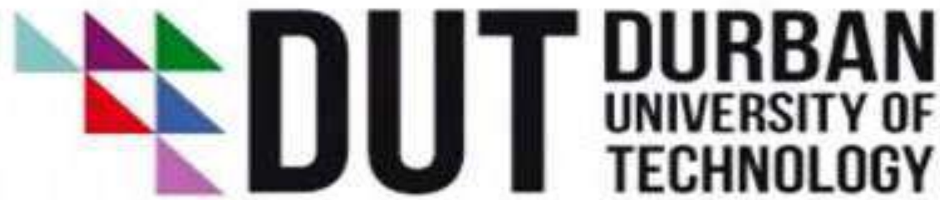

CHARACTERISATION OF *OPUNTIA* PHENOLIC EXTRACTS AND ENZYMATIC MODIFICATION OF SELECTED COMPOUNDS

By

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Submitted in fulfillment of the requirements for the degree of Doctor of Philosophy (PhD): Biotechnology

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2019

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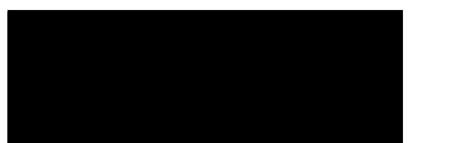
DECLARATION

I hereby declare that the work reported in this thesis titled '**Characterisation of *Opuntia* phenolic extracts and enzymatic modification of selected compounds**' and submitted at the Department of Biotechnology and Food Technology at Durban University of Technology for a Doctoral Degree in Biotechnology is my original work. I confirm that it has not been previously submitted for a degree at any Higher Education Learning Institution.

Christiana E. Aruwa 19/08/2019

Student **Date**

As the candidate's supervisors we agree to the submission of this thesis.



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Dr. Stephen Amoo 19/08/2019

Co-Supervisor **Date**

DEDICATION

This doctoral thesis is dedicated to the master of the universe, God the Almighty, who has been my mainstay and strength despite all the challenges I encountered during my doctoral research, and to my entire family who assisted both financially and morally.

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I do not mince words as I return all the glory, honour, power and adoration to my Creator, Heavenly Father and King, the omnipotent, the author and finisher of my faith, my ever-present help, He who fills my cup to overflowing, the beginning and end, for seeing me through, one day at a time.

ABSTRACT

Opuntia species are utilised as local medicinal interventions for chronic diseases and as food sources. The phytochemical profile varies within and across *Opuntia* species and has been related to differences in cultivar and geographical location. Macromolecular antioxidant (MA) fractions are also largely ignored from most conventional extractive processes compared to the well-known extractable polyphenol fractions. This study characterised subtropical spineless cladode, fruit pulp and peel extracts and selected phenolic compounds for enzymatic modification using a laccase from *Trametes pubescens*. MA extracts were also characterised in comparison with extractable fractions. The effects of drying methods and extraction solvent on extract yields and bioactivities were also studied. Extracts were assayed for phenolic content and antioxidant activities were determined using standard 2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,2,-azinobis3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP) assays. Antimicrobial activities and mode of antibacterial action were assessed against type-bacterial cultures. Minimum inhibitory concentration (MIC) values were recorded for the extracts and compounds. Compound profiling was achieved using liquid chromatography-time of flight-mass spectrometry (LC-TOF/MS) in negative ionisation mode.

Antibacterial and antioxidant activities were higher in MA, hydrolysed and hydroalcoholic cladode and fruit extracts than in aqueous fractions. Ethanolic, methanolic and hexane extracts of freeze-dried *Opuntia* cladode, MA and peel samples showed higher total phenolic content, and *in vitro* antioxidant and antimicrobial activities than the oven-dried extracts. Cladode extracts inhibited growth of both Gram-positive and Gram-negative microorganisms (MIC range of 25 to 250 mg/mL). Likewise, fruit extracts inhibited both Gram-positive and Gram-negative microorganisms (MIC range of 2.5 to 18.75 mg/mL). Cladode and fruit extract profiles showed the presence of mainly phenolic acids and flavonoid derivatives. Isovitexin 7-*O*-xyloside-2"-*O*-glucoside, polyhydroxypregnane glycoside and neohancoside C in *Opuntia*

cladode, and pinellic acid in *Opuntia* fruit were identified for the first time in this study. Some compounds, however, remained unidentified. Thereafter, selected *Opuntia* cladode and fruit phenolic compounds (isorhamnetin and luteolin) were used for enzymatic (laccase) transformation after preliminary screening reactions. Laccase-catalysed oxidation of luteolin in a monophasic system containing sodium acetate buffer (pH 5.0) and ethanol (50%, v/v) as co-solvent, resulted in the production of a dimer (*m/z* 569, M=570). Using a similar approach, oxidative coupling of isorhamnetin produced two main products, IP1 which was a dimer (*m/z* 629, M=630) and IP2 (*m/z* 457, M=458) which was most likely a result of coupling of an oxidative cleavage product and the isorhamnetin monomer. Dimers showed up to two-fold improvement in antioxidant and antimicrobial activities, compared to their respective substrates. The synthesised products showed a bactericidal mode of action as demonstrated by time-kill and bacterial cell integrity assays. The bactericidal action was further confirmed by scanning electron microscopy (SEM) which showed that treatment of bacterial cells with the synthesised compounds resulted in deformed, pitted, broken or fragmented cells, indicating strong bactericidal action.

In conclusion, this study showed that *Opuntia* fruit pulp, peel and cladode extractable and MA extracts have potential as sources of phenolic compounds with antioxidant and antimicrobial activities. Laccase catalysis has potential to transform the phenolic compounds into coupling products with higher biological activities. The synthesised products have potential for application in the food, nutraceutical and other relevant industries.

PREFACE

This thesis is organised into eight chapters and the experimental work is presented in manuscript format. Chapter one gives the general introduction to the thesis. Chapter two provides a critical review of literature on the *Opuntia* plant and its locations worldwide. The main aspects covered include *Opuntia* cladodes, fruits and peels and their associated compounds, the relevance of the *Opuntia* plant to health and the growing global trends in antimicrobial resistance and non-communicable disease linked with oxidative stress. Finally, a brief review of fungal laccases and their applications in organic synthesis is provided, focussing mainly on enzymatic modification of monomeric phenolics to improve biological activities as well as structure-activity relationship (SAR) in phenolic antioxidants. Chapter three presents the biological activities and phenolic compound profile of *Opuntia* cladode as a potential source of new bioactive compounds. Chapter four reports on the compound profile of extracts of *Opuntia* fruit pulp and peel and their biological activities. The utilisation of laccase in enzymatic modification of selected *Opuntia*-related phenolic compounds is covered in Chapters five (luteolin) and six (Isorhamnetin). Chapter seven is a general discussion of the entire findings. Chapter eight concludes the thesis and suggests recommendations for future studies.

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ABBREVIATIONS

ABTS: 2,2'-azinobis3-ethylbenzthiazoline-6-sulfonic acid

ADF: Antioxidant dietary fibres

AHA: American Heart Association

AM: Acidified methanol

AMR: Antimicrobial resistance

ANOVA: Analysis of variance

AQ: Acidified water

ATCC: American Type Culture Collection

ATP: Adenosine triphosphate

BDE: Bond dissociation enthalpies

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

CAM: Crassulacean Acid Metabolism

CMV: Cucumber Mosaic Virus

CSAs: Chemically synthesised antioxidants

CTC: Carbon tetrachloride

DNA: Deoxyribonucleic acid

DPPH: 2,2'-diphenyl-1-picrylhydrazyl

E: Ethanol

EACC: Ehrlich Ascites Carcinoma Cells

EP: Extractable polyphenol

FAO: Food and Agricultural Organisation

FRAP: Ferric reducing antioxidant power

GABA: γ-Aminobutyric acid

GMFs: Genetically Modified Foods

HAT: Hydrogen atom transfer

IP1: Predicted dimer product(s) from isorhamnetin

IP2: Unidentified laccase oxidation product from isorhamnetin

LAB: Lactic Acid Bacteria

LC-TOF/MS: Liquid Chromatography-Time of Flight-Mass Spectrometry

LP: Predicted dimer product(s) from luteolin

M: Methanol

MA: Macromolecular antioxidant

MAW: Methanol:acetone:water extraction solvent
MIC: Minimum inhibitory concentration
NCDs: Non-communicable diseases
NEPAC: Non-extractable proanthocyanidins
NEPP: Non-extractable polyphenol
NLM: National Library of Medicine
NMR: Nuclear Magnetic Resonance
OD: Optical density
PBP: Penicillin-binding proteins
PG: Propyl gallate
PI: Polarity index
ROS: Reactive oxygen species
RSA: Radical scavenging activity
SAR: Structure-activity relationship
SD: Standard deviation
SEM: Scanning Electron Microscopy
SET: Single electron transfer
TBHQ: Tertiary-butylhydroquinone
TEM: Transmission Electron Microscopy
TMV: Tobacco Mosaic Virus
TPC: Total phenol content
TPTZ: 3(2,4,6-tripyridyl-s-triazine
UNDPI: United Nations Department of Public Information
WHO: World Health Organisation

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- Aruwa, C. E., Amoo, S. O., Kudanga, T. 2018. *Opuntia* (Cactaceae) plant compounds, biological activities and prospects – A comprehensive review. *Food Research International*, 112, 328-344 (Appendix A).
- Aruwa, C. E., Amoo, S. O., Kudanga, T. 2019. Extractable and macromolecular antioxidants of *Opuntia ficus-indica* cladodes: phytochemical profiling, antioxidant and antibacterial activities. *South African Journal of Botany*. Accepted.
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Submitted manuscripts

- Aruwa, C. E., Amoo, S. O., Koorbanally, N., Kudanga, T. 2019. Laccase-mediated modification of isorhamnetin improves antioxidant and antibacterial activities, submitted.
- Aruwa, C. E., Amoo, S. O., Koorbanally, N., Kudanga, T. 2019. Enzymatic dimerization of luteolin enhances antioxidant and antimicrobial activities, submitted.

Conferences

- Aruwa, C. E., Amoo, S. O., Kudanga, T. 2018. Antioxidant and antibacterial activity of extractable and macromolecular antioxidants from *Opuntia ficus-indica* cladodes. South African Society for Microbiology (SASM), Johannesburg, South Africa, April 2018.

CHAPTER ONE

1.1. Introduction

The present global threats of oxidative stress and antimicrobial resistance (AMR) have necessitated increased research interests channelled toward the discovery of new bioactive phytochemicals with antioxidant (Zrira *et al.*, 2016) and antimicrobial properties (Koubaa *et al.*, 2015a). Oxidative stress arises from the overproduction of free radicals and has been linked with non-communicable diseases (NCDs) such as cancer, cardiovascular diseases, and type-2 diabetes (Hofman, 2014). In recent years there has been an upsurge in incidences of NCDs (Mayosi *et al.*, 2009). In southern Africa and other developing countries, it was predicted that seven out of every ten deaths would be attributed to NCDs by the year 2020 (UNDPI, 2011). The incidences of AMR have increased over the past decade and this is linked mainly to the overprescription and abuse of currently available antibiotics/antimicrobial agents or compounds (Spellberg *et al.*, 2011). AMR is a serious threat to public health and has been estimated to cause 10 million deaths and $100 billion in economic losses by the year 2050 (Spellberg *et al.*, 2011; WHO, 2019).

In a bid to stem the global trends in oxidative stress and AMR, chemically synthesised antioxidants (CSAs) are frequently used to fight oxidative stress (Wojcik *et al.*, 2010), while available antimicrobial compounds derived mainly from microorganisms are used to combat AMR (Martins *et al.*, 2011). However, CSAs are carcinogenic, cause sperm abnormalities, nausea, fatigue, asthma and degenerative diseases as a result of their accumulation in biological systems over time (Wojcik *et al.*, 2010). Consequently, CSAs are increasingly becoming unpopular with consumers due to their adverse effects and unnatural form (Wojcik *et al.*, 2010). On the other hand, new antimicrobial compounds from microorganisms are not being produced

fast enough and new antibiotic discovery and identification are at an all time low (Scarafile, 2016). In addition, problems such as malaise, diarrhoea, as well as prolonged cost of treatments and hospital stays have been associated with AMR (AHA, 2018). These associated problems have necessitated the search for new approaches and investigations into new, but safe and natural bioactive molecules with antioxidant (Zrira *et al.*, 2016) and antimicrobial properties (Rasoulpour *et al.*, 2017). The shift to plant bioactives and phenolics may be the most likely solution to reduce current trends in oxidative stress and AMR. The *Opuntia* plant, which has recently attracted a lot of research interest as an underutilised crop in southern Africa (Khan and Giridhar, 2015), may be integral to the discovery of new and natural plant bioactive compounds (Tesoriere *et al.*, 2005).

The *Opuntia* plant is mainly used as fodder and is underutilised in southern Africa (Khan and Giridhar, 2015). *Opuntia* species are capable of growth in almost all climates (Lallouche *et al.*, 2015). Although *Opuntia* bioactivities have been related to content of flavonoids, phenolic acids, vitamins and other components (Vulic´ *et al.*, 2014), its bioactive compound profile can change with variations in species, cultivar, geographical location and climatic conditions (Paiva *et al.*, 2016). The variation in compound profile has also been used as a genetic fingerprint for the identification and taxonomic classification of *Opuntia* species in different locations worldwide (Moussa-Ayoub *et al.*, 2014). Therefore, there is a high probability of reporting new phenolic compounds or bioactive compounds from *Opuntia* species especially from areas that have not previously attracted much attention such as southern Africa.

A wide array of monomeric phenols are present in *Opuntia* species but have low biological activities (Matsuura and Ohkatsu, 2000), short half-life in the body (Kudanga *et al.*, 2011) and poor physico-chemical properties such as low stability and low solubility (Hierholtzer *et al.*, 2012). Consequently, current research is also focused on the improvement of the biological

activities of natural monomeric phenolic compounds through enzymatic modification (Zwane *et al*., 2012). Laccases have gained much research interest in this regard. Laccases (EC 1.10.3.2) are multi-copper oxidases that catalyse the oxidation of a suitable substrate to its radical form with concomitant reduction of molecular oxygen to water. The radicals formed then undergo coupling to form high molecular weight homomolecular or heteromolecular coupling products (Nemadziva *et al*., 2018; Kudanga *et al*., 2011), which usually have enhanced biological activities compared to the starting substrates (Zwane *et al*., 2012). The improvement in bioactivity is attributed to increase in the number of hydroxyl groups from which hydrogen atoms are abstracted, and/or an increase in electron-donating groups that help to stabilise the antioxidant after donating an electron (Gandia-Herrero *et al*., 2016; Kudanga *et al*., 2017). Peroxidases were previously used for the same purpose, but are now less popular because they require a cofactor, hydrogen peroxide, which makes the process a health hazard and expensive. On the other hand, laccases are now preferred because they are essentially green enzymes that only require oxygen (air) as co-substrate, producing water as the only by-product (Nyanhongo *et al*., 2011; Riva, 2006).

Therefore, the aim of the current study was to characterise southern African *Opuntia* extracts and enzymatically modify selected phenolic compounds for the production of new bioactive compounds with enhanced antioxidant and/or antimicrobial activities.

CHAPTER TWO

2.1. Introduction

In this chapter, literature relevant to this study is presented. Some of the areas covered include: a description of the cactus pear (*Opuntia*) plant; *Opuntia* plant growth locations and general compound profile; antioxidant sources, mechanisms, benefits and purification; biologically active compounds; *Opuntia* cladode compounds, fingerprinting and biological activities; *Opuntia* fruit compounds and biological activities; *Opuntia* peel/skin compounds and biological activities; techniques for the extraction of compounds from *Opuntia* residue; *Opuntia* macromolecular antioxidant biological activities; potential side effects of *Opuntia* species extracts/compounds; the search for new antimicrobial compounds; enzymes as biocatalysts for biotechnological applications; laccases - reactions and applications, and structure-activity relationship (SAR) in phenolic compounds.

2.2. The cactus pear (*Opuntia*) plant

Plants are potential sources of bioactive compounds and have been attracting the attention of scientist globally. In this regard, the cactus pear plant (*Opuntia* spp.), its fruit and cladodes have been shown to be good candidates for the production of health-promoting bioactive compounds (Livrea and Tesoriere, 2006). Although traditionally recognised for its pharmacological properties, *Opuntia* spp. remain underutilised. There is not enough scientific data to back up its bioactivities. Nevertheless, it has been demonstrated that the cactus pear fruit and cladodes could play an integral role in the formulation of new nutraceuticals (Feugang *et al*., 2006).

Cactus pear plants belong to the family *Cactaceae*, order Caryophyllales, and originated from Central America (Felker and Inglese, 2003). Thousands of species of the cactus pear belong to the *Opuntia* genus and produce edible fruits (Abou-Elella and Ali, 2014). Cactus fruits are also referred to as cactus figs or *Opuntia ficus-indica* (prickly pear), and these grow on the prickly cacti plant. The fruit and cladodes (Plate 2.1) are fleshy, oval, and vary in length. Hundreds of the *Opuntia* species are xerophytic (Moβhammer *et al*., 2006) and well-adapted to arid zones where little or no rainfall and poor soil conditions abound due to frequent erosions (FAO, 2001). The plant, however, survives these conditions due to its possession of succulent leaves which store water. It is attractive as food and possesses high dry matter converting potential (Gabremariam *et al*., 2006). The plant accumulates minerals and elements using its shallow root system. The *Opuntia* plant provides the necessary hydration, microelements as well as macronutrients required in animal feeds (Rodriguez-García *et al*., 2007). Biodiversity of *Opuntia* species is still a subject of much investigation (Caruso *et al*., 2010). This is because the cactus pear fruit contains chemical components that are active biologically such as the betalains, ascorbic acid and polyphenols (Livrea and Tesoriere *et al*., 2006). Moreover, the fruit extract has been shown to have antioxidant, antibacterial, antiulcerogenic, anticancer, neuroprotective, as well as hepatoprotective activities (Livrea and Tesoriere *et al*., 2006; Madrigal-Santillán *et al*., 2013).

Plate 2.1: *Opuntia ficus-indica* purple and yellow fruits varieties growing on pads called cladodes. Source: Rice-Evans *et al*. (1996).

