegnoides	0,3635	1,278	0,1093	1,33	
C. erythrophyllum	0,3295	0,5851	0,8023	0,6368	0,6929
C. imberbe	1,089	2,003	0,6161	2,055	0,7254
C. krausii	0,7418	1,656	0,269	1,708	0,3784
C. mkuzense	0,6324	1,547	0,1596	1,599	0,2689
C. pardoides	0,8865	1,801	0,4137	1,853	0,523
C. vendae	0,7599	1,674	0,2871	1,726	0,3964
C. zeyherii	1,873	0,9589	2,346	0,9072	2,237

Mg

Test for equal means					
	Sum of sqrs	df	Mean square	F	p (same)

Between groups:	2276,39	11	206,944	0,963	0,5031

APPENDIX III: CHAPTER 6 STATS RESULTS

ANOVA

1000ug/ml

Test for equal means				
	Sum of sqrs	df	Mean square	F
Between groups:	8100,08	11	736,371	0,9945
Within groups:	17770,7	24	740,444	Permutation p
Total:	25870,8	35	0,4691	
Components of variance (only for random				
Var(group):	-1,35774	Var(error	740,444	ICC:
omega2:	0			
Levene´s test for	p (same):	0,00479		
Levene´s test, from	p (same):	0,932		
Welch F test in the case of unequal variances: F=1.215, df=9.364,				

	C. adenogdnium	*apiculat*	*C. bracteosum*	*C. caffrum*
C. adenogdnium		0,9989	0,9679	0,9995
C. apiculatum	1,231		1	1
C. bracteosum	1,867	0,6365		1
C. caffrum	1,125	0,1061	0,7426	
C. elaegnoides	0	1,231	1,867	1,125
C. erythrophyllum	1,655	0,4243	0,2122	0,5304
C. imberbe	1,591	0,3607	0,2758	0,4668
C. krausii	1,803	0,5729	0,06365	0,679
C. mkuzense	3,14	1,91	1,273	2,016
C. pardoides	3,034	1,803	1,167	1,91
C. vendae	1,973	0,7426	0,1061	0,8487
C. zeyherii	2,504	1,273	0,6365	1,379

500ug/ml

Test for equal means				
	Sum of sqrs	df	Mean square	F
Between groups:	4860,89	11	441,899	0,7224
Within groups:	14680,7	24	611,694	Permutation p
Total:	19541,6	35	0,706	

Components of variance (only for random				
Var(group):	-56,5985	Var(error	611,694	ICC:
omega2:	0			
Levene´s test for	p (same):	0,07739		

Levene´s test, from	p (same):	0,9302		
Welch F test in the case of unequal variances: F=0.565, df=9.273,				

	C. adenogdnium	*. apiculat*	*C. bracteosum*	*C. caffrum*
C. adenogdnium		0,9998	0,9954	1
C. apiculatum	1,027		1	1
C. bracteosum	1,447	0,4202		0,9996
C. caffrum	0,3502	0,677	1,097	
C. elaegnoides	0,6069	0,4202	0,8404	0,2568
C. erythrophyllum	1,564	0,5369	0,1167	1,214
C. imberbe	2,381	1,354	0,9338	2,031
C. krausii	1,681	0,6536	0,2334	1,331
C. mkuzense	2,615	1,587	1,167	2,264
C. pardoides	2,498	1,471	1,05	2,148
C. vendae	1,681	0,6536	0,2334	1,331
C. zeyherii	0,8637	0,1634	0,5836	0,5136

250ug/ml

Test for equal means				
	Sum of sqrs	df	Mean square	F
Between groups:	5407,89	11	491,626	0,8109
Within groups:	14550	24	606,25	Permutation p
Total:	19957,9	35	0,6268	
Components of variance (only for random				
Var(group):	-38,2079	Var(error	606,25	ICC:
omega2:	0			
Levene´s test for	p (same):	0,511		
Levene´s test, from	p (same):	0,9991		
Welch F test in the case of unequal variances: F=0.5864,				

	C. adenogdnium	*. apiculat*	*C. bracteosum*	*C. caffrum*
C. adenogdnium		0,9999	0,9983	1
C. apiculatum	0,891		1	0,9999
C. bracteosum	1,29	0,3986		0,998
C. caffrum	0,02345	0,9145	1,313	
C. elaegnoides	0,5628	0,3283	0,7269	0,5862
C. erythrophyllum	1,594	0,7035	0,3048	1,618
C. imberbe	2,626	1,735	1,337	2,65
C. krausii	1,923	1,032	0,6331	1,946
C. mkuzense	2,063	1,172	0,7738	2,087

C. pardoides	2,04	1,149	0,7503	2,063

C. vendae	0,9848	0,09379	0,3048	1,008
C. zeyherii	0,07035	0,8207	1,219	0,09379

125ug/ml

Test for equal means				
	Sum of sqrs	df	Mean square	F
Between groups:	4815,22	11	437,747	0,7552
Within groups:	13911,3	24	579,639	Permutation p
Total:	18726,6	35	0,6779	
Components of variance (only for random				
Var(group):	-47,2971	Var(error	579,639	ICC:
omega2:	0			
Levene´s test for	p (same):	0,409		
Levene´s test, from	p (same):	0,9896		
Welch F test in the case of unequal variances: F=0.5891,				

	C. adenogdnium	*C.*	*C. bracteosum*	*C. caffrum*
C. adenogdnium		1	1	1
C. apiculatum	0,3837		1	0,9999
C. bracteosum	0,8153	0,4317		0,9967
C. caffrum	0,5755	0,9592	1,391	
C. elaegnoides	0,8633	0,4796	0,04796	1,439
C. erythrophyllum	1,103	0,7194	0,2878	1,679
C. imberbe	2,182	1,799	1,367	2,758
C. krausii	1,703	1,319	0,8873	2,278
C. mkuzense	1,463	1,079	0,6475	2,038
C. pardoides	0,9832	0,5995	0,1679	1,559
C. vendae	0,02398	0,3597	0,7914	0,5995
C. zeyherii	0,5516	0,9352	1,367	0,02398

62,5ug/ml

Test for equal means				
	Sum of sqrs	df	Mean square	F
Between groups:	7025,67	11	638,697	1,029
Within groups:	14897,3	24	620,722	Permutation p
Total:	21923	35	0,4536	
Components of variance (only for random				

Var(group):	5,99158	Var(error	620,722	ICC:

omega2:	0,008771			
Levene´s test for	p (same):	0,6706		
Levene´s test, from	p (same):	0,9958		
Welch F test in the case of unequal variances: F=1.024, df=9.426,				

	C. adenogdnium	C.	C. bracteosum	C. caffrum
C. adenogdnium		1	1	1
C. apiculatum	0,3244		1	0,9999
C. bracteosum	0,8111	0,4866		0,9962
C. caffrum	0,6025	0,9269	1,414	
C. elaegnoides	1,135	0,8111	0,3244	1,738
C. erythrophyllum	1,738	1,414	0,9269	2,341
C. imberbe	2,201	1,877	1,39	2,804
C. krausii	1,854	1,529	1,043	2,456
C. mkuzense	1,043	0,7184	0,2317	1,645
C. pardoides	0,5793	0,2549	0,2317	1,182
C. vendae	0,8111	1,135	1,622	0,2086
C. zeyherii	0,6257	0,9501	1,437	0,02317

