Bioactive Components of Australian Native Plant species and their Potential Antidiabetic Application within the Indigenous Community

A thesis submitted in fulfillment of the requirement for the degree of

Doctor of Philosophy (PhD)

By

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Certification

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College of Health and Biomedicine, Victoria University, St Albans campus, Victoria, Australia. This is to certify that the thesis titled "Bioactive Components of Australian Native Plant species and their Potential Antidiabetic Application within the Indigenous Community" submitted by Collise Njume in partial fulfilment of the requirement for the award of Doctor of Philosophy in Food Processing and Technology at Victoria University is a record of bona fide research work carried out by him under my personal guidance and supervision, and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Prof Andrew McAinch

Signature……. Date……..

Student Declaration

I, Collise Njume, declare that the PhD thesis titled "Bioactive Components of Australian Native Plant species and their Potential Antidiabetic Application within the Indigenous Community" is no more than 100, 000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except, where otherwise indicated, this thesis is my own work.

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Thesis abstract

Type 2 diabetes mellitus (T2DM), driven by overweight and obesity linked to unhealthy diets, is the fastest-growing non-communicable disease in Australia. Considering that food is an important parameter in the regulation of blood glucose response, replacing 'junk food' with products that are good regulators of postprandial blood glucose (PPG) may go a long way to reduce the rate of T2DM in Australia. This study was designed to develop new food products that have the potential for use as nutritional preventatives against escalating levels of T2DM within the Australian Indigenous community. Edible portions of eight Australian native plant species namely; *Leucopogon parviflorus*, *Arthropodium strictum*, *Carpobrotus rossii*, *Rhagodia candolleana*, *Typha orientalis*, *Correa alba*, *Dianella revoluta* and *Acacia longifolia* were collected from the coast of Warrnambool, Victoria Australia. The plant species were analysed for proximate, minerals, fatty acids and phenolic composition following the methods of the Association of Official Analytical Chemistry (AOAC), Inductively Coupled Plasma Spectometry (ICP), High performance liquid chromatography (HPLC) and gas chromatography/Mass spectrometry analysis (GCMS). Plant species that exhibited significantly high amounts of nutrients, antioxidants and antidiabetic polyphenols were selected for development of potential antidiabetic food preventatives. The developed food products (*Acabungi* flakes and crackers) were evaluated for acceptability and the cracker was further studied for stability and microbiological load. Its Glycaemic index (GI) was estimated by *in-vitro* enzymatic starch hydrolysis.

All eight plant species were found to be sources of carbohydrates (39.7 - 65.5%), proteins (2.6 - 15.1%), fats (1 - 14.3%), total dietary fibre (1.5 - 17.2%) and contained Ca, Mg, Na and K. The species exhibited consistent antioxidant activity with phyto-components of gallic acid (GA), epigallocatechin (EPC), catechin (CH), epigallocatechingallate (EPG), dihydroquinidine (DHQ),

ρ-coumaric acid (PCA) and luteolin (LT). The betacyanin, betanidin 5-*O*-β-glucoside (BT) was detected in *R*. *candolleana* (700 mg/kg) and *C*. *rossi* (244 mg/kg) while the alkaloid, Dihydroquinidine (DHQ) was detected in *D*. *revoluta* (101 ± 5.7 mg/kg) and *T*. *orientalis* (17 ± 7.1 mg/kg). However, not all the compounds were isolated from a single plant species and except for BT, higher quantities of components were extractable in methanol than water ($P<0.05$). Palmitic, oleic and linoleic acids were the dominant of 10 fatty acids detected in the native species with record quantities of 4.2 - 39%, 12.3 - 39% and <0.5 - 44% respectively. No single species had all 10 fatty acids. The cracker, with a record overall consumer acceptability of 70.5% remained stable and unchanged with no microbial growth after 35-days storage at room temperature under light and in the dark. The cracker contained significant amounts of total dietary fiber, proteins, complex carbohydrates and exhibited a low GI of 47.7. *R*. *candolleana* and *A*. *longifolia* were found to have high contents of betanin and linoleic acid respectively, two components with potentially wide industrial application. Further studies to determine the antidiabetic mechanism of action of the cracker would shed more light on its potential application.

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List of Publications and Manuscripts

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Abbreviations and Terms

AA	Arachidonic acid
AAE	Ascorbic Acid Equivalence
ABTS	2, 2'-Azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
ADEA	Australian Diabetes Education Association
AHEI	Alternate Healthy Eating Index
AOAC	Association of Official Analytical Chemistry
AMPK	Adenosine Monophosphate-Activated Protein Kinase
ANOVA	Analysis of variance
AUC	Area under the curve
BA	Behenic acid
BT	Betanin
BMI	Body Mass Index
CH	Catechin
CVD	Cardiovascular disease
CC	Control cracker
CDA	Canadian Diabetic Association
$CuSO_4$	Copper sulphate
DA	Diabetes Australia
DAA	Dietitians Association of Australia
DASH	Dietary approach to stop hypertension

DPP	Dipeptidyl-peptidase
DMT	Dimethyltryptamine
DHQ	Dihydroquinidine
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DW	Dry Weight
EA	Erucic acid
EPC	Epigallocatehin
EPG	Epigallocatechingallate
FA	Ferulic acid
FRAP	Ferric reducing antioxidant power
FSANZ	Food Standards Australia and New Zealand
GA	Gallic acid
GAE	Gallic acid equivalence
GC	Gas chromatography
GCMS	Gas chromatography mass spectrometry
GI	Glycaemic index
GIP	Glucose-dependent insulinotropic polypeptide
GLP	Glucagon-like peptide
GLUT	Glucose transporter
HAT	Hydrogen atom transfer
HCl	Hydrochloric acid
HI	Hydrolysis index
HPLC	High Performance Liquid Chromatography

H_2SO_4	Sulphuric acid
IDF	International Diabetic Federation
KOH	Potassium hydroxide
LA	Linoleic acid
LT	Luteolin
MA	Myristic acid
MS	Mass spectrometry
MUFA	Monounsaturated fatty acids
NaOH	Sodium hydroxide
NHMRC	National Health and Medical Research Council
NRS	Non-reducing sugar
NPH	Neutral Protamin Hagedorn
OA	Oleic acid
PA	Palmitic acid
PPAR-γ	Peroxisome proliferation activated receptor-γ
PCA	ρ-coumaric acid
PDA	Photodiode Array
PUFA	Polyunsaturated fatty acids
Q	Quinidine
RP-HPCL	Reverse-Phase High Performance Liquid Chromatography
RS	Reducing sugar
SA	Stearic acid
SET	Single Electron Transfer

SGLT-2	Sodium glucose-linked transporter 2
SU	Sulfonylurease
TDF	Total Dietary Fibre
TC	Test cracker
TEAC	Trolox Equivalent Antioxidant Capacity
T2DM	Type 2 Diabetes Mellitus
TPC	Total Phenolic Content
TZD	Thiazolidinediones
UR	Underground stems and rhizomes
USR	Underground stems and rhizomes
VUHREC	Victoria University Human Research Ethics Committee
WHO	World Health Organization
X	Xanthorrhoea
YPD	Young people with type 2 diabetes
Zn	Zinc

Table of Contents

List of Figures

List of Tables

List of Appendices

CHAPTER 1

INTRODUCTION

This chapter presents a brief overview of the situation of type 2 diabetes mellitus in Australia and the importance of diet in the management and prevention of the disease. Problems associated with treatment of type 2 diabetes mellitus are cited as justification for other alternative control and preventive strategies, followed by presentation of the study objectives.

1. 1 Introduction

Type 2 diabetes mellitus (T2DM) linked to unhealthy food consumption is increasing in Australia (Kaldor et al., 2015). About 2 million Australians have pre-diabetes and are at risk of developing T2DM (Johnson et al., 2015). It is estimated that 275 Australians develop diabetes daily (Gulati et al., 2015; Hill et al., 2017). One million Australians have already been diagnosed with diabetes, type 2 being the most common with a prevalence that seems to rise with age and is higher in men than women (Dunstan et al., 2002). Approximately 3.5 million Australians will have T2DM by 2033 (Gulati et al., 2015; Hill et al., 2017) and food choice is a major contributing factor to this increase (Kaldor et el., 2015).

In addition to its high prevalence, approximately 65% of cardiovascular disease deaths in Australia occur in people with diabetes or pre-diabetes (Barr et al., 2007). The situation is worse in the Indigenous population (McDermott et al., 2010). Indigenous Australians are up to 20 times more likely to have T2DM and twice as likely to be obese than other groups (AIHW, 2006; McDermott et al., 2010; De Abreu et al., 2013; Titmuss et al., 2019). According to Diabetes Australia, diabetes is the epidemic of the 21st century and the biggest challenge confronting Australia's health system

(AIHW, 2018). Uncontrolled diabetes therefore, has dire consequences for the health and well-being of the population including development of complications and depletion of financial resources.

The role of diet in the etiology of T2DM cannot be over emphasized. People in India observed that the disease was common among rich people who consumed oil, flour, and sugar in excessive amounts (Seidell, 1998; Sami et al., 2017). This observation is not too different in Australia where frequent consumption of energy-dense foods has been linked to the disease (Dendup et al., 2018). Avoiding the temptation of eating foods that might worsen glycaemic control has been a major challenge in the management of T2DM. That is why modifications that will see the replacement of simple sugars with fructan or inulin-type carbohydrates and saturated fats with unsaturated fats in human food may help control the alarming rates of T2DM in Australia.

For T2DM to be brought under control, consumption of "junk food", i.e., energy-dense products high in saturated fats, sugar and salt may have to be brought to a minimum and development of new food products that are rich in antidiabetic polyphenols encouraged (Tutelyan et al., 2016). However, it is obvious that the use of food preventatives will be most effective only when other risk factors such as overweight/obesity, inactivity and sedentary lifestyle are under control. Some phenolic antioxidants in native plant species may improve dyslipidemia and insulin sensitivity at the peripheral tissues, attenuate oxidative stress and inflammatory processes that could prevent the development of T2DM complications including cardiovascular disease, neuropathy, nephropathy and retinopathy (Mirmiran et al., 2014). By developing food products with native plant species, it

could be possible to tap into the reservoir of functional properties of many of the species to mitigate the escalation of T2DM in Australia.

1. 2 Rationale for study

T2DM, arising from overweight and obesity linked to unhealthy diets, is the fastest-growing non-communicable disease in Australia (Kaldor et al., 2015). A number of pharmacological agents including metformin/thiazolidinedione (improve insulin sensitivity); exenatide (incretin-mimetic agents that suppress gastric emptying, glucagon secretion and reduction of appetite); Vildagliptin (dipeptidyl peptidase -4-inhibitor) and dapaglifloxin (sodium glucose-linked transporter-2-inhibitor) are available for treatment of T2DM (Rojas and Gomes, 2013; Larvernia et al., 2015; Wu et al., 2016). However, monotherapy with a single agent such as metformin may not achieve adequate glycaemia control (Rojas and Gomes, 2013), requiring the use of 2 or more drugs to manage the disease. Despite adverse effects, cost, lengthy treatment regimens and high pill burdens associated with combination therapy, T2DM elimination is yet to be attained. This calls for renewed efforts to increase investments for prevention and complementary treatment options, especially within the Indigenous population where the condition is at epidemic and severe proportions (O'Dea et al., 2007).

In recent times, coexistence of obesity and T2DM, otherwise known as diabesity (Colagiuri, 2010) has exacerbated management problems for patients and physicians, making it much more difficult to limit its escalation. Considering that food is an important parameter in the regulation of blood glucose response, replacing 'junk food' with native plant products loaded with phyto-components

that are good regulators of postprandial blood glucose (PPG) may go a long way to reduce the rate of diabesity in Australia. The use of native plant species as sources of nutrition, health and general wellbeing is an age-old tradition that has coexisted with the Australian Indigenous population for centuries (O'Dea et al., 1991; Clarke, 2008; Mohanty et al., 2012; Konczak et al., 2014). Some members of the Indigenous community, especially the older generation believe that native species are medicinal and more nutritious than convenience energy-dense products, yet, not much attention is given to them. In fact, with the exception of *Carpobrotus rossii* (Haw.) Schwantes (pig face), a literature search of the native species studied herein did not produce any scientific information on their nutritional and phenolic properties. This is surprising, considering that most native species, being resistant to pests and diseases are able to survive unfavorable environmental conditions, thus accumulating polyphenols and antioxidants that could be used to benefit the control of T2DM in the country. This thesis was designed therefore, to develop antidiabetic food products with native plant species that are rich in antioxidants and antidiabetic polyphenols, in a bid to limit the escalation of T2DM in Australia.

1. 3 Project objectives

1. 4 Overall goal

To investigate the potential application of native plant species as nutritional preventatives of T2DM within Indigenous communities.

1. 5 Specific Objectives

1. To determine proximate composition of the selected plant species; *Leucopogon parviflorus* (Andrews) Lindl*, Arthropodium strictum* R. Br., *Carpobrotus rossii* (Haw.)Schwantes, *Rhagodia candolleana* subsp. *candolleana*, *Typha orientalis* C. Presl, *Correa alba* Andrews, *Dianella revoluta* R. Br. and *Acacia longifolia* subsp. *sophorae* (Labill.) Court.
2. To determine phenolic composition of the selected plant species.
3. To determine free radical scavenging activity of the selected plant species.
4. To develop food products with plant species that have displayed high amounts of antioxidants and antidiabetic polyphenols.
5. To determine acceptability of the food products by conducting sensory evaluation.
6. To determine glycaemic index of most acceptable food products.

It is hoped that potential antidiabetic food preventatives made with edible portions of selected Australian native plant species may constitute part of a national culturally acceptable strategy which employs target nutrition as a means to control the rising level of T2DM within the Indigenous population. However, given that T2DM is a health problem within other groups of the Victorian community (older Australians, rural remote communities and mental health consumers), the food products will target both Indigenous and non-Indigenous populations.

Chapter 2 a

LITERATURE REVIEW

This chapter has been published: Njume, C., Donkor, O., McAinch, A. J. (2019). Predisposing factors of type 2 diabetes mellitus and the potential protective role of native plants with functional properties. Journal of Functional Foods, 53, 115 - *124. The section appears as per the publication, however to assist with clarity and continuity the section, figure and table numbers have been changed, citations reformatted, abbreviations standardised and the language has been standardised to English (Aus). The chapter discusses type 2 diabetes mellitus as a global problem and the commonality of the risk factors. It then goes ahead to describe different plant species within Australia that may be regarded as potential sources of antidiabetic components. It also presents a table of some plant species with antidiabetic properties from different parts of the world.*

2 a. 1 Abstract

Type 2 diabetes mellitus (T2DM) affects at least 285 million people globally and is at epidemic proportions within Indigenous communities in Australia. This review creates awareness about populations most at risk of T2DM, factors that accelerate rise of the disease and the potential protective role of plants with functional properties including non-native (*Moringa oleifera*, *Momordica charantia*, *Panax ginseng* and *Curcuma longa*) and native Australian (*Terminalia ferdinandiana*, *Smilax australis* and *Xanthorrhoea johnsonni*) plants. While risk factors of age, ethnicity and genetics are hard to control, environmental factors of inactivity, lifestyle and diet have to be meticulously tailored to curb the prevalence of T2DM in many parts of the world. Plants with functional properties may be regarded as sources for alternative control measures against

T2DM in a bid to alleviate the burden of disease within Indigenous populations and compensate for limitations of current pharmacological strategies.

2 a. 2 Introduction

Type 2 diabetes mellitus (T2DM) is one of the major threats to global public health in the 21st century (Florez, 2016). It is the fourth or fifth leading cause of death in first world countries and morbidity and mortality are in an upward trajectory in emerging or newly industrialized economies (Tuei et al., 2010; Hu, 2011). T2DM alone accounts for at least 90% of all diagnosed cases of diabetes (WHO, 2016). Many researchers are of the opinion that the global estimate of 285 million cases of this disease may almost double by 2030 (Hu et al., 2011; Patel et al., 2012). Since its discovery, the prevalence of T2DM has always been on the rise, and coupled with obesity is projected to go higher if measures are not taken to arrest its escalation (van Dieren et al., 2010). It has been hypothesized that developing countries witnessing economic boom characterized by rapid changes from traditional to 'westernized diet' and nomadic to sedentary lifestyle are most at risk of a T2DM epidemic by 2030 (Tuei et al., 2010; Hu, 2011). This hypothesis is already almost confirmed in some developing countries and within some Indigenous communities of a few developed nations such as Australia, Canada and the United States (Naqshbandi et al., 2008; Unnikrishnan et al., 2017).

In developed countries there is improved longevity due to improved sanitation and social services accompanied by urbanisation and industrialization. However, infectious diseases seem to have been overtaken by disturbing increases in the number of some non-communicable diseases such

as cardiovascular disorders (CVD), cancer and T2DM (Allen, 2017). According to Diabetes Australia (DA) and the Australian Institute of Health and Welfare (AIHW), 65% of CVDs deaths occur in people with diabetes or pre-diabetes. Diabetes is a major cause of illness, disability, death and therefore public health priority in Australia (AIHW, 2012). At least 8% of men and 6.8% of women have T2DM in Australia resulting in an overall prevalence of 7.4 % (Dunstan et al., 2002; Tran et al., 2014). The disease is at epidemic proportions within the Australian Indigenous population (McDermott et al., 2010; Shukla, 2010). Aboriginal Australians are 5-10 times more likely to have T2DM than the rest of the population (de Abreu et al., 2013).

It is believed that a rapid dietary transition from bush animals, seeds, unrefined carbohydrates, of high dietary fibre and protein content to a 'westernized diet' with elevated levels of fats, sugar and salt could be accelerating the unfavourable health transition characterized by high prevalence of T2DM within the Australian Indigenous population (AIHW, 2012). This situation is not unique to Aboriginal Australians or Torres Strait Islander people. In some developing nations, rapid economic and social development with concomitant shifts in life style habits and dietary structure consistently work together with sedentary lifestyle to increase body weight and central adiposity, thus increasing the risk of T2DM (Hu, 2011). A few Indigenous groups in different parts of the world are also showing similar trends in the prevalence of T2DM and other non-communicable diseases as they make the transition from unrefined staples to 'convenience foods' (Oster and Toth, 2009; Tuei et al., 2010). Latest reports from some parts of Africa, Eastern Mediterranean region, Western Pacific region, South and Central Asian regions where economic improvements have affected the diet and lifestyle of the population are witnessing an increase in the prevalence of T2DM (Zimmet et al., 1990; Tuei et al., 2010; WHO, 2016). For example, the Nauruans in the

South Pacific (Zimmet et al., 1990) and some Indigenous groups of Sub Saharan Africa have seen significant increases in rates of T2DM in the presence of concomitant rapid nutritional and lifestyle changes coming with improved national economy (Tuei et al., 2010). According to Oster and Toth (2009), Canadian Indigenous groups of Mètis, Inuit and First Nations are considered high risk populations of T2DM. The prevalence rates within First Nations are 2 - 5 times higher than the rest of the Canadian population. In the United States of America, American Indians and Alaskan native adults are 2.3 times more likely to have diabetes, mostly T2DM than the rest of the population (O'Connell et al., 2010). O'Connell et al. (2010) also reported higher rates of diabetes co-morbidities and complications such as hypertension, cerebrovascular disease, renal failure and lower extremity amputations in these groups accompanied by mortality rates that are 4 times higher than the rest of the American population. Rapid nutritional transition thus seems to be one of the precipitating factors of T2DM within Indigenous communities around the world.

According to AIHW, advances in food technology alongside increases in the number of large supermarkets have led to growing preference for 'convenience foods' leading to an increase in a number of chronic diseases such as T2DM, CVDs and cancer that are major causes of death in the country. Groups that are most at risk of these conditions include older Australians, Aboriginal and Torres Strait Islander people, rural and remote Australians, and socioeconomically disadvantaged groups. Communities making a transition from rural to urban settings have a tendency to consume high-calorie foods containing high amounts of sugar, salt and fats. From the days of 'early man' to 'modern man', the nature, storage and preparation of the human food has been changing rapidly. Demand for extra nutritious food has encouraged rapid production of generic products also as means to assist people on low incomes to meet dietary guidelines (Chapman et al., 2013). It may

require several generations for the human metabolic machinery to completely adapt to some new brands of foods. It is important to note therefore that despite variability and differences in cultures and traditions among the world's Indigenous groups, the consequences of rapid nutritional transition, chronic over nutrition and adoption of sedentary lifestyles seem to be presenting similar metabolic patterns characterised by high risk of T2DM, obesity and associated co-morbidities. These patterns are likely to be the result of interactions between environmental changes and genetic susceptibility as postulated by Neels' thrifty genotype hypothesis (Yu and Zinman, 2007; de Abreu et al., 2013) stated as follows: an energy-conserving genotype that was previously adaptive in a hunter-gatherer era of ''feast or famine'' is now maladaptive in our current sedentary era of ''feast'' but not famine, resulting in the phenotype of diabetes (Yu and Zinman, 2007; Tuei et al., 2010). It would be appropriate therefore to say that the metabolic machinery of *Homo sapiens* was naturally not designed to cope with the excesses of sugar, salt and fats that are the facets of some diets of modernity or extra nutritious products. Rather, a species that lives on berries, wild fruits, tubers and bush animals hunted in long distance walks and high speed chases.

In 2007, the International Diabetic Federation (IDF) released a 3-step approach to the management of T2DM within nations; firstly, to identify populations most at risk of the disease, measure the risk and, intervene to arrest the onset of the disease through health behavioural strategies and modification of risk factors. Some risk factors such as age, ethnicity and genetic predisposition are almost always impossible to manage but modifiable factors of lifestyle (smoking, alcohol-intake, demand for high energy extra nutritious foods and physical inactivity) can be meticulously pursued. Prevention of T2DM within the Australian Aboriginal community would require more than just a pharmacological intervention. Creating awareness on modifiable risk factors should be

fundamental in the campaign for cost effective strategies against T2DM. A strategy that makes use of Indigenous/natural foods with unrefined carbohydrates, high in dietary fibre and protein contents could be readily acceptable and relatively healthier. This approach will be worth pursuing because for many people with diabetes, the challenge has always been to avoid foods that may cause spiking of their blood glucose levels despite their attractive taste, aroma and flavour. Considering that dietary changes and food choices are important parameters in the regulation of blood glucose levels, it becomes imperative therefore for governments and research institutions to pursue control/preventive measures in food therapy or dietary intervention strategies.

Australia's Strategy against T2DM is relatively new and found wanting in its failure to emphasize the importance of tailoring control measures towards cultures and traditions of Aboriginals and Torres Strait Islanders most affected by this disease. Equally important is the need for adequate action plans to reduce overweight and obesity (WHO, 2016), which are important co-morbidities of T2DM. Indigenous populations most affected by this disease would require health promotion, disease prevention and management strategies that are culturally appropriate to facilitate acceptability and compliance. This paper examines major risk factors of T2DM within the population while bringing to public attention the potential preventive role of native plants with functional properties.

2 a. 3 Risk Factors of Type 2 Diabetes Mellitus

2 a. 3. 1 Genetic predisposition

Genetic variants contributing to susceptibility of T2DM have been identified (McCarthy 2010; Hu and Jia 2018). Due to high concordance rates seen with identical twins, siblings and ethnic groups (Valdez, 2009), there is reason to believe that T2DM is genetic with strong family history. Generally, in the Western world, Hispanics, South Asians and Indigenous populations are considered to be at high risk of T2DM epidemic (CDA, 2013). In the United Kingdom, on average if either parent has T2DM, the risk for the offspring developing the disease is 15% and 75% if both parents have it (Valdez, 2009). Screening people for family history of T2DM as recommended by WHO is a suitable way of identifying populations most at risk and providing informed choices that would minimise the risks. It is important to note that genetic determinants alone are not enough to develop the disease. They work in consonance with environmental factors, some of which are described herein.

2 a. 3. 2 Age

T2DM was formerly known as "adult-onset" diabetes because it was uncommon before the age of 40 years, an indication that the risk of getting the disease increases with age probably due to progressive increases in insulin resistance (Ramlo-Halsted and Edelman, 2000; Fletcher et al., 2002). However, as much as this is true, there has been a rising prevalence in younger age groups (Fletcher et al., 2002; Suastika et al., 2012). Middle age people with abnormal glucose tolerance have a huge possibility of developing the disease later in life, a troubling issue considering that 17.8% of Australia's population will be over 65 years old by 2021. The situation is worse within

the Australian Indigenous population with increase in prevalence from 0.5% for 2-14 year age group to 40% for those ≥55 years respectively (Table 2 a. 1; Burrow and Ride, 2016).

Table 2 a.1**: Age distribution of type 2 diabetes mellitus within the Australian Indigenous population**

Age group (years)	**% Prevalence**
2-14	0.5
15-24	1.4
25-34	5.3
35-44	11
45-54	23
55+	40

Source: (Burrow and Ride, 2016).

Pathophysiological studies of glucose tolerance in the elderly indicate insufficiency in β-cell functionality in the presence of increasing insulin resistance and hyperglycemia as major causes of this problem (Chang and Halter 2003). Approximately 0.7% decrease in insulin secretion occurs per year with age in normal individuals and may be twofold in people with impaired glucose tolerance (Szoke et al., 2008). Unfortunately, age, ethnicity and genetic predisposition are the three main risk factors of T2DM that are not modifiable.

2 a. 3. 3 Overweight/obesity

Obesity is the result of imbalances between energy intake and energy expenditure (Colagiuri, 2010). According to WHO, 2.8 million people die annually as a result of overweight or obesity, a preventable epidemic. Close to 2 billion people worldwide are overweight (BMI 25.0 - 29.9 kg/m^2) and 650 million are obese (BMI ≥ 30 kg/m^2). The prevalence of overweight or obesity in the world's adult population is predicted to rise from 33% in 2005 to 57.8% by 2030 (Chen et al., 2012). About 65% of people live in countries where overweight and obesity kills more than underweight (WHO 2013; Ahmed et al., 2014). According to AIHW (2017), 63% of Australian population (≥18yrs old) are overweight or obese; the majority of them are men. Again, the situation is not any better within the Indigenous population where 66% of the population (≥15 years) are either overweight or obese (Esler et al., 2016). This is very disturbing considering that overweight /obesity is a major risk factor of T2DM. Excessive accumulation of fat as observed in visceral adiposity, a common feature of obesity has been associated with insulin insensitivity, supporting development or worsening an already existing case of T2DM (Gohda et al., 2008; Colagiuri, 2010). Compared to those who have a healthy body weight, those with a body mass index (BMI) of 25.0-29.9 kg/m^2 have 3 times greater risk of developing T2DM while those with a BMI over 30 kg/m^2 have a 20 fold increased risk (Field et al., 2001). With compelling evidence of this association now accumulating, the word ‘diabesity’ has been coined to describe it (Colagiuri, 2010).

2 a. 3. 4 Diet

Good nutrition contributes to better quality of life, helps maintain healthy body weight, protects against infections, and reduces the risk of chronic disease and premature death (AIHW, 2012). Since the 1960s, Australia has witnessed a nutrition transition, resulting in the adoption of a 'westernized' diet with a shift away from unrefined foods (Naughton et al., 2015). This nutritional transition has also affected the Indigenous communities of various countries, with the Australian Aboriginal community witnessing a troubling increase in the prevalence of T2DM and associated co-morbidities and complications (Burrow and Ride, 2016). This unfortunate situation is believed to be the result of rapid dietary transition and sedentary lifestyle which has also been reported with other Indigenous communities around the world that have made similar changes (Zimmet et al., 1990; Tuei et al., 2010; WHO, 2016). It is understood that dietary patterns are consistently associated with risks of T2DM even when other lifestyle factors are under control (McEvoy et al., 2014). Foods, especially those rich in carbohydrates ultimately have an effect on the blood glucose levels (Georgoulis et al., 2014; Esposito et al., 2015).

The type and/or amount of food eaten greatly impacts on other lifestyle factors such as overweight and obesity which are well known contributors of T2DM. In a study of the Mediterranean diet and T2DM, Esposito et al. (2015) reported that higher adherence to specialized Mediterranean diet significantly reduced the risk of disease. They concluded that the Mediterranean diet was associated with better glycaemic control and cardiovascular risk factors than control diets including a lower fat diet and suggested that it was suitable for management of T2DM. The Mediterranean diet involves high consumption of olive oil, vegetables, whole grains, fruits and

nuts, moderate consumption of fish and chicken accompanied by low consumption of full fat dairy products and meat, topped with low to moderate amounts of wine (Sofi, 2009). A few other dietary plans including DASH (dietary approach to stop hypertension) and AHEI (alternative healthy eating index) and vegetarian diets have been found to be associated with significant reduction in T2DM (Esposito et al., 2015). The mechanism of protection of these diets may be, at least partially, due to high anti-inflammatory and anti-oxidative content of the diets due to the presence of certain vitamins, high dietary fibre, high in antioxidants and plant phenols. It is worth mentioning that although different diets have been associated with control of T2DM, the American Diabetic Association does not recommend any specific diet for prevention of the disease. That notwithstanding, a low intake of dietary fibre, low GI carbohydrates and whole grain cereals has been associated with greater risk of T2DM (Vessby et al., 2001).

2 a. 3. 5 Inactivity and sedentary life style

About two thirds of the major causes of death are, to a significant degree, lifestyle-related (Knight, 2012). A sedentary or inactive lifestyle is a major risk factor for T2DM owing to the fact that it might cause accumulation of fats that might impact negatively on insulin sensitivity. The importance of physical activity for improving glucose homeostasis, insulin sensitivity, overweight and obesity are well established (Blair, 2009; Silverman and Deuster, 2014). Exercise improves normal physiological functions, reduces abdominal adiposity and improves circulation and mental alertness (Gerber and Puhse, 2009). High physical fitness is inversely related to metabolic stress-related disorders such as T2DM, obesity and overweight (Gerber and Puhse, 2009). Exercise may also offer anti-inflammatory effects, offering protection for β-cells against glucotoxicity,

lipotoxicity, chronic inflammation, obesity and overweight (Codella et al., 2015). A more active lifestyle in combination with physical exercise may prevent the accumulation of abdominal fats and has been reported to improve glucose metabolism by improving insulin sensitivity in people with T2DM (Knight 2012).

2 a. 3. 6 Cigarette-smoking and alcohol-intake

Smoking is a risk factor of many non-communicable diseases (CVDs, cancer and lung disease) and therefore gravely detrimental to human health. It causes insulin insensitivity, dislipidaemia and inflammation (Chang, 2012; Seet et al., 2012). There are reports of its consistent association with obesity (Ding et al., 2015), oxidative stress, and diabetes (Sairenchi et al., 2004; McGuire et al., 2014), probably due to nicotine's ability to cause loss of pancreatic β-cell mass and destruction (Bruin et al., 2008). Being a risk factor of other chronic conditions (e.g., CVD), smoking is known to precipitate mortality of people suffering from T2DM by increasing their risk of coronary heart disease and stroke (Chang, 2012). According to the Australian Government Department of Health, Australia's smoking rate is about 13% nationally. However, the smoking rate within Aboriginal and Torres Strait Islander community (a high risk population of T2DM) is close to 50% (Willcox, 2014), which puts them at a greater risk of developing the disease and complications than the rest of the Australian population. It is therefore not surprising that the rate of CVD deaths is equally on the rise within Aboriginal and Torres Strait Islander communities.

Excessive alcohol intake is associated with a huge burden of disease in Australia; especially among Indigenous Australians where the burden of disease due to alcohol is almost double that of the

general Australian population (Wilson et al., 2010). It has been reported that high alcohol-intake and tobacco-smoking work together with young maternal age to provide a poor intra-uterine environment for many Indigenous Australian babies, and contribute significantly to high perinatal morbidity and future disability (McDermott et al., 2009). Alcohol, when taken in moderation (5 - 29.9 g/day in men and 5 - 19.9 g/day in women) may be protective and at those levels is actually associated with reduced incidence (≈30%) of T2DM compared with lower intakes (Carlsson et al., 2003; Koppes et al., 2005). Unfortunately, no risk reduction has been documented for consumptions ≥48 g/day (Koppes et al., 2005). Inhibition of lipolysis by moderate alcohol consumptions may cause reduction in free fatty acid levels, offering more protection against T2DM (Metcalf et al., 2014). On the other hand, higher intakes may stimulate increases in body weight (especially abdominal mass), blood pressure and triglyceride concentrations (Metcalf et al., 2014) which may negatively affect insulin sensitivity. With correlations of excessive alcohol intake and high incidence of T2DM now established, any anti-diabetic preventive strategy within the Aboriginal community will have to address the issue of high alcohol intake.

2 a. 4 Application of native plants in the prevention of T2DM

Many studies have indicated the salient role of plants in the fight against T2DM (Sakthiswary et al., 2014; Yin et al., 2014; Dey and Mitra, 2013; Salim, 2014). Compounds with α-glucosidase inhibitory activities seem to be ubiquitous in many plants (Vuong et al., 2014; Yin et al., 2014; Zhang et al., 2014). Considering that α-glucosidase is an important enzyme in glucose metabolism, its inhibition will ultimately affect the amount of carbohydrates absorbed from the gut and consequently the amount of glucose in the blood stream. It is also interesting to note that the active

ingredient of metformin, a popular drug that has shown significant effectiveness in arresting disease progression of T2DM was initially isolated from the herb *Gallega officinalis* L. (Patade and Marita, 2014). There is need therefore for research institutions to intensify investigations into the application of alternative therapeutic and preventive measures such as the use of locally available functional plants with novel mechanisms of action. Such measures may be important in remote rural Aboriginal communities where their use is acceptable and highly regarded as a pivotal customary practice. Some Australian native plants that are available within Indigenous communities yet ignored due to changing social/feeding habits have been discussed herein. These could hopefully offer informed choices that would alleviate the burden of T2DM within the Australian Aboriginal community and increase the armamentarium of anti-diabetic efficacy in their daily staples.

2 a. 4. 1 Kakadu plums (*Terminalia ferdinandiana* Exell.)

Kakadu plum is a native Australian plant which produces pale green fruits renowned for their high vitamin C content (≈5100 mg/100 g), more than 100 times the amount found in oranges and 900 times in blueberries (Netzel et al., 2007). The plant is also known as Gubinge, the Aboriginal name, billygoat plum or Murunga (Cock, 2015). It belongs to the family Combretaceae and grows as a small semi-deciduous fruit-bearing tree, 5 - 10 m high (Konczak et al., 2014). It is found in the Northern and Western regions of Australia. It has been an essential part of Aboriginal culture in Western Australia, consumed as a fruit during hunting; otherwise the succulent fleshy part is removed, pounded, soaked in water and used to make a refreshing drink (Konczak et al., 2014). The fruits of this plant are rich in phenolic compounds (flavonoids, anthocyanins, tannins and

gallic acid), many of which have demonstrated antimicrobial, antioxidant, anti-inflammatory and anticancer properties (Cock, 2015). However, scientific evidence to support its application in the treatment or prevention of T2DM is lacking. The nutritional composition indicates presence of proteins, carbohydrates, fibre, high potassium/sodium ratios, neutral sugars, reducing sugars and absence of fats (Konczak et al., 2014). These properties together with its rich antioxidant content may favour its application in the prevention of non-communicable diseases such as cancer, T2DM and co-morbidities within Australian Aboriginal communities. Fortunately, the use of this plant is no longer limited within the Indigenous population. In fact, while Aboriginal uses of Gubinge seem to be declining in the light of urbanisation, local industries are producing nutraceuticals, beverages, chutneys, jams, cosmeceutical and pharmacological products for national and international markets (Zhao and Agboola , 2007; Gorman et al., 2016). Despite the fact that this may be of enormous economic potential, awareness campaigns about its potential health benefits and support for inclusion in daily staples could prove useful in limiting the development of T2DM and complications within the Australian Indigenous population.

2 a. 4. 2 Illawarra Plum (*Podocarpus elatus* R. Br)

Illawarra plum is a tall to medium size ornamental tree with a spreading crown found growing in the rainforest of the Eastern region of Australia and around the border between New South Wales and Victoria. It is also known as pine plum or brown pine and belongs to the family Podocarpaceae (Tan et al., 2011). It produces purple seed cones with fleshy base that can be eaten fresh or cooked. The fruits constitute a source of food and medicine in the traditional Aboriginal population (Vuong et al., 2014). The bi-segmented fruit is composed of a hard seed about 1 cm wide, and a large,

fleshy, purple-black, seedless, grape-like stalk about 2.5 cm in diameter (Netzel et al., 2006). Illawarra plum is plumlike in taste with a resinous flavour. The fruit pulp is used as bush tucker, condiment and sometimes made into jams, jellies and sauces (Low, 1991). The plant is reported to have high content (122.77 μmolTE/ g FW) of anthocyanin-rich phenolics (Netzel et al., 2007; Konczak et al., 2008), responsible for its high antioxidant properties demonstrated by its ability to reduce proliferation of colon cancer cells through alteration of cell cycle, increased apoptosis and possible induction of autophagy (Symonds et al., 2013). Its rich phenolic and antioxidant potential may be an indication of its therapeutic and prophylactic protection against cancer, diabetes and thus validation of its prior Indigenous uses.

2 a. 4. 3 Davidson's plum (*Davisonia jerseyana* F. Muell and *D. Pruriens* F. Muell)

D. jerseyana and *D. Pruriens* are the 2 most common species found in the Australian rain forest growing as beautiful ornamental trees 8 m to 20 m in height with symmetrical crown, hairy stems and leaves. They produce large crimson red fruits with a tart to sour taste (Jensen et al., 2011). Unripe fruits have a very unpleasant taste, almost non-palatable. They belong to the family Cunoniaceae and are noted for their high antioxidant and phenolic content (Miller et al., 1993; Sommano et al., 2013). There is a huge commercial interest for ripe fruits due to their use in production of jams, jelly and wine. They are also useful as ice-cream colorants owing to their 95˚C-stable anthocyanin-based content. The fruits of *D. jerseyana* and *D. Pruriens* are low in sugar and high in antioxidants (Jensen et al., 2011), yet very little information is available on their medicinal properties and potential application in the management of T2DM within the Indigenous population.

2 a. 4. 4 Wattle seeds (*Acacia* species)

Acacia species are flowering plants ranging from low-growing to large shrubs and shade trees. They are dominant in central Australia and the seeds of some species are consumed by the Aboriginal population as a staple food (Miller et al., 1993; Konczak et al., 2010). They have a nutty flavour and varieties of these seeds from Australia's *Acacia* species are edible. Wattle seeds are used as food-flavouring agents and incorporated into various foods such as cream, granola, chocolate and bread (Maslin et al., 2004). Commonly traded species include *Acacia colei* (Cole's wattle), *A. victoriae* (Bardi bush) *A. Arabica* (Indian gum) *A. coriacea* (wiry wattle), *A. melanoxylon* R. Br (blackwood acacia), *A. pycnantha* Benth (Golden Wattle), *A. murrayana* (Sandplain Wattle), *A. retinodes* (Silver Wattle) and *A. sophorae* (Coastal Wattle).

They belong to the family Mimosaceae and close to 1000 different species exist in Australia, many of which have not been fully investigated (Soumyanath, 2005). However, they are widely known in the community due to the fact that Australia's national floral emblem is *A. pycnantha* Benth (golden wattle), with National 'wattle day' celebrated on 1st September every year. Seeds of *Acacia* species are consumed fresh or dried, ground into flour and baked into damper, a type of 'local bread' produced by the Australian Aboriginal community (Ee et al., 2011; Ee and Yates, 2013). Sometimes, gum produced by *A. melanoxylon* R. Br, is also harvested and used as food. The seeds contain about 18.2% proteins, are gluten-free, and the flour has a low glycemic index (Hegarty et al., 2001). In a study of *A. victoriae* Bentham, Ee et al. (2011) reported a decrease in total and soluble carbohydrates of roasted seed samples without a corresponding decrease in protein content. However, the benefits of such findings on the management of T2DM are yet to be investigated.

Not much has been reported on the application of Australian wattles in the fight against T2DM. However, in Indian traditional medicine, *A. arabica* is used as a secretagouge to release insulin and reduce complications of diabetes (Prakash et al., 2015).

2 a. 4. 5 Quandongs (*Santalum acuminatum* R. Br)

Quandong is a hemi parasitic plant belonging to the family Santalaceae (Howes et al., 2004). Other species in Australia include *S. album*, *S. lanceolatum*, *S. murrayanum*, *S. obtusifolium* and *S. spicatum* (Applegate and McKinnel, 1993). *S. acuminatum* is commonly found in Southern Australia where it grows on nitrogenous plants such as *Acacia*, *Casuarina* or on legumes and shrubs (Lock, 2010). It is a slow-growing perennial plant (Loveys et al., 2002). It produces fruits that are initially green but become increasingly attractive with a bright red colour when fully ripe. Fruits were eaten fresh by Aboriginal Australians or stored to be consumed later in the year. Oil is extracted from the kernel within the fruit and used for medicinal purposes by Aboriginal Australians (Misra and Dey, 2012). They introduced the plant to early European settlers, who made jams, sauces, ice creams and cakes generating a huge commercial interest for the fruits (Loveys et al., 2002). The fruits contain relatively high amounts of potassium (629 mg/100 g), important in the regulation of blood pressure levels (Brand and Cherikoff, 1985). The kernel oil is likely to contain components of Sandalwood oil such as α and β-santalols with antimicrobial and anti-inflammatory effects as previously reported in other members of the genus (Sindhu et al., 2010).

2 a. 4. 6 Bush tomatoes (*Solanum centrale* J. M. Black)

S. centrale is a fast growing shrub found in the arid and semi-arid areas of Australia (Miller et al., 1993; Lee, 2012). It belongs to the family Solanaceae and is locally known as bush tomato or Australian desert raisin (Robins and Ryder, 2008). Its green fruits measure 1 - 3 cm in diameter and are yellow in colour when fully ripe (Lee, 2012). They are rich in vitamin C and have been used as food by Indigenous Australians for many years (Konczak et al., 2009). However, the fruits are not consumed fresh, they are usually sundried, powdered and used as a condiment in food. *S. central* contains selenium, an important rare element and a high amount of iron, approximately 26.5 mg/100g (Konczak et al., 2009). The fruits have a strong pungent taste of caramel and have recently been used as ingredient in chutneys, sauces and spice sprinkle. The dried ground fruit is sold as 'Kutjera powder' and added to bread mixes, salads, sauces and cheese dishes (Cleary et al., 2008). In some parts of Central Australia, the fruits of *S. centrale* are commercially grown by Aboriginal Australians in a bid to meet the huge commercial interest and also rural developmental needs.