According to the Food and Agricultural Organisation (FAO) report for 2013, the *Opuntia ficus-indica*, as well as other species such as *Opuntia streptacantha*, *Opuntia xoconostle*, *Opuntia megacantha* and *Opuntia amyclae* are mostly cultivated. Other wild types cultivated include *Opuntia hyptiacantha*, *Opuntia robusta* and *Opuntia leucotricha.* However, the most common and widely cultivated is *Opuntia ficus-indica.* In the Mediterranean basin the plant has been adapted for different uses/purposes. The botanical characteristics vary from species to species. For example, shape of the cladodes, presence/absence of spines, the size and colour of the fruit. Scientifically, cactus plant stems are called cladodes (fleshy leaves, paddles). Photosynthetic functions occur within the cladodes. The pads/paddles are made up of a waxy cuticle which serve to reduce moisture loss (Nobel *et al*., 1992). Cladodes may have spines with few stomas per unit surface area of the stem. Stomas do not open during the day, thereby helping to conserve moisture within the plant. Carbon dioxide (CO_2) is however absorbed at night for photosynthetic activities. The specific type of photosynthesis that occurs in *Opuntia* is commonly referred to as the crassulacean acid metabolism (CAM) (Nobel, 1998).

Freshly harvested cactus pear cladodes (green parts of the plant) are used as vegetables. They have high water content, little amounts of dietary fibre, calcium and proteins. Other antioxidant constituents include beta-carotene and ascorbic acid (Betancourt-Domínguez *et al.*, 2006). Flours made from cactus cladodes have been used as food supplement, ingredients and alternatives. The dried flours are microbiologically stable, thereby helping to greatly reduce storage and transport, and highly concentrate nutrients (Torrecilla *et al*, 2005). Furthermore, immature spineless *Opuntia ficus-indica* cladodes of 15±5cm in length were analysed by López-Cervantes *et al*. (2011). In their study, cladodes were dried at 60°C to 80°C followed by profiling for amino acid and other chemical components. The analyses showed no significant difference in proximate content of the flours, which was attributed to the effect of drying temperature. Nevertheless, at 80°C drying temperature, colour (green) intensity reduced as well as water holding capacity. On the other hand, there was increased concentration of glutamic acid and proline amino acids, while oleic, palmitic and linoleic fatty acids concentrations were highest. The best drying temperature for flour production with regard to nutrient composition and retention was 60°C. They surmised that the physiological effects of the cactus plant could be attributed to the functional properties of its constituents (Feugang *et al*., 2006).

2.2.1. *Opuntia* plant: growth locations and general compound profile

Opuntia ficus-indica is a slow growing perennial shrub which can grow up to 3-5 m high. Some arid growth locations for *Opuntia* species include Saudi Arabia, Egypt, Pakistan, Middle East, Algeria, Iran, Libya, Mexico, South Africa, Spain (Feugang *et al.*, 2006; Moussa-Ayoub *et al.*, 2014). The temperate regions where the plant may be found include Tunisia (Ayadi *et al.*, 2009), while tropical countries include Brazil (Paiva *et al.*, 2011), Ethiopia (Chiteva and Wairagu, 2013), Kenya (Kunyanga *et al.*, 2014), and the Mediterranean country of Morocco (Castellanos-Santiago and Yahia, 2008). Some *Opuntia* species and their locations of growth

are shown in Table 2.1, but most species are native to Mexico and the Americas. Some *Opuntia* species have spread to some African and Asian regions and show significant biodiversity within and across species (Caruso *et al*., 2010). This biodiversity determines the component profile of each species, as well as other factors such as growth location, prevailing climatic conditions and soil conditions, among others (Ammar *et al*., 2012).

Cactus (*Opuntia*) plants contain carotenoids, amino acids, vitamins, fibres and antioxidant phenol components (Table 2.2). Its phytochemical compounds such as phenolic acids, flavonoids and derivatives, sterols, esters, coumarins, terpenoids, alkaloids bring about several health benefits such as hypoglycaemic (Paiz *et al*., 2010), and antioxidant activities (Osorio-Esquivel *et al*., 2011). *Opuntia* also contains water-soluble nitrogenous betalain pigments (Table 2.2) which are classified as betacyanins (red to purple coloured) and betaxanthins (yellow and orange coloured) (Khan and Giridhar, 2015). Betalains serve as chemo-systematic markers for members of the Caryophyllales family (Slimen *et al*., 2017). Esters, alkaloids, phenolic acids, essential fatty acids (Ramadan and Mörsel, 2003b), polysaccharides, sugars can also be found in *Opuntia* species (Benayad *et al*., 2014; Yeddes *et al*., 2014a,b) as well as alkanes, carotenoids, amino acids, among others (Manzano *et al*., 2017). The profile of phenolic and non-phenolic components (Table 2.2) reported to be present in *Opuntia* species plant parts constantly evolve from one location to another (Butera *et al*., 2002). Therefore, it is important to investigate the phenolic compound profiling of southern African *Opuntia* species.

Table 2.1: Selected *Opuntia* spp. and their locations

Species	Locations
Opuntia basilaris	Southwest United States (US) and northwest Mexico
Opuntia chlorotica	Native to southwest US, and the Sonoran and Mojave deserts
Opuntia engelmannii	Mexico
Opuntia ficus-indica	Originally in south-central Mexico; cultivated in warm parts of the world for its edible fruit
Opuntia fragilis	Northern Great Plains and as far west as British Columbia, also found in the southern Great Plains
Opuntia humifusa, Opuntia compressa var. humifusa	Throughout the US, east of the Great Plains and into southern Ontario
Opuntia leucotricha	Mountains of Central Mexico
Opuntia macrocentra	Southwest US and Northern Mexico
Opuntia macrorhiza	Throughout the Great Plains except for the northernmost areas (not found in North Dakota), and extending sporadically eastward as far as Kentucky.
Opuntia ficus-indica	Spain, Italy
Opuntia microdasys	Mexico (Hidalgo)
Opuntia ficus-indica (prickly and spineless), *Opuntia stricta, Opuntia macrorhiza, Opuntia microdasys*	Algeria, Tunisia, Morocco, Kenya, Egypt, South Africa (Northern, Eastern and Southern Africa)
Opuntia dillenii, Opuntia ficus-indica, Opuntia humifusa	India, Iran, Korea, Saudi Arabia
Opuntia santa-rita	Texas, Arizona and Northern Mexico
Opuntia stricta *Opuntia polyacantha* *Opuntia phaeacantha* *Opuntia lindheimeri, Opuntia engelmanii* var. *lindheimeri* *Opuntia littoralis* *Opuntia erinacea* *Opuntia pusilla*	Coastal regions

Compiled from Griffiths, 1915; MacMahon and Wagner, 1985; Stiling *et al.*, 2000; Bobich and Nobel, 2001; Van Sittert, 2002; Goettsch and Hernández, 2005.

Table 2.2: Phenolic and non-phenolic compounds in *Opuntia* cactus plant parts

Major Compounds	Cacti part	References
Gallic acid Quercetin 3-*O*-Rutinoside Quercetin 7-*O*-Rutinoside Kaempferol 3-*O*-Rutinoside Quercetin 3-*O*-Glucoside Isorhamnetin 3-*O*-Robinobioside Isorhamnetin 3-*O*-Galactoside Isorhamnetin 3-*O*-Rutinoside Isorhamnetin 3-*O*-Glucoside	**Flower**	Ammar *et al*., 2015; De Leo *et al.,* 2010; Yeddes *et al.*, 2014a, b
Isorhamnetin 7-*O*-rutinoside Isorhamnetin 3-O-rhamnosyl 7-O-rutinoside Kaempferol 3-*O*-arabinoside		Ammar *et al*., 2015; Benayad *et al.,* 2014; De Leo *et al.,* 2010
Rhamnetin 3-*O*-rutinoside		Merina *et al.,* 2011
Other compounds Terpenes: (limonene, linalool, germacrene D, aromadendrene, squalene) Esters and alkaloids (ethyl linoleate) Sterols Carboxylic acids (linoleic, palmitic, octanoic, butanedioic, pentanedioic)		Ammar *et al.,* 2012
Phenolic acids: Hydroxycinnamic acid derivatives: (5-Hydroxyferulic acid-rhamnosidehexoside; Caffeoyl methoxycinnamoyl quinic acid; 1,4-Diferuloyl syringic acid; 4-p-Coumaroyl caffeic acid; 1,5-Dicaffeoyl ferulic acid; 1,4-Syringicferuloyl 4-coumaroyl caffeic acid)		
Flavonoids Rutin, Myricetin Quercetin, Isorhamnetin Kaempferol, Luteolin Isorhamnetin glycosides Kaempferol glucosides Catechin	**Whole fruit/pulp**	Bensadón *et al*., 2010; Cha *et al.*, 2013; Fernández-López *et al*., 2010; Mabrouki *et al*., 2015; Osorio-Esquivel *et al*., 2011
Phyllocactin Protocatechuic acid		Osorio-Esquivel *et al*., 2011; Tesoriere *et al*., 2004
4-hydroxybenzoic acid, Ferulic acid Vanillic acid (also isovanillic acid) Trans-coumaric and trans-cinnamic acids		Galati *et al*., 2003; Serra *et al*., 2013

Compounds	Part	Reference
Syringic, Fukic, piscidic, and eucomic acids		
Betalains Taxifolin, Orientin Vitexin		Khatabi *et al*., 2011
Volatile organic compounds (VOCs) e.g. 2-decanynoic acid, γ terpinene, linalool, α farnesene etc.		Zrira *et al*., 2016
Other compounds		
Carotenoids [Lutein- 5,6- epoxide, (all-E)-b-criptoxanthin, (all-E)-a-carotene, (all-E)-b-carotene, (9Z)-b-carotene, lycopene]. Ascorbic acid and Tocopherols Amino acids (taurine, cystine) Biothiols (glutathione)		Feugang *et al*., 2006; Tesoriere *et al*., 2005
Phenolic acid Feruloyl-sucrose isomer 1 Feruloyl-sucrose isomer 2 Sinapoyl-diglucoside Flavonoids Tannins	**Seed**	Chougui *et al*., 2013; Tlili *et al*., 2011
Fatty acids (linoleic, oleic, palmitic, stearic, vaccenic); sterols, vitamin E		Ghazi *et al*., 2013; Tlili *et al*., 2011; Zakynthinos and Varzakas, 2016
Total phenolic acid Flavonoids – Kaempferol, Quercetin, Isorhamnetin, Isorhamnetin glucosides	**Peel/Skin**	Kuti, 2004; Moussa-Ayoub *et al*., 2011; Ndhlala *et al*., 2007
Gallic acid, Rutin, Catechin, Epicatechin, Vanillin		Guzman-Maldonado *et al*., 2010
17-decarboxybetanin, betanin		Abou-Elella and Ali, 2014
Xanthophylls		Cano *et al*., 2017
Terpene alcohols		Koubaa *et al*., 2015b
Fatty acids; Minerals (Fe, Mn, Mg, Ca, Zn); Glucose, fructose		El-Salid *et al*., 2011; Guzman-Maldonado *et al*., 2010
Polysaccharides		Koubaa *et al*., 2015b
Phospholipids and glycolipids		Ramadan and Mörsel, 2003a, b
Phytoestrogens		Diaz-Vela *et al*., 2013; Elleuch *et al*., 2011; Federici *et al*., 2009

Phenolic acids - Gallic acid, Coumaric acid, 3,4-dihydroxybenzoic, 4-hydroxybenzoic; Ferulic, Salicylic, Vanillic Syringic, Synaptic acids; Protocatechuic acid etc.		Bensadón *et al*., 2010; Gallegos-Infante *et al*., 2009; Guevara-Figueroa *et al*., 2010; Valente *et al.,* 2010; Wright and Setzer, 2014
Flavonoid - Isorhamnetin, Quercetin, Kaempferol, Isoquercetin, Nicotiflorin, Rutin, Narcissin		
Terpenoid volatiles (cis-linalool oxide, trans-linalool oxide)		Avila-Nava *et al*., 2014; Gallegos-Infante *et al*., 2009; Ginestra *et al*., 2009; Guevara-Figueroa *et al*., 2010;
Fatty acids and tocopherols	**Cladode**	Chahdoura *et al*., 2014
Other components		
Alkanes (heptadecane) Ascorbic acid β-carotene, Lutein		Avila-Nava *et al*., 2014; Betancourt-Domínguez *et al*., 2006
Alpha-pyrones, opuntiol, opuntiosides		Qiu *et al*., 2007; Siddiqui *et al*., 2016
Pectic polysaccharides Lignans		Panico *et al*., 2007; Rocchetti *et al*., 2018
Flavonoids Glycosides Coumarins		Jiang *et al*., 2006; Manzano *et al.,* 2017
Terpenoids: Triterpenes, Labdane-type diterpenes		de las Heras and Hortelano, 2009; Jamal *et al*., 2009; Manzano *et al.,* 2017
Tannins Polysaccharides Fatty acids (azelaic acid, palmitelaidic acid)	**Stem**	Manzano *et al.,* 2017
C_{29}-5β-sterols (Taraxerol)		Jiang *et al*., 2006

Reports from different areas have frequently shown differences in compound profile. For example, Galati *et al.* (2003) carried out the analysis of fruit juice of prickly pear cultivars from San Cono, Sicily and reported ferulic acid as the major phenolic acid present. Other compounds detected include isorhamnetin derivatives like isorhamnetin-3-rutinoside, isorhamnetin-3-

glucoside, kaempferol-3-rutinoside (all flavonols) as well as rutin. In a study of *Opuntia* in Tunisia, Yeddes *et al.* (2013) showed that flavonoid glycoside derivatives dominated the phenolic compound profile of *Opuntia* fruit pulp and peel, and that they were mostly isorhamnetin derivatives. They also indicated that more flavonoids were identified in the spiny cultivars compared to the spineless varieties. Compared to the fruit pulp, prickly pear peels contained more flavonoids. Prickly pear cladodes also contain biochemical compounds such as glucose, galacturonic acid, calcium oxalate, eucomic and piscidic acids; and flavonoids such as keampferol and isorhamnetin glycosides, isoquercitrin, quercetin and myricetin (Ginestra *et al.*, 2009). Besides the presence of betalains, Tesoriere *et al.* (2005) reported the presence of amino acid taurine as well as tocopherols (α-tocopherols, β-tocopherols and γ-tocopherols), carotenoids and thiols such as glutathione and cysteine (Table 2.2) in the pulp of red, yellow and white Silician *Opuntia ficus-indica* cultivars. They, however, surmised that industrial processing of the fruit pulps reduced the contents of cysteine, ascorbic acid, beta-carotene, and glutathione. However, tocopherols, betalains and taurine appeared to be less affected by industrial processing. Taurine is an amino acid also referred to as 2-aminoethanesulfonic acid and is considered a cell protective compound and suspected to be involved in inflammatory response modulation (McCarty, 1999). While considerable amounts of taurine were reported in *Opuntia* cultivars from Mexico and South Africa (Tesoriere *et al.*, 2005), the concentrations were lower than those reported by Stintzing *et al.* (1999). However, Ali *et al.* (2014) analysed the amino acid content of prickly pear fruits and observed that taurine was absent in the different prickly cultivars sampled from the Near East, Italy and South Africa. The same was the case for the commercially available juices which were purchased from the market. This led to their summary that the relative presence and amount of taurine that has been reported in literature for *Opuntia* fruits may have resulted from the confusion of this amino acid with γ-Aminobutyric acid (GABA). This misrepresentation was linked to the analytical methods utilised. GABA and proline constituted some of the most abundant amino acids in all the fruits tested.

Chiteva and Wairagu (2013) assessed the nutrient composition of prickly pear fruits from Laikipia, Kenya and compared results with Egyptian, American and Ethiopian samples. They reported no significant differences in moisture contents of fruits. Ascorbic acid content varied with samples, that is, 13.7 mg/100 g for Egyptian samples and 5.17 mg/100 g for the Kenyan samples. In agreement with Lee *et al.* (2002b), it was submitted that increased light intensity during growing seasons contributed to the increase in the vitamin C contents of the prickly pear fruits. Other contributory factors to higher vitamin C content in fruits include good storage, low harvest temperatures, and reduced irrigation (Lee and Kader, 2000). They also recorded low vitamin C content in physically damaged fruits (Lee and Kader, 2000). Results for proteins ranged from 0.2 to 2.0%, but only very few fruits contained some proteins. *Opuntia* sugar content as low as 0.11% and as high as 59.40% have also been reported for Kenyan samples (El Samahy *et al.*, 2006). Seasons also determine fruits sugar content (Chiteva and Wairagu, 2013). In light of the nutrient variations in cactus pears, it will be interesting to investigate fruits from *Opuntia* species from sub Saharan Africa/South Africa, which have largely been neglected.

2.3. Antioxidants: sources, mechanisms, extended benefits and purification

Free radicals or reactive oxygen species (ROS) are highly unstable molecules that can cause oxidative stress especially when their presence in the body outweighs the concentration of antioxidants present (Jerome-Morais *et al.*, 2011). In an oxidatively stressed state, the probability of developing non-communicable diseases (NCDs) such as cardiovascular and respiratory diseases, diabetes and cancer, increases. Body inflammations or tissue trauma, and external inducers such as pollution, smoking, ultraviolet (UV) exposure, industrial solvents, ozone are also contributory factors that may lead to the generation of free radicals and then oxidative stress. Free radicals seek to stabilise themselves by damaging other cells leading to

diseases and premature aging (Song *et al*., 2009; Wilson *et al*., 2017). The structure of a free radical has at least one unpaired electron. The unpaired electrons are very reactive and cause cellular damage and/or genetic changes. Some examples of free radicals include hydroxyl (HO•), hydroperoxyl (HOO•), alkyloxyl (ROO•), superoxide anion (O2−•), nitric oxide (NO•), thiyls (RS•) and disulfide anions (RSSR−•), and carbonate (CO3−•) group radicals (Ayala *et al*., 2014). In order to prevent or inhibit the continuous production of free radicals, antioxidants are required. Studies have shown that the probability of contracting NCDs may by reduced by improving eating habits and lifestyles such as the increased consumption of antioxidant-rich foods and exercise (Song *et al*., 2009; Wilson *et al*., 2017). While the body has its own innate antioxidative defense systems, it may require additional supply of antioxidants to facilitate the prevention or inhibition of ROS generating chain reactions and to halt the progression of associated diseases. The extra antioxidants may be derived from antioxidant-rich diets or supplements (Ayala *et al*., 2014; Wilson *et al*., 2017).

An antioxidant has been described as a substance that can inhibit the initiation or progression of an oxidative process (Wilson *et al*., 2017). Antioxidants have been used successfully to reduce degradation and loss of nutrients in foods during processing and storage. Antioxidants may be natural or synthetic. Natural antioxidants imply those derived from natural sources such as plants/foods and are usually naturally present as dietary constituents. Synthetic antioxidants on the other hand are produced from non-natural processes or from chemosynthetic industrial processes (Song *et al*., 2009). Endogenous antioxidants produced by the body also fall under natural antioxidants. Flavonoids, phytoestrogens, polyphenols and catechins are examples of natural plant-based antioxidants (Jerome-Morais, *et al*., 2011; Song *et al*., 2009). Plant sourced antioxidants are also usually referred to as phytochemicals or phytonutrients (Nahar *et al*., 2009). The demand for natural antioxidants has increased all over the world as synthetic antioxidants cause unhealthy side effects. Globally there is also an increase in consumer

preference and sensitisation for clean labels/natural flavours and reduction in use of synthetic food additives. In addition, natural antioxidants may have added benefits for health, and they are also readily absorbed *in vivo*. Synthetic antioxidants/food preservatives are usually used to mitigate oxidative food spoilage during processing or storage. They help in maintaining food properties such freshness, nutrients and shelf life due to their high stability (Wilson *et al*., 2017). However, some bring about nausea, as well as allergenic and carcinogenic effects and they may have no added benefits to health (Embuscado, 2015).