31,25ug/ml

Test for equal means				
	Sum of sqrs	df	Mean square	F
Between groups:	8286,22	11	753,293	1,095
Within groups:	16509,3	24	687,889	Permutation p
Total:	24795,6	35	0,4083	
Components of variance (only for random				
Var(group):	21,8013	Var(error	687,889	ICC:
omega2:	0,02823			
Levene´s test for	p (same):	0,6953		
Levene´s test, from	p (same):	0,9951		
Welch F test in the case of unequal variances: F=1.246, df=9.421,				

	C. adenogdnium	C.	C. bracteosum	C. caffrum
C. adenogdnium		1	0,9999	1
C. apiculatum	0,2421		1	1
C. bracteosum	0,9245	0,6824		0,9946
C. caffrum	0,5503	0,7925	1,475	

C. elaegnoides	1,497	1,255	0,5723	2,047

C. erythrophyllum	1,937	1,695	1,013	2,487
C. imberbe	2,157	1,915	1,233	2,708
C. krausii	1,937	1,695	1,013	2,487
C. mkuzense	0,7264	0,4843	0,1981	1,277
C. pardoides	0,6164	0,3742	0,3082	1,167
C. vendae	0,8145	1,057	1,739	0,2642
C. zeyherii	0,5723	0,8145	1,497	0,02201

15,6ug/ml

Test for equal means				
	Sum of sqrs	df	Mean square	F
Between groups:	8801,56	11	800,141	1,087
Within groups:	17659,3	24	735,806	Permutation p
Total:	26460,9	35	0,4127	
Components of variance (only for random				
Var(group):	21,4453	Var(error	735,806	ICC:
omega2:	0,02602			
Levene´s test for	p (same):	0,5128		
Levene´s test, from	p (same):	0,9614		
Welch F test in the case of unequal variances: F=2.159, df=9.182,				

	C. adenogdnium	apiculat	C. bracteosum	C. caffrum
C. adenogdnium		1	1	1
C. apiculatum	0,2554		1	1
C. bracteosum	0,6598	0,4044		0,9986
C. caffrum	0,596	0,8514	1,256	
C. elaegnoides	1,469	1,213	0,8088	2,065
C. erythrophyllum	1,767	1,511	1,107	2,363
C. imberbe	1,873	1,618	1,213	2,469
C. krausii	1,767	1,511	1,107	2,363
C. mkuzense	0,3831	0,1277	0,2767	0,9791
C. pardoides	0,2767	0,02128	0,3831	0,8727
C. vendae	1,213	1,469	1,873	0,6172
C. zeyherii	0,6811	0,9365	1,341	0,08514

7,5ug/ml

Test for equal means				
	Sum of sqrs	df	Mean square	F

Between groups:	8240,08	11	749,098	0,9349

Within groups:	19230,7	24	801,278	Permutation p
Total:	27470,7	35	0,5255	
Components of variance (only for random				
Var(group):	-17,3931	Var(error	801,278	ICC:
omega2:	0			
Levene´s test for	p (same):	0,6669		
Levene´s test, from	p (same):	0,9585		
Welch F test in the case of unequal variances: F=1.745, df=9.16,				

	C. adenogdnium	*C.*	*C. bracteosum*	*C. caffrum*
C. adenogdnium		1	1	1
C. apiculatum	0,2244		1	1
C. bracteosum	0,6935	0,4691		0,9985
C. caffrum	0,5711	0,7954	1,265	
C. elaegnoides	1,53	1,305	0,8362	2,101
C. erythrophyllum	1,326	1,101	0,6323	1,897
C. imberbe	1,836	1,611	1,142	2,407
C. krausii	1,632	1,407	0,9382	2,203
C. mkuzense	0,3059	0,08158	0,3875	0,877
C. pardoides	0,204	0,0204	0,4895	0,7751
C. vendae	1,122	1,346	1,815	0,5507
C. zeyherii	0,6119	0,8362	1,305	0,04079

3,9ug/ml

Test for equal means				
	Sum of sqrs	df	Mean square	F
Between groups:	8856,33	11	805,121	1,05
Within groups:	18394,7	24	766,444	Permutation p
Total:	27251	35	0,4319	
Components of variance (only for random				
Var(group):	12,8923	Var(error	766,444	ICC:
omega2:	0,01518			
Levene´s test for	p (same):	0,4141		
Levene´s test, from	p (same):	0,9394		
Welch F test in the case of unequal variances: F=1.444, df=9.208,				

	C. adenogdnium	*. apiculat*	*C. bracteosum*	*C. caffrum*

C. adenogdnium		0,9998	0,9944	1
C. apiculatum	1,043		1	1
C. bracteosum	1,481	0,4379		0,999
C. caffrum	0,2711	0,7716	1,21	
C. elaegnoides	2,419	1,376	0,9385	2,148
C. erythrophyllum	2,002	0,9593	0,5214	1,731
C. imberbe	2,607	1,564	1,126	2,336
C. krausii	2,398	1,356	0,9176	2,127
C. mkuzense	0,9385	0,1043	0,5422	0,6673
C. pardoides	0,9385	0,1043	0,5422	0,6673
C. vendae	0,3128	1,356	1,793	0,5839
C. zeyherii	0,1043	0,9385	1,376	0,1668

1,95ug/ml

Test for equal means				
	Sum of sqrs	df	Mean square	F
Between groups:	8484,31	11	771,301	0,9821
Within groups:	18848	24	785,333	Permutation p
Total:	27332,3	35	0,4818	
Components of variance (only for random				
Var(group):	-4,67761	Var(error	785,333	ICC:
omega2:	0			
Levene´s test for	p (same):	0,3084		
Levene´s test, from	p (same):	0,9206		
Welch F test in the case of unequal variances: F=1.233, df=9.232,				

	C. adenogdnium	apiculat	C. bracteosum	C. caffrum
C. adenogdnium		0,9999	0,996	1
C. apiculatum	0,9889		1	1
C. bracteosum	1,422	0,4326		0,9994
C. caffrum	0,2678	0,7211	1,154	
C. elaegnoides	2,307	1,319	0,8859	2,04
C. erythrophyllum	1,937	0,9477	0,5151	1,669
C. imberbe	2,452	1,463	1,03	2,184
C. krausii	2,246	1,257	0,8241	1,978
C. mkuzense	0,9065	0,08241	0,5151	0,6387
C. pardoides	0,7005	0,2884	0,7211	0,4326
C. vendae	0,3296	1,319	1,751	0,5975