Despite its potential commercial interest, Australian Aboriginals believe that the fresh fruits of *S. centrale* are poisonous, especially the green ones and have been used by Australian Aboriginal women as a herbal agent for abortions. There are scanty reports on its high alkaloid content with solanine being one of its toxic alkaloids, but these are inconclusive (Konczak et al., 2009). Solanine is found in other members of the genus including *Solanum tuberosum* (potatoes), *Solanum lycopersicum* (tomatoes), *Solanum melongena* (eggplants) and possibly many other members of the Solanaceae family (Manrique-Moreno et al., 2014). It is a steroidal glycoalkaloid and in some

parts of the world has been included as an ingredient in the production of contraceptives (Cham, 2007). Apart from that, solanine is reported to demonstrate anticancer activity by apoptosis and necrosis of cancer cells (Cham, 2007; Manrique-Moreno et al., 2014).

2 a. 4. 7 Tasmanian pepper (*Tasmannia lanceolata* Poir A. C. Sm)

T. lanceolata is a medium to large shrub (2 - 5 m tall) found in the temperate rain forest of Tasmania and the South Eastern region of Australia (Cock, 2013). It is also known as Tasmanian or Mountain pepper and belongs to the family Winteraceae (Read and Menary, 2000). The name comes from its location and its production of small fleshy black-lobed berries with spicy pepper-like flavour. The berries are harvested, dried, crushed and used by Aboriginal Australians as flavourings in traditional food and other parts of the plant for treatment of colic, skin infections and stomach ache (Cock, 2013). European settlers also used the berries as a food flavouring (Cock, 2013). The plant is rich in terpenes and phenolic compounds, especially antioxidants such as anthocyanins and anthocyanin glycosides (Cock, 2013; Sakulnarmrat et al., 2013). The plant fruits are rich in lutein, a carotenoid antioxidant compound associated with eye health (Cock, 2013). Its extracts are reported to demonstrate broad antimicrobial activities against bacteria and fungi (Winnette et al., 2014). The plant is rich in flavonoids, many of which could protect against cancer and CVDs (Cock, 2013). The volatile constituents of this plant are mostly made of sesquiterpenes, some of which have demonstrated anticancer activity *in vitro* (Tatman and Mo, 2002). Beta-caryophyllene and camphene purified from *T. lanceolate* both demonstrate suppressive growth activity towards B16 melanoma and human HL-60 leukemia cells (Tatman and Mo, 2002).

However, these compounds and others in the plant are yet to be scientifically evaluated for potential application in the management of T2DM within the Indigenous population.

2 a. 4 .8 Sarsaparilla vines (*Smilax australis* R. Br and *S. glyciphylla* Sm)

S. australis is a tough, long and slender Australian vine found in the rain forest areas and shady bushes of New South Wales (Packer et al., 2012). Its stems have sturdy prickles, thus the name, barb wire vine. It is also referred to as sweet sarsaparilla because of the sweet taste of the leaves. *S. smilax* and *S. glyciphylla* are native to New South Wales and both belong to the family Smilaceae (Cox et al., 2005). *S. glyciphylla* produces narrow leaves and is known as narrow leaf sarsaparilla. The young soft red leaves are a popular snack among Aboriginal Elders. The leaves are used by the Indigenous population to treat diabetes, arthritis and rheumatism (Packer et al., 2012). However, there is no scientific validation of their anti-diabetic properties in the literature. Solvent-extracts of both plants have been tested for antimicrobial activity but did not demonstrate any activity against the tested organisms (Packer et al., 2015).

2 a. 4. 9 Grass trees (*Xanthorrhoea johnsonni* A. T. Lee)

Xanthorrhoea johnsonni is a slow-growing endemic Australian perennial plant, commonly known as the grass tree because of its long grass-like leaves usually at the tip of its trunk (Cock and Kalt, 2012; Zalucki et al., 2013). Sometimes it is also called black bully because of its black trunk resulting from the after effect of bush fires on its unshed thatch of dead leaves. It grows naturally on gravel or sandy soil around hills with good drainage and forms thick trunks that only increase

by about 1 cm a year. The plant is able to grow to about 4 m tall and can survive for more than five centuries. It belongs to the family Xanthorrhoeaceae and produces a long spectacular flowery stalk which may be 2 - 3 m in height and bear seeds (Cock and Kalt, 2010). The plant has several uses within the Australian Indigenous population. It is used to make tools, weapons, resin and its nectar is harvested for making sweet drink while the seeds are ground and mixed into dough to produce a local form of bread called 'damper' (Cock and Kalt, 2010). It is hypothesized that *Xanthorrhoea* species must have developed chemical deterrents to prevent foraging herbivores, allowing juvenile plants to develop to maturity (Cock and Kalt, 2012). Gas Chromatography/Mass Spectrometry (GCMS) and High Performance Liquid Chromatography (HPLC) analysis indicate that the plant contains O-hydroxycinnamic acid as a likely compound responsible for most of its bioactivity. However, the nutritional composition and anti-diabetic properties of *X. johnsonni* remains unknown despite its use as an important source of food within the Australian Aboriginal community. Other species in this genus include *X. australis*, *X. arenaria*, *X. nana* and *X. minor*.

2 a .5 Water Cumbungi (*Typha* Species)

These are semi aquatic plants with grass-like leaves found in slow flowing streams, ponds and dams. They are locally known as Water Cumbungi or Bulrush and consist of *T. latifolia* (weed), *T. orientalis* C. Presl. (broad leaf species) and *T. domingensis* Pers. (narrow leaf species) commonly found in South Eastern or South Western Australia (Gott, 1999). They belong to the family Typhaceae (Gallardo-Williams et al., 2002). Aboriginal Australians use the plant pollen to make a form of traditional bread. The plants produce thick white roots and rhizomes which are harvested in spring or summer, peeled, roasted, baked or boiled and consumed by Aboriginal

Australians. Its uses as food are also popular in New South Wales, South Australia, Western Australia and Victoria. However, the nutritional composition and the anti-diabetic potentials of the pollen, fleshy roots and rhizomes are still to be investigated.

2 a. 6 Non-native plants with proven antidiabetic potential

Globally, many plants have been found to be important reservoirs of anti-diabetic compounds, some of which are already being formulated into medicine for the treatment of T2DM (Coman et al., 2012). For example; one of the world's most prescribed antidiabetic drugs, metformin has the guanide active agent galegine (an alkaloid) initially isolated from G. *officinalis* (Coman et al., 2012; Patade and Marita, 2014). Other compounds isolated from plants include, curcumin from the rhizomes of *Curcuma longa*, momordicine from *Momordica charantia*, strictinin from *Psidium guajava* and anthocyanins from *Aronia melanocarpa* (Coman et al., 2012; Patel et al., 2012). Their mechanisms of action may range from repair and maintenance of pancreatic β-cell function to stimulation of peripheral glucose utilization. S-allyl cysteine sulfoxide from *Allium sativum* acts as insulin secretagogue while some sesquiterpine glycosides exhibit α-glucosidase inhibitory activities, thereby delaying the digestion of carbohydrates in the gut. Equally important is the fact that some prebiotics such as galactooligosaccharides, fructooligosaccharides and inulin are believed to have insulin and glucose regulatory properties (Al-Sheraji et al., 2013). Some of the most important plant families which have been scientifically proven to be sources of antidiabetic compounds have been summarised on Table 2 a. 2.

Table 2 a. 2: **Non-native Australian plants with antidiabetic properties**

Botanical/Common name	**Family**	**Parts used**	**Bioactive principles**	**Antidiabetic Mechanism of action**	**References**
Capsicum frutescens L. (chilli)	Solanaceae	Fruit seeds	Capsaicin	Suppresses inflammatory responses and enhances fatty acid oxidation in adipose tissue and/or liver, both of which are important at peripheral tissues affecting insulin resistance. The effects of capsaicin in adipose tissue and liver are related to its dual action on PPARα and TRPV-1 expression/activation. It also has Insulin-mimetic or secretagogues activities and hypoglycemic activities.	Kang et al., 2010

Rosa rugosa Thunb (beach rose)	Rosaceae	Roots	Sesquiterpene glycosides	Possess α-glucosidase inhibitory activities.	Yin et al., 2014
Allium sativum L. (garlic)	Liliaceae/ Alliaceae	Bulbils	S-allyl cysteine sulfoxide	Acts as an insulin secretagogue and also demonstrate hepato-protective effects	El-Dermadash et al., 2005
Curcuma longa L. (turmeric)	Zingiberaceae	Rhizome	Curcuminoids	Possess α-glucosidase inhibitory activities.	Yin et al., 2014
Zingiber officinale Roscoe (Ginger)	Zingiberaceae	Rhizome	Zingiberine, coumarins and gingerols	Hypoglycaemic activity and improve insulin sensitivity at the peripheral tissues	Al-Amin et al., 2006; Salim, 2014
Allium cepa L. (Onion)	Liliaceae/ Alliaceae	Bulbs	S-methylcysteinesulfoxide	Acts as an insulin secretagogue and also demonstrate hepato-protective effects	El-Dermada

					sh et al., 2005
Glinus oppositifolius L.	Molluginaceae	Aerial parts	Triterpene saponins	Compounds exhibit antioxidant activity and α-glucosidase inhibitory activities.	Behera et al., 2010; Yin et al., 2014
Camellia sinensis L. Kuntze (green tea)	Theaceae	Leaves	Epigallocatechingallate, epigallocatechin and epicatechin	Reduction of gluconeogenesis and enhancement of insulin secretion, protection of pancreatic β-cells by amelioration of cytokine- induced cell damage.	Patel et al., 2012
Piper sarmentosum Roxb (cha phluu)	Piperaceae	Leaves	Phenylpropanoylamides;chaplupyrrolidones A and B.	Exhibit α-glucosidase inhibitory activities	Zar et al., 2012; Yin et al., 2014

Psidium guajava L. (guava)	Myrtaceae	Fruit peels and leaves	Strictinin, isostrictinin and pedunculagin	Compounds possess insulin secretagogue activities and α-glucosidase inhibitory activities.	Patel et al., 2012;Manikandan et al., 2013
Aloe vera L. Burm.f.	Liliaceae	Leaves	Pseudoprototinosaponin A111 and prototinosaponin A111	Compounds enhance secretion of insulin from pancreatic β-cells and inhibit intestinal glucose absorption	Patel et al., 2012Sakthiswary et al., 2014
Ipomea batatas L. Lam. (sweet potatoes)	Convolvulaceae	Tubers	Flavones and acidic glycoproteins	Hypoglycemic effects and improves insulin sensitivity. Decrease output of lipid peroxidation	Kusano et al., 2001;

					Zhao et al., 2007;
Acacia Arabica Lam.	Leguminosae	Seeds	Quercetin compounds	Antioxidant activities, hypoglycemic and hypolipidemic properties	Hegazy et al., 2013
Rubia cordifolia L.	Rubiaceae	Rhizome	Anthraquinones	Exhibit α-glucosidase inhibitory activities	Yin et al., 2014
Gingko biloba L. (Maiden hair tree)	Ginkgoaceae	Leaves	Kaempherol, isorhamnetin and Gingko-flavone glycosides of quercetin.	Free-radical scavenging activities of antioxidants and other phenols	Saravana muttu and Sudarsan am, 2012;
Glycine max L. Merr	Fabaceae	seeds	Genistein	Modulate hepatic glucose and lipid regulating enzyme activities	Park et al., 2006; Gaikwad

					et al., 2014
Solanum melongena L. (eggplant)	Solanaceae	Fruit	Solanine and solanoflavone	Inhibition of α-glucosidase activity	Kwon et al., 2008; Das and Barua, 2013
Fagara tessmannii Engl. (African satinwood)	Rutaceae	Aerial parts, stem bark and roots	2,6-dimethoxy-1,4-benzoquinone, 3β-acetoxy-16β-hydroxybetulinic acid	Possess α-glucosidase inhibitory activities.	Mbaze et el., 2007; Yin et al., 2014
Vernonia amygdalina Delile (bitter leaf)	Asteraceae	Leaves	1,5-dicaffeoyl-quinic acid, dicaffeoyl-quinic acid, and chlorogenic acid	Suppression of gluconeogenesis and potentiation of glucose oxidation through the pentose phosphate pathway.	Ong et al., 2011; Atangwho et al., 2014

Panax ginseng L (Ginseng)	Araliaceae	Roots, leaves and berries	Malonyl-ginsenosides; protopanaxadiol (PPD) and protopanaxatriol (PPT)	These compounds have antioxidant activity and may improve peripheral insulin sensitivity and blood insulin levels	Wu and Basilla, 2005; Liu et al., 2013
Moringa oleifera Lam (Moringa)	Moringaceae	Every part of this plant is used	Isothiocyanate glycosides, moringine and moringinine.	Compounds exhibit antioxidant activity, capable of normalization of high levels of hepatic pyruvate carboxylase enzyme; regeneration of damaged hepatocytes and pancreatic β-cells	Anwar et al., 2007
Momordica charantia L (bitter gourd)	Cucurbitaceae	Fruits	Cucurbitane-type triterpenoids, charantin, polypeptide-p (p-insulin), momordicine II, 3-hydroxycucurbita-5, 24-	Insulin secretagogue-like effect, stimulation of skeletal muscle and peripheral cell glucose utilization, inhibition of intestinal glucose uptake, inhibition of adipocyte	Krawinkel and Keding, 2006; Fernande

			dien-19-al-7, 23- di-O-β-glucopyranoside, Charantin, p-insulin and vicine	differentiation, suppression of key gluconeogenic enzymes, stimulation of key enzymes, HMP pathway and preservation of pancreatic islet cells and their functions. Polypeptide p is an insulin-like hypoglycemic protein. Momordicine II, 3-hydroxycucurbita-5, 24-dien-19-al-7, 23- di-O-β-glucopyranoside have insulin secretary activities from β-cells. Charantin, p-insulin and vicine have hypoglycemic properties through enhancing of glucose uptake at the peripheral tissues.	s et al., 2007; Joseph and Jini, 2013;

2 a. 7 Conclusion

Prevention of T2DM in Australia remains enigmatic amidst rising prevalence within the Indigenous Community. Considering that pharmacotherapy alone is insufficient in limiting this disease, the need to intensify research into alternative management strategies becomes imperative. To encourage acceptability and compliance of antidiabetic regimens, control strategies have to be incorporated with customary practices of Indigenous populations most at risk of the disease in Australia. Non-native Australian plant species including *Momordica charantia*, *Panax* ginseng and *Moringa oleifera* have shown promising results as sources for novel antidiabetic compounds owing to their hypoglycemic effects, insulin secretagogue-like activity, α-glucosidase inhibitory activity and stimulation of peripheral glucose utilization. However, Australia's native biodiversity which constitute about 10 % of the world's plant population, well adapted to harsh environmental conditions and upon which the Indigenous population has relied for nutritional and primary health care for thousands of years remains relatively unexplored. Most of Australia's native plants may constitute a reservoir of functional properties still unknown to the scientific world and have the potential of revealing antidiabetic compounds with novel mechanisms of action.

Chapter 2 b

SUPPLEMENTARY LITERATURE REVIEW

This chapter describes treatment options of T2DM, different classes of drugs and their mechanisms of action. Pictures and descriptions of Australian native plant species that were selected for the study have also been presented.

2 b. 1 Treatment of T2DM

Several pharmacological agents and approaches are available for treatment of T2DM (Gao et al., 2009; Pernicova and Korbonits, 2014). It is advisable that treatment be commenced early enough to curb progression of pathophysiological conditions. The intention is to eliminate the effects of glucose toxicity and preserve pancreatic β-cell function (Kaku, 2010). A sizeable proportion of T2DM patients may require insulin to maintain longterm glycaemic control either as a monotherapy or in conjunction with oral antidiabetic therapy (Pernicova and Korbonits, 2014). With the objective of maintaining patient compliance, protecting patients against complications and co-morbidities, combination therapy has become a regular feature of modern day antidiabetic treatment (Lajara, 2019). Combinations of metformin with other anti-diabetic agents such as thiozolidinediones, incretin-based agents, insulin secretagogues and dipeptidyl-peptidase (DPP)-4 inhibitors are not uncommon (Larvenia et al., 2015). However, monotherapy with metformin is still a suitable drug strategy in most T2DM patients, especially in patients with diabesity because it improves insulin sensitivity, stimulates weight loss and has beneficial effects on several cardiovascular disease risk factors (Crofts et al., 2016).

Major classes of anti-diabetic agents include thiozolidinediones, sulfonylureases, α-glucosidase inhibitors, DPP-4 inhibitors, glucagon-like peptide (GLP)-1-agonist, glinides and amylin agonists (Tuei et al., 2010). Thiazolidinediones reduce peripheral insulin resistance, enhance glucose uptake in skeletal muscle and reduce hepatic glucose output, in addition to anti-inflammatory and some lipid-lowering effects (Saltiel and Olefsky, 1996; Pontarolo et al., 2013). Gliflozins such as dapagliflozin, canagliflozin and empagliflozin act as sodium-glucose linked transporter (SGLT-2) inhibitors (Rosenwasser et al, 2013). Alpha-glucosidase inhibitors (e.g., acarbose, voglibose and miglitol) slow down the digestion of carbohydrates in the gut. The mechanisms of action and adverse effects of different classes of drugs are presented in Table 2 b. 1

Table 2 b. 1: **Major Classes of Anti-diabetic Agents**

Drug class/examples	**Mechanisms of Action**	**Possible Adverse Effects**	**References**
Thiazolidinediones (TZD); Ciglitazone, Pioglitazone, Rosiglitazone, Troglitazone, Englitazone	Agonists of the peroxisome proliferation-activated receptor γ (PPAR-γ), which regulate transcription of a variety of genes encoding proteins involved in glucose homeostasis and lipid metabolism. TZD suppresses hepatic glucose output and triglycerides synthesis while improving glucose utilization in muscles. Lowers basal insulin secretion rates. TZDs also exhibit anti-inflammatory, antioxidant and anti-proliferative properties which have potential for protection against T2DM-associated co-morbidities.	Peripheral oedema and congestive heart failure. Fluid retention due to increased fluid re-absorption in distal nephron and increased vascular permeability in the adipose tissue. Weight gain. Troglitazone was withdrawn from the market because of liver toxicity.	Saltiel and Olefsky, 1996; Lincoff et al., 2007; Yang and Soodvilai, 2008; Haper et al., 2013; Pontarolo et al., 2013.

Sulfonylureases (SU); Tolbutamide, Acetohexamide, Tolazamide, Chlorpropamide, Glimepiride, Glipizide, Gliclazide, Glibenclamide.	Interact with the drug-biding subunit of the pancreatic β-cell ATP-sensitive K-channel at the plasma membrane to trigger a sequence of events leading to release of insulin. They can also stimulate the secretion of somatostatin and suppress secretion of glucagon in δ and α cells.	Hypoglycaemia, increased risk of CVD deaths, cholestatic jaundice, thrombocytopenia, skin rash, weight gain, haemolytic anaemia and sometimes agranulocytosis. All SUs are not the same and adverse effects seem to be agent specific and not necessarily group-based. Second and third generation SUs have less adverse effects.	Ashcroft, 1996; Aquilante, 2010; Sola et al., 2015; Kalra et al., 2015.
Meglitinides; Repaglinide, Nateglinide, Mitiglinide.	These are insulin secretagogues with similar mechanism of action to SUs. They act by stimulation of endogenous insulin.	Minimal cases of hypoglycaemia.	Pontarolo et al., 2013.

	Secretion by β-cells mainly affecting postprandial hyperglycaemia (PPG).		
Alpha glucosidase inhibitors; Arcabose, Voglibose, Miglitol.	These prevent the degradation of complex carbohydrates by competing to inhibit α-glucosidase enzymes located at the brush border of enterocytes in the small intestines, slowing down the digestion of carbohydrates in the gut. Examples of α-glucosidase enzymes inhibited include; α-amylase, glucoamylase, sucrase, maltase and dextranase.	About 80% of the patients have gastrointestinal side effects such as flatulence, abdominal pain and diarrhoea. Elevated transaminases.	Derosa and Maffioli, 2012; Kao et al., 2016.
Incretin-based Agents; DPP-4, GLP-1-receptor antagonist. E.g., Sitagliptin,	Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are the major hormones involved in endocrine signalling of the gut making them suitable targets for	Upper respiratory tract infection, nasopharyngitis and headache with Sitagliptin; and upper respiratory tract infection, urinary tract infection, and	Drucker et al., 2010; Schwartz, 2014.

Saxagliptin, Linagliptin and Vildagliptin.	management of T2DM. They exert their action by potentiating incretin- receptor signalling. DPP-4 inhibitors slow down the inactivation and degradation of GLP-1 and GIP by inhibiting DPP-4. These inhibitors prevent their degradation and keep them at high levels such as those seen during fasting.	headache with Saxagliptin. Sitagliptin-use has been associated with allergy and Stevens-Johnson's syndrome. Side effects of GLP-1 receptor agonists include nausea, vomiting, diarrhoea, headache, and injection site reactions.	
Insulin; NPH, Detemir Aspart Glulisine.	Regulates metabolism of carbohydrates, proteins and fats through activation of specific receptors.	Hypoglycaemia and weight gain.	Haper et al., 2013.
Biguanides; Metformin, Phenformin.	Suppression of hepatic glucose output by up to 30%, improves insulin sensitivity, enhances glucose uptake by	About 30% of patients have diarrhoea, faecal urgency, metallic taste and nausea in the	Olokoba et al., 2012; Pontarolo et al., 2013.

	phosphorylating GLUT-enhancer factor by up to 40%, increases fatty acid oxidation, and decreases the absorption of glucose from the gastrointestinal tract. Inhibits lipolysis and availability of free fatty acids.	first 1-2 weeks of treatment. Anorectic effects. Phenformin has been withdrawn because of severe and sometimes fatal adverse effects.	
SGLT-2 Inhibitors; Dapaglifloxin, Empagliflozin, Canagliflozin and Ipragliflozin.	Decreases re-absorption of glucose from the renal proximal tubule leading to increased urinary excretion of glucose and consequently loss of calories.	Urinary tract infections due to glucosuria.	Pontarolo et al., 2013.

2 b. 2 Plant species selection for this study

A number of native Australian plant species are used by the Indigenous community for nutrition, health and general wellbeing (Gott, 1983; Pascoe, 2014). Following consultation with an Indigenous Elder and an Indigenous community member, as well as availability of the plants, the species detailed in the following section were selected for further analysis. .

2 b. 2. 1 *Acacia longifolia* subsp. *sophorae* (Labill.) Court (coast wattle)

Acacia longifolia subsp. *sophorae*, commonly known as coast wattle is a low-growing shrub or small tree (0.5-3 m) that may occasionally grow to a height of about 5 m. It produces small elongated green leaves and densely arranged clusters of elongated golden yellow flowers (Entwisle et al., 1996). It flowers in late winter or early spring and bears pods that are initially green but become brown as they mature, holding 3 - 5 hard black seeds which drop to the ground as the pod dries and cracks open (Fig 2 b. 1). Seeds are available late November to early April and are eaten and dispersed by birds through their droppings.

The plant is distributed along the Victorian volcanic plain, Victorian Riverina, Warrnambool plain, spreading in abundance along the coast (Entwisle et al., 1996). *A*. *longifolia* subsp. *sophorae* belongs to the family Fabaceae (Entwisle et al., 1996). Its hard black seeds with brown fleshy arils have been used as food by members of the Australian Indigenous community for centuries (Brand-Miller & Holt, 1998). The seeds are consumed raw or roasted and sometimes used to make the local bread known as ‘damper’ (Maslin and McDonald, 2004). Ground seeds are added to chocolate, granola, ice creams and cakes as flavourings mostly by Australian European settlers (Maslin and McDonald, 2004). The seed arils are rich in oils,

mostly unsaturated fatty acids mainly in the form of linoleic or oleic acid (Brown et al., 1987; Seigler, 2002; Njume et al., 2019). However, seeds may contain 0.2% dimethyltryptamine (DMT), a psychoactive hallucinating alkaloid commonly known as 'the spirit molecule'. When taken orally, the psychoactive threshold for DMT is 0.2 mg/Kg body weight which usually sets in after 30 minutes and can last for several hours, yet it is important to note that most times there are no psychotropic effects with oral ingestion of DMT (Barker, 2018).

Fig 2 b. 1: Brown pods, black seeds and brown arils of *A. longifolia* collected from the coast near Warrnambool, Victoria Australia.

2 b. 2. 2 *Rhagodia candolleana* subsp. *candolleana* (sea berry salt bush)

R. candolleana is a sprawling coastal halophytic shrub, about 2 m in height (Walsh, 1996). The plant stems bear branches that are distinctly ribbed and the leaves are semi-succulent, glossy green above and paler below (https://friendsofvenusbaypeninsula.org.au/wp-content/uploads/2017/08/Seaberry-Saltbush-Rhagodia-candolleana.pdf). The plant produces small glossy dark red-purple attractive edible berries that are flattened and dimpled at the top and which upon touch/squeezing produce a red/purple juice that readily stains the hands (Fig

2 b. 2). The berries are distributed along the Victorian volcanic plain, Bridgewater, Warrnambool and are consumed by birds and members of the Indigenous community. *R. candolleana* is pest- resistant and relatively fire-retardant due to its high salt content. The salt content has been attributed to its habitat, close to the sea where the plant becomes occasionally inundated with salty waters (Loganathan et al., 2017). It belongs to the family Chenopodiaceae which is shared with *Beta vulgaris* L., another heavy red/purple pigment-producing species and *Chenopddium album* L. (Walsh, 1996).

Fig 2 b. 2: *Rhagodia candolleana* subsp. *candolleana* showing whole plants and pigment-producing berries.

2 b. 2. 3 *Carpobrotus rossii* (Haw.) Schwantes (pig face)

C. rossii is a coastal halophytic ground cover plant species with succulent distinctly curved leaves that are 3.5 - 10 cm long and 6 - 11 mm thick (Walsh 1996). The leaves are green to glaucous and the flowers are lightly purple merging to white at the base. It belongs to the family Aizoaceae (Walsh, 1996). The plant produces fruits that are purple to red in colour when fully ripe in spring and summer (Fig. 2 b.3). The fruits are known as Karkalla and are harvested by members of the Indigenous community and consumed directly or used for treatment of throat

infections, gastrointestinal upset, spider and tick bites, and as an astringent used externally on wounds, as well as to treat burns, eczema, bluebottle and jellyfish stings (Geraghty et al., 2011). The plant has been reported to demonstrate significant antioxidant, antiplatelet, anti-inflammatory and low-density lipoprotein-lowering activities in vitro (Geraghty et al., 2011; Pirie et al., 2013; 2014). The high salt content of its leaves has been attributed to its coastal habitat where it is occasionally inundated with salty seawater. The species is found in Western Australia, South Australia, Tasmania, and in Victoria along the volcanic plain, Warrnambool plain, Gippsland plain and Bridgewater.

Fig 2 b. 3: *Carpobrotus rossii* (Haw.) Schwantes (pig face) showing fruits (left), succulent leaves and purple flowers (right).

2 b. 2. 4 *Arthropodium strictum* R. Br. (chocolate lily)

A. *strictum* is a slender perennial herb, about 1.2 m in height with narrow grass-like leaves (Fig. 2 b. 4). It produces purple flowers, usually from October to December which smell like chocolate when crushed. It belongs to the family Asparagaceae (Conran, 1994) and produces small tubers for storage of carbohydrates, most of which have been reported to be fructans

(Incoll et al., 1989). Tubers are a delicacy and are harvested in spring, roasted and consumed by members of the Indigenous community (Incoll et al., 1989). It is a common plant species in many states including Queensland, New South Wales, Victoria, Tasmania, South Australia and Northern Territory.

Source: https://vicflora.rbg.vic.gov.au/flora/search

Fig 2 b. 4: *Arthropodium strictum* R. Br. (chocolate lily) whole plant (left) and tubers (right).

2 b. 2. 5 *Leucopogon parviflorus* (Andrews) Lindl (native currant)

L. parviflorus is an erect coastal shrub or small tree 1 - 5 m in height belonging to the family Ericaceae (Powell et al., 1996). It produces small white flowers that are hairy inside and are bell-shaped and bears hard narrow pale green leaves. It flowers most times of the year and produces spherical berries, 4.5 cm in diameter (Powell et al., 1996). The berries are initially green but turn to white when fully ripe (Fig 2 b. 5). *L. parviflorus* is found along the Victorian volcanic plain, Wimmera, Bridgewater, Warrnambool plain, Otway ranges and Victoria Alps. The plant is commonly known as native currant and its fleshy ripe berries are collected and consumed directly by members of the Indigenous community.

Fig 2 b. 5: *Leucopogon parviflorus* (Andrews) Lindl (native currant) berries. Green berries are unripe while the milky-white berries are ripe and edible

2 b. 2. 6 *Correa alba* Andrews (white correa or cape barren tea)

C. alba is a spreading shrub 1.5 m by 1.5 m with coriaceous leaves (Duretto, 1999). The leaves are 1.5 - 3.5 cm long and 1 to 2.7 cm wide with a hairy undersurface and lightly hairy upper surface (Fig. 2 b. 6). The plant is able to grow in low-nutrient soils and along the coast where it becomes inundated with salty seawater. It produces 4 white petal-flowers usually between April and June. It belongs to the family Rutaceae and is a common plant in all states of Australia except in the Northern Territory (Duretto, 1999). It is commonly known as white correa or cape barren tea. The leaves are harvested by members of the Indigenous community, dried and used as a tea substitute (Njume et al., 2019).

Fig. 2 b. 6: *Correa alba* Andrews (white correa or cape barren tea) leaves.

2 b. 2. 7 *Dianella revoluta* R. Br. (black anther flax lily)

The Australian National Botanic Gardens and Australian National Herbarium describes *D. revoluta* as an erect, hairless, perennial herb with a lifespan of many years, forming clumps and growing from rhizomes underground (Robyn, 2007). *D. revoluta* grows to about 1 m in height and is a drought and frost-resistant plant (Robyn, 2007). The leaves are leathery, long and linear, varying in length from 1.5 - 8.5 cm and width from 4 - 15 mm (Fig. 2 b .7, left). It belongs to the family Asphodelaceae. It flowers in spring to summer with a star-like purple flower which develops into a blue to purple berry 4 - 10 mm in diameter containing 3 - 10 seeds. The flowers and berries are held at the tip of the long thick filament that bears brown to black anthers, thus the name black anther flax lily. The blue/purple fruits are harvested and consumed directly by members of the Indigenous community and the black seeds provide a slightly oily delicious snack (Fig. 2 b.7, right). Black anther flax lily is used as an ornamental

plant in home and school gardens (Hanks, 2001). It is distributed along the Victorian volcanic plain, Warrnambool plain, Wimmera, Victorian Alp and Gippsland plain.

Fig. 2 b. 7: *Dianella revoluta* R. Br. (black anther flax lily) whole plant (left) and blue/purple berries (right).

2 b. 2. 8 *Typha orientalis* C. Presl (water cumbungi or bulrush)

Typha orientalis species are semi aquatic perennial grass-like plants (2 - 4 m tall) commonly known in most Australian communities as reeds or cattails (Fig 2 b. 8, left). *T. orientalis* produces thick white roots and rhizomes (Fig 8, right) which are used as food by Indigenous Australians (Gott, 1999). *T. orientalis* is an invasive plant species, spreading freely at the roots and growing through canals, ditches, reservoirs, cultivated fields, farms and ponds.

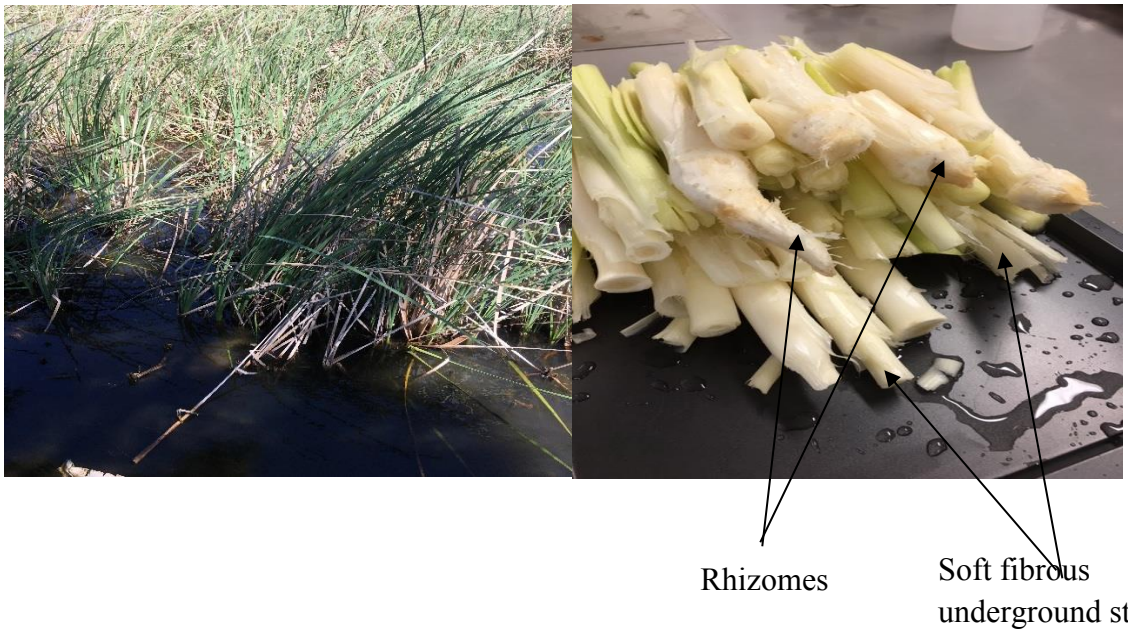


Fig 2 b. 8: *T. orientalis* underground stems and rhizomes

Chapter 3 a

PROXIMATE AND PHENOLIC COMPOSITION OF SELECTED NATIVE AUSTRALIAN FOOD PLANTS

This chapter has been published: Njume, C., McAinch, A. J., Donkor, O. (2019). Proximate and phenolic composition of selected native Australian food plants. International Journal of Food Science and Technology, https://doi.org/10.1111/ijfs.14400. The section appears as per the publication, however to assist with clarity and continuity the section, figure and table numbers have been changed, citations reformatted, abbreviations standardised and the language has been standardised to English (Aus). The chapter gives detailed information about the methods and the results obtained for proximate and mineral analysis, total phenolic contents, fatty acids and antioxidant polyphenols of the selected plants species.

3 a .1 Abstract

Edible portions of *Leucopogon parviflorus*, *Arthropodium strictum*, *Carpobrotus rossii*, *Rhagodia candolleana*, *Typha orientalis*, *Correa alba*, *Dianella revoluta* and *Acacia longifolia* were collected in 2017 and 2019 from Warrnambool, Victoria Australia, and studied for phenolics and proximate composition. The plant species recorded consistent antioxidant activity with no nutritional alterations. Except for *A. longifolia*, total phenolic contents (TPC) were higher in 2019 than 2017 ($P<0.05$). All the plant species contained Ca, Mg, Na, and K. Except for betanin, isolated from *R. candolleana* (700 mg/kg) and *C. rossi* (244 mg/kg), higher quantities of polyphenols were extractable in methanol than water ($P<0.05$). The plants fatty acid composition was predominantly palmitic, oleic and linoleic acids with *A. longifolia*

containing 44% linoleic acid. While the plant nutrients and fatty acids were fairly stable, TPCs, some of the minerals and polyphenols varied between the years.

3 a .2 Introduction

Australia is known for its rich biodiversity (Horwitz *et al*., 2008) and edible wild plant species that are endowed with remarkable ability to survive harsh environmental conditions (Cock, 2013). *Xanthorrhoea johnsonni* A. T. Lee, one of Australia's endemic species, for example has been known to survive for more than 5 centuries (Cock & Kalt, 2012), while *Lomatia tasmanica* W.C. Curtis. is known as one of the world's oldest plant species (Lynch & Balmer, 2004) and *Terminalia ferdinandiana* Exell is reported as the world's richest source of Vitamin C (Konczak *et al*., 2010; Njume et al., 2019). The country is known for changing climatic conditions with some regions unfavourable for human habitation and survival of its rich biodiversity. Despite these changes, many native food plant species relied upon by members of the Indigenous community have continuously thrived and survived (Clarke, 2007), with rich polyphenol and antioxidant contents, some of which have exhibited useful therapeutic properties *in vitro*; extracts of the native Australian bush fruit Illawarra plum (*Podocarpus elatus* Endl.) have been shown to reduce the proliferation of colon cancer cells by altering the cell cycle, increasing apoptosis and possibly inducing autophagy (Symonds et al., 2013). The active ingredients in Illawarra plum therefore, may have the potential to provide an alternative chemoprevention strategy to conventional chemotherapy.

Some native Australian plant species including cinnamon myrtle (*Backhousia myrtifolia* Hook. & Harv.), anise myrtle (*Syzygium anisatum* (Vickery) Craven & Biffen), lemon myrtle

(*Backhousia citriodora* F. Muell) and Tasmannia pepper leaf (*Tasmannia lanceolata* R. Br.) with rich antioxidant and anti-inflammatory polyphenols are reported as potential sources of lead molecules for production of anti-inflammatory drugs (Guo et al., 2014; Rupesinghe et al., 2016). There is reason to believe therefore that such species that have survived unfavourable climatic conditions over the years and have accumulated a huge amount of bioactive compounds would likely have good nutritional and health benefits within Australian Indigenous communities. This philosophy is stimulated by the historical fact that native plant species have provided a primary source of food and general wellbeing for Australia's Aboriginal population for thousands of years (Brand-Miller & Holt, 1998). Some of the species include *Leucopogon parviflorus* (Andrews) Lindl (native currant), *Arthropodium strictum* R. Br. (chocolate lily), *Carpobrotus rossii* (Haw.) Schwantes (pig face), *Rhagodia candolleana* Moq.subsp. *candolleana* (seaberry saltbush), *Typha orientalis* C. Presl (water cumbungi or bulrush), *Correa alba* Andrews (white correa or cape barren tea), *Dianella revoluta* R. Br. (black anther flax lily) and *Acacia longifolia* subsp. *sophorae* (Labill.) Court (coast wattle). Knowledge regarding their application is still fresh in the minds of the elders of martang Djab Wurrung Country, an Indigenous community in the Western region of the state of Victoria in Australia.

With the coming of modernity and industrialization, many communities in Australia have moved away from traditional staples to 'convenience energy-dense foods' in a nutrition transition era that is believed to be responsible for the high rate of non-communicable diseases within the community (Naughton et al., 2015). According to the Australian Institute of Health and Welfare (AIHW 2016), 69% of adult Indigenous Australians are either overweight or obese (Vos et al., 2009; Stevens, Egger, & Morgan, 2018). Sedentary life styles and consumption of diets low in fruits and vegetables are some of the contributing factors to Australia's obesity

problem (Sevoyan et al., 2019; Njume et al., 2019). This partly explains why Indigenous Australians who are overweight or obese may be more likely to also have type 2 diabetes mellitus (T2DM) than the rest of the population (de Abreu et al., 2013). The economic, health and social effects of overweight and obesity including increased risk of cardiovascular disorders and T2DM accompanied by reduction in quality of life are becoming increasingly disturbing. Indigenous food plants are now being considered as possible sources of raw materials, nutrients and antioxidant polyphenols for the production of healthier food options. The species selected for this study are still of value to the Indigenous community, especially the older generation yet they are largely ignored by the younger generation due to changing social/dietary habits.The people of martang Djab Wurrung community continue to use and cherish these traditional foods despite popularity and availability of generic energy-dense convenience products. In this community, the white part of the underground stem and rhizomes of *Typha orientalis*, a member of the family Typhaceae is harvested in spring or summer, peeled, roasted, baked or boiled and consumed (Gott, 1999). Owing to its semi-aquatic nature, *T. orientalis* is readily available from ponds, standing water and wetlands.

The seeds of *Acacia longifolia*, fruits of *Carpobrotus rossii*, berries of *Leucopogon parviflorus* and *Rhagodia candolleana* subsp. *candolleana* are harvested along the Victorian coastline and consumed directly while tubers of *Arthropodium strictum* are usually baked in hot ash or roasted before consumption (Ee & Yates, 2013). The round blue-purple fruits of *Dianella revoluta*, a popular ornamental and understorey plant (Duncan et al., 2004) are collected and consumed directly while the hairy thick leaves and shoots of *Correa alba* are collected, dried and used as a tea substitute. Some members of this community believe that consuming these species is a healthier and more nutritious choice than fast foods. Despite such beliefs, a literature search of the nutritional and bioactive composition of many of the species generated

scanty information, most of which has not been investigated scientifically except for studies conducted on the halophyte *C. rossii* by Pirie et al. (2013) and (2014) which indicated that the plant crude extracts possess high *in vitro* antioxidant and *in vivo* low-density-lipoprotein-lowering activities. However, details of the plant bioactive compounds responsible for the above mentioned characteristics remain unknown. This study therefore, investigates the bioactive and nutritional quality of selected plant species in an attempt to highlight potential sources of raw materials for production of healthier food options within Australian Indigenous communities

3 a .3 Materials and methods

3 a .3 .1 Collection and preparation of samples

A permit to collect native plant species for this study was obtained from the Department of Environment, Land and Water Planning (Appendix 1). In February 2017 and 2019, with the help of the elders of martang Djab Wurrung Indigenous community, edible portions of eight plant species were collected. The white part of the underground stems and rhizomes (USR) of *T. orientalis* were collected by uprooting the species from a group of the plants growing close together around a pond while tubers of *A. strictum* were harvested by digging up the plant from the ground and sniping the tubers from the stems with a pair of scissors. The tubers were collected from individual plants of the same species growing about a meter from each other.

The black seeds of *A. longifolia* were collected from a group of individual plants of the same species growing next to each other along Warrnambool beach and fruits of *C. rossii* commonly known as karkalla were harvested from different clusters of the same plant species in the same area. The white-coloured berries of *L. parviflorus* were collected from a single mainland plant,

2 km from the beach while the greyish hairy leaves of *Correa alba* and the glossy dark red berries of *R. candolleana* were collected from the Correa heath and sea berry salt bush respectively along the foot path of the beach of Warrnambool. The blue/purple berries of *Dianella revoluta* were collected from individual plants of the species growing about a meter from each other at Tower Hill, Warrnambool, Victoria Australia. About 300 g of edible plant material was collected for each species. It is important to note that approval to collect *D. revoluta* and *C. alba* was only granted in 2019 by the Indigenous Elders and even though a permit was granted by the government, their approval was still required before collection. Berries of *L. parviflorus* were not collected in 2019 due to scarcity. Pictures of the plant species were also taken. Samples were collected in zip lock bags, labeled with date, name, parts and place of collection. The samples were transported on ice in a cooler box to Victoria University food laboratory, Werribee West Campus. The samples were identified using descriptions gathered at the time of collection and comparison of photographs and plant location to entries in the reference website of Flora of Victoria (https://vicflora.rbg.vic.gov.au/flora/search). The plants were authenticated with assistance from a botanist at Victoria University and vouchers were prepared for future reference. Samples were cleaned by removing dirt, soil, residue and debris from other plant species and then washed with distilled water. Each of the plant material collected was subsampled while the rest of the material was stored at -80 ºC for long term storage.