Antioxidative compounds are a principal line of defence in protecting food quality from farm to market and homes. There is an increase in consumer consciousness with respect to quality and health and these affect the food choices. The farmers and producers of foods are therefore challenged with supplying high-quality goods. Oxidation of foods which occur is a major bane in tackling this challenge (Shahidi and Zhong, 2015). Oxidation in lipid-based foods affects texture, toxicity, nutrient quality and flavour. Antioxidants are required to achieve extended shelf-life for lipid-based foods such as animal fat, vegetable oils, nuts, processed meats, and margarines among others (Shahidi and Zhong, 2015). Antioxidative compounds act by inhibiting oxidative processes. Preservation of foods by added antioxidants is achieved through this inhibitive process. Many nutraceuticals and dietary supplements boost and promote health through the antioxidative action of their components (Shahidi and Zhong, 2015). Antioxidants carry out their function using various modes of action.

Free radicals attack important macromolecules such as proteins, lipids and DNA, resulting in cell destruction/damage and homeostatic imbalance. Targets of free radicals include all kinds of molecules in the body (Huang *et al*., 2005; Lobo *et al*., 2010). Mechanisms of antioxidant action include hydrogen or electron donation, peroxide decomposition, singlet oxygen quenching, enzyme inhibition, synergism (co-antioxidants), metal-chelation and gene

expression regulation. Most of these functions fall under the chain-breaking and/or chain-initiation quenching modes of action (Lobo *et al.*, 2010; Moon and Shibamoto, 2009). The antioxidant power and mechanism of action of antioxidant compounds vary from molecule to molecule. Phenolic acids scavenge free radicals more than they chelate metals, while flavonoids efficiently carry out their functions using both mechanisms. Also, as an added benefit, many phenolics are not prone to attack by molecular oxygen (Brewer, 2011). Once free radical generation is initiated, body cells protect themselves against oxidative stress using its cascade of antioxidant enzymes within the body. Enzymatic and nonenzymatic antioxidants exist in the intracellular and extracellular environment to detoxify ROS. Intracellularly, an enzyme like superoxide dismutase is involved with the breakdown of the superoxide anion into oxygen and hydrogen peroxide, while catalases facilitate the breakdown of hydrogen peroxide to water and oxygen (Lobo *et al.*, 2010). Also, glutathione, glutathione reductase, glutathione peroxidases, and glutathione S-transferases which make up the glutathione enzyme system, catalyse the decomposition of hydrogen peroxide and organic hydroperoxides (Nyanhongo *et al.*, 2011). Tocopherols and tocotrienols (Vitamin E), melatonin, uric acid, and ascorbic acid are examples of non-enzymatic antioxidants. Lipophilic or hydrophilic endogenous antioxidants may act through any of the mechanisms previously mentioned (Brewer, 2011; Lobo *et al.*, 2010). Hydrophilic antioxidant examples include thiols, uric acid, albumin and bilirubin. Ubiquinol and tocopherol are lipophilic antioxidants. Certain *de novo* and repair antioxidants such as proteinases, proteases, and peptidases also act as lines of defense to prevent oxidised proteins accumulation in the body (Brewer, 2011). Glycosylases and nucleases which are known as deoxyribonucleic acid (DNA) repair enzymes/antioxidants protect the DNA against oxidative damage.

Before exogenous antioxidants can be used, however, they may need to be purified from their sources for use in assays and various application. Antioxidants may be purified using

chromatographic techniques. In thin layer chromatography (TLC), substances are separated on thin adsorbent plate layer based on polarity and affinity of the compounds for the stationary adsorbent, usually silica gel. Following the purification of different components, several assays can be carried out. Nevertheless, some tests can be performed during the chromatographic isolation/separations (Adelakun *et al.,* 2012a). Pure concentrates of bioactive compounds may be obtained by utilising selective extraction methods depending on the target compound or group of compounds. Some isolated compounds contribute more to the bioactivity of plant formulations/extracts and only bioactivity assays for each component can determine which compounds are more active (Prior *et al*., 2005). The degree to which a bioactive compound or antioxidant can scavenge free radicals constitute the basis of some *in vitro* assays. Some of the mechanisms for detecting antioxidant activity include hydrogen atom transfer (HAT), single electron transfer (SET), reducing power, and metal chelation, among others (Leopoldini *et al*., 2004; Mishra *et al*., 2012; Pinela *et al*., 2016). Antioxidant substances decrease or block the oxidation reactions induced by free radicals (Costa *et al*., 2012). Ennouri *et al.* (2005) reported that antiradical activity of cactus pear extracts was consistent with the presence of some phenolic flavonoids such as quercetin and carotenoids, and several vitamins, among them ascorbic acid. In addition, as regards *Opuntia* antioxidants/extracts roles, Galati *et al*. (2003) demonstrated that rats treated with extracts from *Opuntia ficus-indica* peel and fruit were protected against ethanol-induced gastric ulcer. *Opuntia ficus-indica* extracts have also shown liver protective capabilities. Extracts reduced liver damage induced by carbon tetrachloride (CTC)-induced poisoning when administered orally two hours after the toxic substance. Prior to inducing toxicity in the liver, hepatoprotective effect was also demonstrated by application of the juice consecutively for nine days (Livrea and Tesoriere, 2006). Prickly pear fruits have also been applied in cancer treatment and prevention. Aqueous extract from Arizonan *Opuntia ficus-indica* fruits successfully suppressed ovary tumors (Zou *et al*., 2005). The fruit extract was injected intraperitoneal a day before the injection of tumor cells. Significant tumor

suppression and modulation of tumorigenic genes expression showed similar effect with that of N-(4-hydroxyphenyl) retinamide (4-HPR), a known chemo-preventive agent. The same extract effectively treated immortalised ovarian, cervical and bladder epithelial and cancer cells (Zou *et al*., 2005). The extract initiated an increase in cancer cell growth inhibition, death and affected cancerous cells cycle in a time and dose-dependent manner. *Opuntia ficus-indica* methanol extract had also earlier been shown to have neuroprotective effects on radical-mediated (superoxide and hydroxyl-) neural damage (Ha *et al*., 2003).

2.4. Biologically active compounds

Bioactive compounds occur in nature, have varying effect on living organisms and are sometimes called nutraceuticals (Biesalski *et al*., 2009). The presence of natural bioactive components in food and plants such as the *Opuntia* sp. provide added health functions while providing basic nutrients. On the other hand, nutraceuticals may be packaged as dietary supplements, isolated nutrients, genetically modified foods (GMFs), and also as processed beverages and/or cereals (Biesalski *et al*., 2009). Moon and Shibamoto (2009) defined biological antioxidant as any substance that, when present in low concentrations compared to those of an oxidisable substrate, significantly inhibits oxidation of that substrate. A good example of natural bioactive antioxidant compound is phenols. Phenolic compounds are able to inhibit lipid peroxidation (Kristinova *et al*., 2009) and lipooxygenation *in vivo*. This is mainly attributed to the reducing properties of the chemical structures that enable neutralisation or sequestration of free radicals, as well as the chelation of transition metals (Moon and Shibamoto, 2009).

Phenols are a group of bioactive substances with structures consisting one or more hydroxyl groups (-OH) directly bonded to an aromatic ring. They are produced and found in plants and

microorganisms. The simplest of the class is phenol (carbolic acid - C_6H_5OH). Phenols may be monophenolic or polyphenolic in nature depending on number of phenol units within each molecule (Sant'Anna *et al*., 2013). Phenolic compounds contribute to sensory attributes such as astringency, colour and bitterness of many foods (Sant'Anna *et al*., 2013). Phenolic antioxidants are able to donate electrons or hydrogen which form the backbone of their reductive capabilities and the basis for predictions on their free radical scavenging potential. The structural chemistry of polyphenols is directly linked with their ability to scavenge free activities (Rice-Evans *et al*., 1996). In addition, the inclination of polyphenols to chelate copper and iron in metal chelation mechanisms facilitate their function as pre-emptive antioxidants when they obstruct transition metal-catalysed free radical formation (Rice-Evans *et al*., 1996). A polyphenol is regarded an antioxidant if when present in low concentration compared to the oxidisable substrate it is able to prevent free radical-mediated oxidation. Secondly, after scavenging, free radicals generated must be stable upon further oxidation (Rice-Evans *et al*., 1996). A large number of phenolic compounds and antioxidants are derived from plant sources such *Opuntia* spp.

Thousands of different phenolic compounds are ubiquitous in plant materials. In vegetables, onions, cereals and *Opuntia* fruits, cladodes and peel by-product, phenolic acids, lignans and flavonoids abound. Teas and fruits also contain major constituents of the human diet (Stahl *et al*., 2002). However, prior to the absorption of phytochemicals like phenols which are trapped within plant tissues, there is need for the application of processing methods that would serve to release these valuable compounds for health and nutrition, and other potential applications (Stahl *et al*., 2002). The type of phenolics targeted and their solubility in various solvents go a long way to determine which processing/extractive methods are applied. Usually, water soluble phenols have lower molar masses compared to the more insoluble high molecular weight phenolic compounds (Saura-Calixto, 2012). During absorption *in vivo*, phenols bind with

themselves and proteins irreversibly, while direct or enzymatic oxidation of native phenols may also occur during mastication or raw plant material preparation. High molecular weight compounds may also be produced through several condensation reactions. Phenolic compounds condensation may however be inhibited if a reducing agent like ascorbic acid is present. Reducing agents are able to reduce the condensed compound to the original forms. A low pH environment, as well as the presence of sulphur dioxide may also affect oxidation and condensation reactions of phenols *in vivo* (Stahl *et al*., 2002).

A study by Spencer *et al*. (2000) demonstrated that phenolic dimers are more likely to be better absorbed in the small intestine compared to higher oligomeric forms such as trimers or tetramers. In other words, the higher the molecular size the lower the probability of being absorbed *in vivo*. For example, high molecular weight procyanidin oligomers were reduced to the native epicatechin monomer and other dimer/oligomeric units as well and were unstable in acidic gastric juice *ex vivo* (Spencer *et al*., 2000). Although insignificant levels of oligomer absorption across the intestine was observed, the significant levels of epicatechin released suggested that higher molecular weight oligomers could ensure a constant and significant supply of the monomeric and dimeric forms that can be absorbed. This may play a critical role in the *in vivo* action of procyanidins (Lin *et al*., 1999). The probability of the phenols being metabolised by native colon microflora subsequent to metabolites absorption, as well as biliary excretion determine the rate and extent of phenolic compounds absorption (Lin *et al*., 1999). Also, the time it takes for the compound to pass through the intestine and gastric region, and lumen pH constitute biological factors that influence phenols absorption *in vivo*. In addition, solubility, hydrophobicity, molecular weight and pKa are some other factors that can affect phenolic compounds absorption in the small intestine (Stahl *et al*., 2002).

Phenolic compounds are regarded as primary antioxidants. They are able to delay the progression of oxidation in lipids such that ketonic and aldehyde decomposition products are not formed. The decomposition products give rise to the phenomenon known as rancidity which is associated with lipid spoilage/denaturation (Alamed *et al.*, 2009). Propyl gallate (PG), butylated hydroxyanisole (BHA), tertiary-butylhydroquinone (TBHQ) and butylated hydroxytoluene (BHT) are some of the approved synthetic phenolic antioxidants which may be used in food products (Makahleh *et al.*, 2015). However, reports of toxicity and other adverse effects attributed to the use of synthetic antioxidants have raised global concerns and calls for the use of natural alternatives to safeguard public health (Aremu *et al.*, 2011). In addition, besides scavenging of free radicals, phenolic antioxidants also act by reducing peroxides to the stable hydroxyl derivatives or act as metal chelators (Frankel and Finley, 2008; Sadiq *et al.*, 2017). Food manufacturers are encouraged to use natural antioxidative ingredients in place of synthetic antioxidants (Shahidi and Ambigaipalan, 2015). Naturally occurring, plant-sourced, antioxidant compounds include tocopherols, flavonoids, lignans, phenolic acids, and poly-functional organic acids and betalains (Shahidi and Ambigaipalan, 2015). Some of these natural ingredients and compounds are found in *Opuntia* plant parts such as the cladode, fruit and peels and show various biological activities. The biological activities of pure *Opuntia* bioactive compounds are, however, low. Enzymatic modification could be an option for improving the biological activity of *Opuntia* phenolics.

2.5. *Opuntia* cladode: compounds, fingerprinting and biological activities

Cladodes are the spiny or spineless paddle-like, oblong (up to 70-80 cm), thick and succulent parts of the *Opuntia* plant with varying widths. They possess a waxy, water repellent epidermis and are capable of photosynthesis and asexual reproduction. Small bristles (glochidia) may be present with barbs in the areoles (Heuzé and Tran, 2017). Several studies have shown that

Opuntia cladodes compounds profile change with species type, post-harvest treatment, environmental conditions and plant age (Contreras-Padilla *et al*., 2011; Astello-García *et al*., 2015). Nevertheless, some studies have demonstrated that different cladode cultivars from distinct locations have similar flavonol profiles which may be used in taxonomic identification/classification of cultivars (Moussa-Ayoub *et al*., 2014). Only isorhamnetin aglycon (m/z 315) was detected which was in partial agreement with results published by Santos-Zea *et al*. (2011) and Ginestra *et al*. (2009) from cactus *O. ficus-indica* cladodes collected from Mexico and Italy, respectively. Flavonoid glycosides such as isoquercitrin, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-glucoside and isorhamnetin-3-*O*-rutinoside were detected in wild and commercial *O. ficus-indica* cladodes cultivated in Mexico (same location and close phylogenetic proximity), but no kaempferol was detected (Guevara-Figueroa *et al*., 2010). Sugars, fatty acids, tocopherols and organic acids were reported in Tunisian*O. microdasys* and *O. macrorhisa* cladodes varieties (Chahdoura *et al*., 2014). Eucomic acid, kaempferol 3-*O*-robinobioside-7-*O*-arabinofuranoside and isorhamnetin 3-*O*-rhamno-side-7-*O*-(rhamnosyl-hexoside) dominated the phenol profile of *O. ficus-indica* (highly domesticated), *O. albi-carpa* (intermediate domestication), and *O. hyptiacantha* and *O. streptacantha* (wildest). Lesser amounts of quercetin 3-*O*-rhamnosyl-(1-2)-[rhamnosyl-(1-6)]-glucoside) were reported in domesticated species, and kaempferol 3-*O*-arabinofuranoside in wild cultivars (Astello-García *et al*., 2015). In other studies, wild cladode varieties had similar or higher amounts of pigment compounds such as β-carotene, lutein and xanthophylls compared to commercial ones (Jun *et al*., 2013).

Cladode extracts of *O. ficus-indica* have been shown to lower cholesterol levels and have antiulcer and anti-inflammatory activities. Aqueous extracts also improved wound healing (Laughton *et al*., 1991). Nopal extracts containing quercetin, isorhamnetin, kaempferol and vitamin C also showed *in vitro* (IC_{50} of 0.8 mg/mL) and *in vivo* (20% increase in plasma

antioxidant activity after 3 days of subjects feeding) antioxidant activity (Avila-Nava *et al.*, 2014) and hepatoprotective activities (Zourgui *et al.*, 2008). Antidiabetic and antioxidant activities of cladode extracts have been attributed to significant amounts of polysaccharides, as well as lignans, flavonols and phenolic acids in the cladode extracts (Valente *et al.*, 2010; Rocchetti *et al.*, 2018). *Opuntia ficus-indica* nopal extracts rich in polysaccharides and polyphenols were also able to decrease the hydroxyl radical induced oxidation of linoleic acid and DNA (IC_{50} of 9.3 µg/mL, better than 3.2 µg/mL for ascorbic acid) (Lee *et al.*, 2002a,b; Panico *et al.*, 2007). Mucilage, pectin and total pectic polysaccharide fractions of *Opuntia ficus-indica* cladodes were also characterised and showed antioxidant activity (Bayar *et al.*, 2016). Cladode extracts radical scavenging abilities may be linked to presence of phenolic antioxidants and vitamin content (Stintzing and Carle, 2005). Cladode cultivars have further demonstrated useful anticarcinogenic selenium-enriched chemotherapeutic properties and could serve as food providing advanced dietary selenopharmacology to help fight human diseases (Bañuelos *et al.*, 2012). Cladode extracts may find use as surfactant and natural antioxidants in food and pharmaceutical industries.

Recently, *Opuntia dillenii* cladodes were shown to contain α-pyrones, and novel opuntiol and opuntioside (opuntiol glucosides) compounds (Siddiqui *et al.*, 2016). Opuntiol and opuntioside have been successfully utilised in the reduction of pain, but opuntioside was a more effective analgesic ($IC_{50} = 26 \pm 0.9$ mg/kg). In future design of novel analgesics, both compounds may serve as integral constituents (Siddiqui *et al.*, 2016). *O. humifisa* cladode extract also protects against UVB-induced skin degeneration achieved by reducing associated enzymes gene expression (Park *et al.*, 2017). In addition, azelaic acid and labdane-type diterpene were recently detected in cactus cladodes (Manzano *et al.*, 2017). Azelaic acid shows good antibacterial activity (Sieber and Hegel, 2014). Diterpenes demonstrate broad biological effects (de las Heras and Hortelano, 2009). Bioactivities may be linked to terpenoids possession of isoprene structure

with added elements such as oxygen and acetate units, as well as the branched, cyclic structure and the presence of hydroxyl groups (Ciocan and Bara, 2007; Wu *et al.*, 2016). It is interesting also that under stressed growth conditions, *Opuntia* cladodes can biologically accumulate compounds (Lallouche *et al.*, 2015).

Algerian *O. engelmanii* and *O. streptacantha* cladode cultivars accumulated significant amounts of proline and soluble sugars which ensured osmotic adjustment under saline stress conditions (Lallouche *et al.*, 2015). This phenomenon was also observed in other parts of the cactus plant, but to lesser extent as salinity increased (Hadjadj *et al.*, 2011; Lallouche *et al.*, 2015). This bioaccumulative potential may pave the way for the application of cladodes in atmospheric pollution and bioremediation by heavy metal (lead) bioaccumulation (El Hayek *et al.*, 2017). *Opuntia* cladodes are also able to bioaccumulate inorganic constituents such as magnesium oxide, calcium carbonate, calcium oxalate monohydrate, calcium-magnesium bicarbonate, potassium chloride and potassium peroxydiphosphate [$K_4P_2O_8$] using diffractive, microscopic, and spectrometric techniques (Contreras-Padilla *et al.*, 2011).