C. zeyherii	0,0206	1,01	1,442	0,2884

p (same)
0,4786
-0,00184

elaegno	*erythro*	*imberbe*	*krausii*	*mkuzen*	*pardoid*	*vendae*	zeyherii
1	0,9866	0,9901	0,9748	0,5516	0,5988	0,9534	0,8181
0,9989	1	1	1	0,9626	0,9748	1	0,9985
0,9679	1	1	1	0,9985	0,9993	1	1
0,9995	1	1	1	0,9466	0,9626	1	0,9969
	0,9866	0,9901	0,9748	0,5516	0,5988	0,9534	0,8181
1,655		1	1	0,9943	0,9969	1	1
1,591	0,06365		1	0,992	0,9955	1	0,9999
1,803	0,1485	0,2122		0,9976	0,9989	1	1
3,14	1,485	1,549	1,337		1	0,9993	1
3,034	1,379	1,443	1,231	0,1061		0,9997	1
1,973	0,3183	0,3819	0,1697	1,167	1,061		1
2,504	0,8487	0,9124	0,7002	0,6365	0,5304	0,5304	

p (same)
0,7069
-0,10196

elaegno	*erythro*	*imberbe*	*krausii*	*mkuzen*	*pardoid*	*vendae*	*zeyherii*
1	0,9914	0,8588	0,985	0,7771	0,8201	0,985	1
1	1	0,9974	1	0,9903	0,9947	1	1
1	1	0,9999	1	0,9993	0,9997	1	1
1	0,999	0,9439	0,9977	0,8924	0,9208	0,9977	1
	0,9999	0,9776	0,9997	0,9479	0,965	0,9997	1
0,9571		1	1	0,9997	0,9999	1	1
1,774	0,817		1	1	1	1	0,9932
1,074	0,1167	0,7003		0,9999	1	1	1
2,008	1,05	0,2334	0,9338		1	0,9999	0,9797
1,891	0,9338	0,1167	0,817	0,1167		1	0,9879
1,074	0,1167	0,7003	0	0,9338	0,817		1
0,2568	0,7003	1,517	0,817	1,751	1,634	0,817	

p (same)
0,6297
-0,06726

elaegno	erythro	imberbe	krausii	mkuzen	pardoid	vendae	zeyherii
1	0,99	0,7725	0,9608	0,938	0,9423	0,9999	1
1	1	0,9809	0,9998	0,9993	0,9994	1	1
1	1	0,9976	1	1	1	1	0,9989
1	0,9887	0,7633	0,9575	0,9335	0,938	0,9998	1
	0,9998	0,938	0,9973	0,9938	0,9945	1	1
1,032		0,9998	1	1	1	1	0,993
2,063	1,032		1	1	1	0,9874	0,7992
1,36	0,3283	0,7035		1	1	0,9999	0,9696
1,501	0,469	0,5628	0,1407		1	0,9997	0,9503
1,477	0,4455	0,5862	0,1172	0,02345		0,9997	0,954

0,	0,	1,	0,	1,	1,		0,
0,	1,	2,	1,	1,	1,	0,	

elaegno	. erythro	. imberbe	krausii	mkuzen	. pardoid	vendae	zeyherii
0,9994	0,9807	0,9084	0,9694	0,9998	1	1	1
1	0,9962	0,9667	0,9928	1	1	0,9994	0,9999
1	0,9999	0,9967	0,9998	1	1	0,9885	0,9957
0,9807	0,8711	0,6998	0,8344	0,9872	0,9992	1	1
	1	0,9997	1	1	1	0,9574	0,9788
0,6025		1	1	1	0,9993	0,8017	0,8641
1,066	0,4635		1	0,9993	0,9885	0,6084	0,6899

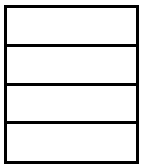
0,7184	0,1159	0,3476		1	0,9984	0,7573	0,8265
0,09269	0,6952	1,159	0,8111		1	0,9694	0,9858
0,5562	1,159	1,622	1,275	0,4635		0,9967	0,9991
1,947	2,549	3,013	2,665	1,854	1,39		1
1,761	2,364	2,827	2,48	1,668	1,205	0,1854	

p (same)
0,4056
0,03072

elaegno	erythro	. imberbe	krausii	mkuzen	pardoid	vendae	zeyherii
0,9939	0,9588	0,9187	0,9588	1	1	1	1
0,9986	0,984	0,9618	0,984	1	1	0,9997	1
1	0,9998	0,9988	0,9998	1	1	0,9806	0,9939
0,941	0,8237	0,7401	0,8237	0,9984	0,9993	1	1
	1	1	1	1	1	0,8795	0,9369
0,4403		1	1	0,999	0,9979	0,7219	0,816
0,6604	0,2201		1	0,9958	0,9923	0,6266	0,731
0,4403	0	0,2201		0,999	0,9979	0,7219	0,816
0,7705	1,211	1,431	1,211		1	0,9923	0,9982
0,8805	1,321	1,541	1,321	0,1101		0,9958	0,9992
2,311	2,752	2,972	2,752	1,541	1,431		1
2,069	2,509	2,73	2,509	1,299	1,189	0,2421	

p (same)
0,4108
0,02832

elaegno	erythro	imberbe	krausii	mkuzen	pardoid	vendae	zeyherii
0,9948	0,9783	0,9672	0,9783	1	1	0,999	1
0,999	0,9934	0,9888	0,9934	1	1	0,9948	0,9999
1	0,9996	0,999	0,9996	1	1	0,9672	0,9976
0,9378	0,8645	0,8301	0,8645	0,9999	1	1	1
	1	1	1	0,9996	0,9991	0,7505	0,9204
0,298		1	1	0,9968	0,9941	0,623	0,8373
0,4044	0,1064		1	0,9941	0,9899	0,5756	0,7999
0,298	0	0,1064		0,9968	0,9941	0,623	0,8373
1,085	1,383	1,49	1,383		1	0,9899	0,9997
1,192	1,49	1,596	1,49	0,1064		0,9941	0,9999
2,682	2,98	3,086	2,98	1,596	1,49		1
2,15	2,448	2,554	2,448	1,064	0,9578	0,5321	



C.	C.	C.	C.	C.	C.	C.	C.
0,9928	0,9978	0,9715	0,988	1	1	0,9995	1
0,9981	0,9996	0,9891	0,9963	1	1	0,9975	1
1	1	0,9994	0,9999	1	1	0,9736	0,9981
0,9307	0,9642	0,8507	0,9081	1	1	1	1
	1	1	1	0,9989	0,9978	0,7626	0,9222
0,204		1	1	0,9998	0,9995	0,8373	0,9587
0,3059	0,5099		1	0,9928	0,988	0,6329	0,8373
0,102	0,3059	0,204		0,9978	0,9959	0,7211	0,8979
1,224	1,02	1,53	1,326		1	0,9959	0,9999
1,326	1,122	1,632	1,428	0,102		0,9978	1
2,651	2,448	2,957	2,753	1,428	1,326		1
2,142	1,938	2,448	2,244	0,9178	0,8158	0,5099	

p (same)
0,4369
0,01654

elaegno	erythro	imberbe	krausii	mkuzen	pardoid	Cvendae	zeyherii
0,8467	0,9488	0,78	0,8534	0,9999	0,9999	1	1
0,997	0,9999	0,9914	0,9973	1	1	0,9973	0,9999
0,9999	1	0,9995	0,9999	1	1	0,9758	0,997
0,9208	0,9813	0,8725	0,9253	1	1	1	1
	1	1	1	0,9944	0,9944	0,7301	0,8785
0,4171		1	1	0,9997	0,9997	0,8785	0,9641
0,1877	0,6048		1	0,9858	0,9858	0,6496	0,8185
0,02085	0,3962	0,2085		0,995	0,995	0,7386	0,8844
1,481	1,064	1,668	1,46		1	0,9987	1
1,481	1,064	1,668	1,46	0		0,9987	1
2,732	2,315	2,92	2,711	1,251	1,251		1
2,315	1,898	2,503	2,294	0,8342	0,8342	0,4171	

p (same)
0,4881
-0,00599

elaegno	erythro	imberbe	krausii	mkuzen	pardoid	vendae	zeyherii
0,8806	0,9589	0,836	0,8974	0,9999	1	1	1