3 a .3 .2 Determination of Moisture content

Moisture content was determined gravimetrically by oven- and freeze- drying according to standard procedures (AOAC, 2002; Donkor et al., 2012; Al-Abdulkarim et al., 2013). Fresh plant material, 25 g was weighed and placed in an oven (Thermoline Scientific, Wetherill Park,

NSW) set at 60 ºC until constant weight was obtained. The same amount of each fresh plant material was weighed, rapidly frozen in liquid nitrogen at -196 ºC and freeze-dried (Airvac, Rowville, VIC) for 48 hours. The procedure was performed in triplicate. Differences in weight between fresh and dried samples were recorded and used to compute the percentage moisture for each plant.

3 a. 3 .3 Determination of Ash

Ash content of selected species were determined gravimetrically by burning the samples in a muffle furnace (Labec, Merrickville, NSW) at 600 ºC for 5 hour according to the method of Ee & Yates (2013), with modifications. Exactly 15 g of plant material was separately weighed into heat-resistant crucibles. The crucibles were placed over a bunsen flame in a class 2 bio-safety cabinet and the samples were ignited and burnt until no smoke (removing as much soot as possible). Burning was then ceased and crucibles were allowed to cool at room temperature before placing in the muffle furnace. After 5 hours, the furnace was switched off, allowed to cool for 2 hour, and the samples weighed and recorded.

3 a .3 .4 Determination of crude fat-content

Crude fat-content was determined by Soxhlet extraction following standard procedures (AOAC 1984; Bhattacharjee et al., 2013). Briefly, 3 g of dried plant material was ground into powder and placed in labelled thimble filters (MicroAnalytix Pty Ltd, Taren Point, NSW). The filters were placed in 250-mL boiling flask (with known weight) containing 100 mL of petroleum ether (Sigma Aldrich, Castle Hill, NSW). The flasks were heated at 50 ºC and refluxed on Soxhlet apparatus for 5 hour. The thimble filters were carefully removed. The extracts were

concentrated to dryness using a rotary evaporator (Rikakikai Co., LTD, Tokyo, Japan), allowed to dry in a class 2 bio-safety cabinet for 48 hour with the fan on for complete evaporation of residual solvents and then weighed. The % fat for each sample was calculated as follows;

Fat (%) = (wt. of fat/3) x 100 (Bhattacharjee et al., 2013).

3 a .3 .5 Determination of crude protein

The Kjeldahl method of protein determination involving digestion, distillation and titration (AOAC 2002; Magomya et al., 2014) was employed to determine the amount of protein in the selected species. Exactly 1g of freeze-dried ground sample was wrapped in non-nitrogen containing filter paper and weighed into Kjeldahl digestion tube. This was followed by addition of Copper sulphate ($CuSO_4$) tablets, (digestion catalyst) and 12.5 mL concentrated sulphuric acid (H_2SO_4). A few anti-bumping granules were also added into the tube and digestion was performed at 420 ºC for 1 hour. A control tube with non-nitrogen containing filter paper, $CuSO_4$ tablets, 12.5 mL H_2SO_4 acid and anti-bumping granules only was used as a blank. After digestion, the tubes were allowed to cool at room temperature for 10 min. A total amount of 50 mL Milli-Q water was added to the tubes and connected to the distillation unit. Exactly 60 mL of 45% sodium hydroxide solution was added to the digestion tube and ammonia was steam distilled for 5 min into a 250 mL conical flask containing 25 mL of 4% boric acid and indicator (0.1% methyl blue and 0.3% methyl red in ethanol). The resulting distillate (green in colour) was titrated against 0.1M HCl acid with methyl red as an indicator. The volume of acid recorded for neutralization was used to calculate percentage nitrogen and crude protein in the sample as per the equation below:

$$\text{Percentage nitrogen (N)} = \frac{(\text{mL standard acid} - \text{mL blank}) \times \text{Molarity of acid} \times 1.4007}{\text{Weight of sample in grams}}$$

Crude protein = % nitrogen x 6.25 (Mæhre et al., 2018)

Determinations were repeated twice; means and standard deviations (SD) were computed and recorded.

3 a .3 .6 Determination of total dietary fiber (TDF)

The determination of the TDF of the species was performed in accordance with standard procedures of AOAC 985.29 with modifications (McCleary et al., 2015). Briefly, duplicate dried and homogenized plant materials were weighed 1 g each into separate conical flasks containing 100 mL distilled water and 50 µL of heat stable α-amylase (Sigma Aldrich, Castle Hill, NSW) per flask. The flasks were then heated at 100 ºC in a water bath (Thermoline, Wetherill Park, NSW) for 15 min with intermittent shaking at 5 min intervals. The flasks were cooled to room temperature followed by addition of 10 mL 0.275 M NaOH solution and the pH was adjusted to 7.5.

Exactly 100 µL of aminopeptidase (to breakdown proteins) was added to each flask and incubated at 60 ºC for 30 min. The flasks were then cooled to room temperature and 10 mL of 0.325 M HCl was added to adjust the pH to 4.5. This was followed by the addition of 200 µL of amyloglucosidase (to convert starch to glucose) and incubated for 30 min at 60 ºC in a shaker incubator (Victoria, Australia) at 10 x g. An aliquot of 280 mL 95 % pre-heated ethanol at 60 ºC was added to each flask to precipitate soluble fiber and remove depolymerized protein and glucose. The flasks were allowed to stand at room temperature for 1 hour for precipitation to form. The precipitate was washed three successive times with 20 mL portions of 78% ethanol and filtered on a celite-fitted glass under suction/wash followed by two 10 mL portions of 95%

ethanol washes and then finally with two 10 mL portions of 95% acetone. The residues were dried overnight at 105 ºC in a hot air oven (Labquip Technologies Pty Ltd, Ferntree Gully, VIC), cooled in a desiccator and weighed. One residue was analyzed for ash and another for protein. The total dietary fiber was calculated as the weight of the residue minus weight of protein plus ash.

3 a .3 .7 Determination of total carbohydrates

Total carbohydrates were determined spectophotometrically by using the anthrone method described by Ohemeng-Ntiamoah and Datta (2018), with slight modifications. Briefly, 0.1 g of dried ground plant material was weighed into boiling tubes and reducing sugars extracted with 2 x 5 mL aliquots of 80 % ethanol. The mixture was filtered using filter paper No. 1 (pore size between 5 and 10 µm). The filtrate was reserved for analysis of simple sugars and to the residue was added 5 mL of 2.5 N HCl for extraction of starch. The tubes were heated in a water bath at 100 ˚C for 30 min, cooled to room temperature and neutralized by adding solid sodium carbonate until effervescence was ceased.

The volume of each tube was made up to 100 mL by adding Milli Q water and centrifuged at 4000 x g for 5 min. A volume of 1.0 mL was collected from the supernatant and used for analysis of starch. A standard solution of glucose at 20 mg/L, 40 mg/L, 60 mg/L, 80 mg/L and 100 mg/L was prepared for generation of a standard curve (plotting absorbance against concentration). Anthrone reagent (0.1%) was made fresh by adding 0.1 g of anthrone to 100 mL of ice cold 95% H_2SO_4. To each 1 mL sample, 3 mL of freshly prepared anthrone solution was added, vortexed for 3 sec and heated for exactly 11 min at 100 ˚C in a water bath. The

tubes were cooled rapidly to 0 ˚C by putting in ice bath for 5 min, diluted 10 times with Milli Q water and absorbance of the greenish coloured-solution was measured at 630 nm (against water) within an hour. Total carbohydrates were computed using the linear equation generated from the standard glucose curve and was the sum total of the value obtained from simple sugars and starch. The experiment was performed in triplicates.

3 a .3 .8 Determination of mineral content

Total of 10 minerals (Na, K, Ca, Mg, Fe, Zn, Cu, Cr, Se & Mn) were determined in the plant samples using inductively coupled plasma spectrometry (Shimadzu Corporation, Kyoto, Japan) according to standard procedures (Antonious et al., 2011). Exactly 1 g of sample ash was weighed into a conical flask and 10 mL concentrated nitric (HNO_3) acid was added. The mixture was gently swirled, sealed with aluminum foil and allowed to digest overnight at room temperature. The mixture was heated for 1 hour at 160 ºC in an oil bath (Ratek Pty Ltd, Boronia, VIC), cooled to room temperature and then diluted with 50 mL Milli-Q water. The mixture was filtered through cellulose filter paper No. 1 (pore size of between 5 and 10 µM). The filtrate was 10-fold serially diluted and the minerals detected by spectrometry using Shimadzu plasmic atomic emission spectrometer (Shimadzu Corporation, Kyoto, Japan). The sample minerals were tested and quantified by use of standard curves generated from a set of serially diluted reference standards of Ca, Mg, Na, K, Fe, Cu, Zn, Mn, Cr and Se (r- value = 0.999).

3 a .3 .9 Extraction of phenolic compounds

Ten grams of homogenized freeze-dried plant material was soaked in 100 mL of 80% hexane in a conical flask. The flask was placed in a shaker incubator (Thermo Scientific, Scoresby, VIC) set at 2 x g for 24 hour at 30 ˚C. Aqueous extracts were prepared by soaking the same amount of plant material in water and placed in the same incubator. The extracts were centrifuged (Beckman Coulter, Indianapolis, IN) at 4000 x g for 30 min. The supernatants of hexane were concentrated to dryness using a rotary evaporator (Rikakikai Co., LTD, Tokyo, Japan) while the aqueous extract was rapidly frozen in liquid nitrogen at -196 ˚C and concentrated by freeze drying. The procedure was repeated three times with new solvent before sampling the plant material a second time. Plant material used in the hexane extraction was spread in a bio-safety class 2 cabinet for a few hours to allow the solvent to evaporate and then used for the methanol extraction following the same procedure (Massaud et al., 2017). The resulting plant crude extracts of each solvent were combined and left in a bio-safety class 2 cabinet with the fan on for 48 hour to ensure complete evaporation of residual solvents while water was removed by freeze-drying.

3 a .4 Antioxidant capacity

3 a .4 .1 Determination of antioxidant capacity by DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay

This was performed according to the method of Sommano et al. (2013) with slight modifications. Briefly 3.9 mL DPPH (Sigma Aldrich, Castle Hill, NSW) solution (0.075 mM in methanol) was added into test tubes containing 100 µL of 2-fold serially diluted methanol crude extracts beginning from 200 mg/mL-12.5 mg/mL and incubated for 30 min in the dark.

The negative control was DPPH solution (Sigma Aldrich, Castle Hill, NSW) only. The optical density of all the tubes were read at 515 nm using Shimadzu UV-visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan). In order to prepare a standard curve, the reduction in absorbance of DPPH solution with ascorbic acid at different concentrations over an incubation period of 30 min was measured and plotted. DPPH radical scavenging activities of the samples were expressed in ascorbic acid equivalent (AAE) antioxidant capacity. The experiment was repeated twice to provide for n=3 and mean ascorbic acid antioxidant capacity and standard deviation was computed and recorded.

3 a .4 .2 Determination of antioxidant capacity by ABTS (2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) assay

This was performed according to the method of Biskup et al. (2013) with slight modifications. Briefly, 7 mM solution of ABTS (Sigma Aldrich, Castle Hill, NSW) was mixed with 2.45 mM potassium persulphate as stock solution and allowed to stand in the dark for 12 hour at room temperature to generate the ABTS radicals. The working solution was prepared by diluting ABTS stock solution with 50% ethanol to an absorbance of 0.8 at 734 nm. Exactly 0.1 g of Trolox (6-hydroxy-2,5,7,8-tetratmethylchrman-2-carboxylic acid) powder (Sigma Aldrich, Castle Hill, NSW) was dissolved in 100 mL of ethanol and two-fold serially diluted in test tubes. Exactly 1 mL ABTS working solution was added in each tube. The tubes were incubated in the dark for 10 min and the absorbance was read at 734 nm. A plot of the absorbance against concentration was used to generate the standard curve for Trolox. Approximately 1 g of methanol plant extract was dissolved in 3 mL of ABTS working solution and incubated in the dark for 10 min after which the absorbance was read at 734 nm. The experiment was repeated

twice (n=3) and mean antioxidant capacity and standard deviation was computed and recorded. The results were expressed as Trolox equivalent antioxidant capacity (TEAC).

3 a .5 Determination of total phenolic content (TPC)

This was performed according to the method of Maria et al. (2018) with slight modifications. Briefly, 0.005 g of methanol crude extract of each plant species was separately dissolved in 1 mL of methanol followed by addition of 0.5 mL Folin-Ciocalteu reagent. The mixture was vortexed for 1 min and incubated at room temperature for 3 min (Donkor et al., 2012). Exactly 10 mL of sodium carbonate (75 g/L) and 5 mL of Milli-Q water was added to each tube and mixed. The tubes were incubated for 1 hour at room temperature in the dark. The absorbance of the samples was read at 765 nm using Shimadzu UV-visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan). In order to prepare a standard curve, changes in the absorbance of Folin-Ciocalteu reagent solution at different concentrations of gallic acid only after 1 hour incubation was measured and plotted. The TPC was expressed as milligram gallic acid equivalent (GAE). The experiment was performed in triplicates.

3 a .6 Analysis of fatty acids

The hexane crude extracts were used for fatty acids and Gas Chromatography/Mass Spectrometry (GCMS) analysis. The extracts were dissolved in concentrated hexane and analyzed according to previously established procedures (Ee & Yates, 2013), with slight modifications. Briefly, the crude extracts were mixed with concentrated hexane in a ratio of 1:5 respectively and centrifuged at 2147 x g for 10 min. The supernatant was removed, filtered through a 0.45 μm membrane filter and mixed with 0.5 mL of 0.2 M sodium methoxide (2.3 g

of sodium in 200 mL anhydrous methanol). The mixture was vortexed for 15 sec and left to stand for 10 min at room temperature. About 2-3 drops of bromothymol blue was added and mixed. Next, 0.4 mL of 1 M HCl was added drop-wise until a yellow colour was detected. Approximately 600 µL of 1.5 % (w/v) sodium carbonate in water was added drop wise until a blue colour was obtained. About 1500 µL of distilled water was added to bring the hexane layer to the top of the tube. The hexane layer was carefully removed and subjected to GCMS analysis using a Shimadzu MS detector (Shimadzu Corporation, Kyoto, Japan). Composition of the oils was determined using a GC Phenomenex column (30 m × 0.25 mm id, ft. 0.50 µm). The detector was set at 350˚C and the injector port at 250˚C. Separation of fatty acids was achieved using the following column temperature conditions; 80˚C (held for 2 min), heated to 220˚C at 30˚C per min and held at 220˚C for 5.5 min with a total run time of 12.17 min. A mixture of nitrogen, hydrogen and air was used as carrier gas at a linear velocity of 3.5 mL/s. The compounds were identified by comparing their retention times with standard ester derivatives of fatty acids. The relative level and percentage of each fatty acid was estimated from the standard curve prepared for reference standards of palmitic (P), myristic (M), linoleic (L), oleic (O), stearic (S), arachidonic (A), behenic (B) and erucic (E) acids.

3 a .7 Determination of plant components

Detection of polyphenols in the various plant crude extracts was achieved by reverse phase high performance liquid chromatography (RP-HPLC) analysis as described by Donkor et al. (2012) with slight modifications using a photodiode array detector (PDA). Briefly, a Shimadzu HPLC system (Nishinokyo-Kuwabaracho, Kyoyo, Japan) equipped with a C18 Phenomenex Luna column (2.4 mm porosity, 250 x 4 mm, and an UltraSep ES RP18 pre-column) was used

with a 3-steplinear gradient for separation of compounds in the extracts. Two separate analysis were conducted at different times with different mobile phases as described below;

3 a .7 .1 HPLC analysis of betanidin 5-O-β-D-glucoside

The isolation of Betanin was performed with 10 mM sodium phosphate buffer pH 5.5 (A) and 100% HPLC grade methanol (B) as mobile phase. The gradient for separation was as follows; from 10 % to 20 % of B in 30 min, 20 % to 60 % of B in 20 min, then 60 % to 90 % of B for 20 min (flow rate of 1 mL per min, at 20 ˚C. The elution pattern was monitored with a PDA detector at 254 nm (Goncalves et al., 2012). The extracts were prepared in sodium phosphate buffer at pH 5.5 and filtered through 0.45 μm membrane filter and injected with a 10 μL injection volume. Betanin was identified by comparing with the retention time of reference standard (6.0 min) and quantified using a calibration curve generated from the standard. The column was flushed and auto purged with 100 % methanol before and after the analysis.

3 a .7 .2 HPLC analysis of other phyto-components

The mobile phase for isolation of other components consisted of: (A) 0.3 % phosphoric acid and (B) 100 % acetonitrile. The gradient used for separation of components was as follows: from 10 % to 20 % of B in 45 min, 20 % to 60 % of B in 20 min, then 60 % to 90 % of B for 20 min (flow rate: 0.7 mL/min at 20℃). The elution pattern was monitored with a PDA detector at 220 nm. The extracts were prepared in methanol and filtered through a 0.45 μm membrane filter and injected with a 10 μL injection volume. The individual polyphenols were identified by comparing with the retention times of reference standards. Quantitative analysis was performed by calibration curves using the reference standards of gallic acid (GA),

epigallocatechin (EPC), catechin (CH), epigallocatechingallate (EPG), dihydroquinidine (DHQ), Ferulic acid (FA), ρ-coumaric acid (PCA) and luteolin (LT) (Sigma Aldrich, Castle Hill, NSW). Linearity was investigated in the range of 0 - 5 mg at five increasing concentrations. Intra-day analyses of the same solution containing all phenolic compounds tested were used to validate the precision of the chromatographic system (Donkor et al., 2012).

3 a .8 Statistical Analysis

All data including triplicate determinations for proximate analysis, antioxidant capacity, fatty acids, betanin, quantities of other polyphenols, and TPCs of 2017 and 2019 were entered in Excel spread sheet (Microsoft® Excel, District of Columbia, WA) and IBM SPSS statistic software version 25 (IBM SPSS®, Chicago, IL). Means and standard deviations were computed using both software and multiple comparisons between means were performed by one-way analysis of variance test (ANOVA). Differences between means for proximate analysis, antioxidant capacity, fatty acids and TPC were considered significant at $P < 0.05$.

3 a .9 Results and discussion

Despite popular use, rich nutrient content and adaptability to harsh climatic conditions, there is limited scientific data for many useful native food plants to help reduce dependence on unhealthy food choices in Australia. Before the coming of westernization, Indigenous Australians relied on their traditional staples composed mainly of uncultivated plant foods, non-domesticated animals, aquatic foods and insects, most of which were high in dietary fibre and slow digestible carbohydrates (Brimblecombe et al., 2014). Elders of the martang Djab Wurrung Aboriginal community in Victoria, Australia still hold onto their beliefs and claims

that their staples are relatively healthier than the predominantly wheat -flour and sugar diets of modernity. This study was able to provide preliminary scientific evidence that underground stems and rhizomes of *T. orientalis*, berries of *L. parviflorus*, tubers of *A. strictum*, fruits of *C. rossii*, *D. revoluta* and *R. candolleana*, leaves of *C. alba* and seeds of *A. longifolia* used by the people of martang Djab Wurrung community do have nutritional properties that are worth preserving.

3 a .9 .1 Proximate analysis

With the exception of seeds of *A. longifolia*, the moisture content of the plants in 2017 was ≥ 66.8 % for oven- and freeze-dried samples and dropped to ≥ 55.6% in 2019. Generally, all the plant species, except *T. orientalis* exhibited no alterations in moisture contents in 2019 (Table 3.1). The freeze-drying method revealed more water loss in almost all of the plant species tested than oven-drying in both years ($P<0.05$). *T. orientalis* which recorded the highest amount of moisture loss (93.6 %) showed more moisture loss in 2017 compared to 2019 ($P<0.05$). Freeze-drying has been reported as a less aggressive drying method (de Torres et al., 2010; Çoklar & Akbulut, 2017) due to its ability to prevent loss of volatile polyphenols and potential antioxidants, thus maintaining the total phenolic content of the samples during processing (Mediani et al., 2014). Our results indicate that freeze drying in addition to its less aggressiveness in dehydrating the samples is also an efficient moisture remover, consistent with the findings of Puranik et al. (2012) who after a comparative study of different drying techniques on the quality of garlic, concluded that freeze dried samples had maximum water loss, than fluidized bed drying, oven drying and microwave heating methods.

Table 3 a .1: **Changes in proximate composition (%w/w) of native plant species collected over 2-year period**

Factor/ Test	February 2017								February 2019							
	Rc (b)	Lp (b)	Dr (b)	Cr (f)	To (usr)	As (t)	Al (s)	Ca (l)	Rc (b)	Lp (b)	Dr (b)	Cr (f)	To (usr)	As (t)	Al (s)	Ca (l)
Moisture OD	18.7±0.2 (74.8)	18±0.0 (72.0)	-	21.1±0.3 (84.4)	22.7±0.5 (90.8)	16.7±0.2 (66.8)	1.5±0.6 (6.0)	-	18.4±0.3 (73.6)	-	19.8±1.5 (79)	21±0.2 (84)	22.5±0.06 (90.1)	16.4±0.2 (65.6)	1.5±0.5 (6.0)	14.5±1.5 (58.1)
Moisture FD	18.4±1.05 (73.6)	17.9±0.3 (71.6)	-	21.2±1.05 (84.8)	23.4±0.9 (93.6)	17.4±0.7 (69.6)	1.8±0.2 (7.2)	-	18.2±0.3 (72.7)	-	20.0±0.5 (80.1)	21.2±1.6 (84.8)	22.8±0.05 (91.3)	17.1±0.5 (68.4)	1.4±0.02 (5.6)	13.9±0.5 (55.6)
Ash Content	0.18±0.005 (1.2)	0.42±0.05 (2.8)	-	0.39±0.03 (2.6)	0.1±0.0 (0.67)	0.065±0.0 (0.433)	1.2±0.02 (8)	-	0.27±0.0 (1.8)	-	0.15±0.03 (1.0)	0.45±0.04 (3)	0.18±0.05 (1.2)	0.75±0.5 (0.5)	1.3±0.1 (8.7)	0.675±0.2 (4.5)
Total Fats	0.06±0.01 (2)	0.39±0.05 (13)	-	0.03±0.0 (1)	0.03±0.0 (1)	0.02±0.0 (0.7)	0.42±0.2 (14)	-	0.06±0.0 (2)	-	0.18±0.6 (5.9)	0.03±0.0 (1)	0.03±0.0 (1)	0.02±0.0 (0.7)	0.43±0.3 (14.3)	0.45±0.3 (3)
Crude protein	0.054±0.03 (5.4)	0.026±0.0 (2.6)	-	0.039±0.0 (3.9)	0.062±0.03 (6.2)	0.045±0.0 (4.5)	0.151±0.05 (15.1)	-	0.051±0.05 (5.1)	-	0.079±0.02 (7.9)	0.04±0.0 (4.0)	0.068±0.03 (6.8)	0.042±0.02 (4.2)	0.15±0.05 (15.0)	0.085±0.05 (8.5)
Total Dietary Fibre	0.032±0.0 (3.1)	0.042±0.0 (4.2)	-	0.11±0.06 (11)	0.11±0.05 (11)	0.016±0.0 (1.6)	0.172±0.06 (17.2)	-	0.046±0.03 (4.6)	-	0.082±0.02 (8.2)	0.112±0.05 (11.2)	0.107±0.05 (10.7)	0.015±0.04 (1.5)	0.170±0.02 (17.0)	0.101±0.05 (10.1)
Total Carbs	27.2 (59.1)	27.2 (59.1)	-	24.2 (52.6)	22.3 (48.5)	28.5 (61.95)	18.3 (39.7)	-	28.0 (62.2)	-	22 (48.9)	25.8 (57.3)	24.03 (53.4)	29.5 (65.5)	18.2 (40.4)	27 (60.0)

Data are mean ± SD of triplicate determinations of actual amount of each nutrient yield in grams; %w/w, percentage weight by weight; OD, Oven drying; -, not determined; FD, Freeze-drying; Carbs, Carbohydrate; To, *T. orientalis*; Lp, *L. parviflorus*; As, *A. strictum*; Cr, *C. rossii*; Rc, *R. candolleana* subsp. *candolleana*; Al, *Acacialongifolia* subsp. *sophorae*; Dr, *Dianella revoluta*; Ca, *Correa alba*; usr, underground stems and rhizomes; b, berries; t, tubers; f, fruits; s, seeds; l, leaves. Values in brackets represent percentages.

Apart from seeds of *A. longifolia* , leaves of *C. alba* and fruits of *D. revoluta* (with protein contents of 15.1 ± 0.05 g, 8.5 ± 0.05 g and 7.9 ± 0.02 g/100 g Dry Weight (DW) respectively), the rest of the plant species contained ≤ 6.8 g of protein per 100 g DW during both years of the study. Protein contents of individual species did not alter between 2017 and 2019 ($P>0.05$), indicating that the people who depend on these species as a source of proteins are likely to obtain consistent amounts from them.

The ash-content of the species ranged from 0.433 - 8.0 g/100g DW in 2017 and 3 - 8.7 g/100g DW in 2019. *R. candolleana* and *A. longifolia* produced more ash in 2019 than 2017 ($P<0.05$) whereas the ash content of *T. orientalis*, *A. strictum* and *C. rossi* did not change within the years ($P>0.05$). Seeds of *A. longifolia* recorded the highest crude fat-content of 14 ± 0.2 g /100 g DW, followed by *L. parviflorus* with a crude fat-content of 13 ± 0.05 g /100 g DW and *D. revoluta* (5.9 ± 0.6 g/100g DW). The rest of the species recorded fat-contents ≤ 3 g /100 g DW (Table 3 a .1) whereas no significant differences ($P>0.05$) in crude fat-contents were observed in plant samples for 2017 and 2019. *T. orientalis* and *C. rossii* each produced 11 g /100 g DW for TDF. While the TDF of *A. longifolia*, *T. orientalis* and *C. rossii* were higher than the rest of the species ($P<0.05$), those for *R. candolleana* and *L. parviflorus* were similar ($P>0.05$). Apart from *R. candolleana*, TDF of the rest of the species did not change significantly between 2017 and 2019 ($P>0.05$).

Tubers of *A. strictum* contained higher amounts of total carbohydrate ($P<0.05$) whereas seeds of *A. longifolia* had lower carbohydrate content compared to the rest of the species ($P<0.05$ The reducing sugar content of *R. candolleana* berries, *C. rossi* fruits and *T. orientalis* underground stems and rhizomes was consistently higher than their non-reducing sugar-

contents in both years sampled (Fig 3 a .1 & 3 a .2). *L. parviflorus* berries (Fig 3.1) and *C. alba* leaves (Fig 3 a .2) also contained higher amounts of reducing sugars than non-reducing sugars ($P<0.05$) whereas *A. strictum* tubers and *A. longifolia* seeds contained higher amounts of non-reducing sugars ($P<0.05$) (Fig 3 a .1 & 3 a .2). Seeds of *A. longifolia* have exhibited interesting characteristics (e.g., high amounts of proteins, high TDF and low amounts of total carbohydrate) which may be helpful in the management of overweight, obesity and possibly type 2 diabetes within the Indigenous community. Consumption of foods high in dietary fiber has been reported to modulate the absorption of carbohydrates into the blood and improve insulin sensitivity at the peripheral tissues (Galisteo et al., 2008). For people who are overweight or obese, dietary fiber supplementation may also enhance weight loss (Anderson et al., 2009). Furthermore, increased fibre intake is known to alleviate a number of gastrointestinal disorders including gastroesophageal reflux disease, duodenal ulcer, diverticulitis, constipation, and haemorrhoids (Anderson et al., 2009). The high fibre content demonstrated by *A. longifolia*, *T. orientalis*, *C. alba* and *C. rossii* in this study highlights their usefulness as possible candidates for inclusion in foods with minimal fibre contents to meet the Australian recommended daily intake of 28 g and 38 g for women and men respectively (Fayet-Moore et al., 2018).

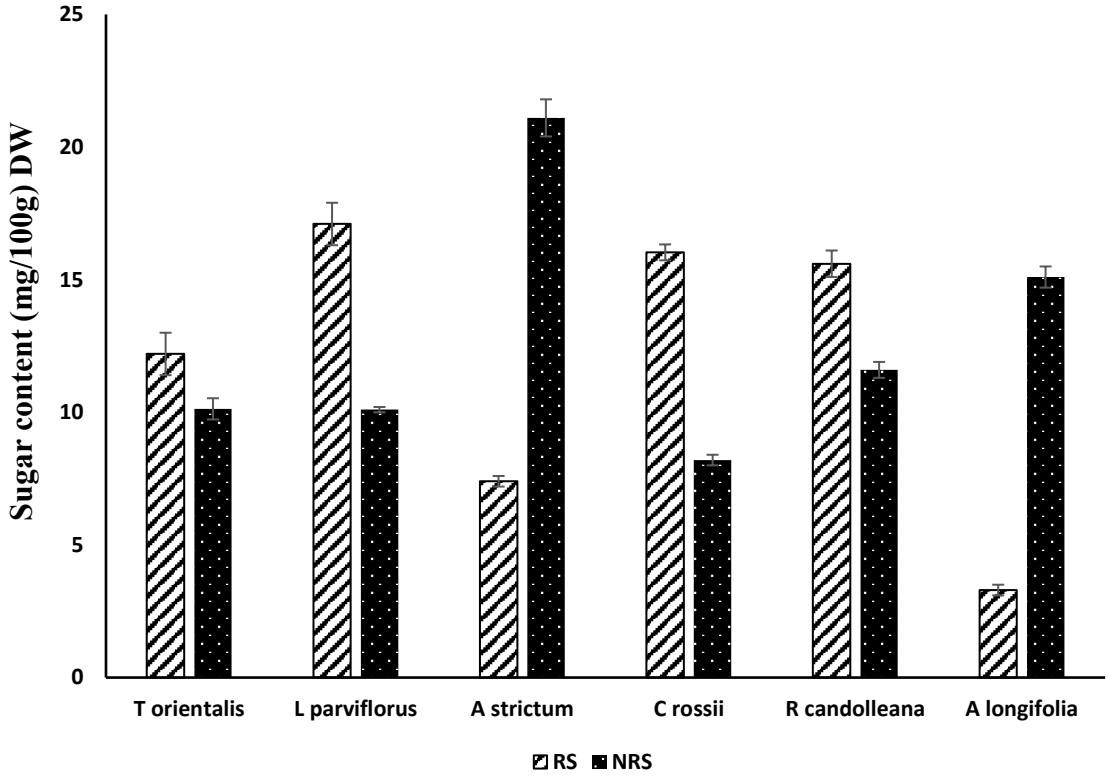


Figure 3 a .1: **Reducing and non-reducing sugar content of native plant species collected in 2017.** Data are means ± SD of triplicate determinations; RS, reducing sugar; NRS, non-reducing sugar; usr, underground stems and rhizomes; b, berries; t, tubers; f, fruits; s, seeds; l, leaves.

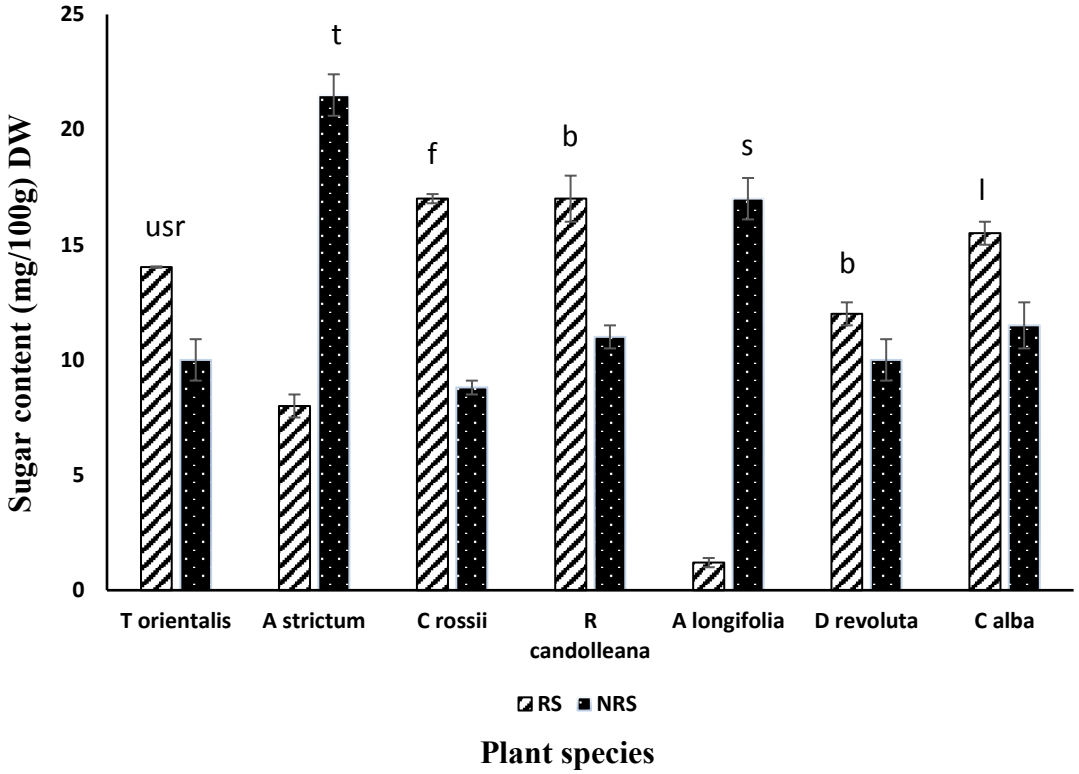


Figure 3 a .2: **Reducing and non-reducing sugar content of native plant species collected in 2019.** Data are means ± SD of triplicate determinations; RS, reducing sugar; NRS, non-reducing sugar; usr, underground stems and rhizomes; b, berries; t, tubers; f, fruits; s, seeds; l, leaves.

3 a .9 .2 Total phenolic content (TPC)

In 2017 the TPC of *A*. *strictum* and *L*. *parviflorus* was higher than the other plant species (Fig. 3 a .3) whereas in 2019 *C*. *alba* recorded higher TPC (Fig. 3 a .4) than the rest of the species ($P<0.05$). The TPC of *L*. *parviflorus* was not determined in 2019 due to unavailability of the berries. Except for *A*. *longifolia*, where TPCs did not change significantly in both years ($P>0.05$), the rest of the species recorded higher TPCs in 2019 than 2017 ($P<0.05$). Biosynthesis of phenolic components is reported to be influenced by agro-climatic conditions of sunshine, rainfall and temperature (Kumar et al., 2017). The significant increase in TPC recorded in 2019 is therefore not surprising considering the reduced rainfall and increased sunshine reported in the study area from 2017 to 2019 (Australian government bureau of meteorology, 2019). A high TPC is likely to indicate richness in free-radical scavenging polyphenols with different plant parts containing different amounts. While the leaves of *C*. *alba* recorded the highest in TPCs in 2019, berries of *D*. r*evoluta* were the lowest, followed by the seeds of *A*. *longifolia* (Figs. 3 a .3 & 3 a .4).

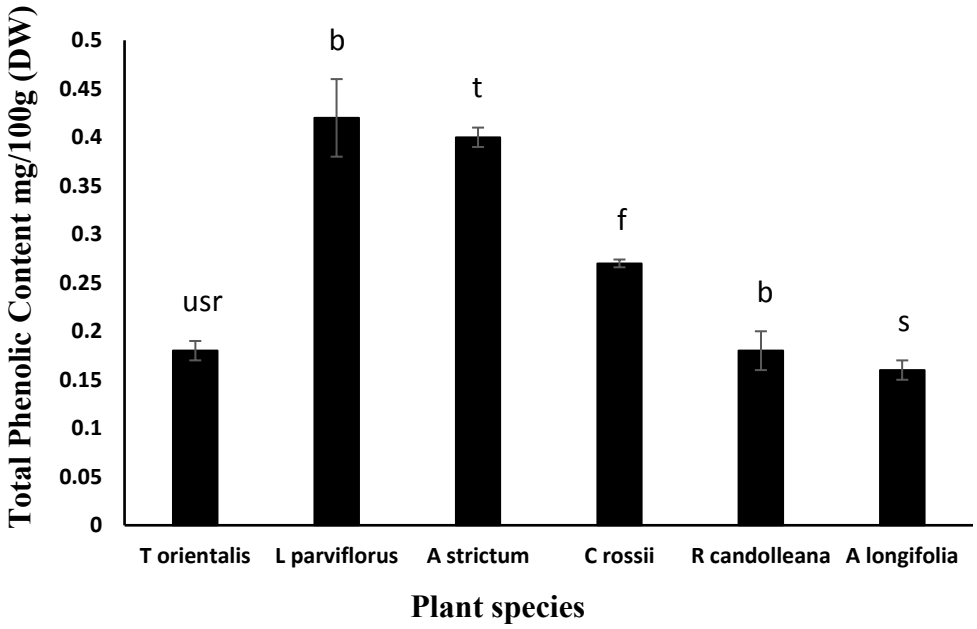


Figure 3 a .3: **Total Phenolic Content mg/100g (DW) of native plants collected in 2017.** Data are means ± SD of triplicate determinations ($P<0.05$); usr, underground stems and rhizomes; b, berries; t, tubers; f, fruits; s, seeds.

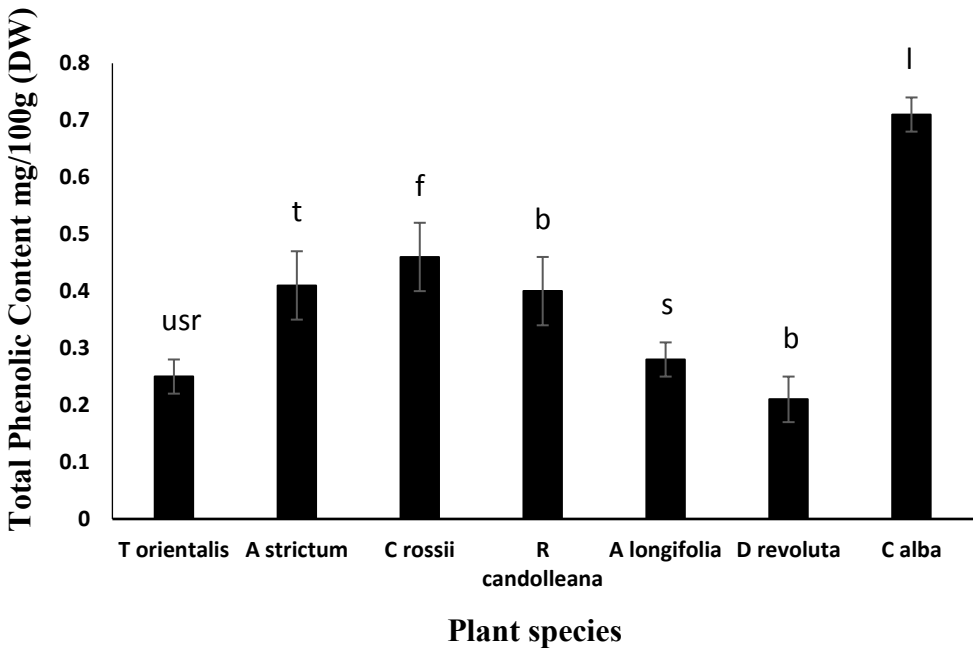
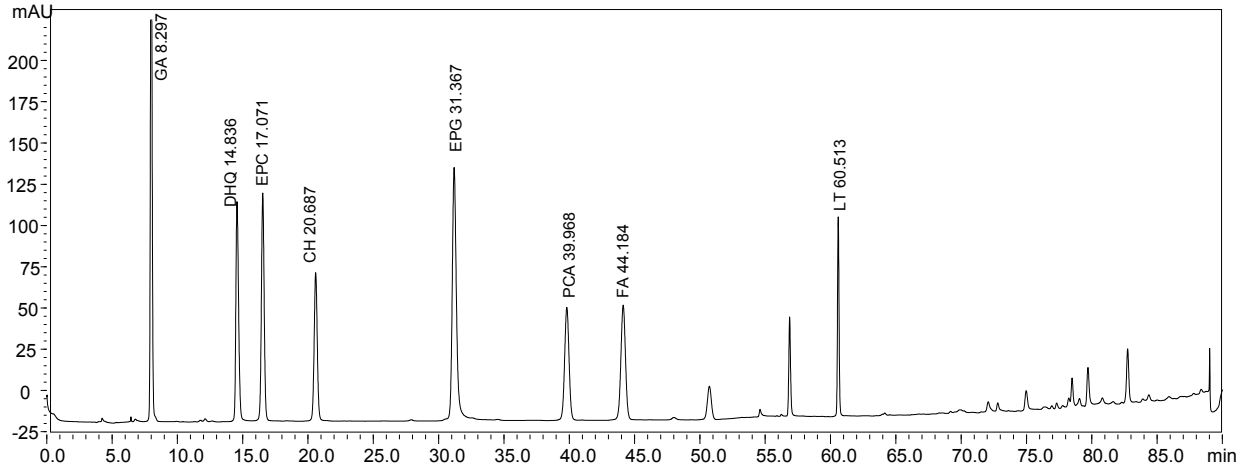


Figure 3 a .4: **Total Phenolic Content mg/100g (DW) of native plants collected in 2019.** Data are means ± SD of triplicate determinations ($P<0.05$); usr, underground stems and rhizomes; b, berries; t, tubers; f, fruits; s, seeds; l, leaves.

3 a .9 .3 Plant component analysis

Figure 3 a .5 a chromatogram depicts the standard mixture of eight reference compounds (gallic acid (GA), Dihydroquinidine (DHQ), epigallocatechin (EPC), catechin (CH), epigallocatechingallate (EPG), ρ-coumaric acid (PCA), Ferulic acid (FA) and Luteolin (LT**)** whereas figure 3 a .5 b, chromatogram depicts phenolic profile of *T*. *orientalis* aqueous extract with six compounds (gallic acid (GA), Dihydroquinidine (DHQ), epigallocatechin (EPC), catechin (CH), epigallocatechingallate (EPG) and ρ-coumaric acid (PCA).