2.6. *Opuntia* fruit: compounds and biological activities

Fruits are the succulent, ellipsoid and edible part of the *Opuntia* plant. They are usually about 7 cm long but can have a wide range of colours which include green, white reddish, purple and yellow (Heuzé and Tran, 2017).

Cactus pear fruits contain significant quantities of vitamin C and polyphenols compared to other *Opuntia* plant parts such as the root, cladodes and seeds (Hahm *et al.*, 2015). Cactus pear fruits also contain significant quantities of minerals (Mabrouki *et al.*, 2015), amino acids (Ammar *et*

al., 2012), flavonoids such as luteolin (Kıvrak *et al*., 2018), phenolic acids and betalains (Cha *et al*., 2013; Osorio-Esquivel *et al*., 2011).

Opuntia fruit/pulp extracts possess antidiabetic, cardioprotective, neuroprotective, anti-inflammatory, and hepatoprotective properties (Serra *et al*., 2013). Betanin in *Opuntia ficus-indica* fruits was reported to reduce chronic myeloid leukaemia cell spread (K562), and cell death was recorded at an IC_{50} of 40 mM betanin (Sreekanth *et al*., 2007). Cactus fruit ethyl acetate extracts containing flavonoids, trans taxifolin, and dihydrokaempferol also suppressed HeLa cervical carcinoma cell proliferation (at ≥ 100 µg/mL concentrations), while normal human BJ fibroblasts were unaffected, which suggests potential application as intervention for human cervical carcinoma management (Hahm *et al*., 2015). Taxifolin has been known to repress prostate cancer cell growth and inhibit HCT116 colorectal cancer cell growth (Woo *et al*., 2012; Zhang *et al*., 2013). Reduction in the viability of hepatic (78.9 - 79.2%), prostate (61.2 - 75.2%) and colon (52.5 - 74.1%) cancer cells have been reported for prickly pear fruit coloured (Chavez-Santoscoy *et al*., 2009). Fruit component compounds and antioxidant properties also evolve with fruit ripening (Cayupán *et al*., 2011). Antioxidant activity have been reported for purified *Opuntia* fruit polysaccharides (Zhong *et al*., 2010). The molar ratio of the polysaccharide sugars differed from those reported earlier (Panico *et al*., 2007). The difference was attributed to different soil types/conditions and climates. Domestication gradients may also affect the content of bioactives and their biological activities.

Opuntia ficus-indica red, purple and orange-coloured fruits grown on agricultural drainage sediment, were shown to possess improved and selenium-enriched chemotherapeutic properties compared to those grown on normal soil (Bañuelos *et al*., 2012). Purple cultivars such as the Mexican *Opuntia robusta* (semi-domesticated) showed the highest total betalain, phenol, carotenoid and vitamin C contents, but with low antioxidant activity compared to other

varieties. Yellow commercial *O. albi-carpa* had no betalains, low total phenolic content and low antioxidant activity (Yahia and Mondragon-Jacobo, 2011). However, in some studies phenol content did not always correlate with good antioxidant activity (Chavez-Santoscoy *et al*., 2009).

Reports on *O. ficus-indica* fruits have shown major variation in total phenolics content. Phenol content of approximately 172 mg/kg (Chavez-Santoscoy *et al*., 2009) and 452 mg/kg (Díaz-Medina *et al*., 2007) have been reported for juice from *Opuntia* spp. Higher levels of phenolic content have also been recorded in juice (Galati *et al*., 2003) and fruit pulp (Moussa-Ayoub *et al*., 2011). Moussa-Ayoub *et al*. (2011) also reported higher antioxidant activity for *O. macrorhisa* pulp compared to whole *O. ficus-indica* fruits. In other studies, the antioxidant activity and vitamin C content varied between different fruit samples. Purple *Opuntia ficus-indica* fruit varieties usually showed the highest antiradical ability (Bañuelos *et al*., 2012). However, Sumaya-Martínez *et al*. (2011) showed that antioxidant activities of differently coloured fruit cultivars (white and yellow) did not show great variance but had reduced activities compared to the purple and red variants. On the contrary, red cactus pears from Mexico had the highest ascorbic acid content/antioxidant activity compared to the purple variety (Stintzing *et al*., 2005; Abdel-Hameed *et al*., 2014). Nevertheless, the presence of betaxanthin in coloured cultivars was; purple > red > yellow > white pear (Stintzing *et al*., 2005).

In another study, El Kharrassi *et al*. (2016) showed that *Opuntia* fruit skin colour did not correlate with ascorbic acid concentration. The differences in these results were attributed to genotype, growth location and environmental contributors affecting the vitamin C content of the *Opuntia* cultivars. However, in *Opuntia* species earlier studied, ascorbic acid concentrations were highest in red fruits than in yellow fruits (Kuti, 2004). Also, *O. streptacantha* (red skinned

cactus pear) carotenoids content was lower (14.6 mg/g fresh weight) than *O. stricta* var. *stricta* (yellow-skinned cactus pear) (23.7 mg/g fresh weight) (Kuti, 2004). Current research has shown that it is possible to concentrate pigments (betalains) and other bioactive fruit components during ripening, compared to the very immature and under ripe cultivars days after anthesis (Pinedo-Espinoza *et al.*, 2017). In addition, a novel, neutral, and water-soluble (1→4)-α-d-glucan polysaccharide from *Opuntia ficus-indica* aqueous fruit extracts was reported by Ishurd *et al.* (2010). The polysaccharide was identified by gel-permeation and anion-exchange chromatographic techniques.

In general, the biological capacities and nutraceutical benefits of *Opuntia* fruit species may be due to the synergistic effects of betalains, flavonoids and other biologically active components present (Stintzing *et al.*, 2005). The highlighted applications suggest that *Opuntia* fruit/extracts have potential for industrial application in the food, agricultural and nutraceutical industries.

2.7. *Opuntia* peel/skin: compounds and biological activities

Peels make up the rinds of the prickly pear fruit. They are often discarded and regarded as a by-product (Ramadan and Mörsel, 2003b). The generation of high value food products with improved nutritional value through food processing methods such as fermentation applied to agro-industrial by-products continues to evolve as an economically viable option (Diaz-Vela *et al.*, 2013). Utilisation of food by-products as food or additives to produce functional foods with improved health benefits is expanding. Fruit peels are a good source of fibre for prebiotic preparations and antioxidants (Diaz-Vela *et al.*, 2013). Studies have shown that non-digestible oligosaccharides from plant by-products with prebiotic potential can undergo fermentative processes involving lactic acid bacteria (LAB) and bifidobacteria to produce new compounds, with enhanced health benefits (Diaz-Vela *et al.*, 2013).

Opuntia peels contain fatty acids, vitamins and antioxidants (Cerezal and Duarte, 2005). Antioxidant components identified include oleuro, pyrogallol, benzoic, 3-OH tyrosol, ellagic, chorogenic, protocatechuic acid, epicatechin, gallic acid and showed 62.14% inhibition of the DPPH radical. *In vivo* study involving hamster diet supplementation with peel extracts increased cholesterol excretionand lowered liver cholesterol levels. Mechanisms of extracts hypocholesterolemic activities include inhibition of hepatic HMG-CoA activity, interference with cholesterol absorption in the intestine and bile acid trafficking and antioxidant activity. These mechanisms work synergistically for coronary heart disease prevention (del Socorro Santos *et al.*, 2017). Fermented flours *Opuntia* peels with higher fibre and carbohydrate content also showed significantly higher antioxidant activities (89% inhibition of ABTS and 2.6 trolox equivalent antioxidant capacity value) compared to pineapple peel flours (69% ABTS radical inhibition and 1.5 trolox equivalent antioxidant capacity (Diaz-Vela *et al.*, 2013).

Opuntia ficus-indica peels contain considerable amounts of neutral glycolipids and phospholipids (Ramadan and Mörsel, 2003b). The presence of xantophylls [(all-E)-lutein, (all-E)-violaxanthin and (all-E)-zeaxanthin], hydrocarbon carotenes (belonging to two types of oxygenated carotenoid derivatives); and chlorophyll (Cano *et al.*, 2017) have also been identified. Flavonoid glycosides dominate the flavonoid profile of cactus peels (Ginestra *et al.*, 2009). Spineless fruit cultivars contain more flavonols than the spiny varieties. Prickly pear peels however contain higher level of flavonoids when compared to the pulp (Yeddes *et al.*, 2013). Polysaccharides have also been reported in *Opuntia* stricta peel extracts and exhibited significant antioxidant activity. It showed 94% DDPH inhibition at 50 mg/ml concentration and an IC_{50} of 6.5 mg/ml (Koubaa *et al.*, 2015a). Other bioactive components include phytoestrogens (Diaz-Vela *et al.*, 2013) and terpene alcohols (Koubaa *et al.*, 2015b).

Anticarcinogenic and antihypertensive activities have also been reported for *Opuntia* peels (Mulaudzi *et al.*, 2017). *Opuntia ficus-indica* fruit peel extracts exhibited anticancer activity in Ehrlich Ascites Carcinoma Cells (EACC). Their potency and total phenolic content were significantly related, with two purified compound fractions having cancer cell killing efficiency of 51.5-76.0 dead cells/100 µg/ml (Abou-Elella and Ali, 2014). By induction of hyperpolarisation in cell membrane, *O. ficus-indica* extract phenolic composition significantly increased calcium levels within human Jurkat T-cell lines (Aires *et al.*, 2004). The extent of molecular branching, chemical structure and composition, presence of sugar moieties and physicochemical attributes of these bioactive molecules are responsible for the biological activities reported for *Opuntia* peel compounds/extracts (Koubaa *et al.*, 2015a).

The components and bioactivities of peel extracts may depend on the extraction method. Recently, ultrasound and pulsed electric field technologies have been successfully used to achieve greater cell denaturation to facilitate better recovery of the intracellular coloured compounds from red prickly pear fruit peels (Koubaa *et al.*, 2016). At higher temperatures, more phenolic compounds are extracted, but with a reduction in antioxidant activity. In some studies, optimal extraction methods for retention of phenols and antioxidant activities was achieved at 80°C with 45% ethanol after 2 hours of extraction (Han *et al.*, 2016). The physicochemical properties of peel fibres can be exploited in the food industry to improve food product parameters such as shelf-life, sensory attributes, staling, and viscosity (Anwar and Sallam, 2016). Peels are cheap and readily available, and these factors enhance the potential for commercialisation (Anwar and Sallam, 2016).). Margarines containing peel extracts resisted oxidation better than vitamin E margarine and physicochemical and microbiological properties of the margarine were unaffected (Chougui *et al.*, 2015).

2.8. Effect of processing conditions on compound profile and bioactivities

Processing techniques applied to foods matrices are integral factors that determine the bioavailability of nutrients and bioactive compounds and bioactivities. The impact of processing technologies on expression of bioactive compounds vary widely and is not a simple cause and effect relationship and remains complex to date (Cilla *et al.,* 2018). However, the major aim of food processing should be to achieve preservation of functional and nutritional characteristics of the foods being processed. Bioactive compounds in *Opuntia* plant may better assert their benefits to health when they are more bioavailable *in vitro* and *in vivo*.

Cladode processing conditions affect the amount and types of compounds present. It has been shown that a combination of 45ºC and 3 ms^{-1} air flow were optimal for the preservation of phenol, flavonoid, ascorbic acid and β-carotene (Medina-Torres *et al*., 2011). Boiling processes affected compound preservation of vitamins and sugars whereas lipids, proteins and fibre were well preserved. Decreased antioxidant activity (38.9 and 31.6 trolox equivalent for raw and boiled samples, respectively) was linked to phytochemical losses due to processing technologies (Ramírez-Moreno *et al*., 2013). Jaramillo-Flores *et al*. (2003) showed 84% retention of cladode carotenoids after heat processing. However, in another study, reduced amounts of carotenoids were reported (Ayadi *et al*., 2009). Lactic acid bacteria (LAB) fermentation of cladode pulp by *Lactobacillus brevis* have also been shown to improve biological properties of the cladode pulp. This enhancement of bioactivity was confirmed using Caco-2/TC7 cells in which fermented pulp demonstrated significant inhibition of related prostaglandin production (Filannino *et al*., 2016).

Many studies on *Opuntia* plant parts and other plants have used methods such as milling/grinding to break down food matrices into smaller particle sizes in order to achieve

improved expression of compounds and nutrients (Medina-Torres *et al*., 2011; Minekus *et al.,* 2014). Following milling of plant material, other processing methods may be applied depending on the required end-product. Thermal treatment such as steaming, boiling, roasting, microwave, have been used to modify food structure and for preservation purposes but with the attendant loss of compounds/nutrients and bioactivities. These effects, however, vary depending on the intensity of the heat applied (Cilla *et al.,* 2018; Wang and Bohn, 2012). In addition, it has been recommended that in the preparatory stages for food/plant materials, freeze-drying or oven-drying of plant materials for short periods at temperatures lower than 50°C may produce more satisfactory results with respect to bioactive compounds expression, yields and biological activities (Torres *et al*., 2010). Freeze-drying minimises loss of functional and nutritional properties of foodstuffs through better retention of phytonutrient and phytochemical properties. Phytochemical losses increase when oven drying is carried out for longer periods or at higher temperatures (Reyes *et al*., 2011). It was therefore of interest to investigate the effect of freeze-drying and oven-drying on extract yields, biological activities and phenolic compound profile.

2.9. Techniques for the extraction of compounds from *Opuntia* residue

Pomaces/residues have been ignored as source of viable phytochemicals which are usually not released from plant matrices (Durazzo *et al*., 2016; Gonzales *et al*., 2015). Therefore, extraction/separation techniques have been developed and utilised to characterise essentially valuable components from *Opuntia* residues which are not released from conventional extractive processes. Separation by adsorption using synthetic resins is usually applied for polyphenols recovery from plant crude extracts. This process is valuable in the food industry (Soto *et al*., 2011). Low operation costs is one of its advantages as well as high adsorption capacities for different classes of compounds and simplicity in handling (Soto *et al*., 2011). Antioxidant and antiproliferative compounds like polyphenols and anthocyanins have been

recovered from apples and cherries through this process (Bobe *et al*., 2006; Schaefer *et al*., 2006).

The use of alkaline hydrogen peroxide treatment (Vilela *et al*., 2016); acid and alkaline hydrolysis and/or sonication; butanolysis and phloroglucinolysis (Arranz *et al*., 2009; Tarascou *et al*., 2010) have been efficient in the release of residue/fibrebound antioxidants (macromolecular antioxidants - MA), also known as antioxidant dietary fibres (ADF) (Durazzo *et al*., 2016). Flavonols undergo degradation (to protocatechuic acid) when subjected to acidic hydrolysis (Moussa-Ayoub *et al*., 2011), and the extent of degradation depends on the concentration of acid utilised, nature of flavonol or plant material as well as hydrolytic temperature and treatment time (Häkkinen and Törrönen, 2000; Tura and Robards, 2002). Enzymatic hydrolysis has however been shown to be milder and more sensitive and yielded significant amounts of quercetin flavonol, and kaempferol compared to acidic hydrolysis. It also released undegraded sugars from flavonol glycosides to yield its corresponding aglycon (Bilyk *et al*., 1984).

2.9.1. *Opuntia* macromolecular antioxidant (MA) biological activities

Macromolecular antioxidants (MA) are high molecular weight antioxidant compounds when compared with extractable phenols, and exhibit significant biological activites (Arranz *et al*., 2009; Cardador-Martínez *et al*., 2011). They are made up of hydrolysable polyphenols, tannins (hydrolysable and condensed tannins) (Cardador-Martínez *et al*., 2011), single phenolic acids and polymeric proanthocyanidins (Pérez-Jiménez and Saura-Calixto, 2015). MA are usually determined in non-extractable polyphenol (NEPP) and non-extractable proanthocyanidins (NEPAC) and expressed in acidified methanol and HCl/butanol (butanolysis) fractions, respectively. The depolymerised hydrolysates are then utilised in quantitative assays (Durazzo

et al., 2016). *In vivo*, MAs are hypothesised to occur mainly in the colon where microbes ferment them to produce absorbable metabolites. MA exhibit different health-related properties which include antiproliferative, antioxidant, and gene expression modification (Tarascou *et al*., 2010). MA have been reported to constitute up to 50-100% of some fruits studied, and showed remarkable biological activities (Arranz *et al*., 2009; Tarascou *et al*., 2010).

Natural anticancer ingredients (ferulic acid, isorhamnetin derivatives, betalains) have been isolated from *Opuntia* fruit residues after hydroalcoholic solvents extraction and separation with microporous resins (Amberlite XAD16 resin) (Serra *et al*., 2013). Juice residues from *O. robusta* and *O. ficus-indica* wild fruits from Portugal contained useful compounds which were responsible for inhibition of human colon carcinoma HT29 cell growth (ED_{50} of 4.6 - 6 mg/mL after 72 h of proliferation). Residue extracts resulted in an increase in reactive oxygen species (ROS) in carcinoma cells, indicating that the prooxidant effect of constituent compounds may induce cancer cell death (Serra *et al*., 2013). The combination of an extractive and resin concentration methods can be used to concentrate bioactive compounds for potential application in pharmarceutical and food industries. The antiproliferative effect was, however, slightly lower than those described by Hahm *et al*. (2015) using highly fractionated *O. humifusa* extracts (hexane, ethyl acetate extracts and water partitioned fractions) in a more sensitive cancer cell line (human glioblastoma cell line-U87MG cells). Compared to doxorubicin (a known anticancer compound), earlier studies on *Opuntia* extracts showed reduced inhibition of cancer cell proliferation. Nevertheless, a combination of these extracts with doxorubicin could improve anticancer efficacy and reduce chemoresistance (Serra *et al*., 2013).