0,9979	0,9999	0,995	0,9986	1	1	0,9979	0,9998
0,9999	1	0,9998	1	1	1	0,9796	0,9955
0,9424	0,9858	0,9126	0,9527	1	1	1	1
	1	1	1	0,9965	0,9893	0,7683	0,8747
0,3708		1	1	0,9998	0,9988	0,8919	0,9559
0,1442	0,5151		1	0,9922	0,9796	0,7094	0,829
0,06181	0,309	0,206		0,9976	0,9922	0,792	0,8919
1,401	1,03	1,545	1,339		1	0,9988	0,9999
1,607	1,236	1,751	1,545	0,206		0,9998	1
2,637	2,266	2,781	2,575	1,236	1,03		1
2,328	1,957	2,472	2,266	0,9271	0,7211	0,309	

APPENDIX V: BIOCHEMICAL IDENTIFICATION TESTS

The GN card is used for the automated identification of 135 taxa of the most significant fermenting and non-fermenting Gram-negative bacilli.

GRAM NEGATIVE TESTS

Well	Biochemical test	Mnemonic	Amount/well (mg)
2	Ala-Phe-Pro- Arylamidase	APPA	0.0384
3	Adonitol	ADO	0.1875
4	L-pryrolydonyl-Arylamidase	PyrA	0.018
5	L-Arabitol	IARL	0.3
7	D-Cellobiose	dCEL	0.3
9	Beta galactosidase	BGAL	0.036
10	H_2S production	H2S	0.0024
11	N-Acetyl.glucosaminidase	BNAG	0.0408
12	Glutamyl Arylamidase pNA	AGLTp	0.0324
13	D-Glucose	dGLU	0.3
14	Gamma-glutamyl-tranferase	GGT	0.0228
15	Fermentation/Glucose	OFF	0.45
17	Beta glucosidase	BGLU	0.035
18	D-Maltose	dMAL	0.3
19	D-Mannitol	dMAN	0.1875
20	D-Mannose	dMNE	0.3
21	Beta-Xylosidase	BXYL	0.0324
22	Beta-Alanine Arylamidase pNA	BAlap	0.0174
23	L-Proline=Arylamidase	ProA	0.0234
24	Lipase	LIP	0.0192
27	Palatinose	PLE	0.3
29	Tyrosine-Arylamodase	TyrA	0.0276
31	Urease	URE	0.15
32	D-Sorbitol	dSOR	0.1875
33	Sucrose	SAC	0.3

34	D-Togatose	dTAG	0.3
35	D- Trehalase	dTRE	0.3
36	Citrate	CIT	0.054
37	Malonate	MNT	0.15
39	5 Keto-D Gluconate	5KG	0.3
40	L-Lactate alkalinisation	ILAKk	0.15
41	Alpha-glucosidase	AGLU	0.036
42	Succinate alkanilisation	SUCT	0.15
43	Beta-N-acetyl galactosaminidase	NAGA	0.0306
44	Alpha galactosidase	AGAL	0.036
45	Phosphate	PHOS	0.050
46	Glycine- Arylamidase	GlyA	0.012
47	Orinthine Decarboxylase	ODC	0.3
48	Lysine decarboxylase	LDC	0.15
53	L-Histidine assimilation	ODEC	N/A
56	Caumarate	IHISa	0.087
57	Beta-Glucoronidase	BGUR	0.0378
58	0/129 Resistance	O129R	0.0105
59	Glu-Gly-Arg- Arylamidase	GGAA	0.0576
61	L-Malate assimilation	IMLTa	0.042
62	ELLMAN	ELLM	0.03
64	L-Lactate assimilation	ILATa	0.186

The GP identification card is based on established biochemical methods and newly developed substrates (Atlas 1993, Barros *et al.,* 2001, Bille *et al.*,1992, Collins *et al.*, 1984a, Collins *et al.*,1984b, Collins and Lawson 2000, Collins *et al.*,2001.

GRAM POSITIVE TESTS

Well	**Biochemical test**	**Mnemonic**	**Amount/well (mg)**
2	D Amygdalin	AMY	0.0384
4	Phosphatidylinsitol-phospholipase C	PIPLC	0.1875
5	D- Xylose	dXYL	0.018

8	Arginine dihydrolase 1	ADH1	0.3
9	Beta galactosidase	BGAL	0.3
11	Alpha glucosidase	AGLU	0.036
13	Ala-Phe-Pro-Arylamidase	APPA	0.0024
14	Cyclodextrine	CDEX	0.0408
15	L- Aspartae-Arylamidase	AspA	0.0324
16	Beta-Galactopyranosidase Resorufine	BGAR	0.3
17	Alpha –Mannosidase	AMAN	0.0228
19	Phosphatase	PHOS	0.45
20	Leucine –Arylamidase	LeuA	0.035
23	L-Proline-Arylamidase	ProA	0.3
24	Beta- Glucoronidase	BGURr	0.1875
25	Alpha-galactosidase	AGAL	0.3
26	L-pryrolydonyl-Arylamidase	PyrA	0.0324
27	Beta- Glucoronidase	BGUR	0.0174
28	Alanine- Arylamidase	AlaA	0.0234
29	Tyrosine- Arylamidase	TyrA	0.0192
30	D- Sorbitol	dSOR	0.3
31	Urease	URE	0.0276
32	Polymixin B Resistance	POLYB	0.15
37	D-Galactose	dGAL	0.1875
38	D-Ribose	dRIB	0.3
39	L-Lactate alkalinisation	ILATk	0.3
40	Lactose	LAC	0.3
44	N-Acetyl glucosamine	NAG	0.054
45	D-Maltose	dMAL	0.15
46	Bacitracin Resistance	BACI	0.3
47	Novobiocin resistance	NOVO	0.15
50	Growth in 6.5 % NaCl	NC6.5	0.036
52	D-Mannitol	dMAN	0.15
53	D-Mannose	dMNE	0.0306

54	Methyl-B-D Glucopyranoside	MBdG	0.036
56	Pllulale	PUL	0.050
57	D-Raffinose	dRAF	0.012
58	0/129 Resistance	0129R	0.3
59	Salicin	SAL	0.15
60	Sucrose	SAC	N/A
61	D Trehalase	dTRE	0.087
62	Arginine dihydrlase	ADH2s	0.0378
63	Optochin Resistance	OPTO	0.0105