Chromatograph a



Retention time

Figure 3 a .5 a: **RP-HPLC Analysis**; **elution profiles of standard mixture showing compounds together with their retention times;** gallic acid (GA), Dihydroquinidine (DHQ), epigallocatechin (EPC), catechin (CH), epigallocatechingallate (EPG), ρ-coumaric acid (PCA), Ferulic acid (FA) and Luteolin (LT**)**. Chromatographic analysis was performed on a Shimadzu HPLC system equipped with a C18 Phenomenex Luna column (2.4 mm porosity, 250 x 4 mm, and an UltraSep ES RP18 pre-column) with a 3-steplinear gradient of solvent A 0.3 % phosphoric acid and solvent B 100 % acetonitrile. The elution pattern was monitored with a photodiode array detector at 220 nm.

Chromatograph b

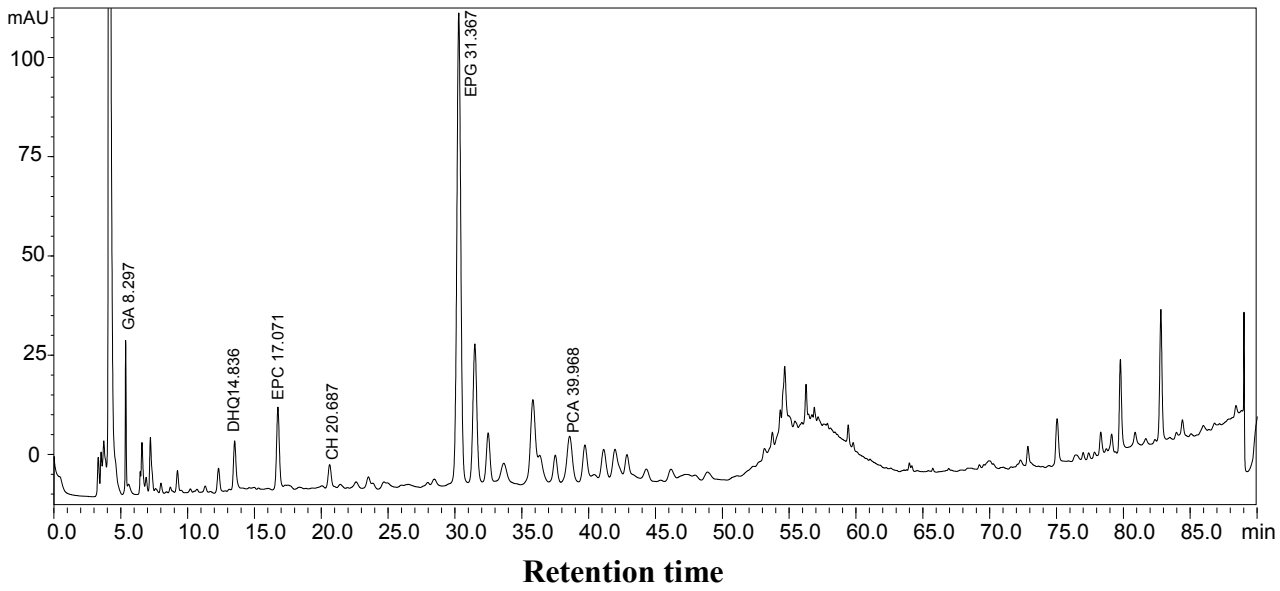


Figure 3 a .5 b: **RP-HPLC Analysis; profile of aqueous extracts of *T. orientalis* showing 6 compounds and their retention times;** gallic acid (GA), Dihydroquinidine (DHQ), epigallocatechin (EPC), catechin (CH), epigallocatechingallate (EPG) and ρ-coumaric acid (PCA). Chromatographic analysis was performed on a Shimadzu HPLC system equipped with a C18 Phenomenex Luna column (2.4 mm porosity, 250 x 4 mm, and an UltraSep ES RP18 pre-column) with a 3-steplinear gradient of solvent A 0.3 % phosphoric acid and solvent B 100 % acetonitrile. The elution pattern was monitored with a photodiode array detector at 220 nm.

The aqueous extract of *C. alba* also contained 6 phenolic compounds (GA, CH, EPC, EPG, PCA and LT) whereas the methanol extract contained 4 (GA, EPC, EPG and LT). Overall, more components were detectable in aqueous extracts of five of the plant species studied (Table 3 a .2). However, except for BT (betanidin 5-O-β-D-glucoside), the methanol extracts produced higher quantities of extractable polyphenols than aqueous extracts ($P<0.05$). GA was detected in all the species except *R. candolleana*. FA was not detected in any of the species.

R. candolleana recorded a very high amount of BT (700 mg/kg) and together with *C. rossi* (244 mg/kg) were the only two species that contained this pigment. It is important to note that samples of *C. rossi* collected in 2017 had a fresh green colour and did not contain BT. However, 2 years later in 2019, samples of *C. rossi* collected from the same spot were red-purple in colour and tested positive for BT. The production of BT in the 2019 samples of *C. rossi* may be attributed to changes in climatic conditions characterized by reduction in rainfall and increased sunshine in the state of Victoria from 2017 - 2019 (Australian government bureau of meteorology 2019), which probably caused the plants to ripen faster and to produce more pigments in adaptation. BT is a photo labile betacyanin with strong antioxidant properties and useful compound in the food, beverage, cosmetic and pharmaceutical industries as a natural colourant (Goncalves et al., 2012; Antigo *et al*., 2018). It has anti-oxidative and anti-inflammatory properties and to the best of our knowledge, extraction and isolation of this compound in *R. candolleana* and *C. rossi* is interestingly being reported for the first time.

Table 3 a .2: **Plant components (mg/kg) detected in methanol extracts and aqueous extracts of native plant species.**

Plant	GA	DHQ	EPC	CH	EPG	PCA	FA	LT	BT
To	127.3±5.7	-	114.5±19.1	115.4±13	160.1±26	-	-	-	-
Lp	111.8±28	-	19±2.8	-	71±28.3	-	-	-	-
As	77±21.2`	-	-	-	59±5.7	-	-	-	-
Cr	91±14.1	-	-	-	-	-	-	69±22.6	-
Rc	-	-	44±14.1	-	-	-	-	83±14.1	-
Al	22±5.7	-	81±10	-	35.5±9.2	-	-	-	-
Dr	102±8.5	-	26±1.4	19±2.8	-	-	-	41±15.6	-
Ca	25±8.5	-	46±14.1	-	52±11.3	-	-	61±18.4	-
	GA	**DHQ**	**EPC**	**CH**	**EPG**	**PCA**	**FA**	**LT**	**BT**
Aqueous extracts									
To	77.1±5.8	17±7.1	91.2±8.5	93.5±31.8	59.4±25.5	93.5±17	-	-	-
Lp	99±10	-	19.1±7.8	-	65±26.9	-	-	-	-
As	59.5±3.5	-	-	-	62.8±5.7	-	-	-	-
Cr	77.4±14.1	-	-	-	-	-	-	51.5±15.6	244±8.5
Rc	-	-	36±5.7	-	-	-	-	76±15.6	700±70.7
Al	22.8±5	-	73.5±14.1	-	33±14.1	-	-	-	-
Dr	88±5.7	101±5.7	22±4.2	17±1.4	-	-	-	33±5.7	-
Ca	17±4.2	-	39±10	31±12.7	47±7.1	34±5.7	-	55±4.2	-

Data are mean ± SD of triplicate determinations; -, Tested but not detected; To, *T. orientalis*; Lp, *L. parviflorus*; As, *A. strictum*; Cr, *C. rossii*; Rc, *R. candolleana* subsp. *candolleana*; Al, *A. longifolia* subsp. *sophorae*; Dr, *Dianella revoluta*; Ca, *Correa alba*.

The high betanin-content of *R. candolleana* is not surprising, as this saltbush plant, adapted to harsh saline environments shares the same family (Chenopodiaceae) with *Beta vulgaris* L., the major source of betanin use for food-colouring purposes and contains ≥ 800 mg/L of the pigment (Goncalves et al., 2012; Wruss et al., 2015; Atigo et al., 2018; da Silva et al., 2019). At 700 mg/kg betanin-content, *R. candolleana* is therefore a likely potential source of the pigment for industrial application.

Apart from their food properties, the selected species also have cultural, medicinal and ornamental uses within the community (Knowles et al., 2014). It is not uncommon to find *R. candolleana*, *C. rossi* or *C. alba* propagated along beaches and foot paths with brightly coloured fruits or flowers. *D.* r*evoluta* is also used as an ornamental plant in home and school gardens (Kjelgren et al., 2009). Whereas the gum of *A. longifolia* is used as glue especially in making Indigenous tools, the leaves of *C. rossii* are used to treat gastrointestinal upsets, and sometimes topically applied to treat scratches and bites (Pirie et al., 2014). It is worth noting that even though most of these species are known to be resistant to diseases and pest, they can as well survive harsh environmental conditions. *L. parviflorus* was not readily accessible in the study area therefore domestication and further propagation may improve its availability.

The compounds detected in this study have been reported in other species including *Camellia sinensis* L. (Kuntze), *Malus prunifolia* (Willd.) Borkh, *Moringa Oleifera* Lam. and *Momordica charantia* L.(Du et al., 2012; Hassan et al., 2011; John et al., 2014; Oboh et al., 2015; Zhang et al., 2016; Perez et al., 2018). Most of the compounds (GA, EPC, LT, PCA, DHQ, CH and EPG) detected in this study have been reported to exhibit strong antioxidant activities and further claimed to have anticancer properties (Du et al., 2012; Hassan et al., 2011; John et al.,

2014; Oboh et al., 2015; Zhang et al., 2016). The detection of luteolin, a flavonoid with strong anti-oxidative, anti-tumourigenic and anti-inflammatory properties in the methanol and aqueous extracts of *C. alba* is of particular interest as it is believed to have multiple cardio-protective effects (Luo et al., 2017). Furthermore, catechins are known to be inhibitors of enzymes involved in carbohydrate metabolism (He et al., 2007; Liu et al., 2016 a), an important factor in the fight against type 2 diabetes. PCA has antioxidant, anti-inflammatory and antidiabetic properties and is known to lower blood glucose by interfering with the activity of glucose-6-phospahatase and fructose-1-6-bisphosphatase (Amalan et al., 2016). However, it is not obvious that such properties would have direct beneficial effects upon consumption of these species as there are likely to be changes during processing or cooking procedures.

3 a .9 .4 Antioxidant capacity (DPPH, ABTS)

The antioxidant activity of methanol and aqueous extracts was similar for 2017 and 2019 ($P>0.05$). However, PCA, BT and DHQ, were only detectable from the aqueous extracts or sodium phosphate buffer at pH 5.5 (Table 3 a .2) but did not change the overall antioxidant activity of the individual species (Figs. 3 a .6 & 3 a .7). Both methanol and aqueous crude extracts of tubers of *A. strictum* exhibited the lowest antioxidant activities in 2017 and 2019 compared to the rest of the species ($P<0.05$) (Figs. 3 a .8 & 3 a .9). Even though it was expected that the aqueous extracts of the species will be more reactive because of the additional compounds, the difference was not significant ($P>0.05$).The antioxidant activity of the aqueous extract of *C. alba* was stronger than *A. longifolia*, *D. revoluta* and *A. strictum* ($P<0.05$), but was not significantly different from *T. orientalis* ($P>0.05$) whereas the halophytic species (*C. rossi* and *R. candolleana* fruits and berries respectively) had similar antioxidant capacities (Figs. 3 a .8 & 3 a .9). The strong antioxidant content of most of the plant species especially *C.*

*alba*and *T. orientalis* may indicate their natural adaptability in the wild Australian climate. Bunea et al. (2011) and Liu et al. (2016 b) reported that species grown under different geographic/climatic conditions exhibited different total phenolic content and antioxidant capacities. However, no significant increase was recorded in antioxidant activity between 2017 and 2019 ($P>0.05$). Further studies that would include longer time frames and more frequent sampling may help detect significant variability in antioxidant activity with the rest of the species.

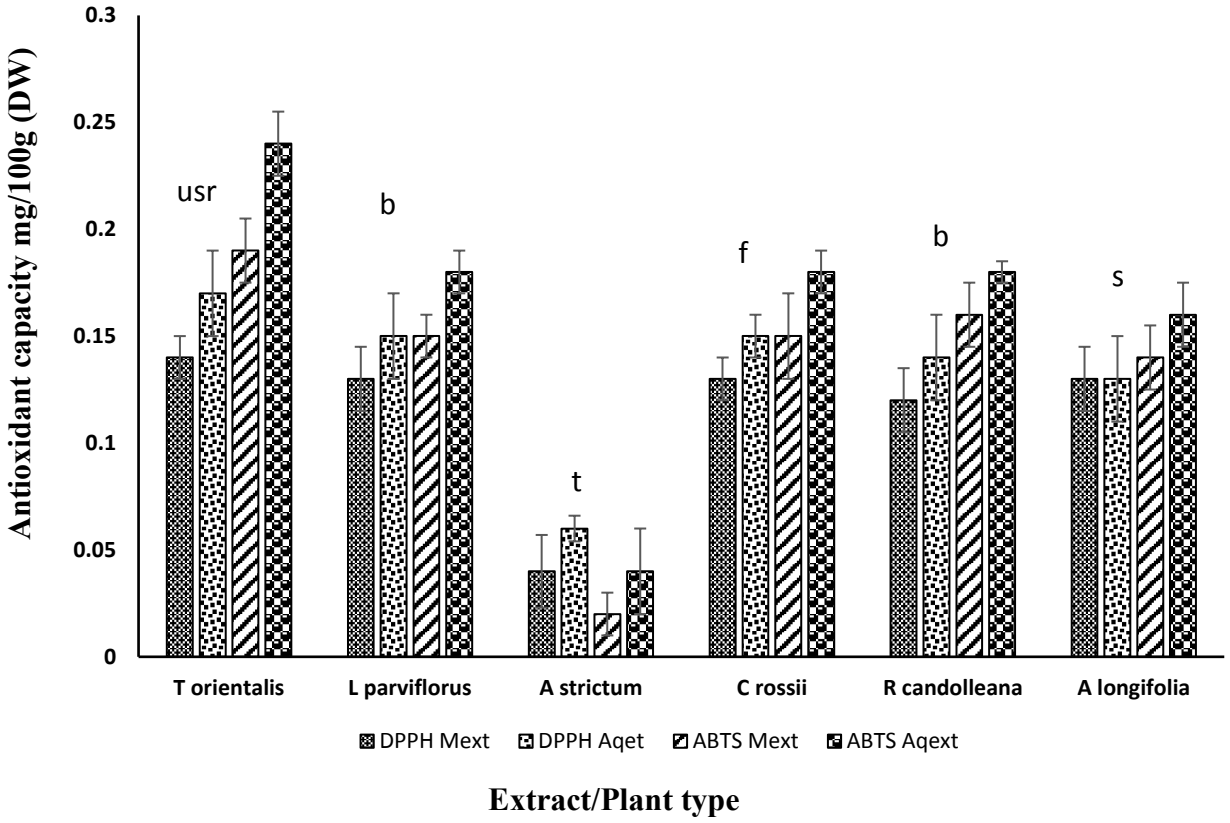


Figure 3 a .6: **Antioxidant activity mg/100g (DW) of native plants collected in 2017.** Data are means ± SD of triplicate determinations (P<0.05); Mext, methanol extract; Aqext, aqueous extracts; usr, underground stems and rhizomes; b, berries; t, tubers; f, fruits; s, seeds.

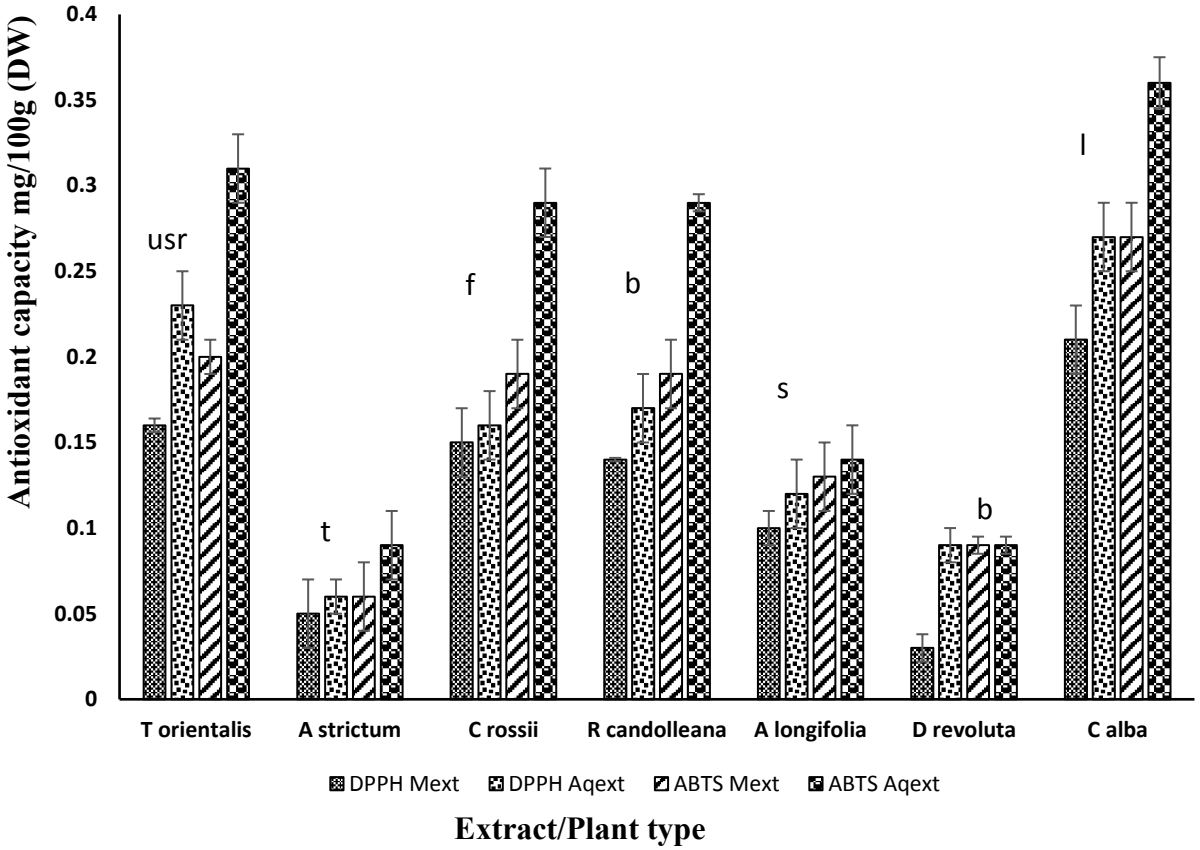


Figure 3 a .7: **Antioxidant activity mg/100g (DW) of native plants collected in 2019.** Data are means ± SD of triplicate determinations (P<0.05); Mext, methanol extract; Aqext, aqueous extracts; usr, underground stems and rhizomes; b, berries; t, tubers; f, fruits; s, seeds; l, leaves.

With the rise in non-communicable diseases (Vos et al*.,* 2009; de Abreu et al., 2013; AIHW 2016), consumption of plant species such as *C. alba*, *T. orientalis*, *L. parviflorus*, *C. rossii*, *R. candolleana* and *A. longifolia* which have demonstrated rich antioxidant potential and TPCs in this study are likely to be beneficial in curtailing alarming increases in overweight/obesity and oxidative stress-related diseases within the Australian Indigenous population. Coupled with their rich antioxidant content, crude extracts of *C. rossi* have been shown to possess hypolipidaemic effects in rats and it is believed that such properties may play a role in reducing cardiovascular risk (Pirie et al., 2013; 2014).

It is worth mentioning that oxidative processes in the body results in production of reactive oxygen species or free radicals such as superoxide ions (O^{-2}), Hydroxyl ions (OH^{-1}) and non-free radical species such as hydrogen peroxide (H_2O_2). These species have been known to play a role in the initiation and progression of degenerative pathological conditions such as cancer, diabetes mellitus, Alzheimer's and cardiovascular diseases (Nita and Grybowski, 2016; Suzen et al., 2017). Physiologically, the human defense mechanism makes provision for the elimination of free radicals from the body. However, sometimes the physiological capacity of the human system is overwhelmed with free radicals, requiring therefore help from externally acquired antioxidants. These antioxidants are generally acquired from food plants with functional properties such as those described in this study. Synthetic antioxidants are readily available, yet their use is not without toxic effects (Saito et al., 2003; Yang et al., 2018) giving preference therefore to natural antioxidants with the advantage of low toxicity, low cost and stronger antioxidant capacity (Anbudhasan et al., 2014; Li et al., 2018). It is believed that the intake of antioxidant-rich foods is inversely related to the prevalence of degenerative diseases (Alissa and Ferns, 2012; Zhang et al., 2016), thus plant species such as *C. alba*, *T. orientalis*,

L. parviflorus, *C. rossii*, *R. candolleana* and *A. longifolia* may be regarded as potential sources of antioxidants within the Indigenous community.

In a study conducted by Varpe et al. (2012), aqueous and methanol extracts of the pollen grains of *Typha angustifolia*, a member of the family Typhaceae were shown to be effective against acute and chronic inflammatory conditions in rats owing to their antioxidant and rich phenolic content. However, none of the three main species of plants in Victoria; *Typha domingensis* Pers. (narrow leaf cumbungi), *T. latifolia* L. (lesser reed-mace) or *T. orientalis* C. Presl (broad leaf Cumbungi) have been reported to exhibit anti-inflammatory properties. The high antioxidant capacity of *T. orientalis* may indicate that most of the phenols in this plant may have strong antioxidant capacities. However, we did not record a significant increase in antioxidant capacity during both years of study and some volatile antioxidant compounds might have been lost during sample preparation, especially during drying (El-Ghorab et al., 2010).

3 a .9 .5 Mineral composition

A total of 8 out of 10 and 9 out of 10 minerals were detected and quantified in 2017 and 2019 respectively (Table 3 a .3). *A. strictum* and *D. revoluta* were the only two species that showed traces of copper (0.05 mg/kg and 0.04 mg/kg) in 2017 and 2019 respectively whereas only *T. orientalis*, *C. rossii* and *C. alba* were found to contain selenium. All the species contained Ca, Mg, K and Na (Table 3 a .3). *T. orientalis* and *A. strictum* recorded the highest detected number of minerals, 7 out of 10 tested. Zn was not detected in any of the species analyzed.*C. alba* recorded the highest amount of Ca whereas *R. candolleana* contained significantly higher amounts of Mg, Na, K and Fe than the rest of the species ($P<0.05$). Samples of *A. longifolia*

collected in 2019 were higher in Na, Ca, and Fe than 2017 whereas samples of *T. orientalis* and *A. strictum* contained higher amounts of K ($P<0.05$). The Na, Mg, Ca, and Fe contents of *C. rossi* and *R. candolleana* were also higher in 2019 than 2017. Overall, 5 of the species exhibited higher mineral contents in 2019 than 2017. It is likely that the dry weather conditions and reduced rainfall in 2019 resulted in increased mineral concentrations in the plant species. Variations in mineral contents of some species due to weather conditions have been previously reported (Sud et al., 1995). *R. candolleana* and *C. rossii* being coastal halophytic species collected from the coast of Warrnambool, Victoria, Australia were among species with the highest mineral content (Table 3 a .3). It has been reported that coastal species and seaweeds may contain 10 - 20 times more minerals and trace elements than some inland species (Akhter et al., 2014), thus the high mineral content of *R. candolleana* and *C. rossii* is therefore not surprising. Considering that seawater is high in salt and minerals (Loganathan et al., 2017), coastal species and seaweeds occasionally inundated with seawater are bound to be high in minerals.

Table 3 a .3: **Variation in mineral composition (mg/kg) of selected native plant species**

	Plant type							
Mineral 2017	**To**	**Lp**	**As**	**Cr**	**Rc**	**Al**	**Dr**	**Ca**
Ca	131.2±1	21.4±0.5	25.6±0.5	444±3.6	844.4±1.5	20.1±1.7	nd	nd
Cr	-	-	-	-	-	-	nd	nd
Cu	-	-	0.05±0.04	-	-	-	nd	nd
Fe	5.84±1	-	1.55±0.6	6.47±0.4	41.4±2.1	-	nd	nd
K	385±3	65.3±6.4	112±1	176±3.5	730±2.3	770.7±2.9	nd	nd
Mg	61.8±1.2	11.6±0.8	11.3±0.7	155.4±5.5	565.8±2.9	130±2.6	nd	nd
Mn	0.11±0.02	-	0.004±0.002	-	0.03±0.01	-	nd	nd
Na	97.8±0.2	114±2.9	5.14±0.2	352±2.5	855±8.1	94.2±0.9	nd	nd
Se	0.71±0.09	-	-	1.41±0.4	-	-	nd	nd
Zn	-	-	-	-	-	-	nd	nd
2019	**To**	**Lp**	**As**	**Cr**	**Rc**	**Al**	**Dr**	**Ca**
Ca	319±1	nd	33±3	615±1	1004.7±0.2	76.1±1.7	8.9±0.4	2010.3±2.3
Cr	-	nd	-	-	-	-	0.08±0.02	-
Cu	-	nd	-	-	-	-	0.04±0.0	-
Fe	6.5 ±0.8	nd	-	17.7±0.8	61.4±0.7	2.14±0.1	-	25.8±0.06
K	590.3±0.5	nd	178±1.5	191±1	1389.3±45.5	1070±6	269±1	763±6
Mg	117.4±2.5	nd	4.7±0.3	349.3±0.6	618.3±1.5	128±2.6	10±0.09	374.3±2.3
Mn	4.4±0.2	nd	-	-	-	-	-	-
Na	150±3	nd	9.4±0.5	704.3±4.5	1025±5.6	167.3±0.6	6.51±0.06	256±26.2
Se	0.28±0.03	nd	-	5.5±0.8	-	-	-	7.1±0.2
Zn	-	nd	-	-	-	-	-	-

Data are mean ± SD of triplicate determinations. -, Not detected. Nd, not determined, To, *T. orientalis*; Lp, *L. parviflorus*; As, *A. strictum*; Cr, *C. rossii*; Rc, *R. candolleana* subsp. *candolleana*; Al, *A. longifolia* subsp. *sophorae*; Dr, *D. revoluta*; Ca, *C. alba*.

Three of the species studied herein (*T. orientalis* , *C. rossii* and *C. alba*) also contained Se, a rare but important mineral in normal human physiology and function as part of the enzyme glutathione peroxidase involved in organic peroxide elimination (Trevisan et al., 2014). Generally, minerals play an important role in the functioning of human physiology and may include structural, catalytic, signaling or osmotic functions. While Na and K ratios are important in the transmission of impulses in the central nervous system, maintenance of osmotic balance across membranes and regulation of blood pressure, Ca and Mg are important components of bones, muscle contraction and enzyme activities (Morris et al., 2010).

The daily intake for Se in Australia is 50 µg/day and 60 µg/day for women and men respectively (NHMRC, 2014). Unfortunately, common foods may not always have adequate concentrations to meet dietary requirements (Multari et al., 2016), and so little amounts from native food plants within meals may be helpful. In a previous study, *Typha latifolia* L., a related species of *T. orientalis* was found to contain Mn in the leaves and Zn and Fe in the roots (Parzych et al., 2016). However, this study detected small amounts of Mn and Fe but not Zn, in the underground stems and rhizomes of *T. orientalis* consumed by the Victoria Indigenous population. The importance of all three minerals is well established and cannot be overemphasized; Mn is a cofactor of arginase, glutamine synthetase and pyruvate carboxylase while Zn is a cofactor of lactate dehydrogenase, alkaline phosphatase, superoxide dismutase, retinene reductase and Fe; an important component of haemoglobin and cytochromes that function in cellular respiration (Soetan et al., 2010; Yamada et al., 2014; Chen et al., 2018).

3 a .9 .6 Fatty acid composition

Fatty acids detected in the standard mix are displayed in figure 3.8 whereas figure 3.9 depicts fatty acid profile of *A*. *longifolia* with linoleic acid, (an omega-6-polyunsaturated compound) as the dominant fatty acid. Total of 10 different fatty acids were identified in the selected species (Table 3 a .4), however not all the fatty acids were found in any single species. The dominant fatty acids were palmitic (P), oleic (O) and linoleic (L) acids. Palmitic acid was the most common fatty acid, detected in 6 of the 8 species studied. The fatty acid content of the species did not differ in 2017 and 2019 ($P>0.05$). The detection of palmitic, linoleic and oleic acids were found to be ≥20 %, 42.4 % and 12.3 % respectively in the seeds of *A*. *longifolia*. These results are consistent with the findings of Brown et al. (1987) who reported these as major fatty acids in Australian *Acacia* species. Also, while working on Tunisian *Acacia* species, Youzbachi et al. (2015) reported linoleic (>52 %) and oleic (15-27 %) acids as major components in Tunisian *Acacia* seed oil. All three studies therefore, have demonstrated that *Acacia* seeds could be regarded as a rich source of oil, mostly polyunsaturated and monounsaturated fatty acids with potential for wide industrial application.

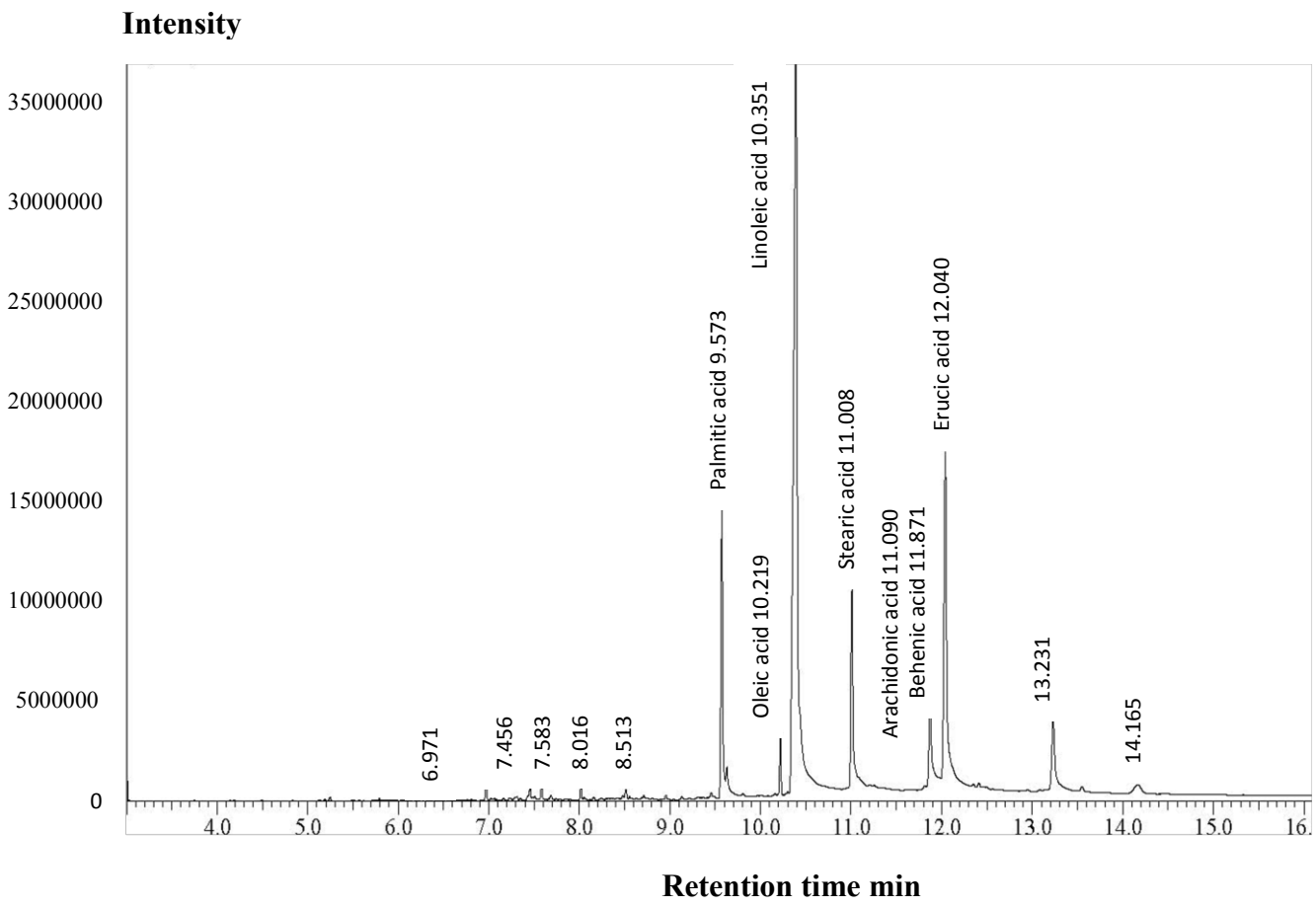


Figure 3 a .8: **GCMS Analysis**; **elution profiles of standard mixture of fatty acids and their retention times**; palmitic acid, linoleic acid, oleic acid, stearic acid, arachidonic acid, behenic acid and erucic acid. Chromatographic analysis was performed on a Shimadzu GC system equipped with a Phenomenex column of 30 m × 0.25 mm id, ft. 0.50 µm and MS detector (GCMS-QP2010 Plus). The detector was set at 350˚ C and the injector port at 250˚ C. Separation of fatty acids was achieved using the following column temperature conditions; 80˚ C (held for 2 min), heated to 220˚C at 30˚C per min and held at 220˚C for 5.5 min with a total run time of 12.17 min. A mixture of nitrogen, hydrogen and air was used as carrier gas at a linear velocity of 3.5 mL/s.

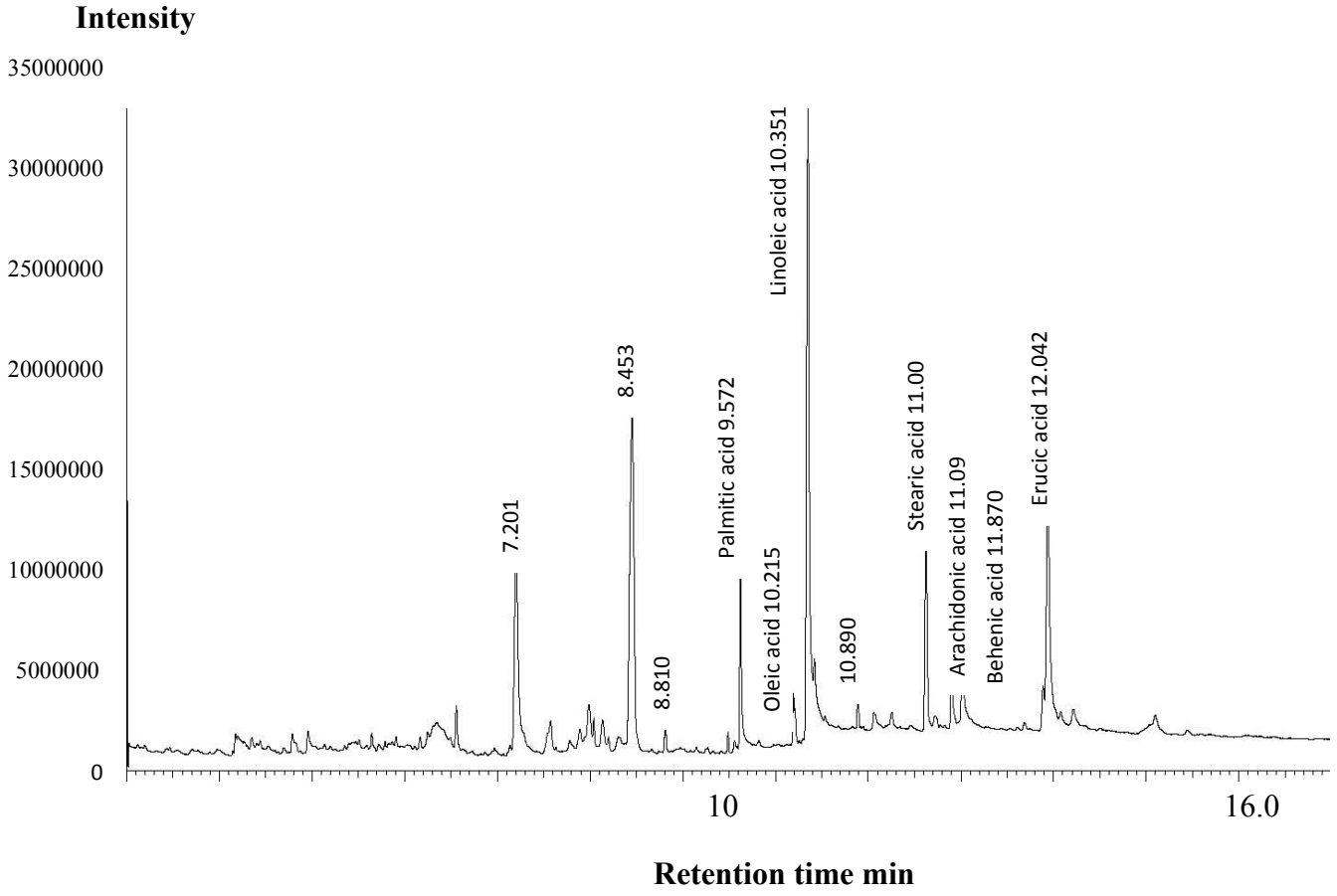


Figure 3 a .9: **GCMS Analysis**; **elution profiles of fatty acids from hexane extracts of *A. longifolia* and their retention times**; palmitic acid, oleic acid, linoleic acid, stearic acid, arachidonic acid, behenic acid and erucic acid. Chromatographic analysis was performed on a Shimadzu GC system equipped with a Phenomenex column of 30 m × 0.25 mm id, ft. 0.50 µm and MS detector (GCMS-QP2010 Plus). The detector was set at 350˚C and the injector port at 250˚ C. Separation of fatty acids was achieved using the following column temperature conditions; 80˚C (held for 2 min), heated to 220˚C at 30˚C per min and held at 220˚C for 5.5 min with a total run time of 12.17 min. A mixture of nitrogen, hydrogen and air was used as carrier gas at a linear velocity of 3.5 mL/s.

Table 3 a .4: **Fatty acid composition of selected native plant species**

Plant name	**February 2017 collections**	**% estimate**	**February 2019 collections**	**% estimate**
T. orientalis	Palmitic acid	37 ± 4.2	Palmitic acid	39 ± 4.2
	Oleic acid	39 ± 6.4	Oleic acid	39 ± 3.5
	Myristic acid	29 ± 1.4	Myristic acid	33 ± 2.8
L. parviflorus	Palmitic acid	38 ± 5.0	nd	nd
	Oleic acid	15.6 ± 5.2	nd	nd
	Linoleic acid	41 ± 4.2	nd	nd
	Arachidonic acid	9 ± 1.4	nd	nd
	Myristic acid	tr	nd	nd
A. strictum	Palmitic acid	17 ± 2.8	Palmitic acid	21.5 ± 1.4
	Oleic acid	37 ± 3.5	Oleic acid	37 ± 4.2
	Linoleic acid	tr	Linoleic acid	tr
	Stearic acid	8.5 ± 2.8	Stearic acid	10 ± 4.2
	Myristic	8.2 ± 1.8	Myristic acid	9 ± 2.1
	Erucic	tr	Erucic acid	tr
C. rossi	Palmitic acid	5.5 ± 0.7	Palmitic acid	7.5 ± 2.8
	Linoleic acid	10.1 ± 1.4	Linoleic acid	10 ± 0.7
	Myristic acid	tr	Myristic acid	tr
R. candolleana	Palmitic acid	17.6 ± 2.4	Palmitic acid	19.5 ± 3.5
	Oleic acid	31 ± 2.8	Oleic acid	31 ± 1.4

A. longifolia	Palmitic acid	20 ± 3.5	Palmitic acid	21 ± 0.7
	Oleic acid	12.3 ± 0.3	Oleic acid	15.2 ± 0.6
	Linoleic acid	42.4 ± 1.4	Linoleic acid	44 ± 4.2
	Stearic acid	24.5 ± 2.1	Stearic acid	22 ± 5.7
	Erucic acid	22 ± 2.8	Erucic acid	23 ± 4.2
	Arachidonic acid	11.2 ± 3.1	Arachidonic acid	13 ± 2.8
	Myristic acid	tr	Myristic acid	tr
	Arachidic acid	ndt	Arachidic acid	ndt
	Behenic acid	tr	Behenic acid	tr
D. revoluta	nd	nd	Linoleic acid	33.1 ± 3
	nd	nd	Linolenic acid	12.6 ± 2.8
	nd	nd	Myristic acid	15 ± 3.5
	nd	nd	Stearic acid	tr
	nd	nd	Erucic acid	tr
C. alba	nd	nd	Oleic acid	27 ± 7.1
	nd	nd	Linoleic acid	13 ± 1.4
	nd	nd	Myristic acid	7.4 ± 1.1
	nd	nd	Arachidonic acid	21 ± 5.7
	nd	nd	Oxalic acid	tr
	nd	nd	Erucic acid	tr

Data are mean ± SD of triplicate determinations; nd, not determined; tr, traces (<0.5%); palmitic acid (PA), myristic acid (MA), linoleic acid (LA), oleic acid (OA), stearic acid (SA), arachidonic acid (AA), behenic acid (BA) and erucic acid (EA)

3 a .9 .7 Conclusion

All eight species (*T. orientalis*, *L. parviflorus*, *A. strictum*, *C. rossii*, *R. candolleana, D. revoluta*, *C. alba* and *A. longifolia*) were found to be sources of proteins, carbohydrates, total dietary fibre, minerals and water. Palmitic, oleic and linoleic acids were the dominant fatty acids isolated from the species in 2017 and 2019 and there were no differences for 2017 and 2019 samples. The aqueous and methanol crude extracts were positive for phenolic compounds and exhibited consistent antioxidant activities in 2017 and 2019, yet TPCs of the species were higher in 2019 than 2017 ($P<0.05$). More components were detectable in aqueous extracts of 5 of the species studied, except for BT, the methanol extracts produced higher quantities of polyphenols than aqueous extracts ($P<0.05$), indicating that both extraction methods are necessary to maximize the extraction of phyto-components. *R. candolleana* to the best of our knowledge is being reported for the first time as a likely source of betanin, an antioxidant pigment with wide industrial application. All the species contained phenolic compounds with consistent antioxidant activities that are likely to be protective against diseases caused by free radical-production in the body. However, the *in vivo* free-radical scavenging activity of most of these compounds are yet to be determined. Whereas the nutritional contents and fatty acids of plant species were fairly stable between 2017 and 2019, some of the minerals, TPCs and some active components varied. Further studies would be required to determine greater degree of variability of different phyto-components within a longer time frame.

Chapter 3 b

FERRIC REDUCING ANTIOXIDANT POWER AND ENZYME INHIBITORY ACTIVITY OF FOOD PLANTS

This chapter presents a continuation of the determination of antioxidant capacity of the selected plant species using ferric reducing/antioxidant power and the continuous evaluation of the antidiabetic properties of the plants through glycosidic enzyme inhibitory assays.