Research has reported high antioxidant activity of MA extracts (52.22 ± 1.07 µmol trolox equivalent/g) even in the absence of some cladode MA compounds such as hydrolysable tannins and proanthocyanidins (Bensadón *et al*., 2010). Hydrolysed cladode extracts were more

effective radical scavengers than the unhydrolysed fractions (Avila-Nava *et al*., 2014). Acid hydrolysis released polyphenols bound to or associated with the insoluble fibre as glycosides (Avila-Nava *et al*., 2014; Ginestra *et al*., 2009). Greek cactus pear fruit seed residue extract also showed good antioxidant activity (69.5 - 95.1 mmol/kg) (Zakynthinos and Varzakas, 2016). Other biological/gastrointestinal activities of antioxidants from non-*Opuntia* residues include antiinflammatory activity of grape proanthocyanidins (Tomás-Barberán and Andrés-Lacueva, 2012), and pomegranate ellagitannins (Larrosa *et al*., 2010). Non-*Opuntia* residue antioxidants also have beneficial microbial interactions [Cocoa flavanols (Tzounis *et al*., 2008)]; and antioxidant effects, that is, they inhibit oxidation of cholesterol and lipids, and proteins and vitamins (Tomás-Barberán and Andrés-Lacueva, 2012). They also interact with α-glucosidase, lipase and alpha-amylase enzymes (Tomás-Barberán and Andrés-Lacueva, 2012). Biological activities of *Opuntia* MAs could have potential applications as pill and capsule supplements; components of cosmetics, food ingredients (prebiotics) and drug excipients. More research into MAs may provide new data on novel antioxidant compounds and their applications (Amoo *et al*., 2014; Aremu *et al*., 2014; Pérez-Jiménez *et al*., 2013). MA analyses and quantification in more *Opuntia*/plant species are still required in the compilation of a broader database. This would be useful in guiding future biological and nutritional research (Arranz *et al*., 2009).

2.10. Potential side effects of *Opuntia* species extracts/compounds

Little information is available on *Opuntia* spp. toxicology and on adverse side effects of its compounds and/or extracts. However, from literature, a low colonic obstruction has been attributed to the consumption of *Opuntia ficus-indica* seeds (Kleiner *et al*., 2002). Nausea, mild diarrhoea, increased stool volume and frequency, headaches and abdominal bloating, have also been reported in books on traditional folk medicine and case reports (De Smet, 2002; Gagnier *et al*., 2006). These effects were, however, not corroborated with results from scientific

research. Although some health care professionals believe that herbal medicines are relatively safe because they are "natural", there is little supporting data to back such an assumption. Although herbal mixtures may contain certain contaminants from any stage of the extraction/production process, side effects may also be linked to plant components and human factors (intrinsic) and non-plant related contaminant factors (extrinsic) (Gagnier *et al*., 2006).

There are possible indications of extreme sensitivity to any component of *Opuntia* as individual pharmacokinetics vary widely. Dermatitis has been reported to be a common adverse reaction to prickly pears (Pawar *et al*., 2017). The presence of opportunistic pathogens/microorganisms or microbial toxins could also affect the content of active components in herbal mixtures and result in adverse health effects. Therefore, plant-derived herbal interventions need to be treated with care, or as containing potentially toxic chemical compounds until proven otherwise. Some of these toxic chemical hazards have been listed in the Maryland National Library of Medicine (NLM) database for harmful substances (Osuna-Martínez *et al*., 2014).

2.11. *Opuntia* plant compounds and the search for new antimicrobial compounds

The rise of new infectious diseases and increased bacterial resistance to available antimicrobials have necessitated plant-related research channelled towards identifying novel compounds with antimicrobial activity (Aremu *et al*., 2011; Silver and Bostian, 1993; Valtierra-Rodríguez *et al*., 2010). Antimicrobial compounds belong in a range of phenolic and non-phenolic classes such as betalains, polyphenols and phenolic acids (caffeic, cinnamic, catechol), quinones, flavones, flavonoids, flavonols, tannins, coumarins, lectins and polypeptides, alkaloids, terpenoids, essential oils, polyamines (spermidine), isothiocyanates, thiosulfinates, glucosides, polyacetylenes and acetylene compounds (Ciocan and Bara, 2007; Tapiero *et al*., 2002). The

antimicrobial activities of *Opuntia* species extracts are attributed to the presence of quite a number of these compounds.

Bactericidal activity has been reported for Cardon Blanco (*O. streptacantha*), Jalpa and Real de Catorce (*O. ficus-indica*) nopal cacti cultivars against *Campylobacter jejuni* (1.1 - 12.5 mg/ml), *Clostridium perfringens* (0.8–16 mg/ml) and *Vibrio cholera* (4.4 - 30 mg/ml). The activities were partly ascribed to the significant amounts of total flavonoids and phenols present in the cultivars (Sánchez *et al*., 2014). *Opuntia ficus-indica* extracts in a wide array of solvents have shown activity against different bacterial strains such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella* spp., *Klebsiella pneumoniae*, *Citrobacter freundii* and *Streptococcus pneumoniae* (Shafiei *et al*., 2013; Wasnik and Tumane, 2016), and against *Bacillus subtilis* (Gnanakalai and Gopal, 2016). Terpenoids, glycosides, saponins, alkaloids and flavonoids were identified in the extracts. Acetone extract compared to n-hexane and petroleum ether extracts showed better antimicrobial activity (Wasnik and Tumane, 2016), while aqueous extracts of both stem and fruit (Gnanakalai and Gopal, 2016) showed the least antimicrobial activity which could be attributed to poor solubility of bioactive components in extraction solvents.

Opuntia flower extracts also showed *in vitro* antibacterial activity against *P. aeruginosa*, *Staphylococcus aureus*, *B. subtilis*, and *E. coli*, and antifungal activity against *Candida lipolytica* and *Aspergillus niger*. Most important was the antibacterial activity which make the extracts potentially suitable for food industry applications, for example, as food additives or preservatives (Ennouri *et al*., 2005). Extracts of *Opuntia matudae* containing betalains inhibited the growth rate of *E. coli* O157:H7 (Hayek and Ibrahim, 2012). In addition, Ammar *et al*. (2015) reported marked antibacterial action (zone of inhibition 25±1.8 mm) of *Opuntia* flower methanol extract on *Listeria monocytogenes*. Recently, first report of a 28.3 kDa protein with

RNase activity purified from *Opuntia* cladode aqueous extract demonstrated antiviral action against cucumber and tobacco mosaic viruses (CMV and TMV) (MIC of 40 mg/mL). Antiviral activity was linked to interaction of the protein with RNase activity with viral nucleic acid (Rasoulpour *et al*., 2017).

Broad spectrum antimicrobial activity of betalain-rich extracts against *Bacillus cereus*, *L. monocytogenes*, *Proteus vulgaris*, *Yersinia enterocolitica* and *Enterobacter aerogenes*, as well as against *Rhizoctonia solani* and *Candida albicans* yeasts (125 - 250 mg/ml), *Fusarium oxysporum* and *Aspergillus flavus* moulds (500 mg/ml), have also been reported (Tenore *et al*., 2012). The broad-spectrum activity of the extracts was attributed to the adverse effect of bioactive components on microbial cell membrane integrity, function and structure (Canadanovic-Brunet *et al*., 2011). *Opuntia stricta* essential oil extracts containing mainly thymol (42.7%) also showed antimicrobial activity against important food and environment borne opportunistic bacterial pathogens such as *E. coli*, *B. cereus*, *P. aeruginosa* (1.25–2.5 mg/ml MICs) and yeast such as *C. albicans* (2.5 mg/ml MIC). The extracts of the *Opuntia* plant therefore have potential for commercialisation as novel drugs for use in antimicrobial therapy (Moosazadeh *et al*., 2014).

Most plant compounds with antimicrobial properties are aromatic organic molecules (Ciocan and Bara, 2007). Phenols and flavonoids are aromatic, hydroxylated biologically active compounds. Presence of either phenols or aldehyde compounds in extracts reportedly show highest antibacterial activities compared to those containing terpene alcohols (Koubaa *et al*., 2015a; Naveed *et al*., 2013). The number and site of the hydroxyl groups on the aromatic molecules determine antimicrobial action. Usually the more hydroxyl groups present, the better the antimicrobial effect. Antimicrobial quinone compounds bind to extracellular and soluble proteins and disrupt the integrity of microbial cell membranes/wall to result in static or cidal

effects. Similar antimicrobial mechanism is observed in coumarins where benzene and α-pyrone rings are bound together. Higher antimicrobial action was reported for polymeric phenols (with multiple number of hydroxyl groups) such as hydrolysable and condensed tannins which act by binding cell walls, inactivating microbial adhesins, enzymes, cell envelope and transport proteins, thereby preventing growth and protease activity (Ciocan and Bara, 2007). Some of the methods used for ascertaining the mechanism of action of a potential antimicrobial agent include measurement of cell conductivity, bacterial rate of kill (time-kill) and cell integrity assays. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) techniques are also been utilised to give a visual observation of cells after treatment with the potential antimicrobial compounds. SEM and TEM confirm changes to cell morphology, cell wall and cell membrane of treated microbial cells compared to untreated cells (Sadiq *et al.*, 2017).

2.12. Laccases as biocatalysts in the synthesis of bioactive compounds and materials

Many phenolic compounds from *Opuntia* cladodes and fruits can serve as good substrates for biocatalytic reactions. Enzymes may be utilised in an array of selective organic transformations, including modification of natural compounds (Adrio and Demain, 2014; Su *et al.*, 2018). Enzymes have been used in many industries for centuries and can act on a wide range of natural and artificial substrates. Enzymes are chemically selective of certain functional groups (chemoselectivity) located in separate regions of a substrate (regioselectivity). These functions are aided by the three-dimensional structure of enzymes (Shoda *et al.*, 2016). Also, most enzymes are composed of L-amino acid units which make them chiral catalysts. In other words, for asymmetric and selective utilisation, chemo- and regioselectivity properties make up a central attribute and determine an enzyme's 'specificity' (Carr, 2004). Enzymes are known to catalyse reactions mostly in the aqueous phase. Generally, for biocatalytic applications, organic

solvents have more advantage over other solvents. This is because they have reduced water activity and allow greater solubility of hydrophobic components; side reactions observed in aqueous reactions are also reduced, there is reduced probability of microbial contamination. Organic solvents also aid isolation/purification in which also enhances yields (Zaks, 2001). The use of enzymes cannot be overemphasised in their biotechnological applications as biocatalysts.

The use of laccase is proposed for use in modifying *Opuntia* related compounds in this study. Laccases are glycoproteins which belong to the oxidoreductase group of enzymes (Riva, 2006). Laccases are copper-containing, *p*-diphenol dioxygen oxidoreductases which carry out one-electron oxidation of appropriate substrate molecules such as aromatic or aliphatic amines, and phenols to the corresponding reactive radical with the subsequent reduction of oxygen to water (Kudanga *et al.*, 2017; Kudanga and Le Roes-Hill, 2014). The mechanism of action of laccase in synthesis of new compounds is shown in Fig. 2.1. Laccase-catalysed oxidative reactions generate radicals from the monomeric substrate units. The radicals may then bind to achieve stability and evolve into a new molecule (Fig. 2.1) or may turn towards degradative processes. Polymers may also be formed if further coupling is allowed to continue. Radicals may evolve in three distinct ways. First, oxidative coupling is the most common path where radical reactions are largely uncontrolled with respect to structure of the desired/end oxidation product. Therefore, optimising reaction parameters cannot be overemphasised if a definite molecule is targeted. In order to achieve this, the frequent application of a short reaction time and defined molar concentrations (Hollmann and Arends, 2012), substrate engineering (Mita *et al.*, 2002), and use of co-solvent (Mita *et al.*, 2004) are sometimes utilised to obtain defined products. Laccases may also catalyse cross-coupling reactions as shown in Fig 1. This may result in new and interesting structural conformations in the end products (Pezzella *et al.*, 2015). Therefore, laccases have been used as a catalyst in organic synthesis for example in drug manufacture and related industries (Adrio and Demain, 2014; Mayer and Staples, 2002; Su *et al.*, 2018).

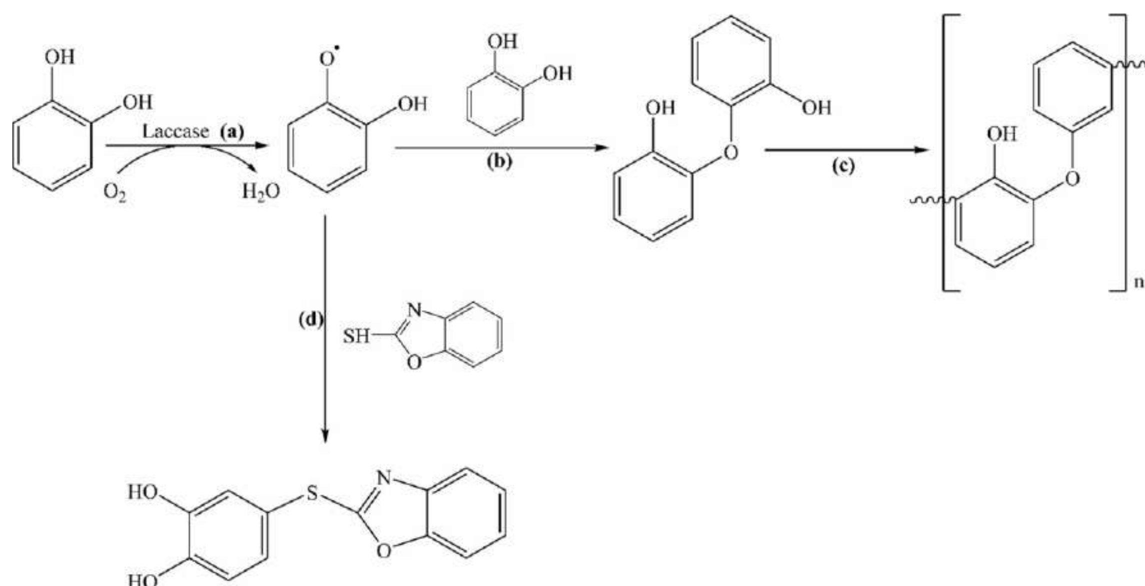

Figure 2.1: Mechanism of action of laccase-catalysed oxidation of substrate to form radicals, **b** radicals undergo oxidative coupling to produce dimers, **c** further coupling results in the formation of polymers through polymerisation, and **d** coupling with a non-laccase substrate to form cross-coupling products.
Source: Abdel-Mohsen *et al.* (2014) and De Regil and Sandoval (2013)

High molecular weight polyphenols have been reported to exhibit enhanced biological properties with longer circulation time *in vivo* (Silva *et al.*, 2011). The oxidative coupling of flavonoids has been used to produce polymers with improved antioxidant capacity. Kurisawa *et al.* (2003) employed laccase to catalyse the oxidative polymerisation of catechin. When compared with the monomeric catechin, the synthesised polymer showed enhanced ability to scavenge superoxide. Jeon *et al.* (2012) also reported a mixture of dimer, trimers and tetramers from a catechin monomer, and these oligomers showed higher anti-glycation and superoxide dimustase (SOD) activities. Oligomers that are insoluble in water have been produced from free laccase, while linear and water-soluble oligomers were derived using a conjugated laccase and endowed with high antioxidative action (Jadhav and Singhal, (2014). In an earlier study, oligomers derived from quercetin and kaempferol monomers also showed enhanced antioxidant activity (Desentis-Mendoza *et al.*, 2006).

In other and more recent applications of laccase, phenolic compounds like flavonoids are oxidatively grafted onto polymers in order to modify/impart new properties to the polymers (Pezzella *et al*., 2015). Polymer terminals contain functional moieties which make it possible for low molecular weight molecules to be coupled to them. The cross-linking may occur either in the side or main functional chains (Kobayashi and Makino, 2009). Using this approach, laccases have been used in the modification and improvement of properties of fibres (cotton and wool) (Hossain *et al*., 2010). In a previous study, Silva *et al*. (2011) used laccase from *Myceliophthora thermophila* to oxidise flavonoids in flax fibres, and covalently bound the quinones produced to chitosan amino groups. The antibacterial activity of the grafted linen was then determined. Chitosan-treated linens where active against *E. coli*, while *S. aureus* showed susceptibility to both catechin and chitosan treated samples. Similarly, laccase has been used to impart antibacterial activity on lignocellulose materials (Widsten *et al*., 2010).

The use of laccase in the modification of materials to produce more active end products has great prospect in various industries (Pezzella *et al*., 2015). In addition, many penicillin and cephalosporin substituents are potential natural substrates for laccase. Hence, laccases has potential to catalyse the production of therapeutics and pharmaceuticals such as antibiotics and anticancer agents/compounds. The use of enzymes to modify compounds with the aim of increasing antioxidant properties and other biological activities is gaining a lot of research interest in biotechnology (Kudanga *et al*., 2017).

2.13. Structure-activity relationship (SAR) in phenolic compounds

In determining the SAR of phenolic compounds, Cai *et al*. (2006) tested different groups of phenolic compounds and found that polymeric tannins showed the best activity compared to most quinones, lignans and isoflavones. The study showed that the number and position of

hydroxyl groups, as well as the presence of other substituents greatly determine the antioxidant capacity in phenolic compounds. In other words, differences in molecular structure also translated into differences in radical scavenging activity which was linked to different hydroxylation patterns, presence and/or absence of methoxylation and glycosylation. The presence of *ortho*-dihydroxy groups conferred high activity on all phenols tested. Where hydroxyl groups were absent no antioxidant activity was recorded, for example in isoflavone and flavanone (Cai *et al*., 2006). Benzoic and cinnamic acids also had no hydroxyl group and no antioxidant activity (Cai *et al*., 2006). Monohydroxybenzoates showed activity because of the presebce of a hydroxyl group (-OH). Vanillic (4-OH, 3-OCH_3) and syringic acids (4-OH, 3,5-OCH_3) showed even better activity than the monohydroxybenzoates that had no methoxy groups. The dihydroxylated protocatechuic acid also demonstrated a lower activity compared to syringic acid which indicated that the presence of methoxy groups increases the ability of the molecules to donate hydrogen and scavenge radicals. However, in the low-density lipoprotein (LDL) system, the hydroxydimethoxy acids had reduced antioxidant capacity compared to the dihydroxy acids (Natella *et al*., 1999).

Cardio-protective effects of flavonoid and other phenolic antioxidants arise from their inhibitory action on lipid peroxidation. They are also able to impair reactive oxygen species (ROS) pathways and chelate redox active metals (Tsao, 2010). Flavonoids have different mechanisms of action. On the other hand, one of the most pronounced features of anthocyanins (flavonoid polyphenols) is their substantial antioxidant capacity. However, as result of their vast structural variations, their bioactivity varies widely (Cai *et al*., 2006). Glycosylation, on the other hand, can reduce free radical scavenging capacity, but enhances molecule stability (Konczak *et al*., 2005). Quite a number of studies have aimed at deriving a common substitution and antioxidant activity hierarchy for flavonoid compounds. Data from these studies have helped scientists make reliable predictions on SARs, as well as improve our general

understanding of flavonoids bioactivities. All additional advancements and progress in this research area could help in the improvement of nutraceuticals and semisynthetic analogues which have little or no side effect and retain significant biological capacities (Heim *et al*., 2002).