Appendix VII BIOCHEMICAL RESULTS

CI		**Grams reaction Negative**				**Identity *Stenotrophomonas maltophilia***				**Percentage 95 %**	
APPA	+	dGLU	-	ProA	+	CIT	-	GlyA	-	BGUR	-
ADO	-	GGT	+	LIP	+	MNT	-	ODC	-	O129R	-
PyrA	-	OFF	-	PLE	-	5KG	-	LDC	-	GGAA	+
IARL	-	BGLU	+	TyrA	-	ILAKk	+	ODEC	-	IMLTa	-
dCEL	-	dMAL	-	URE	+	AGLU	+	IHISa	-	ELLM	-
BGAL	-	dMAN	-	dSOR	-	SUCT	+	BGUR	-	ILATa	-
H2S	-	dMNE	-	SAC	-	NAGA	-	LDC	-	IHISa	-
BNAG	-	BXYL	-	dTAG	-	AGAL	-	ODEC	-		
AGLTp	-	BAlap	-	dTRE	-	PHOS	+				

CEL		**Grams reaction positive**				**Identity *Staphylococcus intermedius***		**Percentage 87 %**	
AMY	+	BGAR	-	AlaA	+	NAG	+	dRAF	-
PIPLC	-	AMAN	-	TyrA	-	dMAL	+	0129R	+
dXYL	-	PHOS	-	dSOR	-	BACI	+	SAL	-
ADH1	+	LeuA	+	URE	-	NOVO	-	SAC	+
BGAL	-	ProA	-	POLYB	+	NC6.5	+	dTRE	+
AGLU	+	BGURr	-	dGAL	-	dMAN	-	ADH2s	-
APPA	-	AGAL	-	dRIB	+	dMNE	-	OPTO	+
CDEX	-	PyrA	+	ILATk	-	MBdG	-		
AspA	-	BGUR	-	LAC	-	PUL	-		

CV, CZ		**Grams reaction positive**				**Identity *Staphylococcus intermedius***		**Percentage 86%**	
AMY	-	BGAR	-	AlaA	+	NAG	+	dRAF	-
PIPLC	-	AMAN	-	TyrA	+	dMAL	+	0129R	+
dXYL	-	PHOS	-	dSOR	-	BACI	+	SAL	-
ADH1	+	LeuA	+	URE	-	NOVO	-	SAC	+
BGAL	-	ProA	-	POLYB	+	NC6.5	+	dTRE	+
AGLU	+	BGURr	-	dGAL	-	dMAN	-	ADH2s	-
APPA	-	AGAL	-	dRIB	+	dMNE	-	OPTO	+
CDEX	-	PyrA	+	ILATk	+	MBdG	+		
AspA	-	BGUR	-	LAC	-	PUL	-		

CI*		**Grams reaction Negative**				**Identity *Aeromonas sobria***				**Percentage 86 %**	
APPA	-	dGLU	+	ProA	-	CIT	-	GlyA	-	BGUR	-
ADO	-	GGT	-	LIP	-	MNT	-	ODC	-	O129R	+
PyrA	+	OFF	-	PLE	-	5KG	-	LDC	-	GGAA	-
IARL	+	BGLU	-	TyrA	-	ILAKk	-	ODEC	-	IMLTa	-
dCEL	-	dMAL	+	URE	-	AGLU	+	IHISa	-	ELLM	-
BGAL	-	dMAN	-	dSOR	-	SUCT	-	BGUR	-	ILATa	-
H2S	+	dMNE	-	SAC	+	NAGA	-	LDC	-		
BNAG	-	BXYL	-	dTAG	-	AGAL	-	CMT	+		
AGLTp	-	BAlap	-	dTRE	+	PHOS	-				

CC,		**Grams reaction Negative**				***Identity Enterobacter cloacae***				**Percentage 50 %**	
APPA	-	dGLU	+	ProA	-	CIT	+	GlyA	+	BGUR	-
ADO	=	GGT	+	LIP	-	MNT	+	ODC	+	O129R	+
PyrA	-	OFF	+	PLE	+	5KG	-	LDC	-	GGAA	-
IARL	-	BGLU	+	TyrA	+	ILAKk	+	ODEC	-	IMLTa	-
dCEL	+	dMAL	+	URE	-	AGLU	-	IHISa	-	ELLM	-
BGAL	+	dMAN	+	dSOR	+	SUCT	+	BGUR	-	ILATa	-
H2S	-	dMNE	+	SAC	+	NAGA	-	LDC	-		
BNAG	+	BXYL	+	dTAG	-	AGAL	+	CMT	-		
AGLTp	-	BAlap	-	dTRE	+	PHOS	-				

CB		**Grams reaction Negative**				**Identity *Enterobacter kobei***				**Percentage 50%**	
APPA	-	dGLU	+	ProA	-	CIT	+	GlyA	+	BGUR	-
ADO	=	GGT	+	LIP	-	MNT	+	ODC	+	O129R	+
PyrA	-	OFF	+	PLE	+	5KG	-	LDC	-	GGAA	-
IARL	-	BGLU	+	TyrA	+	ILAKk	+	ODEC	-	IMLTa	-
dCEL	+	dMAL	+	URE	-	AGLU	-	IHISa	-	ELLM	-
BGAL	+	dMAN	+	dSOR	+	SUCT	+	BGUR	-	ILATa	-
H2S	-	dMNE	+	SAC	+	NAGA	-	LDC	-		
BNAG	+	BXYL	+	dTAG	-	AGAL	+	CMT	-		
AGLTp	-	BAlap	-	dTRE	+	PHOS	-				

CAD		**Grams reaction Negative**				**Identity *Enterobacter hormaechei***				**Percentage 50 %**	
APPA	-	dGLU	+	ProA	-	CIT	+	GlyA	+	BGUR	-
ADO	=	GGT	+	LIP	-	MNT	+	ODC	+	O129R	+
PyrA	-	OFF	+	PLE	+	5KG	-	LDC	-	GGAA	-
IARL	-	BGLU	+	TyrA	+	ILAKk	+	ODEC	-	IMLTa	-
dCEL	+	dMAL	+	URE	-	AGLU	-	IHISa	-	ELLM	-
BGAL	+	dMAN	+	dSOR	+	SUCT	+	BGUR	-	ILATa	-
H2S	-	dMNE	+	SAC	+	NAGA	-	LDC	-		
BNAG	+	BXYL	+	dTAG	-	AGAL	+	CMT	-		
AGLTp	-	BAlap	-	dTRE	+	PHOS	-				

CAP*		**Grams reaction POSITIVE**				**Identity Unidentified**		**Percentage**	
AMY	-	BGAR	-	AlaA	+	NAG	+	dRAF	-
PIPLC	-	AMAN	-	TyrA	+	dMAL	+	0129R	+
dXYL	-	PHOS	-	dSOR	-	BACI	+	SAL	-
ADH1	+	LeuA	+	URE	-	NOVO	-	SAC	+
BGAL	-	ProA	-	POLYB	+	NC6.5	+	dTRE	+
AGLU	+	BGURr	-	dGAL	-	dMAN	-	ADH2s	-
APPA	-	AGAL	-	dRIB	+	dMNE	-	OPTO	+
CDEX	-	PyrA	+	ILATk	+	MBdG	+		
AspA	-	BGUR	-	LAC	-	PUL	-		

APPENDIX IX: CONSENT FORM

Consent to Participate in a Research Study

University of Limpopo

Title of Study:	Improvement of the quality and shelf life of traditionally produced sorghum juice by addition of ashes, dried powdered leaves and stem obtained from *Combretum* spp

Investigators:

Name:	**Miss Morongwa Mathipa**	**Dept:**	BMBT	**Phone:**	015 268 4215
Name:	**Prof P. Masoko**	**Dept:**	BMBT	**Phone:**	015 268 4807
Name:	**Prof M.S Mphosi**	**Dept:**	LATS	**Phone:**	015 268 4619

Introduction

- You are being asked to be in a research study of improvement of the quality of sorghum juice produced traditionally
- We ask that you read this form and ask any questions that you may have before agreeing to be in the study.