3 b .1 Abstract

Antidiabetic control strategies modelled after glycosidic enzyme inhibitory activities of phenolic antioxidants are promising avenues for management of T2DM globally. The objective of this study was to determine the antioxidant and enzyme inhibitory activity of selected plant species namely; *Typha orientalis*, *Leucopogon parviflorus*, *Arthropodium strictum*, *Carpobrotus rossii*, *Rhagodia candolleana*, *Acacia longifolia*, *Dianella revoluta* and *Correa alba*. Crude extracts prepared from edible portions of the plant species were analysed for antioxidant activity by ferric reducing/antioxidant power with ascorbic acid as reference control standard. The plant extracts inhibition of α-glucosidase and α-amylase were performed in 96-well micro titre plates with acarbose as the reference control standard. All the plant species exhibited antioxidant activities measured as ascorbic acid equivalent antioxidant capacity (AAE). The antioxidant capacities of *C. alba* (0.38 ± 0.02 mg/100g DW AAE) and *T. orientalis* (0.33 ± 0.01 mg/100g DW AAE) were higher than the rest of the species ($P<0.05$). Except for *A. strictum* and *D. revoluta*, the rest of the plant crude extracts were found to be inhibitory to α-glucosidase and α-amylase at concentrations of (5.00 - 0.078) mg/mL. The inhibitory activity of acarbose was higher than the plant crude extracts ($P<0.05$) and while its IC_{50} was 2.5 mg/mL, only *R. candolleana* crude extracts produced 49.5% inhibition at 5.0

mg/mL. The plant species exhibited strong antioxidant capacities and their inhibition of 2 enzymes involved in carbohydrate metabolism is a promising lead in the search for control strategies against the rising prevalence of T2DM within the Indigenous community.

3 b .2 Introduction

Postprandial hyperglycaemia, a key feature of T2DM, depends on the amount and speed at which carbohydrates are absorbed into the blood stream (Laoufi et al., 2017). This means biological activities that can interfere with digestion and/or absorption of carbohydrates would ultimately influence the release of glucose in the blood stream. Inhibition of glycosidic enzymes by some plant secondary metabolites have been documented in many plant species from different parts of the world (Deng et al., 2020; Agu et al., 2019; Karakaya et al., 2018). Studies detailing the inhibitory characteristics of some polyphenolic compounds have been reported to play a major role in the development of antidiabetic agents for the management of T2DM (Jung et al., 2006; Kumar et al., 2011; Patade and Marita, 2014; Bailey, 2017). For example, antidiabetic agents of vascicine, vitexin, orientin and pinatin are all α-glucosidase inhibitors initially isolated from some plant species (Kumar et al., 2011). Similarly some active ingredients of metformin were also isolated from plants (Bailey, 2017). However, the plant kingdom is still endowed with enormous amount of resources with limited information on their antidiabetic potential. This is particularly true of Australia's rich biodiversity which constitute approximately 10% of the world's plant population, most of which have not been studied for their application against T2DM. This study was designed therefore to evaluate the antidiabetic potential of selected Australian native plant species by testing their inhibitory activity to α-glucosidase and α-amylase. Both enzymes are involved in regulating postprandial hyperglycaemia by canalizing the release of glucose (Navarro et al., 2019). Considering the alarming rate of T2DM within Australia's Indigenous community (Titmuss et al., 2019),

inhibitors of the enzymes by bioactive components of native plant species would help regulate glucose release and thus could constitute potential antidiabetic agents. The plant species studied herein were selected based on their contribution to nutrition, health and general wellbeing for the Indigenous community (O'Dea et al., 1991; Clarke, 2008; Tan et al., 2010).

Dietary polyphenols, especially those with antioxidant properties found in fruits, vegetables and beverages may offer significant beneficial effects to human health (Ganesan et al., 2017). They have therefore been the subject of increasing scientific interest in the last couple of decades as they are perceived as a means to control alarming increases in non-communicable diseases such as cancer, T2DM, Alzheimer's, hypertension and cardiovascular diseases (Valko et al., 2007; Estruch et al., 2013). Different methods have been applied to determine antioxidant capacity of polyphenols based on hydrogen atom transfer (HAT) and single electron transfer (SET) techniques (Pisoschi et al., 2016). HAT method, measures the capacity of an antioxidant to trap free radicals by hydrogen donation, while SET method relies on an electron transfer reductive ability of an antioxidant compound versus a radical species (Prior et al., 2005; Pisoschi et al., 2016). In the previous chapter, the antioxidant capacity of the selected species were evaluated using DPPH and ABTS (HAT and SET respectively), while TPC was evaluated using Folin-Ciocalteu assay (SET method). However, Folin-Ciocalteu assay is also used in some studies to determine antioxidant capacities of plants and these studies make use of three or more methods for comparison (Palombini et al., 2013; Jemli et al., 2016; Pisoschi et al., 2016; Chaves et al., 2020). The FRAP (SET method) antioxidant assay was therefore employed herein to further evaluate the validity and consistency in the plants antioxidant capacity results especially since antioxidant capacity was one of the factors for selecting plant species to be formulated into a food product.

3 b .3 Materials and methods

3 b .3 .1 Determination of antioxidant activity by Ferric Reducing/Antioxidant Power (FRAP)

The ferric-reducing activities (ferricyanide to ferrocyanide) of methanol and the aqueous extracts of selected plant species were studied according to the method described by Bhalodia et al. (2013), with slight modifications. A 2-fold serial dilution of methanol and aqueous extracts of each plant species was prepared in miliQ water by diluting (200 - 0.39) mg/mL. Ascorbic acid reference standard solution was also prepared (0.5 mg/mL - 0.001 mg/mL). A solution of exactly 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of (1%) potassium ferricyanide was added to each sample in test tubes. The test tubes were then incubated at 50 ˚C in a water bath for 20 min followed by addition of 2.5 mL (10%) trichloroacetic acid. The mixture was centrifuged at 3000 x g for 10 min. Exactly 2.5 mL of the supernatant was mixed with 2.5 mL miliQ water and 0.5 mL of 0.1% freshly prepared ferric chloride solution. The absorbance of each tube was measured at 700 nm against miliQ water as blank. The concentration of the extracts (x) was calculated from the ascorbic acid standard equation ($y = 3.3964x - 0.0027$), $R^2 = 0.999$; y = absorbance. The experiment was repeated twice (n = 3) and mean concentration and standard deviation computed. The results were expressed as ascorbic acid equivalent antioxidant capacity (AAE) in mg/100g dry weight (DW).

3 b. 3. 2 Inhibition of α-glucosidase

Alpha-glucosidase inhibition was performed in 96-well micro titre plates as described by Sagbo et al. (2018), with modifications. Briefly, row A-D wells were filled with 100 µL of miliQ water and another 100 µL of the methanol plant crude extracts prepared at concentration of 10 mg/mL was pipetted into the first A-row well and then serially diluted 2-fold. Exactly 100 µL

of Acarbose (positive control) at 10 mg/mL was pipetted into the first B-row well and 2-fold serially diluted. Twenty microlitres of α-glucosidase (50 µg/mL) previously prepared in 0.1 M potassium phosphate buffer (pH 6.8) was added into rows A, B and C wells. Into C and D wells were added 20 µL of miliQ water instead of plant extract and enzyme as blank and sample blank respectively (Sagbo et al., 2018). This was followed by addition of 60 µL of 0.1 M potassium phosphate buffer (pH 6.8) and incubation for 5 min at 37 ˚C. Exactly 10 µL of 10 mM *ρ*-nitrophenyl-*α*-D-glucopyranoside (ρ-NPG, substrate) prepared in miliQ water was added to all the wells and the plate was incubated for 30 min at 37 ˚C. At the end of incubation, 80 µL of 0.2 M sodium carbonate in phosphate buffer (pH 6.8) was added to the wells to quench the reaction. The amount of ρ-NPG released was measured at 405 nm on a 96-well microplate reader (Tokyo, Japan). The experiment was repeated twice (n = 3) and the percentage inhibition was calculated using the following equation:

$$\% \text{ inhibition} = \left[1 - \frac{\text{Absorbance of A-wells (Test)}}{\text{Absorbance of C-wells (untreated control)}}\right] \text{x } 100$$

(Sagbo et al., 2018)

3 b. 3. 3 Inhibition of α-amylase

Alpha-amylase inhibition was performed in 96-well micro titre plates as described by Sagbo et al. (2018), with modifications. Briefly, rows A-D wells were filled with 100 µL of miliQ water and another 100 µL of the methanol plant crude extracts (10 mg/mL) was pipetted into the first well of the A-row and 2-fold serially diluted. Exactly 100 µL of Acarbose (positive control) at 10 mg/mL was pipetted into the first well of the B-row and 2-fold serially diluted. Five

microliters of porcine pancreatic α-amylase (50 µg/mL) previously prepared in 0.1 M potassium phosphate buffer (pH 6.9) was added into rows A, B and C wells and incubated at 37 ˚C for 10 min. C and D wells were used as sample blank and enzyme blank by adding 20 µL of miliQ water instead of plant extract and enzyme respectively. Twenty microlitres of 1% potato starch solution prepared in 20 mM sodium phosphate buffer (pH 6.9) with 6 mM sodium chloride was added to the wells. The plate was incubated for 30 min at 37 ˚C. The reaction was stopped by adding 10 µL of 1M HCl to each well followed by 75 µL of iodine reagent. Absorbance of the plate was measured at 580 nm on a 96-well microplate reader (Tokyo, Japan). The experiment was repeated twice (n = 3) and the percentage inhibition was calculated using the following equation:

$$\% \text{ inhibition} = \left[1 - \frac{\text{Absorbance of C-wells (untreated control)}}{\text{Absorbance of A-wells (Test)}}\right] \text{x } 100$$

(Sagbo et al., 2018)

3 b .4 Statistical analysis

Values of triplicate determinations for antioxidant capacities of methanol and aqueous extracts, α-glucosidase and α-amylase inhibition were entered in Excel spread sheet (Microsoft® Excel, District of Columbia, WA) and IBM SPSS statistic software version 26 (IBM SPSS®, Chicago, IL). Means and standard deviations were computed using both software and multiple comparisons between means were performed by one-way analysis of variance test (ANOVA). Differences between means for antioxidant capacity and enzyme inhibition activities for acarbose and crude extracts were considered significant at $P < 0.05$.

3 b .5 Results and Discussions

3 b .5 .1 Antioxidant capacity

DPPH, ABTS and FRAP assays are the 3 widely used methods for determination of antioxidant capacities (Jemli et al., 2016). Although these methods have different mechanisms; DPPH and ABTS assays are based on electron and H atom transfer, while the FRAP assay is based on electron transfer reaction; results of all three methods are consistently indicative of the strong antioxidant capacity of most of the plant species studied herein. However, a significant drop in antioxidant capacity of *L. parviflorus* was noticed with the FRAP assay (Fig 4.1). The crude extracts of the rest of the plant species were prepared from fresh samples, yet due to scarcity and unavailability of *L. parviflorus* plant material in the study area, samples of *L. parviflorus* that had been stored at -80 ˚C for 2 years were used in preparing the crude extract. The reduced antioxidant capacity of *L. parviflorus* reported with the FRAP assay could therefore be indicative of loss of antioxidant compounds that must have occurred during the storage period, despite its storage at -80 ˚C.

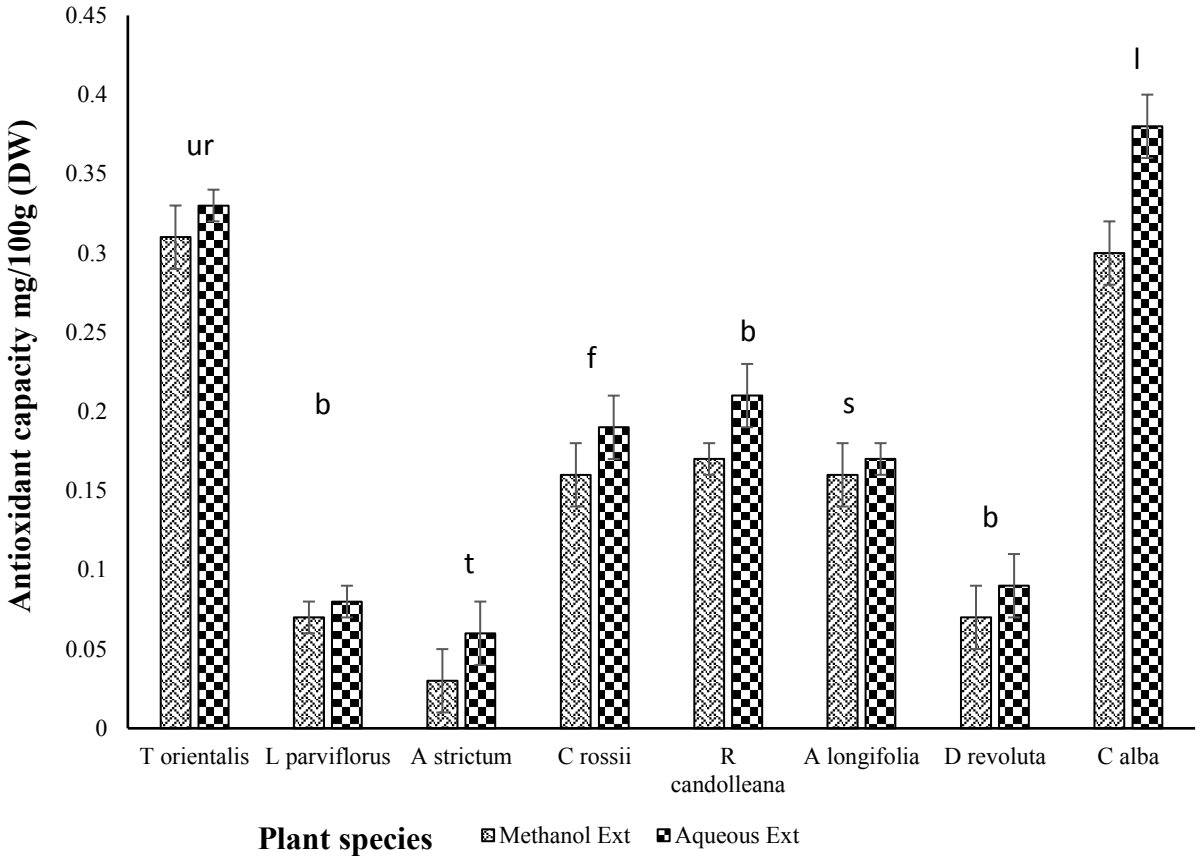


Figure 3 b .1: **FRAP antioxidant capacity mg/100g (DW) of native plant species**. Data are means ± SD of triplicate determinations (P<0.05); Methanol Ext, methanol extract; Aqueous Ext, aqueous extracts; ur, underground stems and rhizomes; b, berries; t, tubers; f, fruits; s, seeds; l, leaves.

All the plant species exhibited antioxidant activity through the reduction of Fe^{3+} to Fe^{2+} in the FRAP assay. Leaves of *C. alba* recorded the highest capacity of 0.38 ± 0.02 mg/100g DW AAE against its aqueous extract while tubers of *A. strictum* recorded the lowest capacity of 0.06 ± 0.02 mg/100g DW AAE (Fig 3 b.1). These results were consistent with previous findings of the DPPH and ABTS assays showing *C. alba* and *A. strictum* as the plant species with highest and lowest antioxidant capacities respectively. The antioxidant capacities of *T. orientalis* and *C. alba* were similar (P>0.05), yet significantly higher than the rest of the plant species (P<0.05). Apart from *T. orientalis* and *C. alba*, the antioxidant capacities of the aqueous

extracts of the rest of the plant species were higher than their methanol extracts. From previous HPLC analysis (chapter 3 a), aqueous extracts of these plant species contained more components with antioxidant properties than methanol extracts which could have contributed to the additional antioxidant capacities reported with the FRAP assay.

Reactive oxygen species (free radicals) have been associated with stress and degenerative diseases such as T2DM (Schiavone et al., 2013; He et al., 2017). Edible portions of some Indigenous food plants with rich antioxidant capacities such as *C. alba*, *T. orientalis, C. rossii*, *R. candolleana* and *A. longifolia* are likely to be useful in mopping up or scavenging for free radicals in the body, thereby mitigating or completely eliminating the deleterious effects of stress, oxidative stress and T2DM.

3 b .5 .2 Enzyme inhibitory activity

Apart from *A. strictum* which exhibited no inhibition to α-glucosidase and *D. revoluta* which recorded zero inhibition against α-amylase, the rest of the plant crude extracts were found to be inhibitory to α-glucosidase (Fig. 3 b .2) and α-amylase (Fig. 3 b .3) activities at concentrations of 5 mg/mL - 0.078 mg/mL. *C. rossii* and *R. candolleana* were the only plant species that were inhibitory to α-glucosidase and α-amylase respectively at concentrations of 0.078 mg/mL. The rest of the plant species were only inhibitory at higher concentrations, indicating that huge amounts of plant material may be required within meal portions to produce any potential antidiabetic effects. However, it is worth mentioning that the antidiabetic effects of the plant species may not be sorely dependent on inhibition of glycosidic enzymes but on

other properties such as fibre content and presence of carbohydrates that are resistant to digestion.

T. orientalis and *C. alba* exhibited significant inhibition of α-glucosidase at 5 - 0.625 mg/mL (Fig. 3 b .2), higher than the rest of the plant species ($P<0.05$) whereas α-amylase inhibition by *R. candolleana* and *C. alba* at 5 - 0.625 mg/mL was higher than the other species. However, none of the plant species recorded up to 50% enzyme inhibition at all concentrations of the crude extracts tested. *R. candolleana* recorded the highest percentage of 49.5% α-amylase inhibition at concentration of 5 mg/mL (Fig. 3 b .3). When this concentration was diluted by half, the inhibitory activity of the extract did not reduce proportionally. Generally, for most of the extracts, dilutions by half did not produce the expected corresponding decrease in percentage inhibition, probably due to presence of mixtures of different compounds in crude extracts, some of which may be antagonistic in action to others. Alpha glucosidase and α-amylase (Fig. 3 b .4) inhibition by acarbose (positive control) was higher than the plant crude extracts ($P<0.05$). For example, the IC_{50} of acarbose was 2.5 mg/mL (Fig. 3 b .4) (Karakaya et al., 2018) whereas the plant species failed to produce 50% inhibition at the highest concentrations tested (Fig. 3 b .2 and 3 b .3). Nevertheless, the plant species have exhibited substantial antioxidant capacities and their inhibition of two enzymes involved in postprandial hyperglycaemia is a promising lead in the search for control strategies against the rising prevalence of T2DM within the Indigenous community.

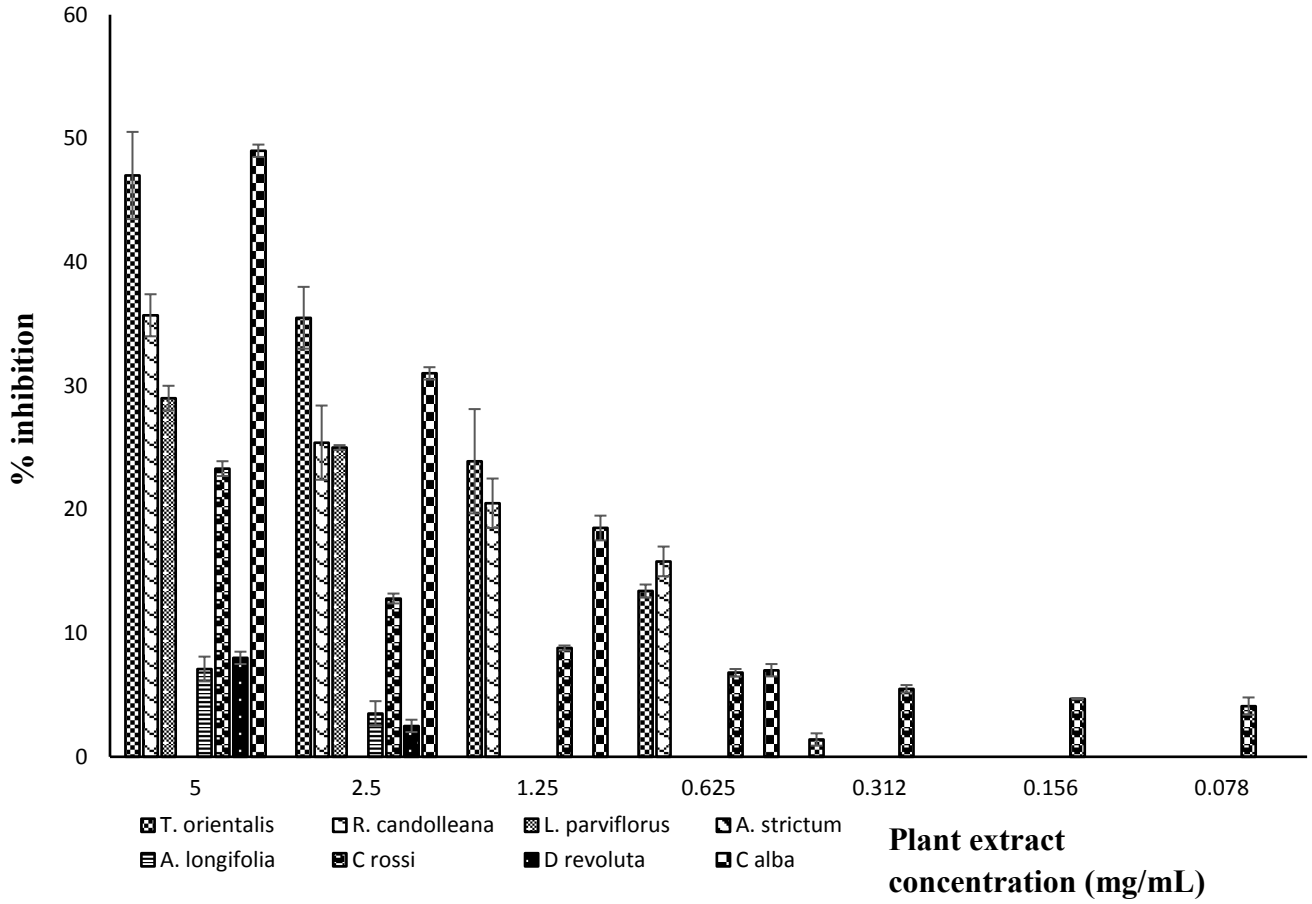


Figure 3 b .2: Alpha glucosidase inhibition of native plant species. Data are means ± SD of triplicate determinations ($P<0.05$). None of the plant species produced inhibition at concentrations ≤ 0.039 (data not shown).

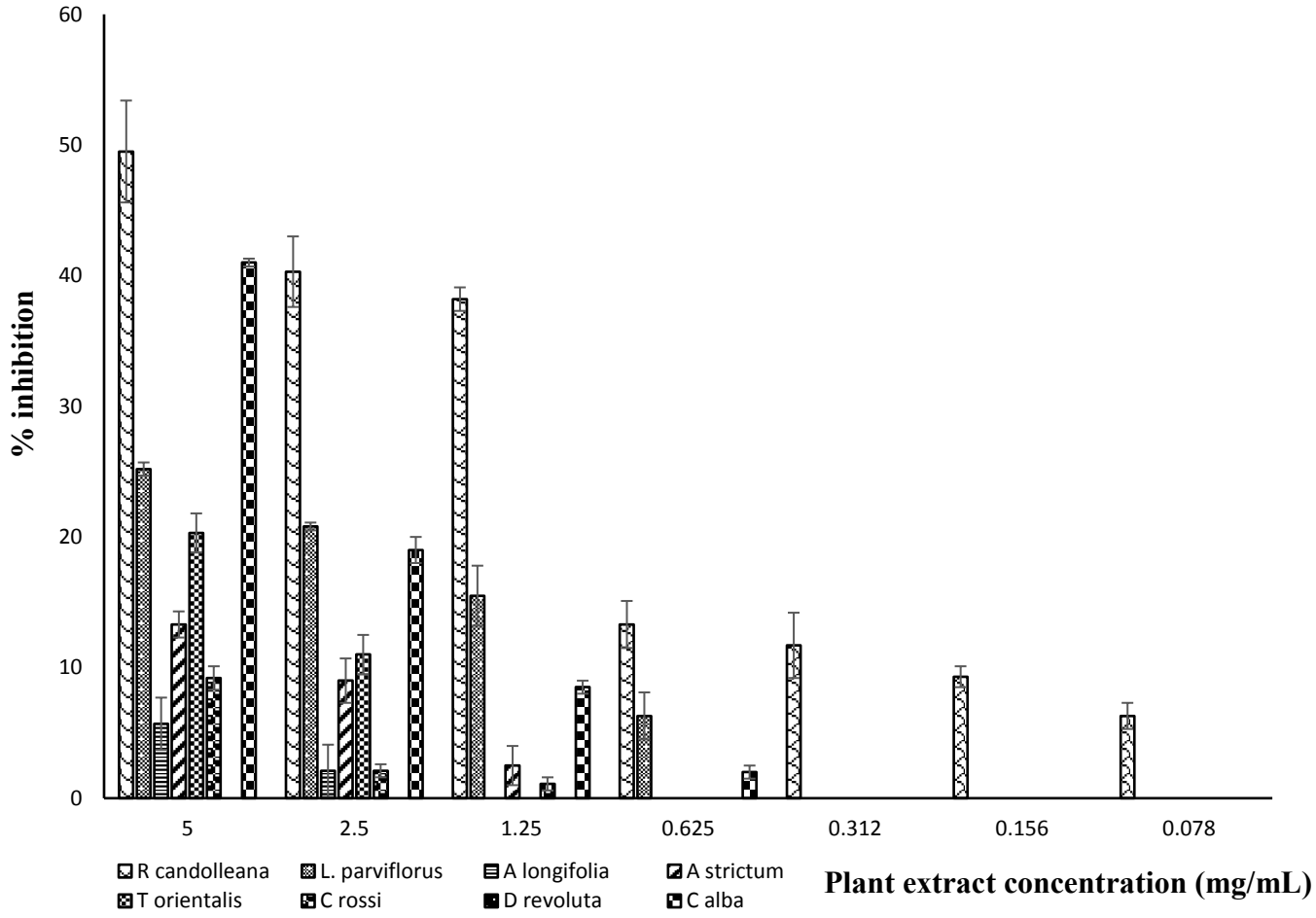


Figure 3 b .3: Alpha amylase inhibition of native plant species. Data are means ± SD of triplicate determinations ($P<0.05$). None of the plant species produced inhibition at concentrations ≤ 0.039 (data not shown).

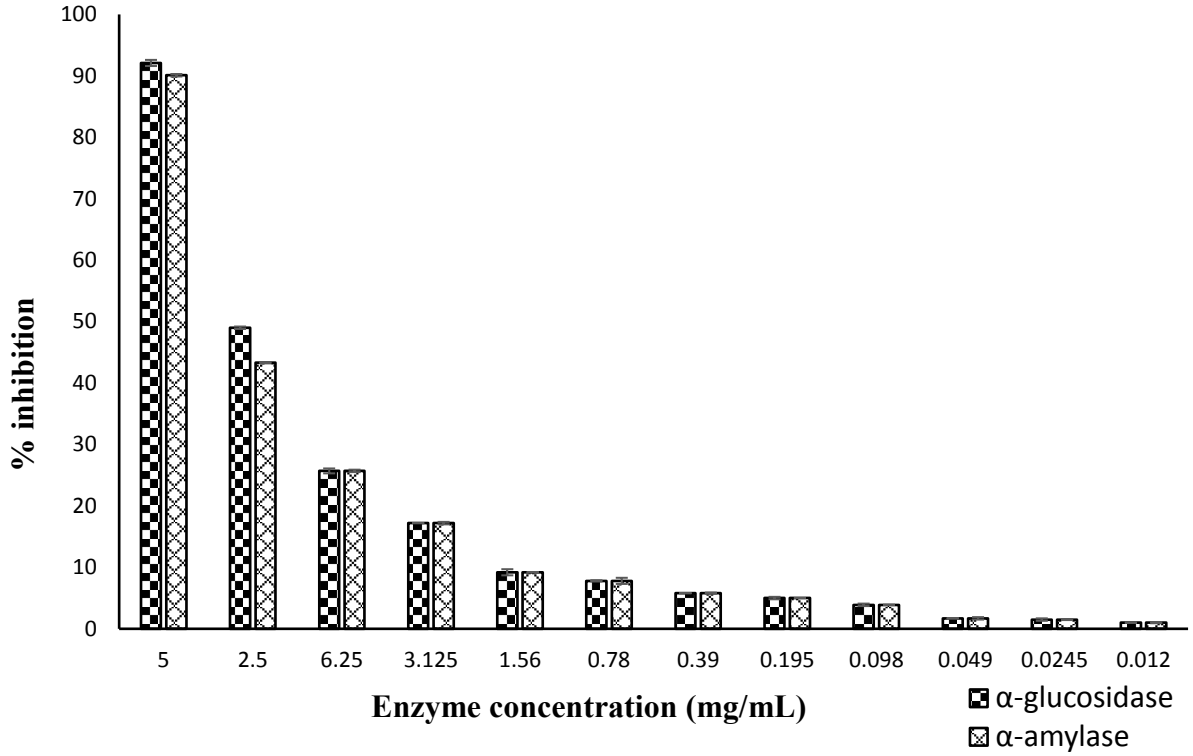


Figure 3 b .4: Acarbose inhibition of α-glucosidase and α-amylase. Data are means ± SD of triplicate determinations.

3 b .6 Conclusion

With the exception of *A. strictum*, *D. revoluta* and *L. parviflorus*, the rest of the plant species demonstrated high antioxidant capacities of ≥ 0.16 mg/100g DW AAE in the FRAP assay. The low antioxidant capacity of *L. parviflorus* in the FRAP assay as opposed to DPPH and ABTS assays could be attributed to long storage period of the plant material likely resulting in loss of some antioxidant compounds. Generally, aqueous extracts exhibited higher antioxidant capacity than methanol crude extracts. A combination of both methods may maximize the isolation of compounds with greater antioxidant and enzyme inhibitory capacity. The crude extracts of *C. alba*, *T. orientalis*, *C. rossii*, *R. candolleana* and *A. longifolia* exhibited considerable ability to inhibit glycosidic enzymes, mainly α-glucosidase and α-amylase, providing a basis for further studies to isolate the individual antidiabetic components in the native plant species.

Chapter 4

PRODUCT DEVELOPMENT AND SENSORY EVALUATION

This chapter covers the production of the antidiabetic food products with three of the eight plant species that were initially selected for study. The chapter also presents sensory evaluation results of the products including overall acceptability of the most acceptable product and goes ahead to evaluate the product for 5-week preliminary stability and shelf life studies.

4.1 Abstract

Unhealthy food choices are major contributing factors to the development of obesity and type 2 diabetes mellitus (T2DM) in Australia. In an attempt to evaluate acceptability of potential antidiabetic food preventatives, 5 flakes and a cracker were developed and tested against a commercially available flake and cracker (as controls) by 44 participants using a 9-point hedonic scale. Participants were blinded to the potential health benefits of the test product. Test and control crackers were subjected to 35-day preliminary stability and microbiological studies to determine shelf life. The highest acceptability of 86.4% was recorded against the control flake, followed by control and test crackers at 84.1% and 70.5% respectively. The flakes recorded overall acceptability of ≥ 54.5% and the acceptability of the mixed flake variety (65.9%) was comparable to the test cracker. Overall, control samples were more acceptable than test samples ($P<0.05$), although acceptability of the test samples was still high. In most cases, flavour ratings for test flakes were better than control flakes ($P<0.05$). Both test and control crackers remained stable and unchanged during the 35-day stability testing period with no microbial growth. Although the overall acceptability of the test cracker was interestingly high, there is the possibility to increase beyond 70.5% should the participants be aware of the potential health benefits of the product.

4.2 Introduction

Food product development and/or improvement of pre-existing food varieties is often performed with the hope of improving the nutritional profile of the food product to help control some non-communicable diseases (Choudhary and Grover, 2012). Functional foods influence specific beneficial physiological functions in the body beyond their basic nutritional role (Granato et al., 2020). Their constituents support health, well-being, or performance beyond regular nutrition, and food products of this nature are marketed and consumed for their value-added properties (Kasbia, 2005; Choudhary and Grover, 2012). The escalation of T2DM within the Australian Indigenous population is pushing research efforts towards the search for local culturally-acceptable strategies to curb the disease.

Dietary polyphenols may constitute possible strategies for mitigation of T2DM considering that they have a record of proven antidiabetic activities (Salim, 2014; Williamson, 2017; Deng et al., 2020). Indigenous food plants that are rich in antidiabetic polyphenols and antioxidants are therefore looked upon as possible sources of raw materials for the development of novel food products for people with T2DM. To ensure maximum activity, plant secondary metabolites are intended to be part of meals in order to have the desired concentrated effects since they function well in the presence of other nutrients (Williamson, 2017). Some polyphenols in food, for example, may slow down the rate of carbohydrate digestion which is a means of regulating postprandial hyperglycaemia (Deng et al., 2020). Thus, if taken out of the meal, the maximum desired effect of the polyphenol may not be achieved.

Results of our previous studies (Chapter 3) indicated that edible portions of some native Australian plant species constitute a rich source of nutrients and antidiabetic polyphenols. These included seeds of *A*. *longifolia* subsp. *sophorae* and underground stems and rhizomes of *T*. *orientalis*. Although both species have been relied upon as sources of food and in some cases primary health care for many centuries, there has been no scientific evidence linking them to the control and management of diseases such as overweight, obesity and T2DM, which now have a high rate of occurrence in the Indigenous community. The level of acceptability of Indigenous foods produced from both species is also lacking. The aim of this study therefore, was to develop antidiabetic food products with *T*. *orientalis* and *A*. *longifolia* subsp. *Sophorae* and to evaluate their acceptability thereafter, as a means to identify local acceptable resources for subsequent control of T2DM within the Indigenous community.

4.3 Methodology

Out of eight native plant species studied in this thesis, three (*A. longifolia* subsp. *Sophorae*, *T. orientalis* and *R. candolleana* subsp. *candolleana*) were selected and assembled into antidiabetic food preventatives based on their polyphenolic constituents and other characteristics (Table 4.1). These plants displayed the highest amounts of dietary fiber and protein (g/100g DW), contained low amounts of total fats, exhibited strong antioxidant capacities and were able to inhibit either α-glucosidase and/or α-amylase. Edible portions of the selected species were developed into 5 varieties of flakes and one cracker at the Footscray Nicholson Commercial Kitchens of Victoria University, Melbourne Australia.

Table 4.1: **Selection of plants for development of food product**

Properties	**Plant type**							
	To	Al	Cr	Rc	Lp	As	Dr	Ca
Rich in dietary fiber (≥10%)	✓	✓	✓	×	×	×	×	✓
High protein content (≥10%)	×	✓	×	×	×	×	×	×
Low in saturated fats (≤10%)	✓	✓	✓	✓	✓	✓	✓	✓
Strong antioxidant capacity (≥0.12mg/mLAAE)	✓	✓	✓	✓	✓	×	×	✓
Inhibition of at least 1 enzyme involved in carbohydrate metabolism (≥40%)	✓	×	×	✓	×	×	×	✓
Rich in antidiabetic polyphenols	✓	✓	✓	×	✓	×	×	✓
Low in simple sugars and carbohydrates (≤50%)	✓	✓	×	×	×	×	×	×
Rich in minerals	✓	×	✓	✓	×	✓	✓	✓
TOTALS	8	6	6	4	3	2	3	6

To, *T. orientalis*; Lp, *L. parviflorus*; As, *A. strictum*; Cr, *C. rossii*; Rc, *R. candolleana* subsp. *candolleana*; Al, *A. longifolia* subsp. *sophorae*; Dr, *D. revoluta*; Ca, *C. alba*; ✓, meets criterion; ×, does not meet criterion.

4.3.1 Production of flakes

Two hundred and fifty grams of whole *Acacia* (*Acacia longifolia* subsp. *sophorae*) seeds were washed, dried at 50 °C for 2 hour and blended. Underground stems and rhizomes of Water Cumbungi (*Typha orientalis*) were washed and oven-dried at 60 °C for 48 hour and milled to flour. The *Acacia* seeds were mixed with 50 g of water Cumbungi flour. Approximately 12.5 mL of vanilla extract, 125 g of *Acacia* gum, 2.5 g of table salt and 500 mL of cold water was added to the mixture and stirred several times to form a paste. The paste was spread over a baking tray that had been previously greased with cooking oil and lined with parchment paper. Another 250 g of *Acacia* seeds that had been pressure-cooked for 50 min (with one third of the seeds scarified) was used to make a separate paste following the above steps. The paste was again spread over a baking tray that had been previously greased with cooking oil and lined with parchment paper. Two more pastes were prepared from five hundred grams of completely scarified pressure-cooked seeds which had been divided into 2 equal parts of 250 g each. To one part was added 1.25 mL of sea berry salt bush (*Rhagodia candolleana* subsp. *candolleana*) water extract, 12.5 mL of vanilla extract, 125 g of *Acacia* gum, 2.5 g of table salt and 500 mL of cold water. The other part was made into a similar paste but without addition of sea berry salt bush water extract. All the pastes were separately spread into baking trays previously lined with parchment paper to the thinnest possible film by using a spatula. The pastes were baked at 120 °C for 25 min and allowed to cool at room temperature for 10 min. The half-baked pastes were manually broken into triangular-rectangular flakes, inverted and baked at 100 °C until crunchy. The flakes were allowed to cool at room temperature, packaged and labelled with code numbers.

4.3.2 Production of crackers

Two hundred and fifty grams of whole *Acacia* seeds were washed, dried at 50 °C for 2 hour and blended. Underground stems and rhizomes of Water Cumbungi were washed and oven-dried at 60 °C for 48 hour and milled to flour. The blended *Acacia* seeds were mixed with 25 g of Water Cumbungi flour together with 25 g of *Acacia* gum, 12.5 mL of vanilla extract, 1 g of table salt and 250 mL of cold water to form the dough. The dough was transferred to a rolling parchment paper and covered with plastic wrap. A rolling pin was used to flatten the dough to a thin layer of desired thickness. The plastic wrap was removed and the dough cut into rectangular shapes by simply drawing lines on it with a sharp knife. The cut pieces of dough were carefully placed in a baking tray that had been previously greased with cooking oil, lined with parchment paper and baked at 120 °C for 30 min. The crackers were allowed to cool at room temperature for 10 min, inverted and baked again at 100 °C until crispy. The crackers were allowed to cool at room temperature, packaged and labelled with code numbers.

4.3.3 Ethical procedures

Approval to conduct the study was granted by Victoria University Human Research Ethics Committee (VUHREC), approval number HRE19-161. Written informed consent was obtained from all participants as follows (Appendix 2): Flyers (Appendix 3) were pasted on notice boards and emails inviting members of the university community were sent out through the Victoria University global mail system. Those who responded to the emails were sent the project information (Appendix 4). After a couple of days, they were invited for a one-to-one information session with the researcher and the study aims and risks were explained.

Participants who indicated an interest in the study were given the consent form (Appendix 5) and time to consider their participation. Participants were requested to read the consent form and ask questions. Given that all doubts were clarified and questions were answered to participant's satisfaction, they were then given the go ahead to sign the consent form. Participants were made to understand that they had the right to withdraw from the study at any time without prejudice. The venue and time of the evaluation was then shared with the participants.

4.3.4 Sensory evaluation of food products

Within a period of 14 days, a total of 44 panel members who had given informed consent performed the evaluation in a sensory room at Victoria University, Melbourne Australia. Panel members were each given, at random 8 sachets (5 test flakes and 1 control flake; 1 test cracker and 1 control cracker) each containing 5 g of the food product (Fig. 4.1 a).

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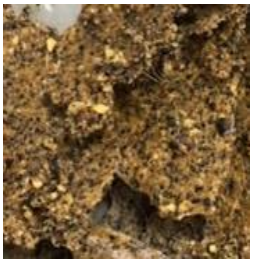

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TC

CC

Figure 4.1a: **Test and control samples submitted for sensory evaluation**; FES, flakes made with scarified seeds and aqueous extracts of sea berry salt bush; FE, flakes made with scarified seeds; FR, flakes made with seeds containing 1/3 seed coat; FW, flakes made with whole seeds; FM, mixture of all the flake varieties; CF, control flake; TC, test cracker; CC, control cracker.

Control samples were purchased from the local supermarket. The products were served at room temperature under white light. Participants were provided with 50 mL of cold full cream milk, 1 spoon and 1 bowl to be used for the flakes. The sachets bore code numbers. Each panel member was given a pencil and 8 evaluation forms (Appendices 6 and 7) with the following parameters; colour, aroma, flavour, taste, aftertaste, texture, shape, appearance and general acceptability of the products according to previously established methods (Watts et al., 1989; Bisla et al., 2014). These methods included the rating of parameters by use of hedonic scale bearing acceptability ratings from 1 (lowest) to 9 (highest) as shown on Fig. 4.1b.

1	2	3	4	5	6	7	8	9
Dislike extremely	**Dislike very much**	**Dislike moderately**	**Dislike slightly**	**Neither like nor dislike**	**Like slightly**	**Like moderately**	**Like very much**	**Like extremely**

Figure 4.1 b: **Hedonic scale used in sensory evaluation of food products**

Panel members' were requested to have a mouth rinse with tap water, pour one of the flakes into the bowl, note colour and appearance and add the desired quantity of milk in the bowl. Panel members were requested to use the spoon to stir contents of the bowl before placing the product in their mouth. Panel members were requested to masticate the product at least 32 times, roll into a bolus and spit out into the container provided for that purpose. Panel members were requested to rate the product on the evaluation form and have another mouth rinse and

non-salted crackers to cleanse the palate before proceeding to the next sample. For test and control crackers, panel members were requested to place a single cracker in the mouth, masticate about 32 times, roll into a bolus and spit out into the container provided for that purpose. At the end of the process, the forms were collected and the data was entered in SPSS and Excel spread sheets for statistical analysis. Parameters rated at ≥6 on the 9-point hedonic scale were considered acceptable.

4.3.5 Preliminary Shelf life and stability studies

Parameters of colour, texture, moisture, water activity and pH of test cracker (TC) and control crackers (CC) were studied weekly for a period of 35 days according to standard procedures (de Morais et al., 2016). There were two groups of samples placed in zip lock polypropylene bags. Each group contained vacuum-sealed (Multivac, Wolfertschwenden, Germany) and non-vacuum sealed samples; group 1 samples (TC1 & CC1) were kept on an open shelf whereas group 2 samples (TC2 & CC2) were kept in a dark cupboard. Colour (luminosity, chroma a* and chroma b*) was measured using a colorimeter (Konica Minolta Sensing, Kyoto, Japan) following the manufacturer's instructions.