In the case of high molecular weight dimers/oligomers generated from laccase-catalysed reaction, different radical linkages are possible. In a study by Adelakun *et al*. (2012b), β-β and β-5 linked dimers were produced using ferulic acid as the monomeric substrate unit. The β-5 dimers showed better enhancement in antioxidant activity compared to β-β dimers. The presence of carboxylic acid group with an adjacent unsaturated C–C double bond, which can provide additional sites for free radical attack. The increase in the number of electron-donating groups on the β-5 dimer, was reported to be responsible for the enhancement in antioxidant activity (Srinivasan *et al*., 2007). In another work which involved the use of a 2,6-dimethoxyphenol (2,6-DMP) as substrate, a symmetrical C–C-linked dimer, 3,3′,5,5′-tetramethoxy biphenyl-4,4-diol, was formed (Fig. 2.2). The antioxidant activity of the dimer doubled compared to the starting substrate, 2,6-DMP (Adelakun *et al*., 2012a). The increased in activity was mainly due to the increase in functional groups with electron donating capacity (Matsuura and Ohkatsu, 2000), the reduction in the O-H bond dissociation energy, and increased stability of radical due to resonance delocalisation (Sánchez-Moreno *et al*., 1998).

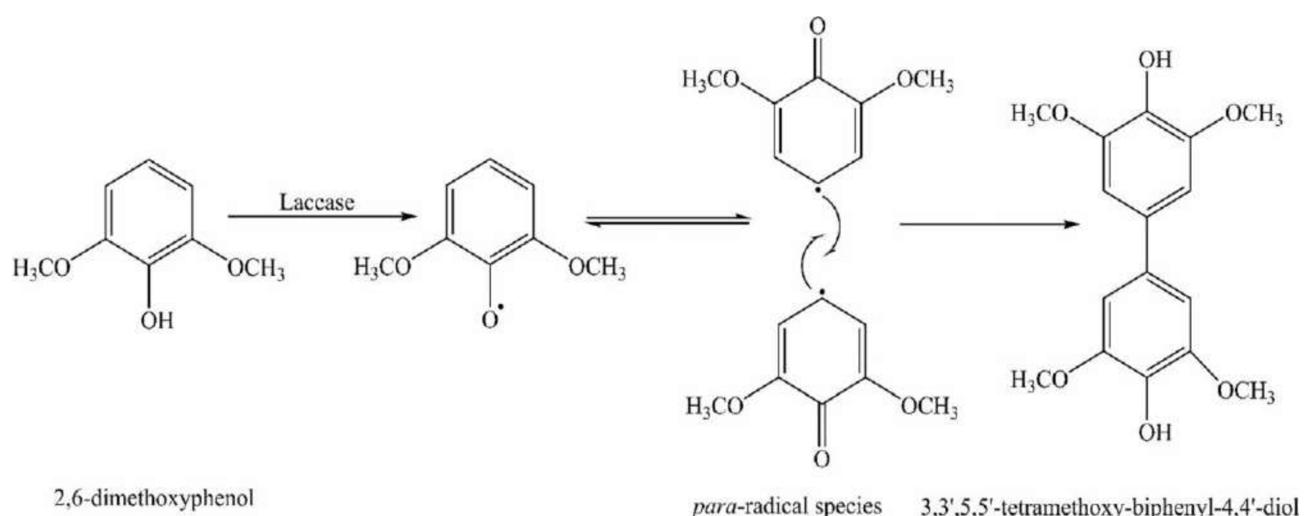

Figure 2.2: Proposed reaction mechanism for the homomolecular coupling of 2,6-DMP to produce the C–Cdimer (3,3′,5,5′-tetramethoxy biphenyl-4,4′-diol) (Adelakun *et al.* 2012a)

The tendency of an antioxidant/compound to release electrons is also dependent on bond dissociation enthalpies (BDEs) of active/functional groups within a compound. In other words, antioxidants containing functional groups with lower BDE would show better antioxidant capacity because they more readily release electrons to radical species (Szymusiak and Zielinski, 2003). A lower antioxidant activity was reported for the β-β linked ferulic acid dimers compared to the ferulic acid substrate (Adelakun *et al*., 2012b). This observation was consistent with earlier findings where bis-ferulic acid (β-β dimers) had a higher BDE (85.76 kcal/mol) than ferulic acid (84.70 kcal/ mol) (Murakami *et al*., 2005). The determination of BDE varies with compounds and experimental conditions. For these reasons, research which have focused on phenolic compounds BDE have published contrasting results (Klein and Lukeš, 2006; Szymusiak and Zielinski, 2003). In general, phenolics are mostly used as antioxidants possibly due to the fact that hydroxyl groups have lower BDE compared to alkyl and aniline groups (Bendary *et al*., 2013).

Antioxidant activity is also affected by hydrophobicity, especially in a multicellular hydrophobic environment (Ishige *et al*., 2001), as well as an antioxidant's ability to produce

stable radicals that can inhibit the progression of oxidative chain reactions (Alov *et al.*, 2015). At sites where free radicals are generated, hydrophobicity increases the bioavailability of the antioxidant (Ishige *et al.*, 2001). For example, hydrophobic antioxidants would effectively scavenge free radicals generated from lipid peroxidation on the lipid bilayer because of their lipophilic properties (Lu *et al.*, 2006). On the other hand, radical stability arises from the resonance delocalisation of lone electrons into the aromatic ring and absence of groups prone to attack by oxygen (Flora, 2009; Shahidi and Naczk, 2004). Large groups on the *ortho* positions of the aromatic ring have been shown to help stabilise antioxidant radicals (Shahidi and Naczk 2004), but they may also reduce antioxidant activity by steric masking of the phenolic hydroxyl group (Murakami *et al.*, 2005). In other words, an ideal antioxidant would consist of a balance of hydroxyl groups that ensure the free radical scavenging ability of the antioxidant, the presence of electron-donating groups that help stabilise the radical after donation of electrons, and hydrophobic groups that ensure the bioavailability of the antioxidant in multicellular systems. Research that focused on improving the hydrophilicity of silybin resulted in its compromised antioxidant activity in lipophilic environments (Gažák *et al.*, 2010; Gažák *et al.*, 2004). These studies emphasised the fact that hydrophobicity is an integral factor if antioxidants are to function effectively in cellular environment.

2.14. Conclusion

The relevance of plant extract compounds profiling cannot be overemphasised. It remains significant for the discovery of new bioactive compounds, especially in unexplored plant species or in different geographical locations and climate. Improving bioactivities remains a challenge for the scientists working with phenolic compounds. Enzymatic modification of plant compounds using an enzyme such as laccase can be an option for the production of new compounds with improved biological activities.

2.15. Research hypotheses, aim and objectives

2.15.1. Research hypotheses

1. *Southern African Opuntia cladode, fruit and peel extracts will show good biological activities and a different phytochemical profile*.

Compound profile of plants have been shown to be affected by biotic and abiotic elicitors (Khan and Giridhar, 2015) and may vary depending on the geographical location (Nascimento *et al*., 2011).

2. *Laccase-catalysed modification of selected phenolic compounds will produce higher molecular weight products with improved antioxidant and antimicrobial activity*.

Phenolic dimers have been reported to possess increased biological activity compared to the monomeric forms (for example, hydroxytyrosol dimer) (Zwane *et al*., 2012). It is expected that oligomeric forms would show improved bioactivities. Oligomerisation usually result in increased number of hydroxyl groups thereby providing more sites to react with free radicals, as well as an increase in electron donating groups (Kudanga *et al*., 2017).

2.15.2. Research aim

The overall aim was to characterise southern African *Opuntia* extracts and enzymatically modify selected phenolic compounds for the production of new bioactive compounds with enhanced antioxidant and/or antimicrobial activities.

2.15.3. Research objectives

The specific objectives of this study were:

- To profile extractable phenols and macromolecular antioxidants in *Opuntia ficus-indica* fruit and cladode extracts and determine the antioxidant and antibacterial activities of the extracts.
- To modify *Opuntia*-related compounds using *Trametes pubescens* laccase.
- To characterise synthesised laccase oxidation products.
- To compare antioxidant and antimicrobial activities of substrates and products.
- To determine the potential mechanism of antimicrobial action of enzymatically (using laccase) synthesised compounds.

CHAPTER THREE

Extractable and macromolecular antioxidants of *Opuntia ficus-indica* cladodes: Phytochemical profiling, antioxidant and antibacterial activities

Abstract

Macromolecular antioxidant (MA) fractions and their antioxidant and antimicrobial activities are largely ignored from most conventional extractive processes compared to the well-known extractable polyphenol fractions. This study investigated subtropical spineless cladode MA extracts in comparison with their extractable fractions; as well as the effect of freeze- and oven-drying methods on extract yields and bioactivities. Cladode extractable phenol (EP) and MA fractions antioxidant capacities, antimicrobial potential against multi-drug resistant bacterial cultures, and phenolic content were evaluated. LC/MS was used to analyse/profile extract components. Freeze-dried samples from all the extracts showed better antioxidant and antimicrobial activities than the oven-dried samples. Antibacterial and antioxidant activities were generally higher in hydrolysed (MA) than in unhydrolysed (extractable) fractions. DPPH radical scavenging activity (RSA) EC_{50} values correlated with phenol contents (R^2=0.9609). LC/MS extract profiles showed the presence of phenolic acids, flavonoid derivatives and other unidentified compounds. Isovitexin 7-*O*-xyloside-2"-*O*-glucoside, polyhydroxypregnane glycoside and neohancoside C were identified for the first time in *Opuntia* cladode extracts. This study showed that cladode EP and MA extracts have potential as rich antioxidant sources and can be key to the discovery of new antimicrobials.

3.1. Introduction

The cactus pear (*Opuntia* spp.) plant has a high genetic diversity with a wide variety of the plant grown worldwide. *Opuntia ficus-indica* is the most common and main commercial variety, with spines or no spines/thorns (Vigueras and Portillo, 2001). Globally, the demand for natural antioxidants has increased due to safety concerns attributed to the use of synthetic antioxidants. For example, synthetic antioxidants have been shown to be allergenic, carcinogenic, and cause nausea, DNA and sperm abnormalities (Wojcik *et al*., 2010). Synthetic antioxidants may also not contribute additional nutritional benefits (Satyanarayana *et al*., 2014). On the other hand, natural antioxidants are readily assimilated by the body and show little or no adverse effect (Wojcik *et al*., 2010). In addition, there is also the alarming trend of multi-drug resistance to available antimicrobials (Valtierra-Rodríguez *et al*., 2010). The safety concerns with synthetic antioxidants and increasing antimicrobial resistance trends have necessitated scientific studies directed towards the search for novel, but safe bioactive molecules in plant extracts with antioxidant (Aremu *et al*., 2011) and antimicrobial properties (Valtierra-Rodríguez *et al*., 2010).

Opuntia nopals (cladodes) have been used in traditional folk medicine (Flores *et al*., 1999). Some reported health benefits of nopal extractable polyphenol (EP) extracts include anticarcinogenic, antihypertensive (Livrea and Tesoriere, 2006) and antioxidative health effects (Avila-Nava *et al*., 2014). The presence of insoluble and soluble fibres (1–2%), as well as biologically active phytochemicals such as polyphenols and flavonoids (Avila-Nava *et al.*, 2014; Stintzing and Carle, 2005) is responsible for nopal health benefits. Ascorbic acid, a natural antioxidant, is also found in considerable quantities in *Opuntia* nopals/cladodes (Stintzing and Carle, 2005). However, the bulk of information on biological activity of cactus pear cladodes comes from the extractable phenol fractions (Livrea and Tesoriere, 2006), while

much less information is available on macromolecular antioxidant (MA) fractions even though there is evidence that both contain biologically active molecules (Avila-Nava *et al.*, 2014). Hydrolysable polyphenols [macromolecular antioxidants (MAs)] isolated in residues are often overlooked in common plant extractive operations (Saura-Calixto, 2012). MAs include hydrolysable tannins, phenolic acids and simple phenols (Pérez-Jiménez *et al.*, 2013). Apart from their antioxidant activity, MA compounds contribute to the beneficial health properties associated with dietary food fibre (Saura-Calixto, 2012).

Several studies have reported antimicrobial (Gnanakalai and Gopal, 2016; Sánchez *et al.*, 2014) and antioxidant (Albano *et al.*, 2015; Galati *et al.*, 2003) activities of extractable polyphenol extracts from *Opuntia* spp. (Livrea and Teosriere, 2006). Studies have also shown that the drying methods applied to plant materials and/or their extracts could affect extract yield and biological functions (Reyes *et al.*, 2011; Torres *et al.*, 2010). However, little data are available on the biological potentials/activities of cladode (*O. ficus-indica*) MA extracts (Bensadón *et al.*, 2010), and none from the subtropical region of southern Africa. Bioactive molecule distribution and antioxidant properties in foodstuffs such as cactus pear fruit and cladodes may also be affected by cultivar/genetic factors, agronomic practices, environmental and climatic conditions (Moussa-Ayoub *et al.*, 2014). Therefore, this study investigated the phenolic composition, antioxidant and antimicrobial activities of subtropical spineless cladode MA extracts. The study also reports on the phenolic content/profile, antimicrobial and antioxidant activities of extractable phenol (EP) extracts for comparative purposes. The effect of drying methods on extracts yield and bioactivities are also reported.

3.2. Materials and methods

3.2.1. Chemicals and reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), TPTZ [3(2,4,6-tripyridyl-s-triazine)], ascorbic acid, and iron (III) chloride ($FeCl_3$) were purchased from Sigma-Aldrich Chemical (Aston Manor, Johannesburg, South Africa), while Folin-Ciocalteu phenol reagent and sodium carbonate were purchased from Merck Chemical Supplies (Damstadt, Germany). All chemicals used, including the solvents, were of analytical grade. Other chemicals (and culture media) unless otherwise mentioned were obtained from Oxoid (UK) and Biolab-Merck (Modderfontein, South Africa).

3.2.2. Culture and maintenance of microorganisms

Pure cultures of the following American Type Culture Collection (ATCC) bacteria were used: Gram-positive *Bacillus cereus* (ATCC 10876), *Listeria monocytogenes* (ATCC 19111), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213), methicillin resistant *Staphylococcus aureus* (ATCC 33591); and Gram-negative *Klebsiella pneumonia* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Salmonella arizonae* (ATCC 13314), *Enterobacter cloacae* (ATCC BAA1143), and *Acinetobacter baumannii* (ATCC BAA1605). Bacterial stock cultures were kept on nutrient agar slants of double strength and stored at 4°C. Overnight nutrient broth culture (18-24 h) of each bacterium was prepared before use in experiments.

3.2.3. Plant material collection and preparation

Opuntia cladodes were manually harvested from a local orchard in Durban, Kwazulu-Natal province, South Africa. The approximate length of nopal leaves used was 25-28 cm. Nopals were washed in distilled water and disinfected using 80% ethanol, sliced (to facilitate drying) and oven dried at 45-50ºC for up to 72 h. Dehydrated cladodes/nopals were mechanically milled in a blender and the powder stored in a closed container at 4ºC. Separate wet milled fractions were also freeze-dried at -80ºC and 1 atm pressure for 24 h and thereafter stored in a closed container at refrigeration temperature (4ºC).

3.2.4. Extraction of cladode phenols/polyphenols

Dried plant material (5.0 g) was placed in separate conical flasks and shaken at 22-25ºC overnight in 50 mL of different extraction solvents [80% methanol (M), 80% ethanol (E), acidified water (AQ), methanol:acetone:water (MAW), and acidified methanol (AM)]. Macerated extracts were filtered using Whatman No.1 filter paper, and the extraction solvents evaporated in a rotary evaporator at 45°C and 60 rpm (Benayad *et al*., 2014).

Polyphenols were extracted with methanol, acetone and water (MAW) according to the method of Avila-Nava *et al*. (2014). Dried samples were extracted with 50% (v/v) methanol at 22-25ºC for 1 h and filtered using Whatman No. 1 filter paper. The residue was extracted with 70% (v/v) acetone under the same extraction conditions. The residue from this acetone extraction was re-extracted in 10 mL of distilled water for 15 min. The resulting filtrates from freeze- and oven-dried samples methanol, acetone and water (MAW) extractions were pooled separately and evaporated to dryness. The freeze- and oven-dried acidified water (AQ) extract were also obtained. The MAW and AQ extracts were separated into two portions (a portion for extractable

phenol, and another for acid hydrolysis). Separated portions of MAW and AQ were thereafter subjected to acid hydrolysis for 3h using 5 mL of concentrated hydrochloric acid. The unhydrolysed/extractable phenol and hydrolysed extracts fractions were then evaporated to dryness. MAW and AQ extracts were hydrolysed because of their viscous nature.

Residue from the MAW extraction process described above was subjected to acid hydrolysis (acid:methanol; 10:90, v/v) to obtain MA extract. A 1:10 (w/v) extraction was done with shaking in a water bath at 85ºC for 20 h (Durazzo *et al*., 2016). Samples were then centrifuged (15 min, 25°C, 3000g) and the supernatants were used for further assays (total phenol and hydrolysable polyphenol, antioxidant and antimicrobial). In order to remove the acidified methanol solvent and concentrate MA extract, the supernatant was evaporated with subsequent three cycle wash with acetone, methanol and acetone. A total of 15 extracts were obtained; 10 unhydrolysed and 5 hydrolysed extracts. The yield (%, w/w) of each extract was calculated using the following equation:

$$\text{Yield (\%)} = (w1 / w2) * 100 \quad (1)$$

where w1 = weight of the extract after solvent evaporation, w2 = weight of the plant powder.

3.2.5. Total phenolic content (TPC) of extracts

Total phenolic content of the cladode extracts (including hydrolysable tannins/polyphenols in MA extract supernatant) was quantified according to a method described by Lee *et al*. (2002a). Plant extracts were dissolved in methanol. Folin-Ciocalteu reagent (0.25 mL of 1:9 dilution) and 0.05 mL of the extract were mixed, followed by 5 min incubation at room temperature. Thereafter, 0.5 mL of 20% (w/v) sodium carbonate were added to the mixture and incubated

for 25 min in the dark at 25°C. The colour formed was read at 760 nm. The experiment was performed in triplicate. A calibration curve was constructed (Appendix B), using gallic acid (0.1-1.0 mg/mL) as standard, and total phenolic content of extracts expressed as milligram gallic acid equivalents per gram of extract (mg GAE/g).

3.2.6. Determination of extracts antioxidant capacities

3.2.6.1. DPPH radical scavenging activity of extracts

In carrying out the DPPH assay, 50 µL of each extract (1-5 mg/mL) and 5 mL of 0.004% (w/v) solution of DPPH (100 µM) were mixed, vortexed and incubated in the dark for 30 min at room temperature. Then, absorbance of the mixtures was read with a UV-visible spectrophotometer (Biochrom Libra S21, England) at 517 nm. The blank was 80% (v/v) methanol, and DPPH in methanol was used as the negative control. Ascorbic acid was used as a positive control. The percentage DPPH inhibition was calculated using the following formula:

$$I\ (\%) = [(A_0 - A_1)/A_0] * 100 \qquad (2)$$

where A_0 = absorbance of negative control, A_1 = absorbance of the extract/standard. The experiment was performed in triplicate. The percentage radical scavenging activity versus extract concentration curve was plotted and the concentration of the sample that was required for 50% radical scavenging activity was determined and expressed as the EC_{50} value. Lower EC_{50} values indicated high antioxidant capacity.