Purpose of Study

- The purpose of the study is to improve the quality of sorghum juice produced traditionally.
- Ultimately, this research may be *published as part of a book on, presented as a paper, etc.*

Description of the Study Procedures

- If you agree to be in this study, you will be asked to do the following things: [taste the juice before and after treatment with the preservatives. The process will take 6-12 months.

Risks/Discomforts of Being in this Study

- The study has the following risks. May cause nausea, vomiting.
- There may be unknown risks.

Confidentiality

- This study is anonymous. We will not be collecting or retaining any information about your identity.

Right to Refuse or Withdraw

- The decision to participate in this study is entirely up to you. You may refuse to take part in the study *at any time* without affecting your relationship with the investigators of this study. Your decision will not result in any loss or benefits to which you are otherwise entitled. You have the right not to answer any single question, as well as to withdraw completely from the interview at any point during the process; additionally, you have the right to request that the interviewer not use any of your interview material.

Right to Ask Questions and Report Concerns

- You have the right to ask questions about this research study and to have those questions answered by me before, during or after the research. If you have any further questions about the study, at any time feel free to contact me, [*Morongwa*] at [Morongwa.Mathipa@ul.ac.za or by telephone at [*015 268 4215*]. If you like, a summary of the results of the study will be sent to you.

Consent

- Your signature below indicates that you have decided to volunteer as a research participant for this study, and that you have read and understood the information provided above. You will be given a signed and dated copy of this form to keep, along with any other printed materials deemed necessary by the study investigators.

Subject's Name (print): _____

Subject's Signature: Date:

_____ _____

Investigator's Signature:	Date:

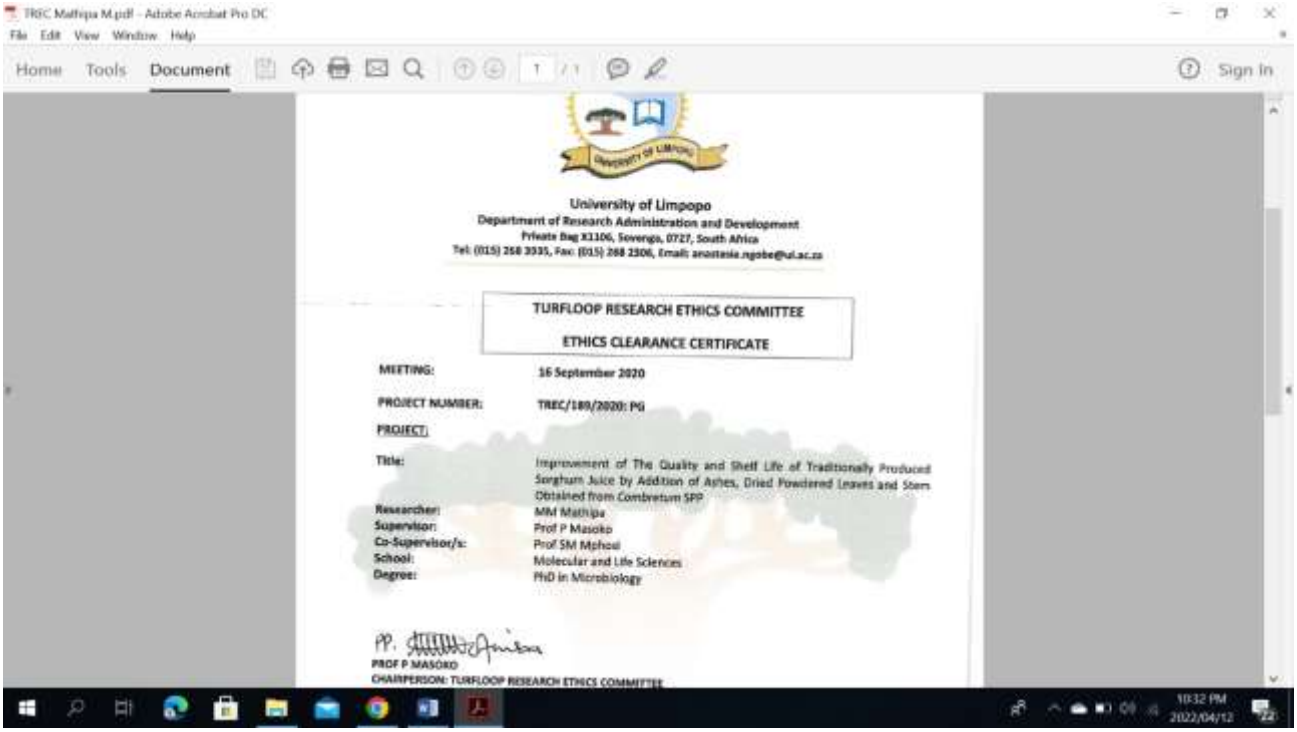
APPENDIX IX: Sensory Evaluation Form

Recipe Name: _____ Category: _____

Directions: Check one rating for each of the following: Appearance, Taste/Flavour, Texture/Consistency, Aroma/Smell, and Overall Acceptability

Rating Scale	Appearance	Taste/Flavour	Texture/ Consistency	Aroma/Smell	Overall Acceptability
9. Like Extremely					
8. Like Very Much					
7. Like Moderately					
6. Like Slightly					
5. Neither Like or Dislike					
4. Dislike Slightly					
3. Dislike Moderately					
2. Dislike Very Much					
1. Dislike Extremely					
Panellist Code: Date:					

Appendix X: TREC Clearence certificate



University of Limpopo
Department of Research Administration and Development
Private Bag X1106, Sovenga, 0727, South Africa
Tel: (015) 268 3935, Fax: (015) 268 2306, Email: anastasia.ngobe@ul.ac.za

TURFLOOP RESEARCH ETHICS COMMITTEE

ETHICS CLEARANCE CERTIFICATE

MEETING: 16 September 2020

PROJECT NUMBER: TREC/189/2020: PG

PROJECT:

Title: Improvement of The Quality and Shelf Life of Traditionally Produced Sorghum Juice by Addition of Ashes, Dried Powdered Leaves and Stem Obtained from Combretum SPP