Texture was measured using a texture analyser (TA plus, Godalming, UK) with probe HDP/3PB (TA plus, Godalming, UK) to determine hardness of crackers horizontally arranged on the platform (TA HD plus, Godalming, UK). The probe height was calibrated to 11.2 mm before testings, test speed 10 mm/s, sensitivity 0.05 N strength. Three readings were performed for each sample on a weekly basis, the mean and standard deviation was computed and recorded. Water activity was determined using a water activity meter (Water Group, Pymble,

NSW) and pH using a pH meter (Wissenschaftlich-Technische Werkstätten GmbH & Co. Weilheim, Germany) both according to the manufacturer's instructions. The moisture content of the samples was determined by heating to constant weight and computing differences in weight before and after heating (AOAC, 2012). The temperature and relative humidity (RH) of the room was also recorded weekly.

4.3.6 Determination of microbial counts

Microbial counts were determined according to standard procedures (Shen and Zhang, 2017). Briefly, 1 g of each sample was homogenised in 9 mL sterile distilled water and ten-fold serially diluted in sterile test tubes. Exactly 1 mL from the 10^{-1}, 10^{-3} and 10^{-5} dilutions was withdrawn from the tubes and inoculated onto nutrient agar plates by pour plate technique (Shen and Zhang, 2017). The plates were allowed to solidify at room temperature for 15 min, inverted and incubated aerobically for 48 hour. Emergent colonies were enumerated with the use of a colony counter. The colonies counted were multiplied by the dilution factor of the corresponding tube and the mean counts computed and recorded as cfu/mL alongside the standard deviation.

4.4 Statistical analysis

All data including the 44 determinations for acceptability ratings, triplicate determinations for stability studies of colour, texture, moisture, water activity, pH and microbial counts were entered in Excel spread sheet (Microsoft® Excel, District of Columbia, WA) and IBM SPSS statistic software version 25 (IBM SPSS®, Chicago, IL). Means and standard deviations were computed using both software and multiple comparisons between means were performed by

one-way analysis of variance test (ANOVA). Differences between means were considered significant at $P < 0.05$. Frequency distribution charts for various parameters on the hedonic scale were also generated from SPSS.

4.5 Results

4.5.1 Sensory analysis

A total of 25 females and 19 males participated in the sensory evaluation and while females found the flavour of the test cracker better than control ($P < 0.05$), the males did not record any significant differences ($P > 0.05$). Except for aroma and mouth feel of the flakes developed with whole seeds (FW) and flakes developed with seeds containing 1/3 seed coat (FR), all the other varieties of flakes and crackers recorded ≥ 54.5% overall acceptability (Table 4.2). The control flake was the most acceptable product with 86.4% acceptability, followed by control and test crackers at 84.1% and 70.5% respectively (Table 4.2). Control samples were generally more acceptable than test samples ($P < 0.05$).

Table 4.2: **Acceptability of different flake varieties and crackers**

Product	Colour and appearance	Aroma	Flavour	Taste & aftertaste	Mouthfeel	Overall acceptability
Flakes						
FW	20 (45.5)	21 (47.7)	24 (54.5)	23 (52.3)	21 (47.7)	24 (54.5)
FR	28 (63.6)	21 (47.7)	23 (52.3)	23 (52.3)	24 (54.5)	26 (59.1)
FE	25 (56.8)	20 (45.5)	24 (54.5)	20 (45.5)	23 (52.3)	26 (59.1)
FES	30 (68.2)	25 (56.8)	31 (70.5)	24 (54.5)	22 (50)	26 (59.1)
FM	27 (61.4)	24 (54.5)	30 (62.2)	32 (72.7)	23 (52.3)	29 (65.9)
CF	40 (90.9)	22 (50)	35 (79.5)	36 (81.8)	37 (84.1)	38 (86.4)
Crackers						
TC	31 (70.5)	35 (79.5)	31 (70.5)	25 (56.8)	32 (72.7)	31 (70.5)
CC	42 (95.5)	32 (72.7)	38 (86.4)	38 (86.4)	41 (93.2)	37 (84.1)

FW, flakes made with whole seeds; FR, flakes made with seeds containing 1/3 seed coat; FE, flakes made with scarified seeds; FES, flakes made with scarified seeds and aqueous extracts of sea berry salt bush; FM, mixture of all the flake varieties; CF, control flake; TC, test cracker; CC, control cracker; overall acceptability is computed based on ratings ≥ 6 on the hedonic scale with total number of participants = 44; Data is presented as total number of participants (out of 44), values in brackets represent percentage of total participants.

Ratings on the hedonic scale for sensory characteristics of different flakes ranged from 5.07 ± 1.99 - 8.59 ± 2.55 whereas the cracker ratings were 6.05 ± 1.61 – 7.71 ± 1.09 (Table 4.3). Except for mixed flake variety (FM), the overall acceptability of test and control crackers was higher than flakes ($P < 0.05$). Flakes made with scarified seeds and sea berry salt bush extracts had improved colour and appearance ratings of 6.25 ± 1.56 ($P < 0.05$) (Table 4.3), yet based on overall acceptability, these were not significantly different from the rest of the flakes ($P > 0.05$).

Table 4.3: **Mean acceptability ratings of different flake varieties and crackers**

Product	Colour and appearance	Aroma	Flavour	Taste & aftertaste	Mouthfeel	Overall acceptability
Flakes						
FW	5.11±2.25 [a]	5.86±1.5	5.68±2.07	5.43±2.11 [a]	5.11±2.12 [a]	5.36±2.2 [a]
FR	6.16±1.77 [a]	5.89±1.54	5.66±1.77	5.61±1.67 [a]	5.43±1.91 [a]	5.64±1.78 [a]
FE	5.88±1.85 [a]	5.46±1.75	5.27±1.91	5.07±1.99 [a]	5.61±1.85 [a]	5.48±2.06 [a]
FES	6.25±1.56 [a]	5.86±1.62	6.09±1.61	5.64±1.69 [a]	5.61±1.62 [a]	5.84±1.83 [a]
FM	5.84±2.03 [a]	6.11±1.48	5.75±1.79	5.75±1.69 [a]	5.36±1.92 [a]	5.79±1.89 [a]
CF	7.34±1.35	6.23±1.87	8.59±2.5	6.75±1.59	6.96±1.43	7.09±1.39
Crackers						
TC	6.29±1.69 [a]	6.41±1.34	6.18±1.89 [a]	5.97±1.79 [a]	6.29±1.59 [a]	6.05±1.61 [a]
CC	7.71±1.09	6.61±1.91	7.21±1.49	7.27±1.45	7.25±1.60	7.29±1.61

FW, flakes made with whole seeds; FR, flakes made with seeds containing 1/3 seed coat; FE, flakes made with scarified seeds; FES, flakes made with scarified seeds and aqueous extracts of sea berry salt bush; FM, mixture of all the flake varieties; CF, control flake; TC, test cracker; CC, control cracker; data are mean ± SD of 44 determinations for each attribute; a, represents significant difference to control sample, CF and CC for flakes and crackers respectively.

The frequency distribution for the overall acceptability of test and control crackers is displayed on Figures 4.2 and 4.3 respectively, showing that both products although different in ratings were highly acceptable as more than 30 participants in each case rated the products at ≥ 6 on the hedonic scale. Thus, the test cracker still presents marketable qualities even though its overall acceptability of 70.5% was lower than the control.

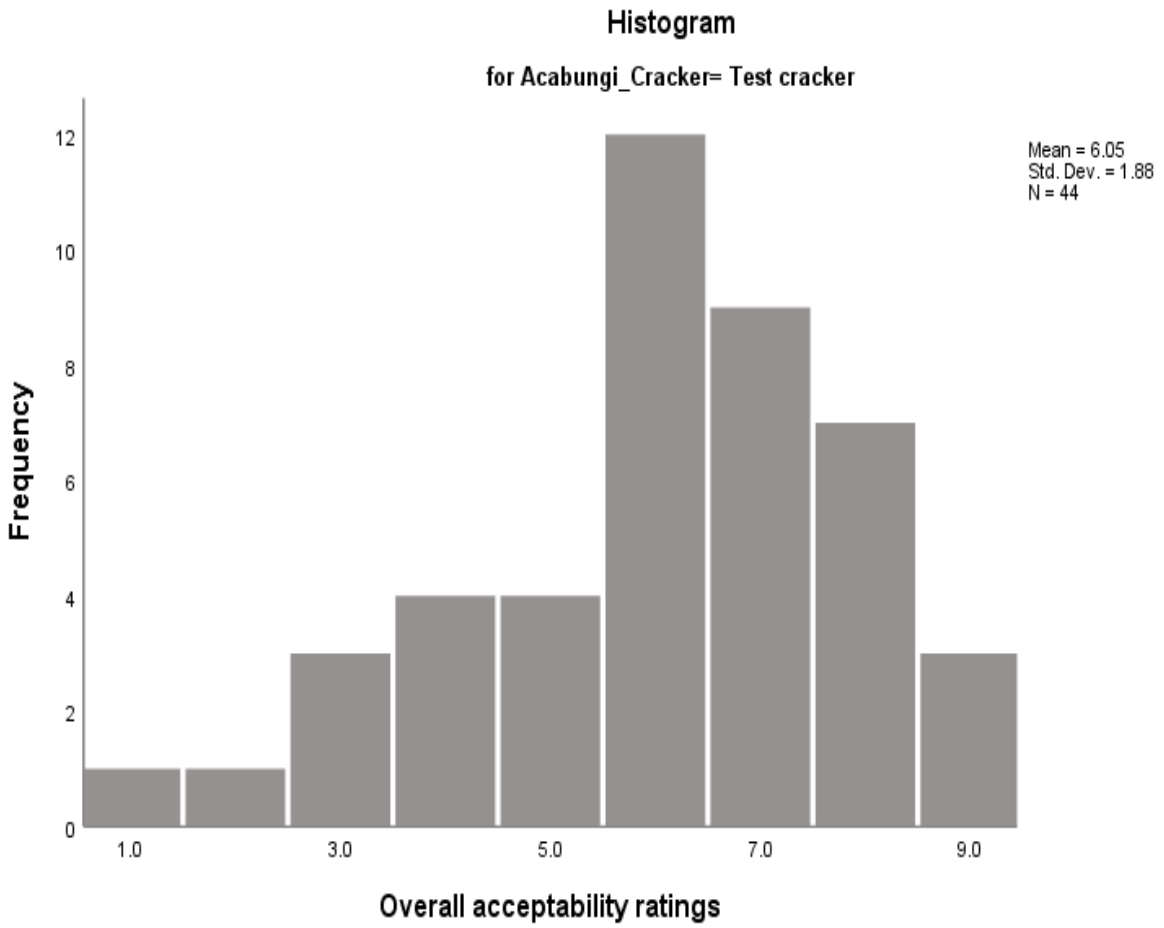

Figure 4.2: **Frequency distribution for the overall acceptability of test crackers**

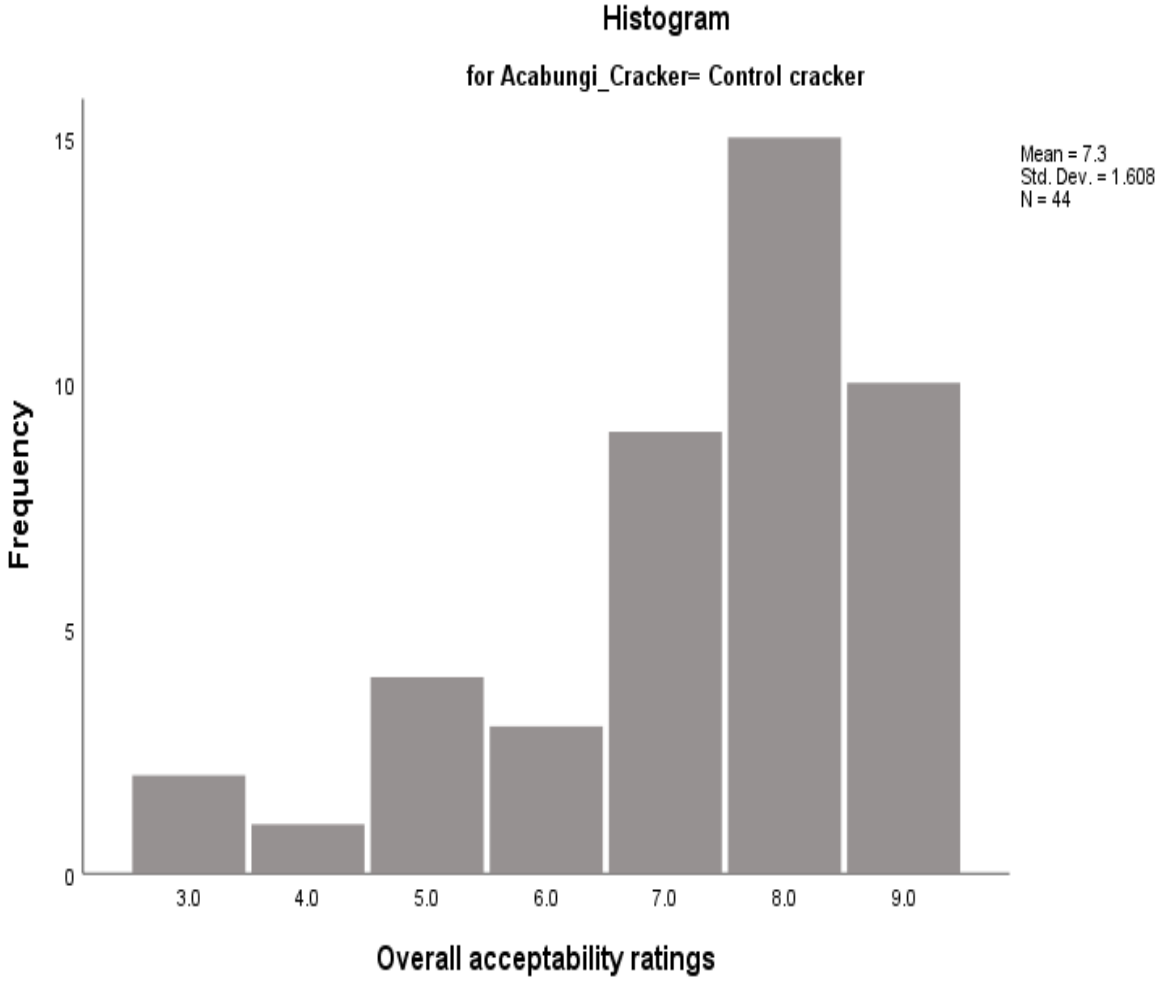

Figure 4.3: **Frequency distribution for the overall acceptability of control crackers**

4.5.2 Preliminary stability findings

The test cracker which recorded highest overall acceptability of 70.5% compared to all test samples was subjected to further studies to determine stability and shelf life. Both test and control crackers remained stable and usable with no significant changes ($P > 0.05$) in texture (Fig. 4.4), colour (Fig. 4.5), moisture (Fig. 4.6), water activity (Fig. 4.7) and pH (Fig. 4.8) during the 35-day study period. The crackers were sterile with no microbial growth recorded from day 1 to day 35. The moisture content and water activity of all the crackers tested was ≤ 1.47% and ≤ 0.45 respectively (Figs. 4.6 and 4.7). The temperature and RH of the room was between 18 ˚C - 19 ˚C and 50% - 59% respectively. The pH ranges for test cracker and control cracker were 5.21 - 5.30 and 6.1 - 6.2 respectively (Fig. 4.8).

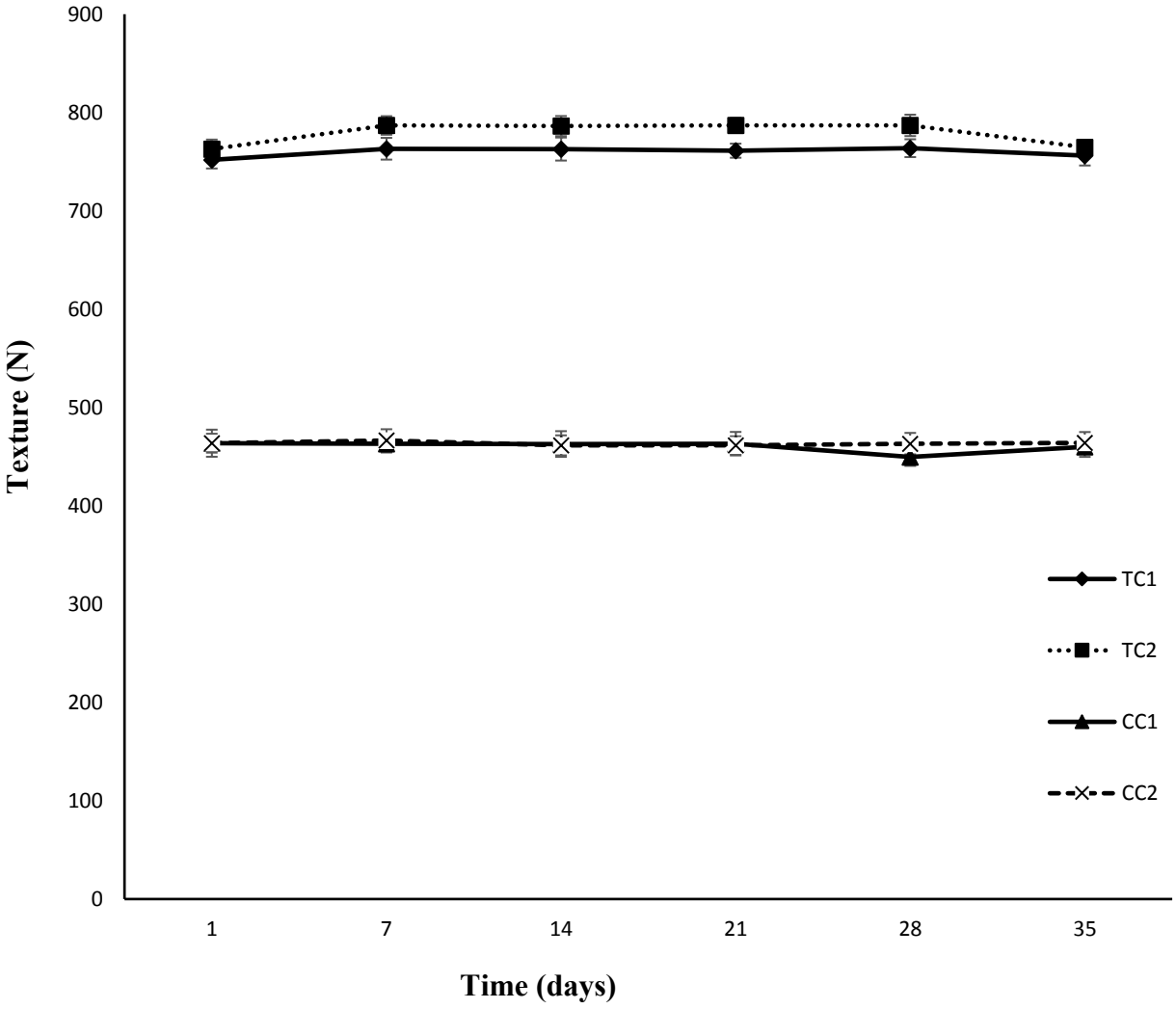

Figure 4.4: **Texture changes of test and control crackers kept on open shelf and dark cupboard**. Data are means ± SD of triplicate determinations; TC1, test cracker kept on open shelf; TC2, test cracker kept in dark cupboard; CC1, control cracker kept on open shelf; CC2, control cracker kept in dark cupboard.

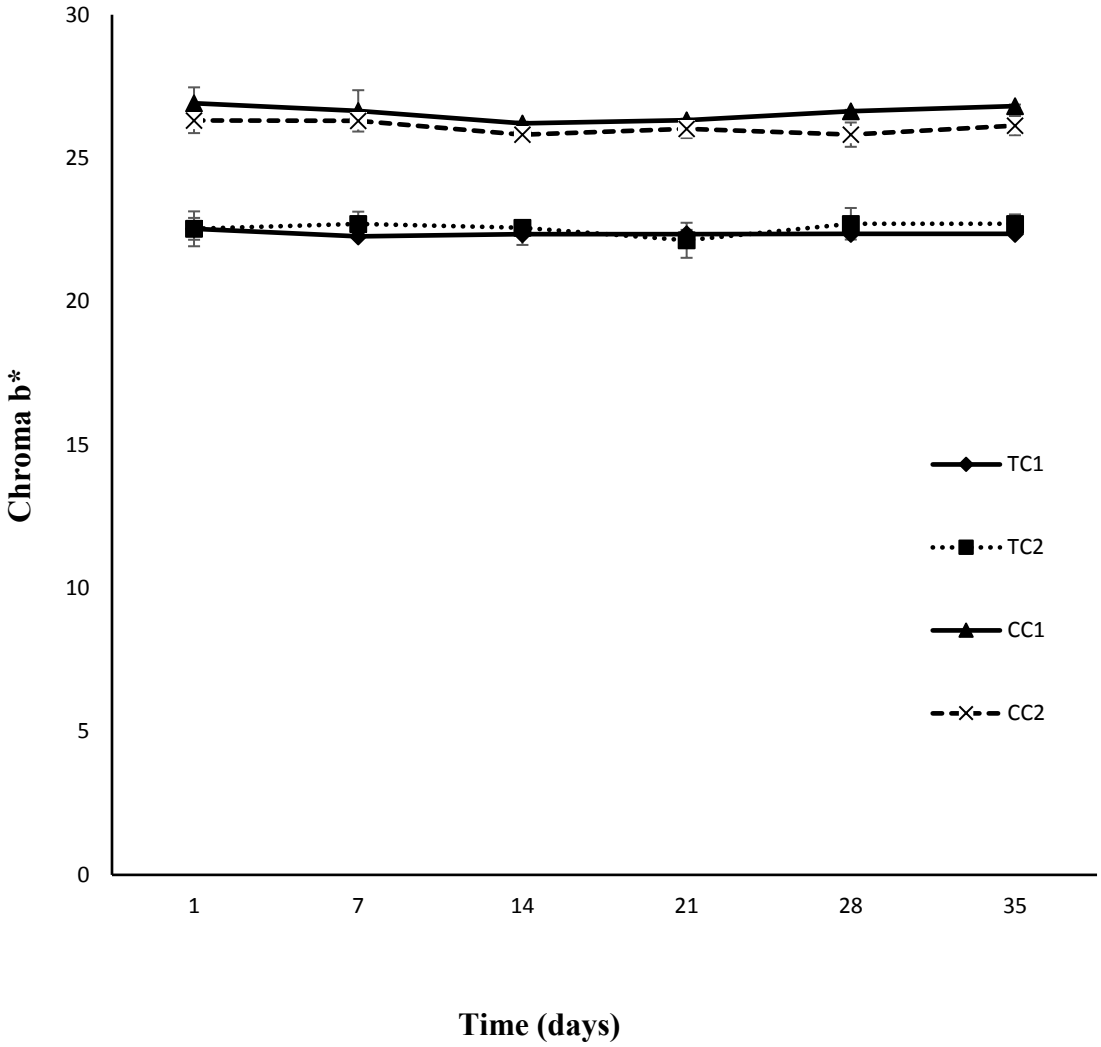

Figure 4.5: **Colour changes of test and control crackers kept on open shelf and dark cupboard**. Data are means ± SD of triplicate determinations; TC1, test cracker kept on open shelf; TC2, test cracker kept in dark cupboard; CC1, control cracker kept on open shelf; CC2, control cracker kept in dark cupboard.

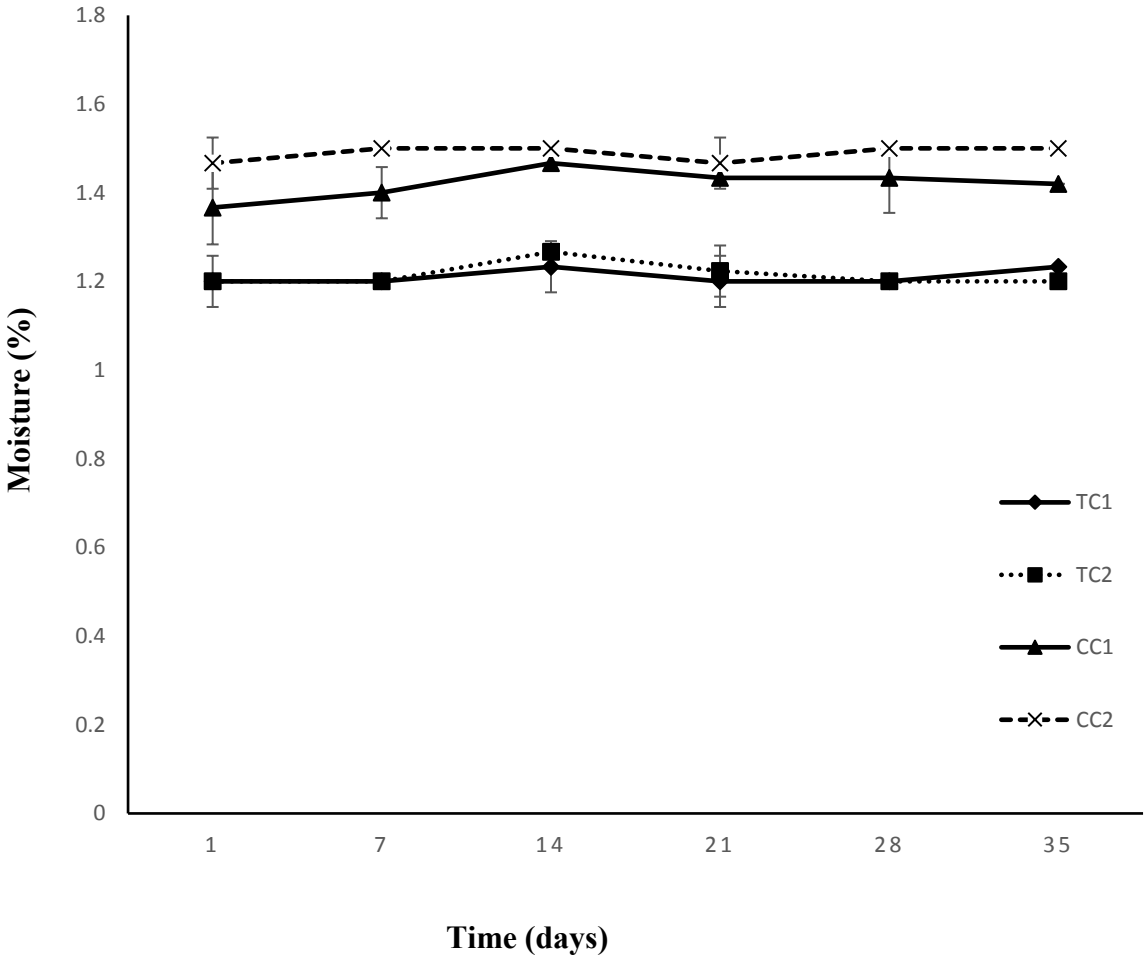

Figure 4.6: **Moisture changes of test and control crackers kept on open shelf and dark cupboard**. Data are means ± SD of triplicate determinations; TC1, test cracker kept on open shelf; TC2, test cracker kept in dark cupboard; CC1, control cracker kept on open shelf; CC2, control cracker kept in dark cupboard.

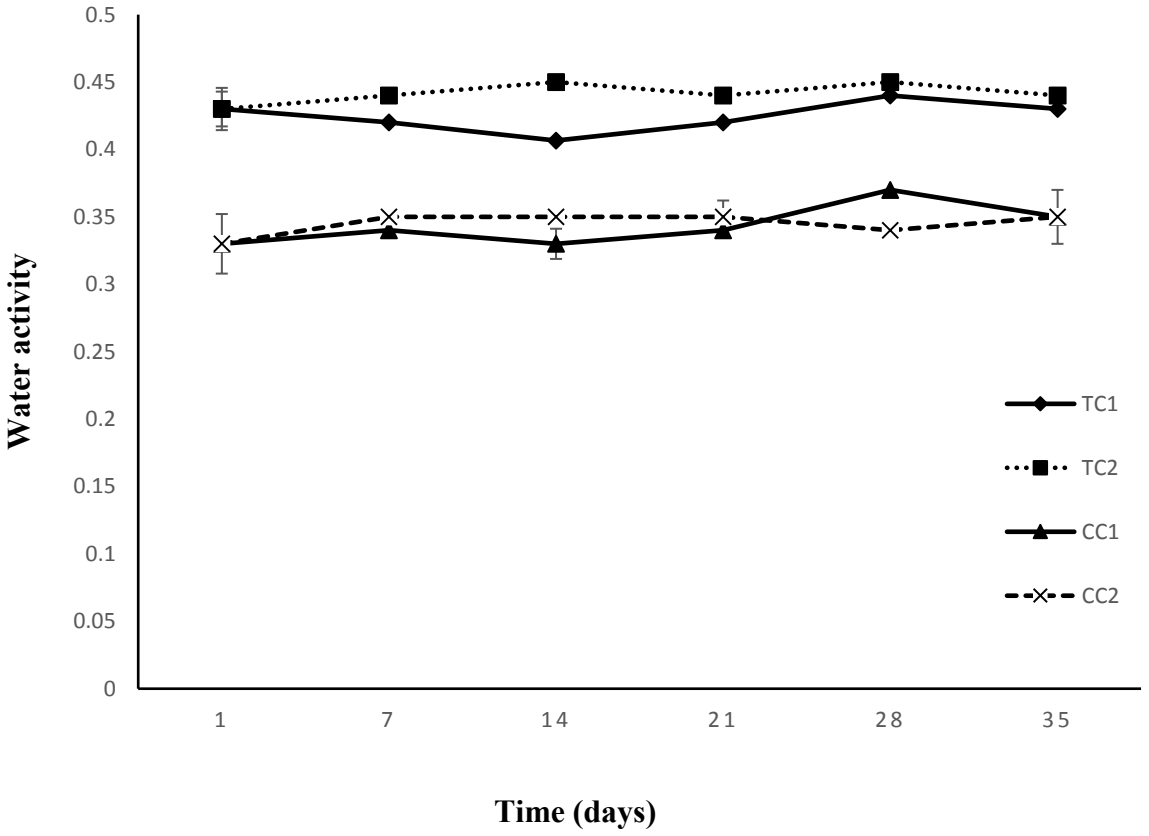

Figure 4.7: **Water activity changes of test and control crackers kept on open shelf and dark cupboard**. Data are means ± SD of triplicate determinations; TC1, test cracker kept on open shelf; TC2, test cracker kept in dark cupboard; CC1, control cracker kept on open shelf; CC2, control cracker kept in dark cupboard.

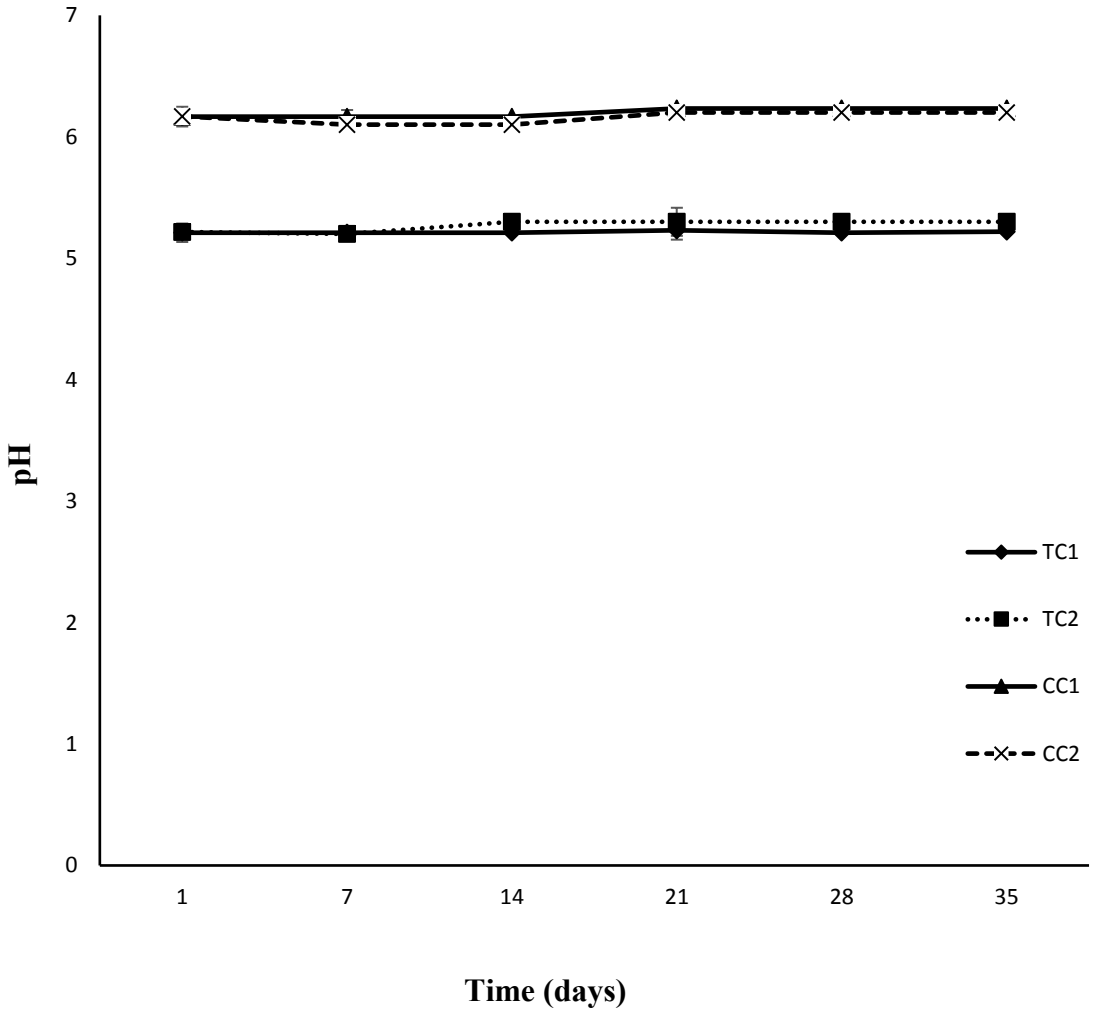

Figure 4.8: **pH changes of test and control crackers kept on open shelf and dark cupboard**. Data are means ± SD of triplicate determinations; TC1, test cracker kept on open shelf; TC2, test cracker kept in dark cupboard; CC1, control cracker kept on open shelf; CC2, control cracker kept in dark cupboard.

4.6 Discussion

Breakfast is one of the most important meals of the day, so it could be beneficial to eat a whole meal product that is filling and provides the nutrients that are required to start the day's activities (Spence, 2017). Despite abundance of breakfast cereals in the shops and supermarkets, choosing a product that would prevent postprandial hyperglycaemia in people with T2DM can be difficult as many products are high in salt and simple sugars. The development of flakes, to be consumed as breakfast cereal and crackers as snack, was performed herein to improve the availability of such products for people with T2DM. Food products developed from traditional Indigenous foods are noted for their unique taste, high nutritional value and functional properties that offer health benefits and protection against T2DM, CVDs and cancer (Calinoiu and Vodnar 2018; Adiamo et al., 2020).

In this study, colour and appearance of some whole grain products produced from Indigenous items seem to have affected their overall acceptability. For example, flakes developed with whole grains (FW) were least acceptable in terms of colour and appearance (Table 4.2) and had the lowest ratings on the hedonic scale (Table 4.3) despite their richness in nutrients. For this reason, it was hoped that reduction or complete scarification of seeds of some flakes (FR, FE and FES) and addition of a colourant (betanin-rich sea berry salt bush water extract) would improve colour and hopefully overall acceptability of the flakes, but this was not the case as the acceptability of FR, FE & FES was still no better than whole grain-containing flakes (Table 4.2). Instead, the test cracker which was also prepared with whole grains was more acceptable and recorded better ratings than most flake varieties (Table 4.3). The high ratings and acceptability of the control samples was also not surprising as these were made with brown rice which are completely different in taste to the test product which was predominantly made

with coast wattle seeds. This could have been a limitation in the selection of the control samples, since these were the closest to the test samples on the market, as there was no product with wattle seed. Overall, the test cracker had higher ratings than flakes and even though its acceptability was comparable to FM, it was selected for preliminary stability testing and further studies over the flakes because of its high overall acceptability of 70.5% (Table 4.2).

Preliminary stability studies were performed to provide information on longevity and shelf life of the product. Generally, crackers have low moisture content, low water activity and are highly crunchy (Yilmaz and Karaman, 2017). These properties are not favourable to support most microbial growth. In fact, most microorganisms will require a water activity of ≥ 0.88 to flourish in food (Leong et al., 2014; Yilmaz and Karaman, 2017), therefore lower moisture content and water activity levels confirm zero microbial growth and stability of the test products during the 35 day storage. This is probably why the products remained sterile with no significant changes in texture, colour and pH throughout the 35-day study period. Both products remained stable at temperature of 18 ˚C - 19 ˚C and RH of 50% - 59%. This finding may be useful within some remote Indigenous communities where longevity of the food product shelf life is valuable (Davy, 2016).

It is worth mentioning that all participants in the study were blinded to the nutritional and phenolic components of the products and health benefits associated with them. That notwithstanding the samples were still highly acceptable, indicating that the commercial value of the food products is likely to increase with additional sensitization of their nutritional and health benefits within the community. Pink Himalayan salt, for example has a less salty taste, less sodium and is rich in minerals (Carapeto et al., 2018). Despite the need for evidence to

establish the health benefits, some people prefer it to regular table salt and this promotes its commercial value owing to its potential health implications (Reddy et al., 2015; Carapeto et al., 2018). Similarly, some people may not like eating leafy vegetables due to perceptions of taste, yet a belief in their health benefits has been shown to increase consumption and commercial value of some fruits and leafy vegetables (Pollard et al., 2002). In a study conducted by Lockie et al. (2004), it was shown that Australians who consume organic foods rated them high because of their naturalness, meaning food free of artificial ingredients, pesticide and other chemical residues, preservatives, hormones and antibiotics. These group of Australians therefore consider foods produced using industrial technologies such as genetic engineering and irradiation to carry significant health risks for consumers (Lockie et al., 2004). For this reason, preference and commercial value of organic products was higher than non-organic products (Lockie et al., 2004). It is logical therefore to think that the highly acceptable craker developed in this study is likely to have significant commercial value.

There is an unacceptable food-related health gap between Indigenous and non-Indigenous Australians, revealing an urgent need to improve food security for Indigenous peoples throughout the country (Davy, 2016). The evidence suggests that food security within the Australian Indigenous community is yet to be attained (Davy, 2016). Despite high poverty levels within remote Indigenous communities (Altman, 2007), they could still be regarded as having available a rich habitat of native plant species, many of which are underutilized and unknown to non-Indigenous Australians. Considering that food insecurity within the Indigenous community is associated with welfare dependency, low incomes, and poverty (Booth and Smith, 2001), native plant resources that are rich in nutrients and dietary polyphenols and are readily available could be developed at industrial levels that could help improve incomes and alleviate poverty. The government has embarked on welfare

quarantining, creating outback stores, closing the social and economic gap (Davy, 2016), yet little has been done on development of antidiabetic food preventatives. The development of some of the native plant species into acceptable food products for commercial purposes could be a huge opportunity for income generation and self-reliance within the Indigenous community, especially given the potential health benefits of the products.

4.7 Conclusion

Food products made with local Indigenous material are highly acceptable as more than 70% of the people who participated in the sensory evaluation rated the cracker at ≥ 6 in 5 of the 6 parameters on the hedonic scale. However, modifications and fortifications with different flavours may have potential to meet wider consumer demands. The cracker remained stable and unchanged with good physical and microbiological parameters after 35-days storage at room temperature in light and in the dark. Although the overall acceptability of the test cracker was interestingly high, there is possibility to increase it even beyond 70.5% should the participants be aware of the potential health benefits of the product.

Chapter 5

PHYSIOCHEMICAL ANALYSIS OF CRACKERS

This chapter covers the properties of the potential antidiabetic food product by determining its nutritional and phenolic composition. The resistant and digestible starch fractions are determined and the glycaemic index of the product is estimated from in vitro enzymatic hydrolysis.

5.1 Abstract

Food choice is an important parameter in the regulation of postprandial hyperglycaemia. In an attempt to investigate the properties of acceptable food products developed for people with T2DM, samples of crackers were tested for phenolic-content by high performance liquid chromatography analysis and evaluated for glycaemic index by *in vitro* enzymatic hydrolysis. A commercially available cracker was included as a control. Phenolic components of Gallic acid (GA) and ρ-coumaric acid (PCA) were detectable from the test cracker while GA was the only compound identified in the control cracker. The total starch (48 ± 0.5%) and digestible starch (39.7 ± 1.5%) contents of the test cracker were lower than the total starch (69.7 ± 5.9 %) and digestible starch (65.7 ± 2 %) contents of the control cracker ($P < 0.05$) whereas the resistant starch-content of the test cracker (8.3 ± 1.2 %) was higher than the control cracker (4 ± 1.5%) ($P < 0.05$). The estimated glycemic indices (GI) for test and control crackers were 47.7 ± 1.3 and 70.3 ± 2.5 respectively. The low GI in the presence of higher amounts of resistant starch, proteins, fats and antidiabetic polyphenols of PCA and GA are promising useful characteristics of the test cracker, qualifying it as a more suitable antidiabetic food preventative than the control crackers.

5.2 Introduction

Diabetes, especially type 2 diabetes mellitus (T2DM) is increasing in Australia (Sainsbury et al., 2018). Many factors have been attributed to this increase including genetic predisposition, overweight/obesity, age, inactivity/sedentary lifestyle, alcohol intake and unhealthy food choices (Dendup et al., 2018; Njume et al., 2019a). According to Dietitians Association of Australia (DAA) and Australian Diabetes Education Association (ADEA), diet and nutritional therapy are first line intervention strategies in both the prevention and management of T2DM (Sami et al., 2017). This is so because food is an important parameter in the regulation of blood glucose response, thus, replacing fast-release carbohydrates in junk foods with resistant or slow release varieties that are excellent regulators of postprandial hyperglycaemia may go a long way to reduce the rate of T2DM in Australia. Native plant species that contain antidiabetic polyphenols, slow digestible carbohydrates and antioxidant compounds are being considered as possible alternative resources for the development of healthy food options in Australia. It is important to note that 63% of adults and 25% of children in Australia are overweight or obese with increasing number of young people with type 2 diabetes, especially within the Indigenous community; e.g., in some parts of the Northern Territory, Indigenous youth represent 88% of youth T2DM diagnoses (Stone et al., 2013; Dalbo et al., 2017; Titmuss et al., 2019). Despite the high percentages of obesity and T2DM, novel food preventatives to mitigate these conditions are not readily available.