3.2.6.2. Ferric reducing antioxidant power (FRAP) assay

The reducing power of 5 hydrolysed (MA, freeze- and oven-dried MAW, freeze- and oven-dried AQ) and 10 unhydrolysed (five freeze-dried, five oven-dried) cladode extracts was determined. Fresh FRAP reagent was prepared by mixing 25 mL of 300 mM acetate buffer pH 3.6, 2.5 mL of 10 mM TPTZ solution made in 40 mM HCl acid and 2.5 mL of 20 mM ferric chloride solution. The reagent was incubated at 37°C for 15 min before use. The FRAP reagent (2.85 mL) was mixed with 150 µL of a plant extract or standard. The mixture was incubated for 30 min in the dark. The coloured product (ferrous tripyridyltriazine complex) was measured at 593 nm. FRAP determination was performed in triplicate. The standard curve (Appendix B) was linear between 100 and 900 µM FeSO4. Results were expressed in µM (Fe(II)/g dry mass.

3.2.7. Antibacterial activity of cladode extracts

3.2.7.1. Agar well diffusion assay

The agar well diffusion method was used to determine the antibacterial activity of the plant extracts. A 100 µL of each standardised bacterium (0.5 McFarland turbidity standard) was aseptically spread on petri dishes containing solidified Mueller Hinton agar. A sterile cork borer was then used to make about 6 mm wells in the inoculated agar medium. Different extract concentrations (80 µL of 25-300 mg/mL for EP and MA) were added to each well and plates labelled accordingly for each bacterium and extract. Plates were then left on the bench for 1 h for adequate diffusion of the extracts and incubated at 37°C for 24 h. Respective extract resuspension solvents were used as negative controls. After incubation, the diameter of the zone of inhibition around each well was measured to the nearest millimetre along two axes (i.e. 90°

to each other) and the mean of the two readings was calculated (Alabi *et al*., 2012). Experimental determinations were done in triplicate.

3.2.7.2. Minimum inhibitory concentration (MIC) of extracts

MICs of extracts were determined using the broth microdilution method of Sen and Batra (2013) in sterile 96-well polystyrene U-shaped microtiter plates (Corning, Costar Cambridge, USA). Turbidity of 18-24 h old bacterial culture suspensions adjusted to a turbidity of 0.5 McFarland standard was used. Wells containing 80 µL of each hydrolysed and unhydrolysed plant extract concentrations ranging from 25 to 300 mg/mL were inoculated with 10 µL of each bacterial suspension. Microtitre plates were properly closed and incubated at 37ºC for 24 h. Thereafter optical density (OD) readings were taken at 600 nm using the microtitre plate reader (Thermofisher-MultiskanGO, South Africa). Extracts in solvent and nutrient broth alone (negative controls), and bacterial suspensions alone (positive growth controls) were also prepared in separate wells. The experiment was done three times. The lowest concentration of the extract that inhibited growth (reduction in OD) was recorded as the MIC.

3.2.8. Extracts profiling by LC-TOF/MS analysis

Samples were filtered through 0.2 mm syringe filters (VWR, Radnor, PA, USA), dried and resuspended in methanol for analysis. LC-TOF/MS work was done at Stellenbosch University Central Analytical Facility (CAF). Individual compounds were identified based on retention time, UV spectra, and mass-to-charge ratio. Chromatographic separations were performed on a Waters Synapt G2 (ESI probe injected into a stream of acetonitrile, ESI negative, Cone Voltage 15) and Dee inlet method, using a Waters Acquity HSS T3 (2.1×150mm 1.8µm) column (Astello-García *et al*., 2015). Elution gradients were performed with solvent A (0.1 % formic

acid in water) and B (0.1 % formic acid in acetonitrile). The gradient was set up as follows: 98% A–0% A (20 min), 0% A–98% A (20–21 min), 98% A (21–23 min) and a flow rate of 0.5 mL/min was used. Complete spectral data were recorded in the range 200-600 nm. For assignation of identity, the database mzCloud and relevant literature were consulted (Astello-García *et al*., 2015).

3.2.9. Statistical analysis

Experimental data/results were presented as mean ± standard deviation (SD) following one-way analysis of variance (ANOVA) and the differences between samples were determined by Duncan's Multiple Range test using Statistical Package for Social Sciences (SPSS) version 16. A value of $p<0.05$ was regarded as statistically significant.

3.3. Results and discussion

3.3.1. Yield of cladode extracts

Yields for freeze-dried extracts (10.5-16.7%) were approximately 10 times higher than those for oven-dried extracts (1.1-2.5%) (Table 3.1). The use of the freeze-drying method and aqueous organic extraction solvents significantly increased extract yields per gram of dried plant powder. Acidified water and pooled methanol: acetone: water extracts had the highest freeze-dried yields. Results were lower compared with that of *O. ficus-indica* flowers, where a percentage of 23.64% was obtained using 50% methanol as solvent (Alimi *et al*., 2011). Drying for a long period of time (48-72 h) may have contributed to the significantly reduced extract yields of oven-dried extracts. Freeze-drying or oven-drying for short periods at temperatures lower than 50ºC have been recommended from a previous study (Torres *et al*., 2010). Freeze-

drying minimises the impact on nutritional properties of foodstuffs and ensures the retention of phytochemical properties (Reyes *et al.*, 2011).

Table 3.1: Percentage yield of *Opuntia ficus-indica* cladode extracts

Extracts	Methanol: Acetone: Water (MAW)	Ethanol (E)	Methanol (M)	Acidified methanol (AM)	Acidified water (AQ)
Freeze-dried yield (%, w/w)	14.9±0.2^{a}	10.5±1.2^{a}	14.7±0.6^{a}	12.3±0.4^{a}	16.7±1.3^{a}
Oven-dried (50°C) yield (%, w/w)	2.5±1.3^{b}	1.1±0.8^{b}	1.2±0.4^{b}	1.3±0.5^{b}	1.9±0.7^{b}

Values are mean ± standard deviation (n=3).
Values with different superscripts (a, b) in the same column are significantly different as shown by Duncan's multiple range test.

3.3.2. TPC and antioxidant activity of extracts

3.3.2.1. TPC of cladode extracts

Extractable polyphenols (EPs) have a low degree of polymerisation and can be extracted using various extractive solvents. Total phenol content for unhydrolysed extracts ranged from 1.07-10.46 mg GAE/g (Table 3.2). EP content was relatively low when compared with values in common dietary foods (Saura-Calixto, 2010).The TPC of young nopals (*O. ficus-indica*) of Mexican origin was recorded as being between 17 to 40 mg/g sample (Medina-Torres *et al.*, 2011). The significantly lower values reported in our study may be attributed to differences in cladode age, climate, growth location, extraction solvent and quantification methods used (Guevara-Figueroa *et al.*, 2010; Kim *et al.*, 2013). Freeze-dried ethanol and methanol extracts had the highest TPC which may be due to the higher polarity of the extraction solvents. In the freeze-dried extracts, AM extract showed good total phenol content (7.04 mg GAE/g), compared to MAW (5.02 mg GAE/g) and AQ extracts (2.12 mg GAE/g) (Table 3.2). The low

phenol content in unhydrolysed MAW and AQ extracts may be due to their viscous nature which did not allow the release of some phenolic constituents. Viscosity of extracts was reduced by application of acid hydrolysis for better determination of phenol content. When applied in our study it, resulted in a 1.4 to 8-fold increase in phenol content of hydrolysed MAW and AQ extracts compared to the unhydrolysed extracts. In previous studies, a similar protocol improved phenol content and bioactivities compared to unhydrolysed fractions (Avila-Nava *et al.*, 2014; Durazzo *et al.*, 2016).

TPC of MA extract was relatively high (11.65 mgGAE/g) (Table 3.2). Acid hydrolysis may have resulted in the release of polyphenols such polyhydroxylated and monohydroxylated phenol compounds from the fibrous nopal extraction residues. However, while this indicated the presence of hydrolysable polyphenols in sample, Bensadón *et al.* (2010) had reported the presence of extractable phenols for cladode MA extract, but not the presence of hydrolysable tannins using the Folin-Ciocalteau method. The variation may be attributed to the use of cladode cultivars from different locations, as well as age and time of harvest (Castellanos-Santiago and Yahia, 2008). Durazzo *et al.* (2016) also reported a high total polyphenol and antioxidant activity for hydrolysable polyphenol fractions from Solina (*Triticum aestivum* L.) residue, compared to the extractable polyphenol fraction.

3.3.2.2. *In vitro* antioxidant activity of cladode extracts

The antioxidant activities of unhydrolysed and hydrolysed (Table 3.2) extracts of nopal were evaluated by the DPPH and FRAP methods. Both extract types exhibited antioxidant activity, although the hydrolysed extracts were more effective than the unhydrolysed extracts. The ethanolic extract had the least DPPH EC_{50} value (65.8 ± 0.05 mg/mL) of all unhydrolysed extracts (Table 3.2). The range of EC_{50} values for hydrolysed extracts (12.34±0.10 - 21.64 ±

0.05 mg/mL), was lower than that of unhydrolysed extracts (65.8 ± 0.05 - 710.4 ± 0.01 mg/mL) (Table 3.2). MA extract demonstrated the highest scavenging activity (12.34 ± 0.10mg/mL). This showed that hydrolysed extracts were more effective DPPH scavengers. This effect may be due to the digestion process during acid hydrolysis that released the polyphenols bound within viscous extract matrix or associated with the insoluble fibres in the case of MA extract (Avila-Nava *et al.*, 2014; Ginestra *et al.*, 2009; Williams *et al.*, 2004). EC_{50} values were higher compared to the ascorbic acid standard (1.1 mg/mL).

The release of polyphenols may be responsible for the improved antioxidant activity in the hydrolysed cactus cladode extracts (Kim *et al.*, 2013), and could also be linked with the phenol types and the number and position of hydroxyl groups present (Gallegos-Infante *et al.*, 2009). The low antioxidant activity of unhydrolysed *Opuntia* fractions may be as a result of the presence of high levels of less active monohydroxylated phenolic compounds (Gallegos-Infante *et al.*, 2009). The high EC_{50} values observed for unhydrolysed AQ and MAW may be attributed to the viscous nature of the extracts which may have bound up phenol components, reducing antioxidant activity (Avila-Nava *et al.*, 2014). Hydrolysis served to release both extracts consistency and phenol constituents (Avila-Nava *et al.*, 2014). The application of hydrolysis thereafter explained the lower EC_{50} values of hydrolysed AQ (21.02-21.64 mg/mL) and MAW (18.39-19.11 mg/mL) extracts, compared to the unhydrolysed fractions (range from 206.4-710.4 mg/mL) (Table 3.2). Avila-Nava *et al.* (2014) also reported lower EC_{50} values for hydrolysed extractable phenol extracts compared to unhydrolysed extracts. Cactus cladode extracts showed a moderate antioxidant activity which could be related, at least partially, to the type and presence of phenolic compounds, phenolic acids and glycosides in the extracts (Khomdram and Singh, 2011).

The lowest FRAP values were observed for oven-dried and unhydrolysed AQ and MAW extracts at 31.42 µmol Fe (II)/g and 41.52 µmol Fe (II)/g respectively. The FRAP values, however, increased to 31.44 µmol Fe (II)/g and 61.60 µmol Fe (II)/g for the oven-dried and hydrolysed AQ and MAW extracts respectively (Table 3.2). This may be attributed to the release of phenols as a result of digestion during acid hydrolysis (Durazzo *et al.*, 2016). Freeze-dried ethanolic extract showed the highest FRAP value of 94.67 ± 2.65µmol Fe (II)/g of all unhydrolysed extracts. The hydrolysed MA extract, however, showed the highest antioxidant activity at 96.20±1.22 µmol Fe (II)/g (Table 3.2). Similarly, high antioxidant activities have been previously reported in nopal MA extracts (52.22 ± 1.07µmol trolox equivalent/g) (Bensadón *et al.*, 2010). It has been reported that *Opuntia* fruits and cladodes (Guevara-Figueroa *et al.*, 2010) contain flavonoids that are common in most fruits and vegetables (Kuti, 2004). The presence of isorhamnetin, kaempferol and other flavonoids has also been qualitatively demonstrated in nopal extracts (Avila-Nava *et al.*, 2014). The mechanism of action for antioxidant compounds in extracts has been linked with their ability to donate hydrogen or transfer electrons which halt/inhibit reactive oxygen species (ROS) generation (Bendary *et al.*, 2013). Generally, this study confirmed that hydrolysed extracts with considerable amounts of total phenolics showed good antioxidant activity compared to unhydrolysed extracts. Acid hydrolysis released phenols from bound extract matrix/residues which is consistent with the work of Avila-Nava *et al.* (2014).

Phenol content and antioxidant activities of the extracts also decreased when the oven-drying method was used compared to the freeze-drying method (Table 3.2). Freeze drying may have helped to achieve significant preservation of cladode phytochemicals. Reyes *et al.* (2011) also reported that there was no significant variation in antioxidant activity of freeze-dried blueberries compared with that of fresh fruits. The freeze-drying method was also found to be less aggressive than oven-drying methods in terms of retention of volatile and non-volatile

components (Torres *et al.*, 2010). In addition, a significant relationship (R^2=0.96) was observed between total phenol content and antioxidant activities of all unhydrolysed extracts (Fig. 3). This indicated that an increase in phenol content correlated with an increase in antioxidant activity in unhydrolysed fractions. A similar pattern was observed in a previous study (Avila-Nava *et al.*, 2014).

Table 3.2: Total phenol and antioxidant values of hydrolysed and unhydrolysed *Opuntia* nopal extracts

TPC of hydrolysed and unhydrolysed cladode extracts					
Unhydrolysed Extracts	Methanol: Acetone: Water (MAW)	Ethanol (E)	MethanoL (M)	Acidified methanol (AM)	Acidified water (AQ)
Freeze-dried (mg GAE/g)	5.02±1.10^{d}	10.46±2.30^{j}	8.61±1.21^{h}	7.04±1.43^{f}	2.12±0.31^{b}
Oven-dried (mg GAE/g)	2.74±0.15^{c}	9.86±1.52^{i}	7.23±2.49^{g}	6.86±0.40^{e}	1.07±0.07^{a}
Hydrolysed extracts	**AQ.FH**	**AQ.OH**	**MAW.FH**	**MAW.OH**	**MA**
Freeze and oven dried (mgGAE/g)	14.79±1.11^{e}	8.53±0.51^{b}	6.84±0.05^{a}	12.40±0.03^{d}	11.65±0.31^{c}
DPPH (EC_{50}) values of nopal extracts					
Unhydrolysed Extracts	Methanol: Acetone: Water (MAW)	Ethanol (E)	Methanol (M)	Acidified methanol (AM)	Acidified water (AQ)
Freeze-dried yield (EC_{50}, mg/mL)	206.4±0.12^{g}	65.8±0.05^{a}	70.9±0.01^{b}	82.5±0.02^{d}	496.1±0.23^{i}
Oven-dried (50°C) (EC_{50}, mg/mL)	310.3±0.03^{h}	73.3±0.02^{c}	87.1±0.10^{e}	98.9±0.02^{f}	710.4±0.01^{j}
Hydrolysed extracts	**AQ.FH**	**AQ.OH**	**MAW.FH**	**MAW.OH**	
DPPH (EC_{50}, mg/mL)	21.02±0.16^{d}	21.64±0.05^{e}	18.39±0.04^{b}	19.11±0.02^{c}	12.34±0.10^{a}
Total antioxidant (FRAP) activity of cladode extracts					
Freeze-dried (μmol Fe (II)/g)	49.68±1.60^{d}	94.67±2.65^{j}	87.17±2.33^{i}	67.17±2.49^{g}	39.19±0.40^{b}
Oven-dried (μmol Fe (II)/g)	41.52±0.15^{c}	68.06±1.52^{h}	62.18±2.49^{f}	56.04±0.40^{e}	31.42±0.07^{a}
Hydrolysed extracts	**AQ.FH**	**AQ.OH**	**MAW.FH**	**MAW.OH**	**MA**
FRAP (μmol Fe (II)/g)	39.51±0.51^{b}	31.44±1.02^{a}	49.73±1.11^{c}	61.60±2.01^{d}	96.20±1.22^{e}

Different superscripted letters ($^{a-j}$) indicate significant difference from one another ($p<0.05$).
Values are mean± standard deviation of three replicate determinations; TPC = total phenol content; AQ.FH = freeze-dried/hydrolysed acidified water extract; AQ.OH = oven-dried/hydrolysed acidified water extract; MAW.FH = freeze-dried/hydrolysed methanol:acetone:water extract; MAW.OH = oven-dried/hydrolysed methanol:acetone:water extract; MA= macromolecular antioxidant extract.

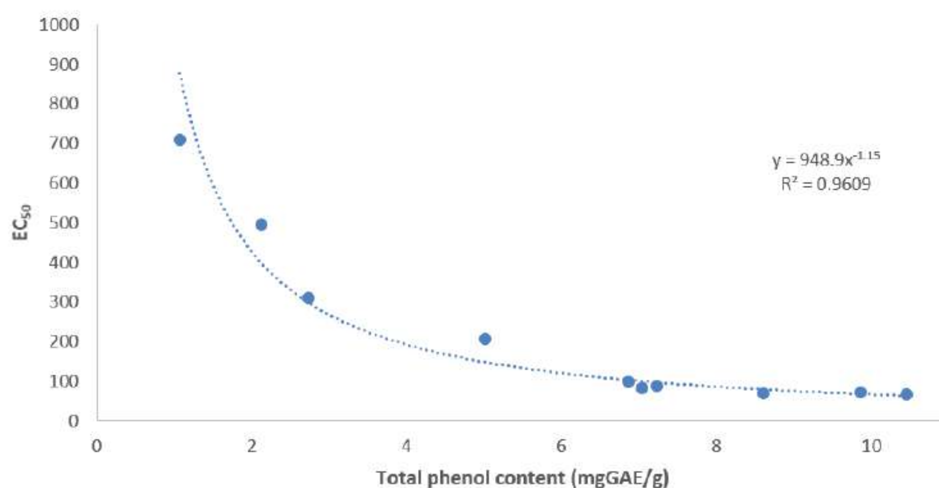

Figure 3: TPC correlation with EC_{50} (DPPH) values for all unhydrolysed cladode extracts

3.3.3. Antibacterial activity and MIC extracts

3.3.3.1. Antimicrobial activity of extracts

Extracts of *Opuntia* nopals showed antimicrobial activity against multi-drug resistant bacteria. Antibacterial zones of inhibition ranged from 10.0-24.0 mm and 12.0-35.1 mm for unhydrolysed and hydrolysed extracts, respectively (Table 3.3). *Klebsiella pneumonia* (20 mm) and *Listeria monocytogenes* (24 mm) were strongly inhibited by ethanol and acidified methanol extracts, respectively. *Enterococcus faecalis* was also strongly inhibited by methanol extract (20 mm) (Table 3.3). *E. faecalis* and MRSA were significantly inhibited with the largest zones at 34 mm and 35.1 mm, respectively, as observed from the MA extract (Table 3.3). For all the selected bacterial cultures, the largest zones (26-35.1 mm) were observed with MA at 300 mg/ml concentrations. Much lower antimicrobial activities were observed in unhydrolysed aqueous extracts compared to hydroalcoholic and hydrolysed cladode extracts.