Researcher: MM Mathipa
Supervisor: Prof P Masoko
Co-Supervisor/s: Prof SM Mphosi
School: Molecular and Life Sciences
Degree: PhD in Microbiology

PP.
PROF P MASOKO
CHAIRPERSON: TURFLOOP RESEARCH ETHICS COMMITTEE

APPENDIX XI: Statistical analysis for the sensory evaluation

Statistix 10.0 12/2/2021 1:21:32 PM

Completely Randomized AOV for Appearanc

Source	DF	SS	MS	F	P
plant	23	377.17	16.3986	7.22	0.0000
Error	456	1036.30	2.2726		
Total	479	1413.47			

Grand Mean 6.7167 CV 22.44

Homogeneity of Variances	F	P
Levene's Test	8.24	0.0000
O'Brien's Test	7.80	0.0000
Brown and Forsythe Test	4.13	0.0000

Welch's Test for Mean Differences

Source	DF	F	P
plant	23.0	11.27	0.0000
Error	165.1		

Component of variance for between groups 0.70630
Effective cell size 20.0

plant	Mean	plant	Mean
CAD Ashes	5.0500	CI Ashes	7.1000
CAD Leaves	5.9500	CI leaves	6.4000
Cap Ashes	7.2000	CK Leaves	6.7500
Cap Leaves	4.5000	CkAshes	6.7000
CB Ashes	8.5500	CM Ashes	6.4500
CB Leaves	7.5500	CM Leaves	6.8000
CC leaves	6.4000	CP Ashes	6.8000

CC Ashes	8.4000	CP leaves	7.3000
CEL Leaves	5.9000	CV Ashes	7.0000
CEL Ashes	6.6000	CV leaves	6.3000
CER Ashes	7.5000	CZ Ashes	7.1000
CER leaves	7.1000	CZ leaves	5.8000
Observations per Mean		20	
Standard Error of a Mean		0.3371	
Std Error (Diff of 2 Means)		0.4767	

Completely Randomized AOV for Taste

Source	DF	SS	MS	F	P
plant	23	393.97	17.1290	8.15	0.0000
Error	456	958.40	2.1018		
Total	479	1352.37			

Grand Mean	6.3083	CV	22.98

Homogeneity of Variances	F	P
Levene's Test	4.46	0.0000
O'Brien's Test	4.22	0.0000
Brown and Forsythe Test	3.58	0.0000

Welch's Test for Mean Differences

Source	DF	F	P
plant	23.0	18.87	0.0000
Error	165.0		

Component of variance for between groups	0.75136
Effective cell size	20.0

plant	Mean	plant	Mean
CAD Ashes	6.0500	CI Ashes	6.5000
CAD Leaves	5.2000	CI leaves	5.2000
Cap Ashes	5.0000	CK Leaves	6.6000
Cap Leaves	4.9000	CkAshes	6.0500
CB Ashes	8.5500	CM Ashes	7.2000
CB Leaves	6.5000	CM Leaves	7.1500
CC leaves	5.3500	CP Ashes	6.0000
CC Ashes	8.2000	CP leaves	6.9500
CEL Leaves	6.3000	CV Ashes	6.1500
CEL Ashes	6.3500	CV leaves	6.1000
CER Ashes	7.1000	CZ Ashes	6.4000
CER leaves	6.3000	CZ leaves	5.3000
Observations per Mean		20	
Standard Error of a Mean		0.3242	
Std Error (Diff of 2 Means)		0.4584	

Completely Randomized AOV for Texture

Source	DF	SS	MS	F	P
plant	23	264.37	11.4942	5.25	0.0000

Error	456	998.00	2.1886
Total	479	1262.37	

Grand Mean 6.1417 CV 24.09

Homogeneity of Variances	**F**	**P**
Levene's Test	5.46	0.0000
O'Brien's Test	5.17	0.0000
Brown and Forsythe Test	3.80	0.0000

Welch's Test for Mean Differences

Source	**DF**	**F**	**P**
plant	23.0	9.40	0.0000
Error	164.8		

Component of variance for between groups 0.46528
Effective cell size 20.0

plant	**Mean**	**plant**	**Mean**
CAD Ashes	6.2000	CI Ashes	5.0000
CAD Leaves	6.1500	CI leaves	5.3000
Cap Ashes	5.6000	CK Leaves	6.2500
Cap Leaves	4.9000	CkAshes	6.5500
CB Ashes	6.7000	CM Ashes	6.8500
CB Leaves	7.1500	CM Leaves	6.6500
CC leaves	5.1500	CP Ashes	5.5000
CC Ashes	7.5000	CP leaves	6.9500
CEL Leaves	5.2000	CV Ashes	6.6500
CEL Ashes	6.3500	CV leaves	5.5500
CER Ashes	6.8000	CZ Ashes	6.5000
CER leaves	6.7000	CZ leaves	5.2500

Observations per Mean 20
Standard Error of a Mean 0.3308
Std Error (Diff of 2 Means) 0.4678

Completely Randomized AOV for Aroma

Source	**DF**	**SS**	**MS**	**F**	**P**
plant	23	451.33	19.6228	10.73	0.0000
Error	456	833.80	1.8285		
Total	479	1285.13			

Grand Mean 6.3125 CV 21.42

Homogeneity of Variances	**F**	**P**
Levene's Test	2.47	0.0002
O'Brien's Test	2.34	0.0005
Brown and Forsythe Test	2.33	0.0005

Welch's Test for Mean Differences

Source	**DF**	**F**	**P**
plant	23.0	16.36	0.0000

Error 165.1

Component of variance for between groups 0.88972
Effective cell size 20.0

plant	Mean	plant	Mean
CAD Ashes	6.0500	CI Ashes	6.2000
CAD Leaves	5.9500	CI leaves	5.0000
Cap Ashes	5.5000	CK Leaves	5.6500
Cap Leaves	5.4000	CkAshes	5.4500
CB Ashes	7.8500	CM Ashes	7.3500
CB Leaves	6.4500	CM Leaves	6.7000
CC leaves	6.1500	CP Ashes	6.8000
CC Ashes	8.2500	CP leaves	7.2000
CEL Leaves	5.0000	CV Ashes	6.1500
CEL Ashes	4.5000	CV leaves	6.8500
CER Ashes	7.9000	CZ Ashes	6.7000
CER leaves	7.1000	CZ leaves	5.3500

Observations per Mean 20
Standard Error of a Mean 0.3024
Std Error (Diff of 2 Means) 0.4276

Completely Randomized AOV for Overall

Source	DF	SS	MS	F	P
plant	23	523.47	22.7594	9.75	0.0000
Error	456	1064.00	2.3333		
Total	479	1587.47			

Grand Mean 6.2167 CV 24.57

Homogeneity of Variances	F	P
Levene's Test	2.89	0.0000
O'Brien's Test	2.74	0.0000
Brown and Forsythe Test	2.61	0.0001