In previous studies (Chapter 3), the nutritional properties of seeds of *Acacia longifolia* subsp. *sophorae* (Labill.) Court (coast wattle), underground stems and rhizomes of *Typha orientalis* C. Presl (water cumbungi or bulrush) and glossy red/purple berries of *Rhagodia candolleana* subsp. *candolleana* (sea berry salt bush) were reported. These are Indigenous plant species that

have supported the health and wellbeing of Australia's Indigenous population for many centuries (Brand-Miller & Holt, 1998). Despite these Indigenous plants resistance to pest and diseases, rich polyphenol and antioxidant content, they are usually not part of the popular food choices in Australia. This is startling considering the ramifications of energy-dense products on the health and wellbeing of the Australian population, especially within the Indigenous community where the rate of T2DM may be 10 - 20 times higher than other groups (De Abreu et al., 2013; Titmuss et al., 2019).

People with obesity and T2DM in Australia may find it difficult to choose food products that are appropriate for managing their condition due to the popularity and abundance of convenience energy- dense food outlets over limited number of healthier food outlets (Hilmers et al., 2012; Oaken et al., 2017). The energy-dense-products of fast food outlets may also be high in salt and loaded with saturated fats (Grunseit et al., 2019). Most often, the metabolic outcome of prolonged consumption of such products is an escalating level of non-communicable diseases including overweight/obesity, T2DM and cardiovascular disorders (CVDs) (Pallazola et al., 2019). As such, the properties of novel food products for people with T2DM are worth investigating as this is pivotal in supporting the health and wellbeing of Australia's 1.7 million people with diabetes. The objective of this study was therefore to evaluate the properties of the most acceptable food products developed in Chapter 4 of this thesis.

5.3 Methodology

5.3.1 Nutritional composition of crackers

Total crude fat, total crude protein, total dietary fibre and total carbohydrates of test crackers were determined according to our previously reported procedures (Chapter 3 a).

5.3.2 Polyphenol analysis by Reverse phase high performance liquid chromatography (RP-HPLC)

Polyphenols were tested in accordance with our previously established procedures (Donkor et al., 2012; Njume et al., 2019b). Briefly, a Shimadzu HPLC system (Shimadzu-LC-2030C, Nishinokyo-Kuwabaracho, Kyoyo, Japan) equipped with a C18 Phenomenex Luna column (2.4 mm porosity, 250 x 4 mm, and an UltraSep ES RP18 pre-column) was used with a 3-steplinear gradient for separation of compounds in the samples. The mobile phase for isolation of components consisted of: (A) 0.3 % phosphoric acid and (B) 100 % acetonitrile. The gradient used for separation of components was as follows: from 10 % to 20 % of B in 45 min, 20 % to 60 % of B in 20 min, then 60 % to 90 % of B for 20 min (flow rate: 0.7 mL/min at 20 ˚C). The elution pattern was monitored with a photodiode array detector (PDA) at 220 nm. One gram each of test and control crackers were crushed in 5 mL of 98 % methanol (HPLC grade) and the sediment was allowed to settle for 15 min. The supernatant was collected, filtered through a 0.45 μm membrane filter and injected in the column with a 10 μL injection volume. The individual polyphenols were identified by comparing with the retention times of reference standards. Quantitative analysis was performed by calibration curves using the reference standards of gallic acid (GA), epigallocatechin (EPC), catechin (CH), epigallocatechingallate (EPG), dihydroquinidine (DHQ) and ρ-coumaric acid (PCA) (Sigma Aldrich, Castle Hill,

NSW). Linearity was investigated in the range of 0 - 5 mg at five increasing concentrations. Intra-day analyses of the same solution containing all phenolic compounds tested were used to validate the precision of the chromatographic system (Donkor *et al*., 2012).

5.3.3 Total, resistant and digestible starch analysis

Starch hydrolysis was performed in accordance with previously established procedures (Siddhuraju and Becker, 2005; Barine and Yorte, 2016), with modifications. For determination of total starch (TS), 200 mg of powdered test and control cracker samples were separately dissolved in 24 mL of 2 M KOH into two 100 mL universal bottles at room temperature. The mixture was vortexed for 30 min. Exactly 20 mg of porcine pancreatic α-amylase (Sigma Aldrich, Castle Hill, NSW) was mixed with 50 mL 0.2 M phosphate buffer (pH 6.9) and 4 mL of the mixture was added to the bottles. The bottles were incubated at 37 ˚C for 45 min in a water bath, with intermittent shaking. The enzyme reaction was halted by adding 4 mL of 3-5 dinitrosalicyclic acid and immediately heated at 100 ˚C for 5 min. The bottles were allowed to cool on ice for 5 min, their contents were transferred into 50 ml Eppendorf tubes and centrifuged for 10 min at 3000 x g. The supernatant was collected and the concentration of glucose determined by anthrone method as previously reported (Njume et al., 2019b). A standard solution of glucose (20, 40, 60, 80 and 100 mg/L) was prepared for the generation of a standard curve (plotting absorbance at 630 nm against concentration mg/mL, r- value = 0.999). The sample glucose concentration was obtained from the standard curve. The experiment was repeated twice and the glucose measurements converted to starch by multiplying the percentage glucose concentration by 0.9 (Barine and Yorte, 2016).

For determination of resistant starch (RS), 100 mg of the residue from the total starch analysis was mixed with 20 mg of pepsin (1 g pepsin per 10 mL KCl–HCl buffer, pH 1.5) and incubated

for 1 hour at 40 ˚C to remove the proteins. Exactly 1 mL of pancreatic α-amylase solution (Sigma Aldrich) containing 40 mg α-amylase per mL tris maleate buffer, pH 6.9 was added into the bottles and incubated for 16 hour at 37 ˚C to hydrolyse the starch. The samples were transferred into 50 mL Eppendorf tubes and centrifuged at 3000 x g for 15 min and the supernatant discarded. The residue was dispensed in 2 M KOH, hydrolysed with amyloglucosidase and the released glucose quantified using the anthrone method as previously reported (Njume et al., 2019b). The experiment was repeated twice and the glucose measurements converted to starch by multiplying the percentage glucose concentration by 0.9 (Barine and Yorte, 2016). Digestible starch (DS) content was obtained by difference, i.e., TS minus RS (Siddhuraju and Becker, 2005).

5.3.4 Determination of hydrolysis index (HI) and estimation of glycaemic index (GI)

The rate of digestion of starch in the test and control crackers and estimation of GI values was performed following the method of Goni et al. (1997) as modified by Siddhuraju and Becker (2005) and Barine and Yorte (2016). Powdered test and control crackers (2 g each) were soaked in 24 mL of 2 M KOH in two 100 mL universal bottles at room temperature for 30 min. The samples were pressure-cooked for 50 min and homogenised in HCl-KCl buffer at pH 1.5. The protein was hydrolysed by mixing with 200 mg of pepsin (1 g pepsin per 10 mL KCl-HCl buffer, pH 1.5) and incubation for 1 hour at 40 ˚C. The pH was adjusted to 6.9 following the addition of 10 mL Tris maleate buffer, and 5 mL of α-amylase solution containing 2.6 IU of α-amylase in Tris maleate buffer (Siddhuraju and Becker, 2005). Exactly 1 mL of sample supernatant was withdrawn at 30 min intervals for 3 hour without disturbing the pellet and placed in separate test tubes. The tubes were immediately heated at 100 ˚C for 5 min to inactivate the enzymes. The test tubes were allowed to cool on ice for 5 min and centrifuged

for 10 min at 3000 x g. The supernatants were collected and the concentrations of glucose were determined by anthrone method as previously reported (Njume et al., 2019b). The rate of starch digestion was expressed as the percentage of TS hydrolysed over time intervals of 30, 60, 90, 120, 150 and 180 min of incubation (Siddhuraju and Becker, 2005). The Hydrolysis Index (HI) was derived from the ratio between the areas under the hydrolysis curve of the crackers and the reference control sample of glucose (Barine and Yorte, 2016).

$$\text{Hydrolysis index (HI)} = \frac{\text{AUC of sample}}{\text{AUC of glucose}} \times 100 \quad \text{(Goni et al., 1997)}$$

Where AUC = Area under the curve

$AUC = C_\alpha (T_f - T_0) - C_\alpha / k) [1\text{-}exp [-k (T_f - T_0)]$,

Where C_α corresponds to the concentration at equilibrium (T180), T_f is the final time (180 min), T_0 is the initial time (0 min), and k is the kinetic constant derived to be:

$$k = \frac{-\ln (1 - C/ C_\alpha)}{t}$$; C=Percentage of starch hydrolysed at time t.

GI = 39.71 + (0.549 x HI). (Goni et al., 1996).

5.4 Statistical analysis

All data including determinations for TS, RS, DS, GI values and quantities of polyphenols extracted from test and control crackers were entered in Excel spread sheet (Microsoft® Excel, District of Columbia, WA) and IBM SPSS statistic software version 25 (IBM SPSS®, Chicago, IL). Means and standard deviations were computed using both software and multiple comparisons between means were performed by one-way analysis of variance test (ANOVA). Differences between means were considered significant at $P < 0.05$.

5.5 Results

5.5.1 Nutritional information of crackers

The test cracker contained mostly non-reducing carbohydrates, proteins, fats (mostly unsaturated) and higher amounts of total dietary fibre whereas the control cracker contained high amounts of carbohydrates, half the amount of proteins, less fibre and more salt (Table 5.1)

Table 5.1: **Nutritional information of crackers (g/100g)**

Nutrients	**Test Cracker**	**Control cracker**
Total Carbohydrates	48.5 ± 0.5	76.5 ± 2
of which:		
Reducing sugar	2.5 ± 0.5	10.1 ± 1
Proteins	18.3 ± 1	9.2 ± 2.5
Total fats	12.7 ± 0.2	6.7 ± 0.5
of which:		
Saturated	3.5 ± 0.2	<1 ± 0
Total dietary fibre	19.6 ± 0.5	3.8 ± 1.5
Sodium	0.05 ± 0	3.1 ± 0.2

Data are means of triplicate determinations ± SD; Comparisons between test and control crackers were significantly different in all cases ($P < 0.05$).

5.5.2 Analysis of plant components

Figure 5.1 (a) chromatogram depicts the standard mixture of six reference compounds (gallic acid (GA), Dihydroquinidine (DHQ), epigallocatechin (EPC), catechin (CH), epigallocatechingallate (EPG) and ρ-coumaric acid (PCA) whereas figure 5.1 (b) chromatogram, depicts phenolic profile of test cracker with 2 compounds (gallic acid (GA) and ρ-coumaric acid (PCA). The control cracker chromatogram (figure 5.1c) identifies 1 phenolic compound (gallic acid (GA). The unknown compound appearing in all 3 chromatographs at 56 minutes was also detected in the solvent blank and as such is likely to be an impurity from the column. The amount of GA and PCA detected in the test cracker was 147 ± 21.1 mg/kg and 41.5 ± 11.3 mg/kg respectively whereas the control cracker recorded lower amounts of GA (18.1 ± 5.2 mg/kg) ($P< 0.05$). PCA was not detected in the control cracker (Fig. 5.1 c).

Chromatograph a

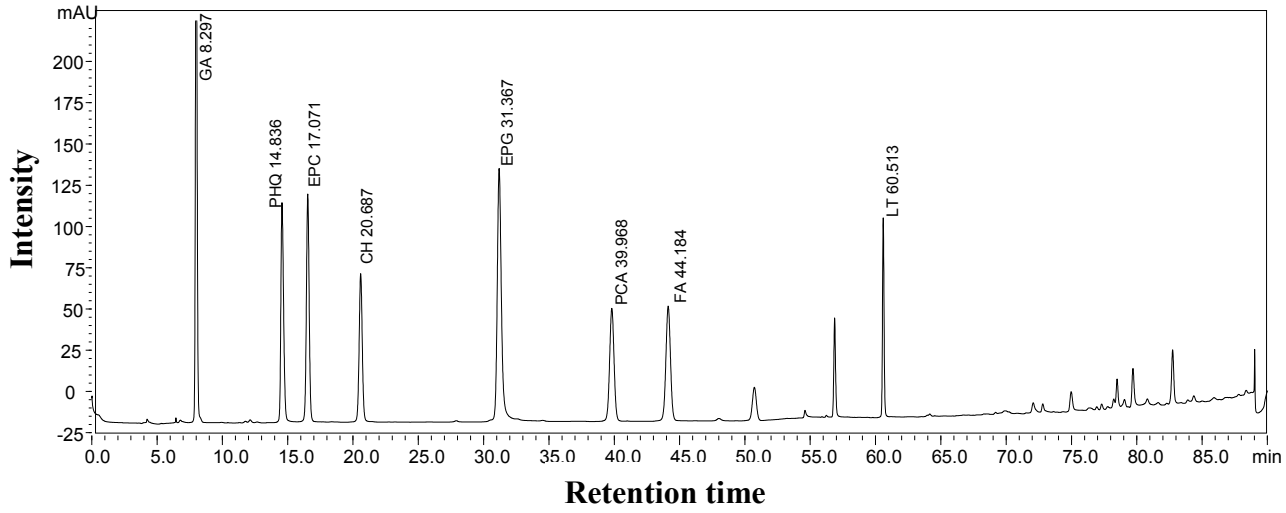

Figure 5.1 a: **RP-HPLC Analysis**; **elution profiles of standard mixture showing reference compounds together with their retention times;** gallic acid (GA), Dihydroquinidine (DHQ), epigallocatechin (EPC), catechin (CH), epigallocatechingallate (EPG) and ρ-coumaric acid (PCA). Chromatographic analysis was performed on a Shimadzu HPLC system equipped with a C18 Phenomenex Luna column (2.4 mm porosity, 250 x 4 mm, and an UltraSep ES RP18 pre-column) with a 3-steplinear gradient of solvent A 0.3 % phosphoric acid and solvent B 100 % acetonitrile. The elution pattern was monitored with a photodiode array detector at 220 nm.

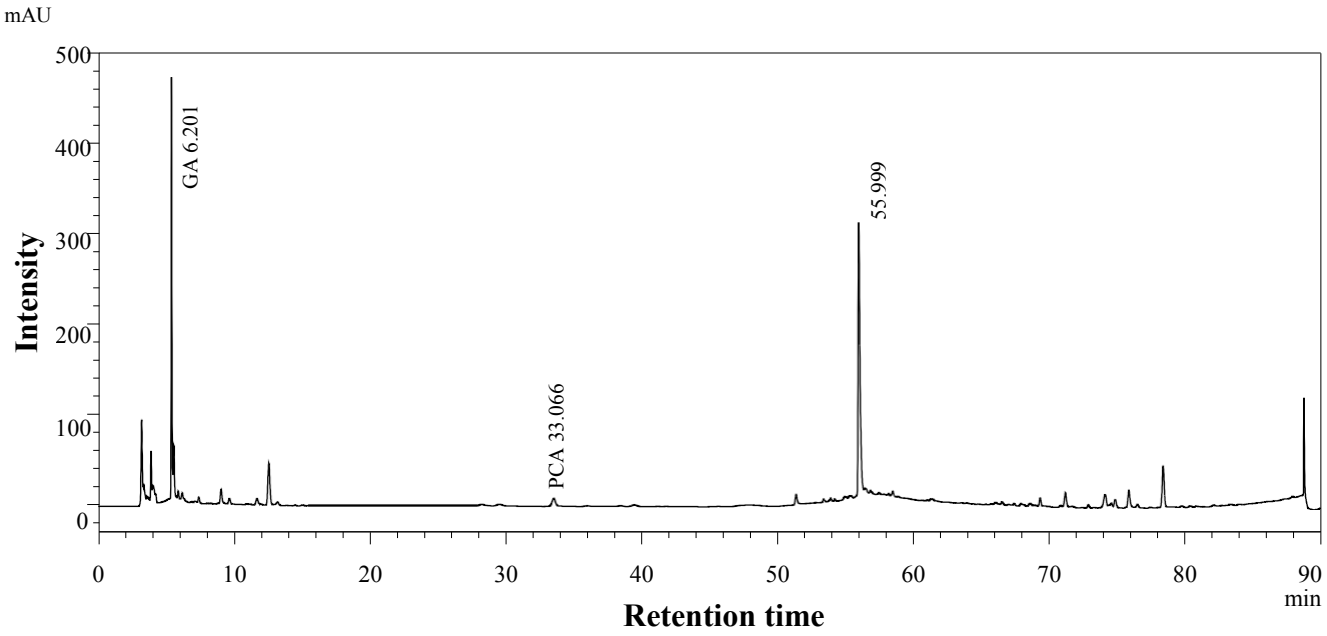

Figure 5.1 b: **RP-HPLC Analysis; elution profile of test cracker showing 2 identified compounds and their retention times;** gallic acid (GA) and ρ-coumaric acid (PCA). Chromatographic analysis was performed on a Shimadzu HPLC system equipped with a C18 Phenomenex Luna column (2.4 mm porosity, 250 x 4 mm, and an UltraSep ES RP18 pre-column) with a 3-steplinear gradient of solvent A 0.3 % phosphoric acid and solvent B 100 % acetonitrile. The elution pattern was monitored with a photodiode array detector at 220 nm.

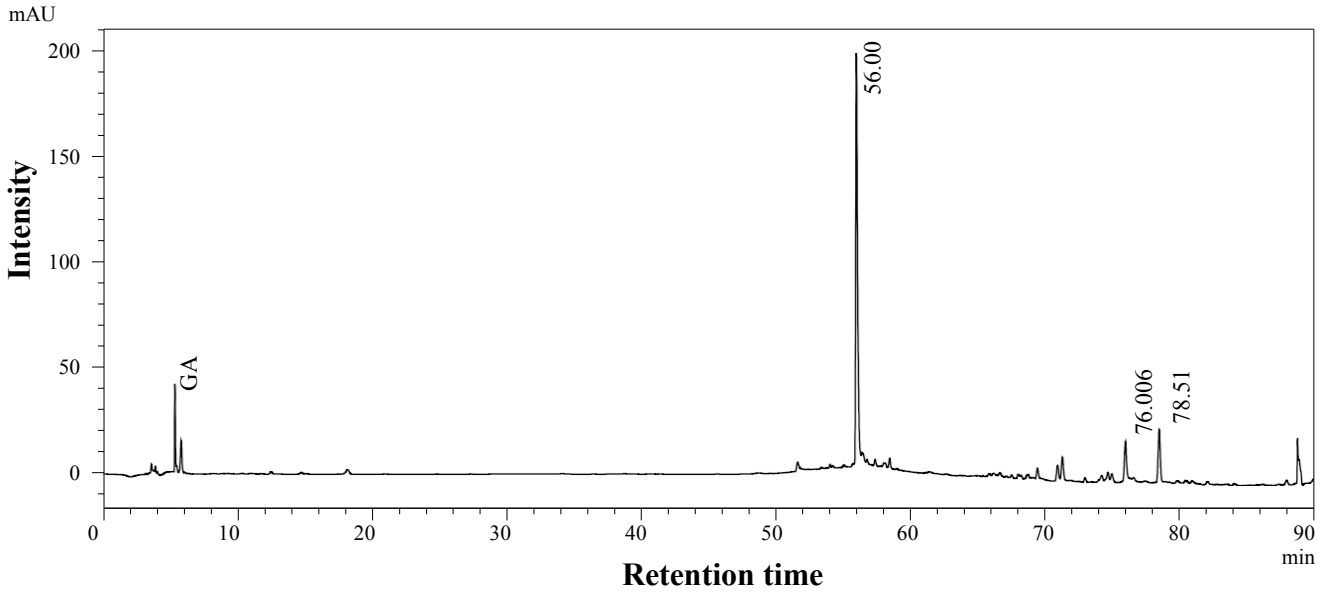

Figure 5.1 c: **RP-HPLC Analysis; elution profile of control cracker showing 1 identified compound gallic acid (GA);** Chromatographic analysis was performed on a Shimadzu HPLC system equipped with a C18 Phenomenex Luna column (2.4 mm porosity, 250 x 4 mm, and an UltraSep ES RP18 pre-column) with a 3-steplinear gradient of solvent A 0.3 % phosphoric acid and solvent B 100 % acetonitrile. The elution pattern was monitored with a photodiode array detector at 220 nm.

The TS and DS-contents of the control cracker were higher than the test cracker ($P < 0.05$) whereas the residual fraction of starch (RS), resistant to enzyme hydrolysis was higher in the test cracker than control cracker ($P < 0.05$) (Table 5.2).

Table 5.2: **Starch composition (%) of test and control cracker**

Sample type	**Total starch**	**Resistant starch**	**Digestible starch**
Test cracker	48 ± 0.5 #	8.3 ± 1.2 #	39.7 ± 1.5 #
Control cracker	69.7 ± 5.9	4 ± 1.5	65.7 ± 2

Data are means of triplicate determinations ± SD; # = difference to control crackers ($P < 0.05$).

The calculated kinetic constant values (k) for test and control crackers were 0.0311 and 0.0881 while the hydrolysis indices (HI) were 14.55 ± 1.5 and 55.7 ± 3.3 respectively. Following Goni et al. (1996) equation for calculation of glycemic index, the estimated glycemic indices (GI) for test and control crackers were 47.7 ± 1.3 and 70.3 ± 2.5 respectively. Hydrolysis of the test cracker began to stabilize after 120 min (Fig. 5.2) whereas the rate of hydrolysis of control cracker was still increasing at time (t=120. t=150, t=180).

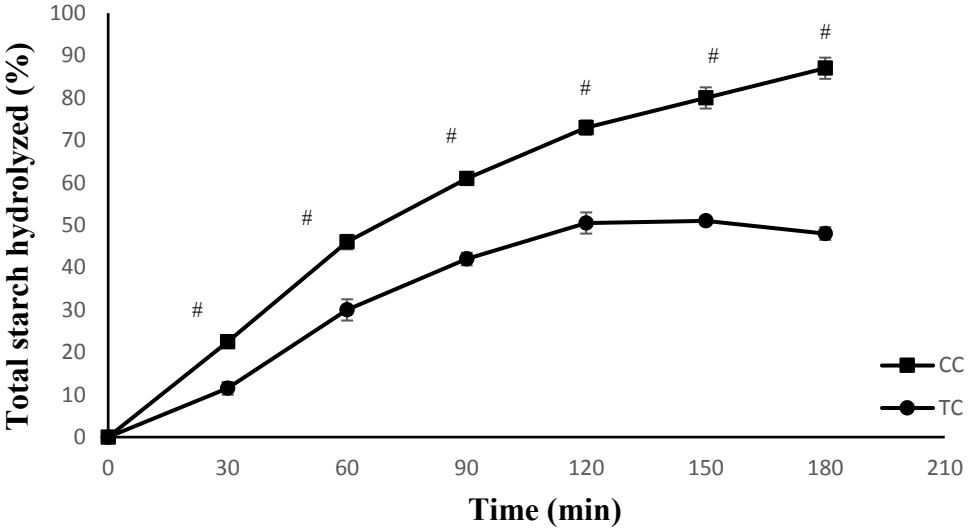

Figure 5.2: **In vitro starch hydrolysis of test and control crackers**; Data are means ± SD of triplicate determinations; CC, control cracker; TC, test cracker; percentage of starch hydrolysed was greater for control cracker than test cracker in all cases ($P < 0.05$). $^{\#}$ = difference to test crackers ($P < 0.05$).

5.6 Discussion

Many diets, including Mediterranean, Flexitarian, Ornish, Vegan and dietary approach to stop hypertension (DASH) have been employed in different parts of the world to manage T2DM, CVDs and hypertension (Gray and Threlkeld, 2019). In most cases, the mechanisms of action of these diets are not fully elucidated, yet the presence of antidiabetic polyphenols, antioxidants and high amounts of total dietary fiber that characterize these diets are likely to be responsible for improvements in the regulation of postprandial hyperglycaemia and protection against glucotoxicity in those who consume them (Gray and Threlkeld, 2019). It is logical therefore to think that a synergistic approach that makes use of total dietary fiber, antioxidants and antidiabetic polyphenols, all in a single food product through a cracker made predominately from Indigenous plants could be applicable in the regulation of postprandial hyperglycaemia within the Australian Indigenous community.

The high fiber-content of the test cracker is particularly interesting as these are non-digestible starches reported to play a role in regulating postprandial hyperglycaemia in people with T2DM (Tutelyan et al., 2016). Generally, the fiber is believed to enfold the food and hinder the action of hydrolytic enzymes in the digestive tract, increasing viscosity of intestinal contents, decreasing accessibility of starch to digestive enzymes, and decreasing the absorption of glucose in the small intestine, thereby reducing the absorption of carbohydrates *in vivo* (Jenkins et al., 1977). Dietary fiber in whole grain products is also known to enhance the growth of non-pathogenic bacteria in the large intestine leading to improvement of gut microflora (Tieri et al., 2020). According to Asif (2014), the objectives of dietary treatment in diabetes are to achieve optimal blood glucose concentrations; achieve optimal blood lipid concentrations; provide appropriate energy for reasonable weight, normal growth and development. The nutritional

properties of *Acabungi* test crackers (Table 5.1) and the results of enzymatic starch hydrolysis (Table 5.2) seem to be pointing to the fact that this product may have the potential to be a useful antidiabetic food preventative.

It has been reported that heating and other food-processing techniques may affect polyphenolic content of food and profile of many ingredients even though many polyphenolic components have high boiling/sublimation points (Zeng et al., 2017; Loncaric et al., 2018). Catechins, for example, will be affected by oxidation, light, high temperature and alkaline environment (Gadkari & Balaraman, 2015) with most changes occurring through epimerisation, hydrolysis, oxidation and polymerisation (Li et al., 2013). This study did not detect any catechins in the finished product, probably due to their instability at high temperatures (Zeng et al., 2017; Loncaric et al., 2018).

Gallic acid was detectable in the test and control crackers (Figs. 6.1 b & c). Gallic acid is one of the most common phenolic antioxidant compounds (Gao et al., 2019) and except for *R. candolleana*, it was detected in all the native species studied herein with amounts ranging from 3.5 - 127.3 mg/kg. Gallic acid is a trihydroxybenzoic acid of plant metabolites that can protect cells, tissues, and organs from damages caused by oxidative stress (Gao et al., 2019). Its detection in the finished products especially in the test cracker is of particular interest and not completely surprising as this antioxidant phenolic is fairly stable with only 30% degradation occurring after heating at 100 ˚C for 250 min (Volf et al., 2014), yet the test cracker was baked at 120 ˚C for only 30 min. The gallic acid content of the fresh plant material of *T. orientalis* and *A. longifolia* was 127.3 mg/kg and 22.8 mg/kg respectively, yet the amount of gallic acid detected in the test cracker was 147 mg/kg indicating that the additional gallic acid could have

come from added ingredients especially canola oil which has been reported to be rich in gallic acid (Farhoosh et al., 2009).

Gallic acid has extensive application in the food and pharmaceutical industries (Kahkeshani et al., 2019). It has antidiabetic, anti-inflammatory, cardiovascular, metabolic, neuropsychological and anticancer properties (Kahkeshani et al., 2019). Its ability to inhibit diet-induced hyperglycemia and hypertriglyceridemia (Gandhi et al., 2014) would be a useful characteristic in the development of food with antidiabetic properties. Its major mechanism of action has been attributed to its ability to reduce the size of adipocytes, and protect pancreatic β-cells by inducing the expression of peroxisome proliferator-activated receptor-γ (PPAR-γ), a nuclear transcription factor that induces differentiation and insulin sensitivity in adipocytes (Gandhi et al., 2014; Kahkeshani et al., 2019). Its presence in food may also influence food properties in relation to microbial growth and absorption of other beneficial components in the gastrointestinal tract (Kardum and Glibertic, 2018; Singh et al., 2019).

PCA (4-hydroxycinnamic acid) was another useful antidiabetic component detected in the test cracker (Fig. 6.1 b). With a thermal degradation temperature of 215 ˚C (Contardi et al., 2019), the detection of PCA in the finished product after 30 min baking at 120 ˚C was not unexpected, though not as high in quantity as gallic acid, PCA was also detected in the aqueous extracts of *T. orientalis* plant material. It is described as an active ingredient in cosmetics, a potent antioxidant and scavenger of free radicals and is commonly found in fruits, vegetables and cereals (Pei et al., 2016; Boo, 2019; Contardi et al., 2019). It is able to modulate the activity of enzymes involved in glucose metabolism, improve β-cell function and insulin action, stimulate insulin secretion and strengthen anti-inflammatory properties (Bahadoran et al., 2013). PCAs

antidiabetic capacity lies in its ability to decrease intestinal absorption of dietary carbohydrates by inhibiting α-amylase, α-glucosidase, β-glucosidase and thus delay the digestion of starch or sucrose to glucose, delaying glucose absorption and protecting against postprandial hyperglycaemia (Bahadoran et al., 2013). The detection of PCA in the test cracker was an additional advantage over the control sample which only exhibited small quantities of GA (Fig. 5.1 c).

With a GI value of 47.7, the test cracker would definitely be placed under the class of low-GI foods, known for their efficient postprandial glucose control, low insulin demand and improved satiety (Ratnaningsih et al., 2017). These properties alongside their rich polyphenolic constituents could be helpful in managing overweight/obesity and T2DM within the Indigenous community. The GI of the test cracker was expected to be low because of the high fiber (Table 5.1), high phenolic and resistant starch-content of the plant species that were mixed together to prepare the product, notably; *Acacia longifolia* seeds, *Acacia* gum and *T. orientalis* rhizomes with fiber contents of 17.2 %, 85% and 11% respectively (Mohamed et al., 2015; Njume et al., 2019b). The presence of high amounts of resistant starch (especially soluble fiber) and fats in foods has been reported to lower their estimated GI values and improve carbohydrate metabolism (Jenkins et al., 1987; Miller et al., 2006; Zabidi and Aziz, 2009). Thus, the low GI value of 47.7 recorded for the cracker could be attributed to its high fats (Table 5.1) and resistant starch contents (Table 5.2). This low value could also be as a result of the presence of PCA and GA, all of which are inhibitory to α-amylase, α-glucosidase and β-glucosidase (Bahadoran et al., 2013; Gandhi et al., 2014). It is important to note that α-amylase was used in the enzyme hydrolysis assay and could have been inhibited by any one or both of the above mentioned compounds.

Acacia gum was used as an additive adhesive and thickener in making the food products, to hold the seed and curtail particles together and its characteristic richness in complex carbohydrates and fiber (Renard et al., 2012), further increased the fiber content of the cracker. The carbohydrates of the gum are slow-release complex polysaccharides consisting mainly of 1, 3-linked β-d-galactopyranosyl units, α-l-arabinofuranosyl, β-d-glucuronopyranosyl, 4-O-methyl-β-d-glucuronopyranosyl and arabinogalactan (Renard et al., 2012). The beneficial effects of these complex carbohydrates is adequate control of PPG via moderation of gastric emptying and reduction in absorption of lipids by increasing their faecal excretion (Tutelyan et al., 2016). They can also initiate inhibition of cholesterol synthesis in the liver through the action of short-chain fatty acids produced during the fermentation of soluble fiber in the colon (Tutelyan et al., 2016). Thus, the high fiber content of the test cracker is advantageous for its possible hypoglycaemic, hypocholesterolemic, and prebiotic effects that may result from the promotion of gut microflora growth including bifidobacteria.

The results of this study are consistent with the findings of Thorburn et al. (1987) and Lister et al. (1996) who after studying seeds of some Acacia species found them to be rich in some carbohydrates, most of which are slow digestible and slow absorbable. These properties can also be noticed on the hydrolysis curve (Fig. 5.2) given that at time, 120 min, the rate of hydrolysis of the test cracker was stabilizing, indicating that digestion of DS was complete or nearing completion whereas hydrolysis of the control cracker was consistently on the rise at time, 120 min, 150 min and 180 min. The control cracker, made with predominantly brown rice, was high in DS and TS (Table 5.2) with a GI value of 70.3 (classifying it as a high GI food) which may not be suitable for use as a preventative for T2DM.

In comparison to most other crackers, the test cracker is made with unique organic ingredients (*Acacia* seeds and underground stems/rhizomes of *T. orientalis*) that are not common in the shops and supermarkets. Considering that one of the ingredients (coast wattle seed) has significant commercial potential (Adiamo et al., 2020), it goes without saying that *Acabungi* crackers which are largely made with these seeds are likely to draw the same or similar commercial attention. Polyphenolic components of the cracker have been scientifically analyzed and found to possess potential antidiabetic properties which could be beneficial in the control of T2DM within the Indigenous community. Thus, the market value of the cracker is likely to be high due to potential health beneficial effects relative to other commonly consumed crackers that lack antioxidant polyphenols, are high in saturated fats, sugar, salt and are likely to be poor regulators of postprandial hyperglycaemia.

The findings of this study are consistent with beliefs that most of the starch in the Indigenous plant species is not readily digestible or absorbable and this might have been protective against T2DM among the Indigenous people of Australia before the era of convenience energy-dense products. Given the high rate of T2DM within the Australian Indigenous community, encouraging more Australians to consume products of Acacia species which are readily and freely available from the community could be beneficial to their health.

5.7 Conclusion

The test cracker which was made of whole grains is rich in proteins, fats, dietary fiber, resistant or slow-release carbohydrates which may prevent rapid release of glucose in the blood stream, thus protecting against postprandial hyperglycaemia in people with T2DM. These

characteristics were consistent with the low GI value of 47.7 recorded against the cracker. Phenolic antioxidant compounds of Gallic acid and ρ-coumaric acid with known antidiabetic properties were still detectable in the test cracker after baking, possibly decreasing the GI even further and ultimately adding to the antidiabetic properties of the product. Based on these findings, the test cracker is less likely to cause spiking of blood glucose in people with T2DM and therefore more suitable for use as an antidiabetic food preventative than the control cracker which was made from brown rice.

Chapter 6

OVERALL DISCUSSIONS, CONCLUSIONS AND FUTURE DIRECTIONS

This chapter presents an overview of the entire study, bringing out the essential findings that may be beneficial in the control of T2DM within the Australian Indigenous community and drawing conclusions from the major findings. Further work to improve the food product commercialization and sustainability has been described as future directions.

6.1 Overall discussion

Food for people with T2DM should not only meet the physiological needs of the body for nutrients and energy, but should also possess preventive and therapeutic bioactivities to help normalize or reduce dysfunctional metabolic processes (Tutelyan et al., 2016). While different control approaches including mandatory front-of-pack food labelling, regulating junk food-advertising and taxing sugar-sweetened beverages are laudable, the development of new food products or supplementation of existing products with dietary polyphenols to serve as preventatives of T2DM has been given little consideration. For T2DM to be effectively managed, reducing excess body weight, ameliorating glycaemic control and monitoring cardiovascular risk factors are crucial (Tutelyan et al., 2016). Reducing energy values of basic foodstuff by replacing simple sugars with slow-release carbohydrates or using sugar substitutes such as sorbitol, lactitol, xylitol or maltitol may not be enough to prevent the disease.

Many of Australia's native plant species though unpopular among the 'westernized' Australian population may contain compounds that could be beneficial to the health and welfare of the population. Having survived harsh environmental conditions under the wild Australian climate for many centuries, native plant species have accumulated bioactive components that may bestow unique tastes, flavours and aromas to food products developed from them (Sultanbawa

et al., 2015). Although native food is not a huge part of mainstream Australian diet, food products developed with native plant species present an opportunity to offer the world a national menu with uniquely Australian taste. With continuing overseas interest in Australia, there is potential for huge economic benefits within the tourism/travel industries with opportunities to create jobs for native food growers and improve livelihoods of Indigenous communities that have preserved the natural habitats of these species. Considering that Australian states have different climatic conditions with different flora, each state has the opportunity to also develop profitable native foods with unique tastes and flavours that are peculiar to its flora. The popularity of some native food products such as wattle seed and lemon myrtle on ice cream, yoghurt, cheese, bread and desserts as seasonings and coatings (Maslin and McDonald, 2004) is an indication of the general acceptability and potential commercial value. Given that more young Australians are eating out (Wellard-Cole et al., 2018), there is bound to be increasing demand for native food products especially in an innovative culinary environment in which professionals take pride in developing a uniquely Australian cuisine.

It is worth noting that food insecurity remains a global nutritional problem and despite Australia's reputation, 4 – 30% of the population is unable to access sufficient, safe and nutritious food (Lindberg et al., 2015; Seivwright et al., 2020). The introduction of native food products hitherto unknown to the general Australian population may benefit people on low income and address problems of food insecurity especially in the rural areas. Rural developmental projects set up to generate income from native food products are likely to be beneficial in creating employment, subsistence and sustainability of livelihoods, especially within Indigenous communities where usage of these products is a customary practice that has coexisted with the people for many centuries. However, more research into the native food

industry would be required to create awareness about the nutritional and health-promoting properties of native species. Programs to reduce stigmatization and improve marketing and youth attitudes towards native food products would help boost popularity of the products. Each state would have to design sustainable business models for development of native food products and market value in collaboration with partner organizations and Indigenous Elders. This would help protect trademarks and produce sharing of benefits agreements that would encourage rural development projects and businesses. Funding for such projects could be sourced from government through the Rural Industries Research and Development Corporation (RIRDC), now known as Agricultures Australia.

Kakadu plum and bush tomatoes for example, are native foods with value added chains made possible by Desert Knowledge Park, Alice Springs for the production of bush tomato ketchup and chutney. Their produce is generated with participation of rural community women who sustain their livelihoods by participating in hand harvesting of fruits, sorting and washing prior to transportation to the manufacturer (McDonald et al., 2006). In Victoria, commercial partnership agreements for Indigenous Australian Foods Supply Chain (IAF) should be developed to a stage of national and global competitiveness so as to provide opportunities for rural developmental projects. By coming together with a common sharing of benefits agreement, Victorian Indigenous enterprises such as martang Djab Wurrung, Worngundidj and Outback bush foods may work with Indigenous community elders and seek funding from RIRDC (now AgriFutures Australia) and Australian Research Council (ARC) to improve supply chain developmental projects that would meet national and global demands for Australian native products.

In this thesis, *Acabungi* flakes and crackers were largely made with the rhizomes and underground stems of *T*. *orientalis* (Water Cumbungi) and seeds of *A*. *longifolia* subsp. *Sophorae* (coast wattle). The name 'Acabungi' was coined from the local names of the 2 major plant species (Acacia and Cumbungi) used in preparing the products. Both species demonstrated rich antioxidant properties, total dietary fiber and *T*. *orientalis* was also rich in antidiabetic polyphenols (GA, EPC, CH, EPG, and PCA) whereas seeds of *A*. *longifolia* subsp. *sophorae* were rich in unsaturated fatty acids, TDF and slow-release carbohydrates. The addition of the aqueous extract of *R*. *candolleana* which is rich in the betacyanin antioxidant food colourant, betanidin 5-*O*-β-glucoside, improved the colour and intensified the antioxidant properties of some of the flakes. It is believable therefore that consumption of these products might enhance antioxidant, anti-inflammatory, insulin sensitivity, and anti-cholesterol functions in the body and prevent the development of T2DM as earlier reported with other plant species that exhibited similar characteristics (Alkhatib et al., 2017). However, the antidiabetic preventative mechanism of action of the products is yet to be determined.

It is worth noting that the flakes were not the most acceptable product although the flavour of most flakes were highly acceptable. More participants found the cracker to be more acceptable than most of the flakes. For this reason, the cracker was selected for further studies of antidiabetic compounds, microbiological quality, preliminary stability studies and enzymatic hydrolysis. The seeds of *A*. *longifolia* subsp. *Sophorae* constituted the major proportion of the cracker. This should not be surprising considering that these seeds are widely consumed within the Indigenous community, have good flavour, high nutritional value, functional properties and seem to have significant commercial potential in Australia where they are used as condiments of many foods and beverage products (Adiamo et al., 2020). The Australian native food

industry has a custom of using roasted ground *Acacia* seeds as value added products, condiments in baked foods, coffee analogues and dairy (Maslin and McDonald, 2004). Even though the seeds were baked into crackers in this study, the enticing flavour of seeds of *A. longifolia* subsp. *Sophorae* are most appreciated when freshly roasted, indicating that freshly roasted seeds may have significant commercial potential as food flavourings.

The low glycaemic index of the cracker is not surprising as this is consistent with its rich fiber, fat, protein content and dietary polyphenols (GA, PCA) which have antioxidant properties and have previously been reported to inhibit α-amylase, α-glucosidase and delay glucose absorption in the intestine (Kim et al., 2016). In other words, the above mentioned polyphenols are known to exhibit specific properties *in vitro* and sometimes *in vivo* which may be helpful in blood glucose regulation (Doan et al., 2015). According to Doan et al. (2015), gallic acid (3, 4, 5-trihydroxybenzoic acid) also plays its homeostatic role through the activation of AMP-activated protein kinase (AMPK) and by regulating mitochondrial function via the activation of peroxisome proliferator-activated receptor-γ coactivator 1 α (PGC1α). GA has been reported to up-regulate the expression of hepatic insulin signal transduction-related proteins, including insulin receptor, insulin receptor substrate 1, phosphatidylinositol-3 kinase, protein kinase B, and glucose transporter 2, in rats fed with high fructose diet (Huang et al., 2016). In the same study, gallic acid also down-regulated the expression of hepatic gluconeogenesis-related proteins, such as fructose-1,6-bisphosphatase, and up-regulated expression of hepatic glycogen synthase and glycolysis-related proteins, including hexokinase, phosphofructokinase, and aldolase, indicating that GA has potential as a health food ingredient to prevent diabetes mellitus (Huang et al., 2016). It was interesting to note that GA was a major phenolic component of the cracker.

It is reported that the rich polyphenolic content of green tea and its glucose regulatory properties are appreciated owing to the CH, EPC and EPG content which may help in improving glycaemic control and lipid profile (Wolfram et al., 2006; Othman et al., 2017; Bakhtiyari et al., 2019). EPG is reported to downregulate genes involved in gluconeogenesis and the synthesis of fatty acids, triacylgycerol, enhancing glucose tolerance in diabetic rats (Wolfram et al., 2006). It is hopeful that with such properties, the antidiabetic efficacy of food products that contain EPG is likely to be better than most other products that do not. However, EPG was not detected in the finished product probably due to its instability at the set baking temperatures (Zeng et al., 2017; Loncaric et al., 2018), but it was one of the components of *T. orientalis*. In fact, EPG, CH and EPC were all detected in the raw plant material of *T. orientalis*. While PCA, BT and LT are noted for their antioxidant and anti-inflammatory properties (Amalan et al., 2016; Antigo et al., 2018), CH and EPC are known to enhance their antidiabetic properties through inhibition of enzymes involved in carbohydrate metabolism (Liu et al., 2016 a), preventing a rapid release of glucose in the blood stream. It is interesting to note that PCA was one of the compounds still detectable in the baked cracker.

Although the mechanism of action of many phenolic-rich food products are not completely elucidated, there is growing evidence that antioxidant compounds in functional food products may improve postprandial hyperglycaemia and modulate carbohydrate and lipid metabolism (Mirmiran et al., 2014). However, it is important to note that taking foods that have rich polyphenol and antioxidant content is not always a guarantee of their bioavailability, as some components are degradable in the gastrointestinal tract (Luminita et al., 2019). Acacia seeds have also been reported to contain anti-nutritional factors such as trypsin and chymotrypsin

inhibitors (Ee and Yates, 2013; Adiamo et al., 2020) which could prevent the absorption of specific nutrients by inhibiting digestive enzymes. Some anthocyanins, for example may be stable in the stomach environment but are degraded during duodenal digestion (Luminita et al., 2019). In some cases, not more than 1 μM concentration is found in plasma for a phenolic that is taken orally (Alkhatib et al., 2017), yet it is important to note that absorption of some phenolics could be improved in the presence of other bioactive compounds. Bioavailability of epigallocatechin-3-gallate for example, was increased (1.3 folds) in the presence of the alkaloid, piperine in mice (Lambert et al., 2004). In a related study, bioavailability of curcumin (a component of tumeric) increased 154% and 2000% in wistar rat and human volunteers respectively when curcumin was administrated together with piperine, a component of black pepper (Gao and Hu, 2010). The bioavailability increase was attributed to glucuronidation inhibition by piperine (Gao and Hu, 2010). Thus, where conditions are conducive, polyphenols taken within the food are likely to be bioavailable and may produce the desired physiological effects, sometimes prompted by synergistic/antagonistic interactions between components.

If vascular/cardiac complications are to be brought to a minimum in people with T2DM, then the amount and type of fats in their food has to be taken into consideration. *T. orientalis*, one of the species used in the production of *Acabungi* crackers had 1% fat while *A. longifolia* subsp. *Sophorae* had 14 % and the cracker has 12.7 %. Even though 12.7 % fat seems to be on the high side for people with T2DM, these are mostly monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Both fatty acids were detected in the seeds of *A. longifolia* in the form of OA (monounsaturated compound) and LA (omega-6 polyunsaturated compound). These compounds are believed not to increase the body mass, total cholesterol and low density lipoprotein in people suffering from coronary heart disease, thus improving the

effectiveness of insulin at the peripheral level (Guadarrama-López et al., 2014; Jiao et al., 2019). Evidence of their improved glycaemic control (decrease of basal glycaemia, postprandial glycaemia, daily glycosuria) and decrease in tissue insulin resistance in people with T2DM has been widely documented (Hu et al., 2001; Sharafetdinov et al., 2003; Guadarrama-López et al., 2014; Jiao et al., 2019). With such properties, it should therefore not be surprising that the glycaemic index values of *Acabungi* crackers were as low as 47.7.

The production of food for people with T2DM would also require utmost consideration of ensuring adequate content and optimal balance of minerals and trace elements. Chromium deficiency is accompanied by the impaired metabolism of glucose and lipids, decreased number of insulin receptors and development of insulin resistance (Tutelyan et al., 2016). A deficiency in Zn may lead to impaired glucose tolerance and mounting evidence now shows that Se levels lower than the recommended daily intake values may sometimes be associated with T2DM (Ogawa-Wong et al., 2016; Wang et al., 2016). This is due to the fact that Se is a co-factor of glutathione peroxidase, which is involved in antioxidant protection. An insufficient supply of the body with biologically active minor food components, many of which may offer chemo-protection or chemo-prevention, is one of the causes of decreases in nonspecific resistance to adverse environmental factors of chemical and biological nature (maladaptation), formation of immunodeficiency states and dysfunction of the antioxidant defense system (Tutelyan et al., 2016). Interestingly, many of the native species studied herein, especially *Rhagodia candolleana* subsp. *candolleana* and *T. orientalis* are rich in minerals and phytochemical compounds that could be helpful in protecting against environmental stresses that predispose people to T2DM within the Indigenous community. In addition to other minerals, *T. orientalis*,

C. rossii and *C. alba* all contained Se, a scarce, yet important mineral which is important for efficient antioxidant protection (Ogawa-Wong et al., 2016).

6.2 Conclusion

Edible portions of the 8 native Australian plant species; *Leucopogon parviflorus*, *Arthropodium strictum*, *Carpobrotus rossii*, *Rhagodia candolleana* subsp. *candolleana*, *Typha orientalis*, *Correa alba*, *Dianella revoluta* and *Acacia longifolia* subsp. *sophorae* have been found to be sources of proteins, carbohydrates, fatty acids, minerals and antioxidant polyphenols. The rhizomes and underground stems of *Typha orientalis* and the seeds of *Acacia longifolia* subsp. *sophorae* which demonstrated rich antioxidant, nutrient and polyphenolic content have been developed into food products, mainly flakes and crackers. The crackers were found to be more acceptable by 31 of the 44 participants who took part in the sensory evaluation. Analysis of the cracker indicated a high total dietary fiber, fat and protein content. The cracker also contained slow-release carbohydrates and two antidiabetic polyphenols of GA and PCA were still detectable after baking at 120 ˚C for 30 min. Based on the above mentioned properties, the highly acceptable cracker may have potential as a useful antidiabetic food preventative, especially given its low glycemic index of 47.7.

The cracker remained stable and unchanged with good physical and microbiological parameters and was still consumable after 35-days storage at room temperature under light and in the dark. The rich Acacia seed flavour of the test sample was rated higher than the flavour rating of the control sample. This product has exhibited promising useful characteristics as potential antidiabetic food preventative and seems to support beliefs that early Indigenous

Australian diet made with these ingredients could have been protective against T2DM. The findings of this study indicate that neglected Indigenous plant species such as *T. orientalis* could constitute a reservoir of nutrients and phytochemicals that may benefit the prevention of dysfunctional metabolic diseases such as T2DM. *R. candolleana* and *A. longifolia* were found to be good reservoirs of betanin and linoleic acid respectively, two components with potentially wide industrial application.

6.3 Future directions

In this study, edible portions of two of the eight plant species (*Typha orientalis* and *Acacia longifolia* subsp. *sophorae*) were developed into flakes and crackers using vanilla extract as the only flavouring. However, there are possibilities to develop different food products including muesli bars, damper and food additives with varieties of different flavours using seeds of *A. longifolia* subsp. *sophorae* to satisfy wider consumer demands. Equally important is the fact that coast wattle seeds are rich in unsaturated fatty acids, indicating that industrial production of healthy oils from these species is possible.

Other commercially valuable products that could be developed from coast wattle seeds include Acacia seed flour and beverage products. Acacia seed flour is rich in protein that have been reported to possess high functional properties such as water and oil holding capacity (Agboola et al., 2007). The emulsifying, foaming, and gelling properties of some Acacia seed proteins have been useful in the preparation of different food formulations (Agboola et al., 2007). However, despite the rich protein content of coast wattle seeds, the protein functional properties have not been put to use in this way. This presents an opportunity to develop high protein

varieties of this species by collecting the seeds and processing the proteins for use as gelling agents in food formulations.

Lightly roasted coast wattle seeds can also be powdered, packaged and commercialized for use in beverages. The seeds of some Acacia species cost about $12 to $25 per kg of clean seed at farm-gate price (Adiamo et al., 2020). Roasted ground Acacia seeds have attractive aroma and flavour which explains their popular use as food flavourings in ice cream, cakes, cheese and desserts (Maslin and McDonald, 2004; Shelat et al., 2019). They have significant commercial potential due to their high nutritional value and functional ingredients and are marketable alone or as ingredients in other products like coffee (Adiamo et al., 2020). Some Acacia seeds are already commercially available in roasted form and are well-known as value added products from the Australian native food industry (Adiamo et al., 2020). However, the aromas and flavours of coast wattle seeds are most appreciated with freshly roasted ground seeds as they readily disappear from the product after a few days of storage at room temperature. Trapping and distilling aromas from the seeds is a relatively unexplored area and could constitute a potential source of cosmetic and pharmaceutical products that may generate economic benefits.

Damper, a traditional bread made from Acacia seeds is largely unknown within the non-Indigenous Australian society, yet damper which is made by crushing a variety of native seeds, and sometimes nuts and roots, into a dough and then baking the dough in the coals of a fire is a healthy bread owing to its high protein, dietary fiber and resistant carbohydrate content. It is possible to produce damper and be able to create a huge market value for it. Although the primary buyers may be Indigenous Australians who constitute only 3.3% of the population (ABS 2018), the healthy benefits of consuming damper may help draw in more buyers from

the non-Indigenous population. Studies investigating sensory attributes of damper within the Indigenous and non-Indigenous Australian society would have to be conducted to help give an indication of the acceptability and overall market potential of this highly nutritious product.

Human intervention studies on the mechanism of action of the antidiabetic cracker produced herein and its components would shed more light on its potential application as an antidiabetic food preventative. Such studies may be centred on the hypoglycaemic, hypolipidaemic, anti-cholesterolmic, anti-triglycaeridaemic and anti-inflammatory effects of the cracker and its components. The studies could be performed as pilot scale by recruiting 5 - 7 healthy and non-healthy volunteers to consume standard amounts of the product for a specified period of time, followed by measurements of blood lipids, blood glucose and blood pressure of both groups (Parikh et al., 2001; Torres-Duran et al., 2007). The measurements are then compared with standard control readings for blood glucose, lipids and blood pressure and analysed statistically to determine differences in parameters between the different groups.

In an attempt to avoid the risks and burdens associated with human intervention studies for the determination of GI (e.g., time consuming, pain and discomfort from needle pricks during blood collection procedures, overnight fasting and high cost) (Argyri et a., 2016), GI values in this thesis were estimated *in vitro* by enzymatic hydrolysis. However, these methods are also limited in that they tend to pulverize, grind or homogenize foods prior to analysis; such treatments breakdown food structures (such as plant cells, tissues, intracellular networks) that are now known to be of great importance with regard to influencing digestion kinetics and measurement of Type 1 resistant starch (*i.e.* starch that is resistant to digestion because it is physically inaccessible to digestive enzymes) (Edwards et al., 2019). It would therefore be logical to perform human GI testing to confirm the *in vitro* laboratory estimations. This can be

achieved by recruiting participants who will consume 6 packs of the crackers (≈50 g carbohydrates) after an overnight fast. Their blood glucose will be measured for 2 hours at 15 minutes intervals (Wolever et al., 2008). The procedure will be repeated on the following day with 50 g of glucose and both sets of results plotted (blood glucose measurements against time) and GI calculated for each individual (Wolever et al., 2008).

Due to the presence of the 'spirit molecule' (dimethyltryptamine) and other possible anti-nutritive factors in coast wattle seeds, toxicity studies would be required to determine the safety of the cracker and other potential food products developed from the species. Cytotoxicity studies of the products and components could be studied by employing various techniques including MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), anti-proliferation, apoptosis or DNA fragmentation assays (Arora et al., 2014). Removal of anti-nutritive factors could be achieved by thermal treatment such as roasting, dry heating and autoclaving which have been reported to significantly reduce the levels of phytic acids and trypsin inhibitors in some species of Acacia seeds and products (Siddhuraju et al., 1996).

Studies on the application of innovative non-thermal methods to reduce any anti-nutrient levels in coast wattle seeds without causing any detrimental effects on the seed's protein quality would have to be investigated. The structure and functional properties of the proteins obtained from the seeds after treatment should be assessed (Adiamo et al., 2020). Studies to determine the presence of allergens in coast wattle seeds would be essential considering that some legumes contain allergic compounds that can pose a high health risk to some categories of people (Adiamo et al., 2020). Protein structure modification of coast wattle seed proteins, achieved

through enzymatic treatments as previously suggested with some other species (Adiamo et al., 2020), could be advantageous in improving their bioactive and health beneficial properties. The bioactive peptides obtained should be further purified to enhance the activity of the peptides and compared with commercially available peptides from legumes such as soybean peptides (Adiamo et al., 2020). Considering that bioactive peptides from plants may exhibit antioxidant, antimicrobial, anticancer, and anti-hypertensive activities, such studies will provide valuable information on possible production of coast wattle seed protein that are both safe for human consumption and exhibit functional and health-beneficial properties. Information gathered could lead to potential application of coast wattle seed protein in food formulations and broaden the market potential of coast wattle and its products in the food industry.

Further research is also required to determine whether supplementation of daily meals with some health-promoting antioxidant polyphenols isolated in this thesis would help curb the escalation of T2DM within the Indigenous community. Should this be the case, the quantities required for each meal would have to be determined. Curcumin, for example is a polyphenol extracted from *Curcuma longa* and a 200 mg curcumin/kg diet has been reported to improve insulin resistance and hyperglycaemia in mice (Aryaeian et al., 2017). A 500 mg/kg curcumin in diet led to a decrease in body weight gain and adiposity of mice (Aryaeian et al., 2017). These findings suggest that curcumin could possibly repress inflammation and obesity and improve the chronic condition in diabetes. In a study on 240 pre-diabetic adults, participants were given 250 mg of curcumin or a placebo every day for 9 months. After intervention, none of those taking curcumin developed diabetes, but 16.4% of the placebo group did (Kim et al., 2009). Curcumin was therefore 100% effective in preventing T2DM during this 9 month trial.

Adding cinnamon to food has also been reported to decrease the rate of stomach emptying from 37% to 34.5% and significantly decreased the rise in blood sugar levels at 0 and 120 minutes compared to ingestion of the reference meal (Kim et al., 2009). Other dietary polyphenols such as resveratrol (from grapes, peanuts, cranberries, blueberries, and Japanese knotweed), capsaicin (from chili peppers), genistein (from soy), catechins and procyanidins (from green tea) have been tested for their antidiabetic properties and the results have been promising (Aryaeian et al., 2017). Similar studies that would see the blood glucose effects of specified quantities of GA, PCA and other polyphenols isolated from coast wattle seeds, Water Cumbungi rhizomes and their products would shed more light on their potential application for dietary polyphenol supplementation. However, it is worth noting that their bioavailability may be affected by their large structural diversity and many undergo intestinal transformation and/or colon microbial digestion (Marin et al., 2015). Flavan-3-ols for example, are acylated by gallic acid and absorbed at enterocyte level without hydrolysis while green tea catechins are absorbed in the small intestines without prior modification (D'Archivio et al., 2010). For most polyphenols, enzymatic transformations include elimination of glycosidic tailoring by gut microbiota of diverse genera (*Lactobacillus*, *Eubacterium*, and *Bifidobacterium*), as well as further transformations in these aglycones' level, giving rise to more stable bioactive compounds that are incorporated into the blood stream (Marin et al., 2015). Consumption of food with high levels of polyphenols, together with having appropriate gut microbiota diversity, is extremely important in facilitating intestinal production of bioactive metabolites from dietary polyphenols, as well as their absorption and bioavailability (Marin et al., 2015).

Participants recruited for the sensory evaluation in this thesis were from diverse cultural groups. Considering that the plant species were collected from the Indigenous community, it

would be appropriate to perform sensory evaluations with members of these communities who are the custodians of cultural properties associated with the species and possibly, primary responders to the commercialized products. It is also worth mentioning that all participants recruited for sensory evaluations in Chapter 4 were blinded to the nutritional and phenolic components of the products and health benefits associated with them. Equally important is the fact that the food products were not labelled with the nutrition-labelling information or statutory requirements of Food Standards code for Australia and New Zealand (FSANZ) and as described under the Legislative Act of 2002/2003. According to these statutory requirements, all manufactured foods must carry nutrition information labels (Curran, 2002; Fabiansson, 2006). Thus, a new sensory study using samples that have been adequately labelled with nutrition information in which the participants are aware of the potential health benefits of the product should be performed and results compared with previous studies (Chapter 4). This would help evaluate the overall commercial potential as participants are likely to choose products that are related to their culture and have health beneficial effects. These aspects have been demonstrated with other similar studies (Pollard et al., 2002; Lockie et al., 2004; Tudoran et al., 2009). In these studies, consumers' perceptions of taste, culture and belief in health properties of some food products influenced choice and overall market potential of the products.

In this thesis, only preliminary shelf life and stability studies were conducted on the most acceptable product and all test samples were studied at room temperature and humidity within 5 weeks. In most cases, the results recorded only minor insignificant changes in texture, colour, pH, water activity and moisture. Microbial counts were zero throughout the testing period. There is need therefore, for detailed studies that would take into consideration different storage

temperatures and longer time frames to investigate the likelihood of changes in texture, colour, pH, moisture, water activity and microbial growth at -15 - 10˚C, 20 - 45˚C and >45 ˚C. These will allow for detection of potential psychrophilic, mesophilic and thermophilic contaminants. Groups of samples would be studied side by side under different humidity conditions and the results reported based on how stable the products remained under the different testing conditions. A repeat of the sensory evaluation with samples stored under different conditions would generate detailed useful information regarding their shelf life and stability. Such studies will give a broader view of the products durability, stability and add to the preliminary shelf life/stability results previously obtained (Chapter 4).

If commercialized, the cracker developed in this thesis may contribute to the economic wellbeing of the Indigenous and non-Indigenous Australian population. However, a number of steps would have to be considered before commercialization. To avoid high chance of market failure and improve chances of product success, it would be helpful to invest time on the product development, design and testing before any intended market launch. According to FSANZ code (standard 3 .2 .2), packaging of the product must take into consideration type of food, storage conditions, weather and risks associated with the specified package material and how these could be eliminated or minimized. The cracker labels will also be produced as recommended by the standards set by the code to protect public health and safety by displaying information such as use by dates, ingredients, certain allergens, instructions for storage and preparation, and advisory and warning statements. The cracker will be submitted for a pre-market safety assessment by FSANZ before marketing.

Performing pre-market testing of the fully labelled packaged variants of the cracker in niche markets, exhibition shows and food ops may provide more information about its market potential and identify customer needs in relation to competitor products (Alexandre et al., 2003), yet these will be done after pre-market testing by FSANZ. Findings of pre-market testing would be used to guide branding, pricing, image, choice of communications and distribution channels of the products. Stake holders and funders from the Indigenous community will be contacted to lobby for funding to pay production experts and consultants. A non-disclosure agreement will be prepared and signed by all stakeholders and a co-packer or co-manufacturer will be contacted for bulk deliveries and packaging in a safe and economical manner. Food regulations will be followed at all stages of the production and distribution process to ensure the safety and quality of the product is maintained at all times by liaising with food testing laboratories. As a highly acceptable native Australian food product, rich in nutrient and functional properties, the crackers could be readily marketed as additional new Indigenous products alongside kakadu plum and bush tomato chutneys which are already witnessing an increasing domestic and export marketing potential (Cooper, 2017). Prospects of such increases are even much more envisaged with the addition of *Acabungi* crackers which have potential to mitigate escalation of T2DM within the Indigenous community, offer a uniquely Australian taste with a flavour that appeals to lovers of local produce, create more opportunities for Indigenous employment and generate revenue for the welfare of Indigenous Australians.

Chapter 7

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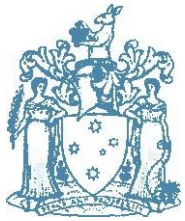
Appendix 1

Permit to conduct study



PermitNo: 10008221
File No: FF383S32

Department of Environment, Land, Water and Planning

FL01u AND FAUNA GUARANTEE ACT 1988
RESEARCH PERMIT
PERMIT TO TAKE / KEEP PROTECTED FLORA

Pursuant to the provisions of the Flora and Fauna Guarantee Act 1988, permission is hereby granted to:

Mr Collise Njume

College of Health and Biomedicine Victoria University Werribee Campus, Hoppers Lane, Werribee 3030

And Prof Andrew McAinch, Dr Osaana Donkor and staff /students/volunteers under the direct supervision of the permit holder in order to take protected and non-protected flora from public land within Warrnambool City Council Parks (Hopkins Rivcr, Thunder Point and Kclly's Swamp), for the purpose of investigating bioactive components or native food ptanl.s and lhcir application in lhc prevention of type 2 diabctcs mellitus within Australian Aboriginal Communities.

Permission is given subject to (he 'Ollowing particular conditions:

1. The researchers are required to notify the local Biodiversity officer of their proposed activities at least three days in advance. He/shc is to be advised, in advance, of each visit made in connection with thc provisions of this permit. Contact the Dcpartmcnt of Environment, Land, Water and Planning (DELWP) Customer Scrvice Centre on 136 186 and ask for thc contact number for thc Biodiversity officer of the nearest DFLWP office where the research is to be undeltaken.

2. Taxa: all except members of the Orchidaceat fåmlly, and thrca(cncd taxa, as listed on Schedule 2 or unclcr Section 10 of the Flora and Fauna Guarantcc Act 1988 Collection or such species tequlres an additional authority. Whcrc field identification is not possible lake photographs for identification rather than plant specimens-e Plant Darts: seed and propagation matetldl

Ouantitv: up to 10% of rcp10ductwe matet•al or 5% of cutting material from any one plant and up 10 25% of any taxon per population and/or 25% of any 30 metrc square area, whichever is less.

3. Prior to commencing the research. approval of the managing authority of lhc Inod must be obtained.

4. Protected (lora must not be taken fiom small populations. As a guide, populations arc considered small whcn they consist or lèwcr than len individuals in lhc case of perenmal ttccs, shrubs and graminoids, and fewer than filly individuals in the case of annuals or perennial forbs or non vascular species.

5. Collection should not takc place in an area from whiCh it is suspected that plants have previously been sampled during that scason, or if the plants appear damaged or in poor hcalth.

6. All research activities should be consistent Vic(oria University's Occupational Health and Safely requirements and the risk assessment and mitigation documentation prepared for specific research activities-

7. It is essenlial that rcscarchers ensure that vehicles travelling on management vehicle ftacks arc free of soil, gravel and plant material prior [o travel/entry to sites. They must also ensurc thal when going ofl:-lmck all boots and any tools used arc free of soil, gravel and plant material, and must be disinfcctcd. This can be done with either Phytoclcan mixed I part Phytoclcan to 1 0 parts water, or with domestic bleach mixed I part bleach to 4 parts water.

8. Provisions or the Flora and Fauna Guarantee Act 1988 are to he fully observed, except where exemption is spccifically provided for in this permit.

9. The direction of any authoriscd officer ofthe DEI,WP, in relation to this permilp must be tòllowed.

10. Work involving authorised site manipulation or specimen collcc{ion is (o be conducted, whcrc possible, away from public view.

ll. The permit holder must provide specific and data rcporls identified and requested by the Environment and Natural Resources, Rcgional Manager, DELWP, within 7 working days ofthe request in both hard copy and cfcctronic format.

' 2. A report of the collection activities, including the name of the collector, spccics, numbers, dates ol' collection and localities, to the nearest onc hundred metres (AMG), must be submitted by the permit holder lo (Inc Environmental Research Co-ordinator, Department of Environment, Land, Water and Planning within 30 days of expiry of this permit.

[redacted]

Merryn kelly

Acting Environment and Natural Resources, Rcgional Manager

(Delegatc of the Secretary)

13. The data must be entered into to the Victorian Biodiversity Atlas (VBA). Your data may remain in the system as 'Draff for up to 12 months from the date of expiration of this permit, at which point it must be submitted for expert review and subsequent publication. There are mandatory fields required to make a VBA record and it must include details of your permit number. Information about how to enter data into the VBA can be obtained from the DEI-AM) website, Biodiversity pages at: http://www.delwp.Vic.gov.au/environment-andwildlife/biodiversity/victorian-biodiversity-atlasor contactvba.help@delwp.vic.gov.au if you require further assistance.

14. Where any rare or threatened species, as listed in the Flora and Fauna Guarantee Act 1988, or listed on the DELWP website advisory lists www.delwp.vic.gov.au), are identified, the exact location should be noted and reported to the relevant Biodiversity Officer of the Department of Environment, Land, Water and Planning, at the earliest possible convenience. These details should also submitted to the VBA as part of your submission. Please contact: vba.help@delwp.vic.gov.au

15. A short research summary of results outlining the key findings must be provided to both Environmental Research Co-ordinator, DELWP, PO Box 137, Heidelberg, 3084 within 30 days of the expiration of this research permit. The publication of any results in connection with this research permit must refer to the fact that they were collected or obtained under the terms of this permit and copies of any reports, theses or published articles must also be provided.

16. The permit must be carried by the permit holder whilst undertaking any work in any area named on the permit and shown on demand to any Authotfsed Officer of the Department of Environment, Land, Water and Planning.

17. Type specimens remain the property of the State Government and must be lodged in the relevant State institution or as directed by the Environment and Water, Regional Manager. All collections suitable for herbarium specimens must be lodged as soon as practicable, and within 30 days upon expiry of this permit, at the National Herbarium of Victoria.

18. Failure to comply with any condition of this permit may result in its cancellation, at the discretion of the Environment and Natural Resources, Regional Manager, Department of Environment, Land, Water and Planning.

19. Requests for the renewal of a research permit will be considered only after these permit requirements are met.

This permit shall, unless revoked, remain in force until 31 December 2019.

[redacted]

Merryn Kelly

23 MAR 2017

Acting Environment and Natural Resources, Regional Manager

(Delegate of the Secretary) Date of issue:

2/2

Dear Collise,

I refer to your application tor a research permit under the Flora and Fauna Guarantee Act 1988 to undertake research Permit number 10008221 (expiry date 31 December 2019) has been granted. If this project IS to continue after that date please wnte to me at the address below requesting a renewal. You are requrred to submit a copy of your final report.

The granting of this research permit is based on the premise that the work will potentially contribute to scientific knowledge, is supported by both the Department of Environment, Land, Water and Planning and broadly by the community. Researchers should note that the issue of this permit is a privilege not available to the general community and therefore the probity reflected in this research permit indicates the responsibility researchers have to properly undertake their authorised work. Close attention must be paid to all conditions on the permit, failure to do so can result in non-compliance.

In particular you should ensure that you discuss your research proposals with the relevant Biodiversity Officer before beginning any field-work. This Will allow DELWP staff to inform you of any local issues that may impact on your work, and also allows them to offer you assistance.

Please note this permit is only valid when signed and dated by the permit holder (see final page of permit).

Should you require further information, please email: environmental.research@delwp.vic.gov.au or telephone 9450 8746. Please quote the file number and permit number in all correspondence relating to this p rmit.

Yours sincerely

[redacted]

Dr Sue Hadden
Environmental Research Co-ordinator

Privacy Statement
Any personal information about you or a third party in your correspondence will be protected under the provisions of the Privacy cnd Data Protection Act 2014. It will only be used or disclosed to appropriate Ministerial, Statutory Authority, or departmental staff in regard to the purposefor which it was provided, unless required or authorised by law. Enquiries about access to information about you held by the Departmentshould be directed to the Privacy Coordinator, Department ofEnvironment, land, water and Planning, PO Box 500. East Melbourne, Victoria 8002

•<ORIA
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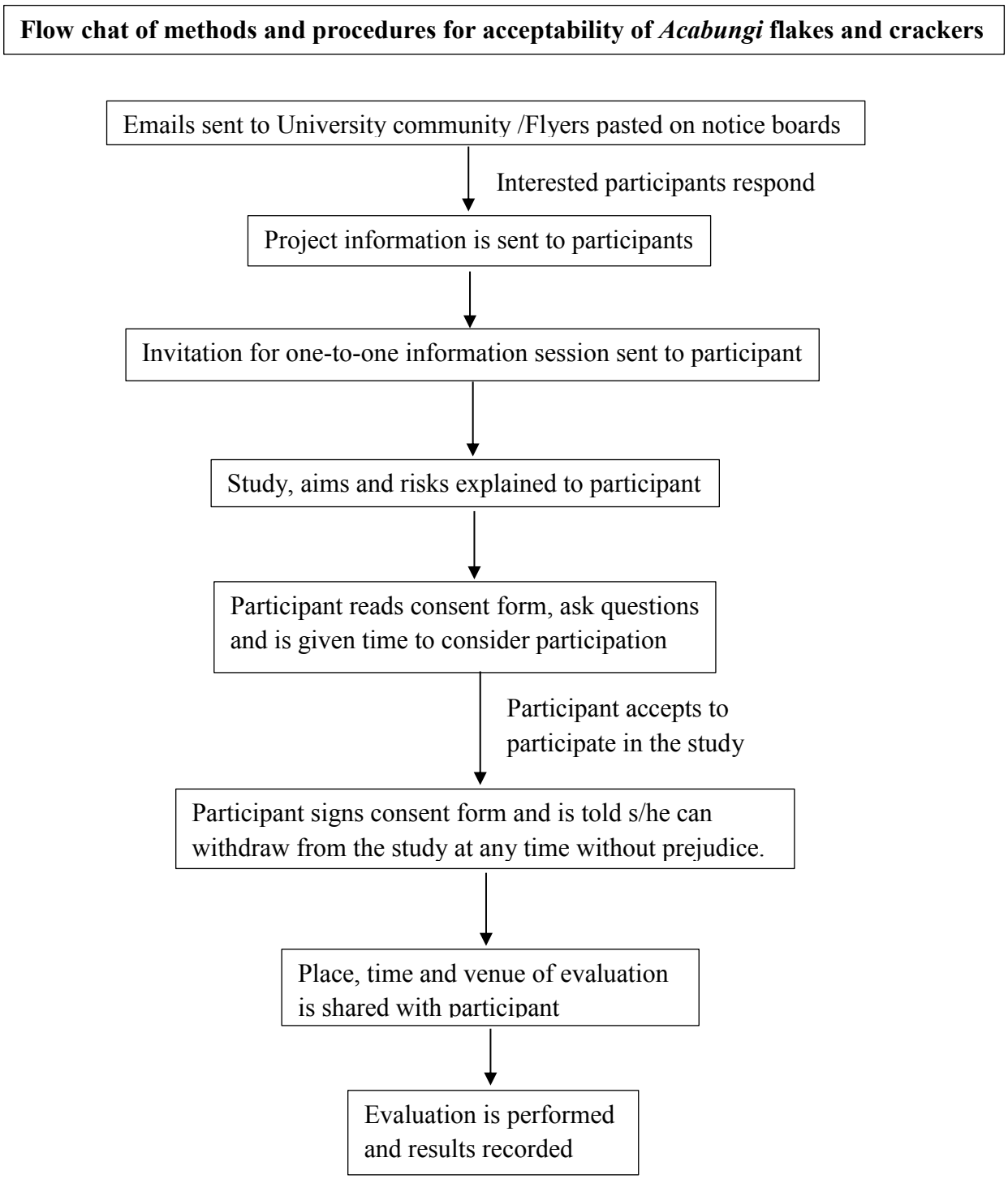
Appendix 2

Informed consent procedures



Appendix 3

Flyers inviting participants for sensory studies

VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

Acabungi flakes and crackers !

Are you curious about new food products? If so, then why not try out a sensory evaluation of *Acabungi* flakes and crackers, newly developed food products rich in polyphenols and antioxidants compounds. If interested, type the word, '**interested**' and send to collise.njume@live.vu.edu.au for more info.

Cheers!

Appendix 4

Information for participants

You are invited to participate

You are invited to participate in a research project *titled "Acceptability of Acabungi flakes and crackers."*

This project is being conducted at Victoria University by: Prof Andrew McAinch, Dr Osaana Donkor and Mr Collise Njume, from the College of Health and Biomedicine and Institute of Sustainable Industries and Liveable Cities.

Project explanation

Acabungi flake is a breakfast cereal while the cracker is a snack. Both products are developed from two of Australia's native food plants; *Typha orientalis* C. Presl and *Acacia longifolia* subsp. *sophorae*. The plants parts (seeds of *A. longifolia* and underground stems and rhizomes of *T. orientalis*) have been used as food by the Australian Indigenous population for centuries. These plant parts were used to develop *Acabungi* flakes and crackers. The name '*Acabungi*' was coined from 'Acacia' and Water Cumbungi, the two plants used in the development of the products. However, it is still not known whether these products will be acceptable as food within the community. Therefore the aim of this study is to determine acceptability of *Acabungi* flakes and crackers through sensory evaluation of flavour, aroma, taste, aftertaste, mouthfeel, texture, appearance and overall acceptance.

If you consent to participate in this study you will be asked to assess 8 samples within 40 minutes provided at the Footscray Nicholson commercial kitchens, sensory room D221, Footscray campus, Victoria University, Melbourne. You will be requested to assess the samples using the following procedure:

Hedonic scale acceptability of *Acabungi* flakes and crackers: You will be asked to evaluate *Acabungi* flakes and crackers for appearance, flavour and mouthfeel using a 9-points scale. The scores obtained will be used to assess the acceptability of the flakes.
(a) Colour and appearance: Visually observe the product and give your assessment.
(b) Aroma: Smell product and give your assessment.
(c) Taste and aftertaste: Place sample in the mouth and chew, then roll it into a small ball and spit it into the container provided. Rate taste, aftertaste and flavour.
(d) Mouthfeel: Place sample in the mouth and manipulate it in a circular motion between the tongue and palate and judge the intensity of mouthfeel as the amount of film remaining in the mouth after disposing of the sample.
(e) Overall acceptance: Give your judgement on the overall acceptability of the product.

You will be provided with water and non-salted crackers to cleanse the palate between tasting. This is important since it eliminates any carry-over and "erases the memory", so you can evaluate the next sample impartially.

You are eligible to participate in this study if you are;

- Between 18-65years

Exclusion criteria:

- Allergy to nuts and seeds
- Allergy to milk and milk products.
- Anyone who is on a diet

Participation is totally voluntary and without prejudice for those who participate and for those who decline. Those who are interested to join the sensory evaluation are free to withdraw from the study at any time.

What will I gain from participating?

You will not gain a direct benefit from this study.

How will the information I give be used?

All information obtained from this research project will remain confidential and will only be used for research purposes. The data collected during the study will be used in conference presentations, and peer-reviewed scientific papers. No personal details will be revealed.

What are the potential risks of participating in this project?

There may be additional unforeseen or unknown risks associated with this project. Before you volunteer to be part of this study, there are some important things to understand:

1. Although rare, food allergy associated with consumption of seeds and / or nuts may occur. If you have an allergy to nuts you should inform the research team and you should not participate in this study.

2. Food allergy associated with milk and milk products may occur. If you are lactose intolerant you should inform the research team and you should not participate in this study.

3. Seeds of *Acacia longifolia* may contain 0.2% dimethyltryptamine (DMT), a psychoactive hallucinating alkaloid commonly known as 'the spirit molecule'. When taken orally, the psychoactive threshold for DMT is 0.2 mg/Kg body weight which usually sets in after 30 minutes and can last for several hours. However, most times there are no psychotropic effects with oral ingestion and the amount of DMT in *Acabungi* flakes and crackers is ≤ 0.1 mg/10 g. Thus your total consumption of DMT would be less than 0.05 mg/kg body weight which is 4 times less than the psychoactive threshold.

4. Risk to gastrointestinal disturbance due to microbial contamination of product. Good hygienic practices and standard procedures will be strictly adhered to. The research student had basic training on food handling practices and is a trained and experienced microbiologist. Food will be prepared and stored within refrigeration temperature at the Footscray Nicholson commercial kitchens to avoid microbial contamination.

Participation in this study is **voluntary**. You may **change your mind or withdraw from the study at any time** or you may be withdrawn if you develop any of the above conditions that would indicate your need to withdraw from the program.

All results will remain confidential throughout and after the completion of the study.

Who is conducting the study?

For more information or to organise a meeting please call or send an email to:

Chief investigator
Prof Andrew McAinch Ph: 9919 2019; Email: Andrew.mcainch@vu.edu.au

Associate investigator
Dr Osaana Donkor Ph: 9919 8059; Email: Osaana.donkor@vu.edu.au

Student researcher
Mr Collise Njume, Ph: 9919 8109; Email: collise.njume@live.vu.edu.au

Any queries about your participation in this project may be directed to the Chief Investigator listed above.
If you have any queries or complaints about the way you have been treated, you may contact the Ethics Secretary, Victoria University Human Research Ethics Committee, Office for Research, Victoria University, PO Box 14428, Melbourne, VIC, 8001, email researchethics@vu.edu.au or phone (03) 9919 4781 or 4461.

Appendix 5

Consent form

INFORMATION TO PARTICIPANTS:
We would like to invite you to be a part of a study looking at *"Acceptability of Acabungi flakes and crackers."*

The nature of the project, procedures and potential risks of participation are detailed in the 'Information to Participants Involved in Research' document.

CERTIFICATION BY SUBJECT

I, _____(Name)
of _____(Suburb)

certify that I am at least 18 years old* and that I am voluntarily giving my consent to participate in the study: *"Acceptability of Acabungi flakes and crackers."* being conducted at Victoria University by: Prof Andrew McAinch, Dr Osaana Donkor and Mr Collise Njume, from the College of Health and Biomedicine and Institute of Sustainable Industries and Liveable Cities.

I certify that the objectives of the study, together with any risks and safeguards associated with the procedures listed hereunder to be carried out in the research, have been fully explained to me by:

_____(Name)

and that I freely consent to participation involving the below mentioned procedures.

- **Forty minute Hedonic Scale evaluation of *Acabungi* flakes and crackers based on appearance, flavour, taste and mouthfeel.**

And to complete the following procedures;

- Colour and appearance: Visually observe the product and give your assessment.
- Aroma: Smell product and give your assessment.
- Taste and aftertaste: Place sample in the mouth and chew, then role it into a small ball and spit it into the container provided. Rate taste, aftertaste and flavour.
- Mouthfeel: Place sample in the mouth and manipulate it in a circular motion between the tongue and palate and judge the intensity of mouthfeel as the amount of film remaining in the mouth after disposing of the sample.
- Overall acceptance: Give your judgement on the overall acceptability of the product.

I certify that I have had the opportunity to have any questions answered and that I understand that I can withdraw from this study at any time and that this withdrawal will not jeopardise me in any way.

I have been informed that the information I provide will be kept confidential.

Signed: _____

Date: ____/____/____

Any queries about your participation in this project may be directed to the researcher
Prof Andrew McAinch; Ph: 99192019; Email: Andrew.mcainch@vu.edu.au
If you have any queries or complaints about the way you have been treated, you may contact the Ethics Secretary, Victoria University Human Research Ethics Committee, Office for Research, Victoria University, PO Box 14428, Melbourne, VIC, 8001, email Researchethics@vu.edu.au or phone (03) 9919 4781 or 4461.

Appendix 6

Hedonic scale evaluation of *Acabungi* flakes

Date……Code number.....

You are provided with a sachet containing 5 g of flakes with code number, a bowl, milk and a spoon. Open the sachet and pour its contents into the bowl.

a.) Colour and appearance: Visually observe the contents of the bowl and give your assessment using the scale below by **circling** the corresponding number.

1	2	3	4	5	6	7	8	9
Dislike extremely	**Dislike very much**	**Dislike moderately**	**Dislike slightly**	**Neither like nor dislike**	**Like slightly**	**Like moderately**	**Like very much**	**Like extremely**

b.) Aroma: Smell the contents of the bowl and give your assessment.

1	2	3	4	5	6	7	8	9
Dislike extremely	**Dislike very much**	**Dislike moderately**	**Dislike slightly**	**Neither like nor dislike**	**Like slightly**	**Like moderately**	**Like very much**	**Like extremely**

Add the desired quantity of milk to the flakes and use the spoon to stir slightly.

c.) Flavour, taste and aftertaste: Place contents in the mouth and chew, then roll into a small ball and spit it into the container provided.

Rate flavour

1	2	3	4	5	6	7	8	9
Dislike extremely	**Dislike very much**	**Dislike moderately**	**Dislike slightly**	**Neither like nor dislike**	**Like slightly**	**Like moderately**	**Like very much**	**Like extremely**

d.) Rate taste and aftertaste

1	2	3	4	5	6	7	8	9
Dislike extremely	**Dislike very much**	**Dislike moderately**	**Dislike slightly**	**Neither like nor dislike**	**Like slightly**	**Like moderately**	**Like very much**	**Like extremely**

e.) Mouthfeel: Place contents in the mouth and manipulate in a circular motion between the tongue and palate and judge the intensity of mouthfeel as the amount of film remaining in the mouth after disposing of the sample.

1	2	3	4	5	6	7	8	9
Dislike extremely	**Dislike very much**	**Dislike moderately**	**Dislike slightly**	**Neither like nor dislike**	**Like slightly**	**Like moderately**	**Like very much**	**Like extremely**

f.) Overall acceptance: Give your judgement on overall acceptability of the product.

1	2	3	4	5	6	7	8	9
Dislike extremely	**Dislike very much**	**Dislike moderately**	**Dislike slightly**	**Neither like nor dislike**	**Like slightly**	**Like moderately**	**Like very much**	**Like extremely**

Comment on areas of improvement

……

……..

Appendix 7

Hedonic scale evaluation of *Acabungi* crackers

Date……Code number.....

You are provided with a sachet containing 5 g of crackers with code number. Open the sachet.

a.) Colour and appearance: Visually observe the contents of the sachet and give your assessment using the scale below by **circling** the corresponding number.

1	2	3	4	5	6	7	8	9
Dislike extremely	**Dislike very much**	**Dislike moderately**	**Dislike slightly**	**Neither like nor dislike**	**Like slightly**	**Like moderately**	**Like very much**	**Like extremely**

b.) Aroma: Smell the contents of the sachet and give your assessment.

1	2	3	4	5	6	7	8	9
Dislike extremely	**Dislike very much**	**Dislike moderately**	**Dislike slightly**	**Neither like nor dislike**	**Like slightly**	**Like moderately**	**Like very much**	**Like extremely**

c.) Flavour, taste and aftertaste: Place contents in the mouth and chew, then roll into a small ball and spit it into the container provided.

Rate flavour

1	2	3	4	5	6	7	8	9
Dislike extremely	**Dislike very much**	**Dislike moderately**	**Dislike slightly**	**Neither like nor dislike**	**Like slightly**	**Like moderately**	**Like very much**	**Like extremely**

d.) Rate taste and aftertaste

1	2	3	4	5	6	7	8	9
Dislike extremely	**Dislike very much**	**Dislike moderately**	**Dislike slightly**	**Neither like nor dislike**	**Like slightly**	**Like moderately**	**Like very much**	**Like extremely**

e.) Mouthfeel: Place cracker in the mouth and manipulate in a circular motion between the tongue and palate and judge the intensity of mouthfeel as the amount of film remaining in the mouth after disposing of the sample.

1	2	3	4	5	6	7	8	9
Dislike extremely	**Dislike very much**	**Dislike moderately**	**Dislike slightly**	**Neither like nor dislike**	**Like slightly**	**Like moderately**	**Like very much**	**Like extremely**

f.) Overall acceptance: Give your judgement on overall acceptability of the product.

1	2	3	4	5	6	7	8	9
Dislike extremely	**Dislike very much**	**Dislike moderately**	**Dislike slightly**	**Neither like nor dislike**	**Like slightly**	**Like moderately**	**Like very much**	**Like extremely**

Comment on areas of improvement

……

……..