Welch's Test for Mean Differences

Source	DF	F	P
plant	23.0	16.12	0.0000
Error	165.0		

Component of variance for between groups 1.02130
Effective cell size 20.0

plant	Mean	plant	Mean
CAD Ashes	6.4000	CI Ashes	6.0000
CAD Leaves	5.3500	CI leaves	4.9000
Cap Ashes	4.0000	CK Leaves	5.7500
Cap Leaves	4.7000	CkAshes	5.8000
CB Ashes	8.2000	CM Ashes	6.4500
CB Leaves	7.5500	CM Leaves	6.7000
CC leaves	6.1500	CP Ashes	6.8000

CC Ashes	8.2000	CP leaves	6.3000
CEL Leaves	5.4000	CV Ashes	6.0500
CEL Ashes	5.4000	CV leaves	6.3000
CER Ashes	7.2500	CZ Ashes	6.6000
CER leaves	7.7000	CZ leaves	5.2500
Observations per Mean		20	
Standard Error of a Mean		0.3416	
Std Error (Diff of 2 Means)		0.4830	

Tukey HSD All-Pairwise Comparisons Test of Appearanc by plant

plant	Mean	Homogeneous Groups
CB Ashes	8.5500	A
CC Ashes	8.4000	AB
CB Leaves	7.5500	ABC
CER Ashes	7.5000	ABCD
CP leaves	7.3000	ABCD
Cap Ashes	7.2000	ABCD
CER leaves	7.1000	ABCD
CI Ashes	7.1000	ABCD
CZ Ashes	7.1000	ABCD
CV Ashes	7.0000	ABCD
CM Leaves	6.8000	BCD
CP Ashes	6.8000	BCD
CK Leaves	6.7500	BCDE
CkAshes	6.7000	BCDE
CEL Ashes	6.6000	CDE
CM Ashes	6.4500	CDE
CC leaves	6.4000	CDE
CI leaves	6.4000	CDE
CV leaves	6.3000	CDE
CAD Leaves	5.9500	CDEF
CEL Leaves	5.9000	CDEF
CZ leaves	5.8000	DEF
CAD Ashes	5.0500	EF
Cap Leaves	4.5000	F

Alpha	0.05	Standard Error for Comparison	0.4767
Critical Q Value	5.142	Critical Value for Comparison	1.7335

There are 6 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Taste by plant

plant	Mean	Homogeneous Groups
CB Ashes	8.5500	A
CC Ashes	8.2000	AB
CM Ashes	7.2000	ABC
CM Leaves	7.1500	ABC
CER Ashes	7.1000	ABC
CP leaves	6.9500	ABCD
CK Leaves	6.6000	BCDE
CB Leaves	6.5000	CDEF
CI Ashes	6.5000	CDEF
CZ Ashes	6.4000	CDEF
CEL Ashes	6.3500	CDEF
CEL Leaves	6.3000	CDEF
CER leaves	6.3000	CDEF
CV Ashes	6.1500	CDEF

CV leaves	6.1000	CDEF
CAD Ashes	6.0500	CDEF
CkAshes	6.0500	CDEF
CP Ashes	6.0000	CDEF
CC leaves	5.3500	DEF
CZ leaves	5.3000	DEF
CAD Leaves	5.2000	EF
CI leaves	5.2000	EF
Cap Ashes	5.0000	EF
Cap Leaves	4.9000	F

Alpha	0.05	Standard Error for Comparison	0.4584
Critical Q Value	5.142	Critical Value for Comparison	1.6670

There are 6 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Texture by plant

plant	Mean	Homogeneous Groups
CC Ashes	7.5000	A
CB Leaves	7.1500	AB
CP leaves	6.9500	ABC
CM Ashes	6.8500	ABCD
CER Ashes	6.8000	ABCD
CB Ashes	6.7000	ABCDE
CER leaves	6.7000	ABCDE
CM Leaves	6.6500	ABCDE
CV Ashes	6.6500	ABCDE
CkAshes	6.5500	ABCDEF
CZ Ashes	6.5000	ABCDEF
CEL Ashes	6.3500	ABCDEF
CK Leaves	6.2500	ABCDEF
CAD Ashes	6.2000	ABCDEF
CAD Leaves	6.1500	ABCDEF
Cap Ashes	5.6000	BCDEF
CV leaves	5.5500	BCDEF
CP Ashes	5.5000	BCDEF
CI leaves	5.3000	CDEF
CZ leaves	5.2500	CDEF
CEL Leaves	5.2000	DEF
CC leaves	5.1500	DEF
CI Ashes	5.0000	EF
Cap Leaves	4.9000	F

Alpha	0.05	Standard Error for Comparison	0.4678
Critical Q Value	5.142	Critical Value for Comparison	1.7011

There are 6 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Aroma by plant

plant	Mean	Homogeneous Groups

CC Ashes	8.2500	A
CER Ashes	7.9000	AB
CB Ashes	7.8500	AB
CM Ashes	7.3500	ABC
CP leaves	7.2000	ABCD
CER leaves	7.1000	ABCD
CV leaves	6.8500	ABCDE
CP Ashes	6.8000	ABCDE
CM Leaves	6.7000	ABCDE
CZ Ashes	6.7000	ABCDE
CB Leaves	6.4500	BCDEF
CI Ashes	6.2000	CDEF
CC leaves	6.1500	CDEF
CV Ashes	6.1500	CDEF
CAD Ashes	6.0500	CDEFG
CAD Leaves	5.9500	CDEFG
CK Leaves	5.6500	DEFG
Cap Ashes	5.5000	EFG
CkAshes	5.4500	EFG
Cap Leaves	5.4000	EFG
CZ leaves	5.3500	EFG
CEL Leaves	5.0000	FG
CI leaves	5.0000	FG
CEL Ashes	4.5000	G

Alpha	0.05	Standard Error for Comparison	0.4276
Critical Q Value	5.142	Critical Value for Comparison	1.5549

There are 7 groups (A, B, etc.) in which the means
are not significantly different from one another.

Tukey HSD All-Pairwise Comparisons Test of Overall by plant

plant	**Mean**	**Homogeneous Groups**
CB Ashes	8.2000	A
CC Ashes	8.2000	A
CER leaves	7.7000	AB
CB Leaves	7.5500	ABC
CER Ashes	7.2500	ABCD
CP Ashes	6.8000	ABCDE
CM Leaves	6.7000	ABCDE
CZ Ashes	6.6000	ABCDEF
CM Ashes	6.4500	ABCDEFG
CAD Ashes	6.4000	BCDEFG
CV leaves	6.3000	BCDEFG
CP leaves	6.3000	BCDEFG
CC leaves	6.1500	BCDEFG
CV Ashes	6.0500	BCDEFG
CI Ashes	6.0000	BCDEFG
CkAshes	5.8000	CDEFG
CK Leaves	5.7500	DEFGH
CEL Ashes	5.4000	EFGH
CEL Leaves	5.4000	EFGH

CAD Leaves	5.3500	EFGH
CZ leaves	5.2500	EFGH
CI leaves	4.9000	FGH
Cap Leaves	4.7000	GH
Cap Ashes	4.0000	H

Alpha	0.05	Standard Error for Comparison	0.4830
Critical Q Value	5.142	Critical Value for Comparison	1.7565

There are 8 groups (A, B, etc.) in which the means
are not significantly different from one another