Bari *et al.* (2012) demonstrated that methanol extracts of *Opuntia monacantha* cultivars showed the best antimicrobial activities against selected microorganisms. Plant extracts and their biologically active components (produced as secondary metabolites for plant defence systems against infection) have been used extensively as natural antimicrobials (Srinivasan *et al*., 2001). In this study, hydrolysed and hydroalcoholic extracts provided more powerful antimicrobial activity compared to unhydrolysed extracts. Low antimicrobial action in unhydrolysed fractions may be attributed to the type of solvent used, unreleased/bound phenols in extract matrices (Avila-Nava *et al*., 2014) and/or inability of extracted compounds to diffuse into the antibacterial assay medium (Kurek *et al*., 2011). On the other hand, the presence of more polar compounds and other non-polar active residues in hydroalcoholic and hydrolysed extracts may have contributed to the better antibacterial abilities observed *in vitro*. Comparable results showing that hydroalcoholic extracts have the best antimicrobial activity have also been reported by Preethi *et al*. (2010) with *Holarrhena antidysenterica* extracts. Seyydnejad *et al*. (2010) also showed that the difference in antimicrobial activity between different alcoholic extracts was due to the different compounds extracted.

Generally, Gram-positive bacterial cells possess peptidoglycan layers in their cell walls which facilitate the penetration of antimicrobial agents. On the other hand, the diffusion of hydrophobic compounds is restricted in Gram-negative bacteria because of the lipopolysaccharide outer membrane which increases antimicrobial resistance. However, the presence of hydrolysable polyphenols in MA extract and phenols in hydrolysed and unhydrolysed extracts could have contributed to the antibacterial activity of extracts on both Gram-positive and Gram-negative microorganisms in this study. Gallic, piscidic, eucomic, ferulic and coumaric phenolic acids, as well as nicotiflorin, isorharmnetin 3-*O*-glucoside and rutin flavonoids have been identified in nopals of *Opuntia* species. Most of these compounds possess antimicrobial activities and are aromatic, hydroxylated bioactive compounds (Guevara-

Figueroa *et al*., 2010; Kurek *et al*., 2011) and saturated organic molecules (Ciocan and Bara, 2007). The site and number of hydroxyl groups in antimicrobial compounds determine microbial toxicity/action (Ciocan and Bara, 2007). Some antimicrobials act by complexing with extracellular and soluble proteins and affect microbial cell membrane and wall integrity causing static or cidal effects. Others like coumarins with fused benzene and α-pyrone rings have similar antimicrobial mechanisms. Tannins/polymeric phenols act by binding cell walls, inactivating microbial enzymes and transport proteins, thereby preventing growth and protease activity (Ciocan and Bara, 2007).

Freeze-dried extracts showed better antibacterial profiles which may be due to better retention of phenolic compounds in extracts compared to oven-dried extracts (Torres *et al*., 2010). Antibacterial activity did not always correlate with the total phenolic content of extracts as observed with AM, AQ and MAW extracts (Table 3.3). Medina *et al*. (2006) also reported that polyphenols content did not always correspond with antimicrobial activities. Poor antibacterial activity has also been reported for *Opuntia* aqueous extracts and linked to poor solubility of biologically active components (Gnanakalai and Gopal, 2016). Results of this study showed a broader spectrum of antimicrobial activity for MA extract against bacteria of food and medical importance compared to EP/unhydrolysed extracts. This may be linked to the presence and/or high content of polyhydroxylated phenols in the MA extract (Ciocan and Bara, 2007). Nopal extracts could be useful in the control of highly resistant foodborne bacteria (Salmonellae, *L. monocytogenes*), as well as those of medical importance (*P. aeruginosa*, MRSA). However, nontoxic techniques must be developed for extraction of active components before they can be used for industrial applications.

3.3.3.2. MIC of cladode extracts

MICs for nopal extracts ranged from 50 to 300 mg/mL for unhydrolysed, and 25 to 250 mg/mL for hydrolysed extracts (Table 3.3). MA extract had the lowest effective MICs between 25 and 50 mg/mL for all bacteria tested, while for other hydrolysed extracts, MICs ranged from 25 (for *K. pneumonia*, A. *baumannii*) to 250 mg/mL (for methicillin-resistant *S. aureus*) (Table 3.3). A similar but closer range of MICs (125-250 mg/mL) have been reported for betalain-rich *Opuntia* extracts against *B. cereus, L. monocytogenes, Proteus vulgaris and Enterobacter aerogenes* (Tenore *et al*., 2012). On the other hand, a lower MIC range (1.25-2.5 mg/ml) was reported for *Opuntia* fruit extract against *E. coli, B. cereus, P. aeruginosa* (Moosazadeh *et al*., 2014) while a 40 mg/mL MIC for *Opuntia* cladode extract against viruses (Rasoulpour *et al*., 2017) was also recently demonstrated. Variation in MIC reports may be attributed to minor differences in techniques applied such as concentration range used, length of incubation and inoculum size used (Kalil *et al*., 2014; Moosazadeh *et al*., 2014). MA extracts from insoluble food pomace/extraction residues have been reported to contain a considerable number of bioactive compounds such as polyphenols (Saura-Calixto, 2010). The release of polyphenols has been reported from fibrous matrices and residues during acid hydrolysis and positively impacted the biological activities of extracts derived from them (Bensadón *et al*., 2010). Extract contents of polyhydroxylated compounds, phenolic acids, flavonoid derivatives and other unidentified compounds may be responsible for the broad-spectrum antimicrobial activity reported in this study.

Table 3.3: Antibacterial inhibition zone and MIC of hydrolysed and unhydrolysed *Opuntia* nopal extracts

Microorganisms	Unhydrolysed freeze-dried extracts [inhibition zone diameter (mm); and minimum inhibitory concentration (MIC - mg/mL)]									
	A		B		C		D		E	
	Zone (mm)	**MIC**	**Zone (mm)**	**MIC**	**Zone (mm)**	**MIC**	**Zone (mm)**	**MIC**	**Zone (mm)**	**MIC**
Klebsiella pneumonia (G-)	20.1±0.0^{a}	100	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa (G-)	16.0±0.2^{c}	100	-	-	14.0±0.1^{b}	200	-	-	-	-
Escherichia coli (G-)	18.0±0.1^{b}	100	-	-	-	-	-	-	-	-
Bacillus cereus (G+)	18.1±0.1^{b}	150	-	-	-	-	-	-	-	-
Listeria monocytogenes (G+)	10.0±0.0^{d}	200	18.1±0.0^{b}	100	16.1±0.0^{a}	150	24.0±0.0^{a}	100	-	-
Acinetobacter baumannii (G-)	-	-	16.0±0.0^{c}	50	-	-	14.1±0.0^{b}	200	-	-
Enterobacter cloacae (G-)	-	-	16.2±0.1^{c}	50	-	-	-	-	16.0±0.1^{a}	200
Enterococcus faecalis (G+)	-	-	20.1±0.0^{a}	100	-	-	-	-	-	-
Staphylococcus aureus (G+)	18.0±0.2^{b}	100	-	-	12.2±0.0^{c}	200	-	-	-	-
methicillin resistant *Staph. aureus* (G+)	18.0±0.1^{b}	200	-	-	-	-	-	-	-	-
Unhydrolysed oven-dried extracts										
P. aeruginosa	-	-	14.0±0.1^{b}	150	-	-	14.0±0.1^{b}	150	-	-
E. coli	-	-	-	-	-	-	12.0±0.1^{d}	250	-	-
B. cereus	20.0±0.1^{a}	50	20.0±0.1^{a}	100	16±0.1^{a}	200	13.3±0.1^{c}	200	-	-
Salmonella arizonae (G-)	12.2±0.0^{c}	200	-	-	-	-	12.2±0.0^{d}	200	-	-
A. baumannii	-	-	-	-	-	-	14.1±0.2^{b}	200	-	-
E. cloacae	-	-	-	-	-	-	12.0±0.1^{d}	150	12.2±0.1^{b}	200
E. faecalis	-	-	-	-	-	-	-	-	-	-
S. aureus	18.1±0.1^{b}	200	12.2±0.0^{c}	150	-	-	20.0±0.1^{a}	100	-	-
MRSA	-	-	-	-	-	-	10.1±0.0^{e}	200	13.2±0.1^{a}	250
Hydrolysed freeze- and oven-dried extracts										
	AQ.FH		**AQ.OH**		**MAW.FH**		**MAW.OH**		**MA**	
	Zone (mm)	**MIC**	**Zone (mm)**	**MIC**	**Zone (mm)**	**MIC**	**Zone (mm)**	**MIC**	**Zone (mm)**	**MIC**
K. pneumonia	19.0±0.1^{c}	25	17.0±0.1^{c}	100	16.0±0.1^{d}	150	13.1±0.0^{c}	150	28.0±0.1^{g}	50
P. aeruginosa	19.1±0.1^{c}	50	17.0±0.3^{c}	100	22.1±0.0^{a}	100	19.2±0.1^{a}	100	32.1±0.1^{d}	25
E. coli	19.0±0.0^{c}	50	20.1±0.0^{a}	50	18.1±0.0^{c}	100	18.1±0.1^{a}	100	26.0±0.2^{h}	50
B. cereus	-	-	-	-	-	-	-	-	34.0±0.1^{b}	25
S. arizonae	20.2±0.1^{b}	50	-	-	-	-	-	-	30.2±0.0^{e}	25
L. monocytogenes	-	-	-	-	-	-	-	-	32.0±0.0^{d}	25
A. baumannii	17.0±0.1^{d}	50	-	-	12.0±0.2^{e}	25	-	-	33.0±0.1^{c}	25
E. cloacae	20.1±0.1^{b}	50	20.0±0.1^{a}	50	18.0±0.0^{c}	100	18.0±0.1^{a}	100	29.2±0.0^{f}	25
E. faecalis	22.0±0.0^{a}	50	20.1±0.1^{a}	100	21.2±0.1^{a}	50	17.2±0.1^{b}	100	34.0±0.2^{b}	25
S. aureus	19.0±0.2^{c}	50	18.2±0.0^{b}	100	16.1±0.2^{d}	100	18.1±0.0^{a}	100	31.1±0.1^{e}	25
MRSA	20.0±0.1^{b}	100	20.1±0.1^{a}	150	19.0±0.1^{b}	100	18.0±0.1^{a}	150	35.1±0.1^{a}	25

MIC = minimum inhibitory concentration; G+ = Gram-positive; G- = Gram negative; A = ethanol extract; B = methanol extract; C = methanol:acetone:water extract; D = acidified methanol extract; E = acidified water;

AQ.FH = freeze-dried/hydrolysed acidified water extract; AQ.OH = oven-dried/hydrolysed acidified water extract; MAW.FH = freeze-dried/hydrolysed methanol:acetone:water extract; MAW.OH = oven-dried/hydrolysed methanol:acetone:water extract; MA = macromolecular antioxidant extract; Values are mean of three replicate determinations.

3.3.4. Mass spectral characteristics of profiled cladode extract compounds

LC/MS is a useful and sensitive method for derivation of elemental composition of ions and accurate mass measurements which are useful for the discovery/recognition of unknown compounds. Molecular ions mass data (elemental compositions and mass errors) were processed using Microsoft Excel program. Phenolic compounds mass spectral data and formulae were shown in Table 3.4. Compounds detected in the MA fraction included polyhydroxylated and/or glycosylated flavonoid derivatives, alkanes or lipids and caffeic acid derivatives. Flavonoid derivatives and phenolic acids were identified in the other extracts. The errors obtained were between -5.6 and 4.6 ppm which fell within the generally accepted threshold (Table 3.4). An acceptable limit for accurate measurement of mass is ≤ 5 ppm for the verification of the elemental compositions (Chen *et al.*, 2011). Almost all extracts contained piscidic and eucomic acids, followed by flavonoid glucosides which were mainly present in hydroalcoholic extracts (Table 3.5). The presence of these compounds with a total percentage occurrence of 68.93% compared to other unidentified components which constituted about 31.07% may be responsible for the antioxidant and antibacterial activities recorded in this study. Phenolic acids (eucomic, piscidic and coumaric acids) (Astello-García *et al.*, 2015), as well as glycosylated flavonoids, have been reported in *Opuntia* cladodes and exhibit *in vitro* biological activities (Santos-Zea *et al.*, 2011). However, this is the first report of isovitexin 7-*O*-xyloside-2"-*O*-glucoside in *Opuntia* cladode EP extract, and pregnane glycoside and neohancoside C in a cladode MA fraction. The pharmacological efficacy (anti-inflammatory and antioxidant activities) of isovitexin in medicinal plants has been reported (Ganesan and Xu, 2017; He *et al.*, 2016). Isovitexin has been associated with rhizosphere soils rich in humic matter (Cesco *et al.*,

2012) and may have been bioaccumulated from the rich garden soil in which the cladodes were grown. The flavonoid profile reported in this study could be used in southern African *Opuntia* cladode cultivar identification. Research has shown that nopal varieties from different regions/locations have different flavonoid types/profiles. This observation has proved to be useful in classification and taxonomic identification of *Opuntia* cultivars (Moussa-Ayoub *et al.*, 2014). However, there is a need for more sensitive analytical method(s) to reduce the number of unidentified compounds. Several new bioactive (neuroprotective) and structurally diverse forms of pregnane glycosides have also been reported earlier but in Asian *Cynanchum otophyllum* weed (Zhao *et al.*, 2013). The novel phenol glycoside, neohancoside C, has been reported, but in Chinese medicinal plants, *Cynanchum inamoenum* and *C. hancockianum* and demonstrated antitumor activity (Konda *et al.*, 1997; Wang *et al.*, 2008). Therefore, cladode extracts contain compounds that may be useful for functional food applications.

Table 3.4: Mass spectral properties of compounds in the *Opuntia* cladode extracts

Retention time (min)	M-H	M-H formula	ppm error (<5ppm)	UV	Tentative Identification
EP extracts					
6.07	353.10	$C_{13}H_{21}O_{11}$	0.6	280	Unidentified
8.43	255.05	$C_{11}H_{11}O_7$	0.4	275	Piscidic acid
10.73	269.06	$C_{12}H_{13}O_7$	1.5	275	Unidentified
12.20	269.06	$C_{12}H_{13}O_7$	1.5	275	Unidentified
12.20	163.03	$C_9H_7O_3$	-0.1	281	Coumaric acid
14.73	303.03	$C_{18}H_7O_5$	4.6	279	Unidentified
12.40	239.05	$C_{11}H_{11}O_6$	0.4	275	Eucomic acid
15.83	363.07	$C_{17}H_{15}O_9$	1.9	292	Unidentified
17.23	725.19	$C_{32}H_{37}O_{19}$	1.8	352	Isovitexin 7-O-xyloside-2"-O-glucoside
17.45	755.21	$C_{34}H_{41}O_{20}$	3.1	352	Quercetin-3-rhamninoside
18.43	609.14	$C_{27}H_{29}O_{16}$	1.8	353	Kaempferol-3,7-*O*-diglucoside
18.64	405.17	$C_{18}H_{29}O_{10}$	0.7	324	Unidentified
19.65	623.16	$C_{28}H_{31}O_{16}$	2.4		Isorhamnetin 3-*O*-glucoside 7-*O*-rhamnoside
MA extract					
4.10	415.12	$C_{18}H_{23}O_{11}$	-1.4	-	Polyhydroxypregnane glycoside
4.81	429.13	$C_{19}H_{25}O_{11}$	-3.3	-	Neohancoside C
5.62	197.05	$C_6H_{13}O_5S_1$	-5.6	-	Caffeic acid hydrate metabolite
9.61	425.22	$C_{19}H_{37}O_8S$	0.2	-	Unidentified
10.80	439.23	$C_{20}H_{39}O_8S$	0.9	-	Isorhamnetin derivative
10.99	473.12	$C_{20}H_{25}O_{13}$	-0.4	-	Glycosyl flavanone
15.60	719.53	-	-	280	Straight chain alkane or lipid
16.81	733.55	-	-	280	Straight chain alkane or lipid
17.32	735.59	-	-	280	Straight chain alkane or lipid

Table 3.5: Identified and unidentified compounds in extractable polyphenol cladode extracts

	353.11	255.05	269.11	269.07	163.04	303	239.05	363	725.19	609.16	405.18	623.16	755.20
Extract/ Compounds	UI.	Piscidic acid	UI.	UI.	p-coumaric acid	UI.	Eucomic acid	UI.	Isovitexin 7-*O*-xyloside-2''-*O*-glucoside	Kaempferol-3,7-*O*-diglucoside	UI.	Isorhamnetin 3-*O*-glucoside 7-*O*-rhamnoside	Quercetin-3-rhamninoside
E.O	-	+	-	-	+	-	+	-	+	+	+	+	-
E.F	-	+	-	-	+	-	+	-	+	+	+	+	-
M.O	-	+	-	-	+	-	+	-	+	+	+	+	-
M.F	-	+	-	-	+	-	+	-	+	+	+	+	-
AM.O	+	+	+	-	+	-	+	-	+	+	+	+	+
AM.F	-	+	+	-	+	-	+	-	+	+	+	+	+
AQ.O	-	+	-	-	-	-	+	-	-	-	-	-	-
AQ.F	-	+	-	-	+	-	+	-	-	-	-	+	-
MAW.O	-	+	-	-	+	-	+	-	+	+	+	+	+
MAW.F	-	+	+	-	+	-	+	-	+	+	+	+	+
AQ.OH	+	+	+	+	-	+	+	+	-	-	-	-	-
AQ.FH	+	+	+	+	-	+	+	+	-	-	-	-	-
MAW.FH	+	+	+	+	-	+	+	-	+	+	+	+	+
MAW.OH	+	+	+	+	-	-	+	-	-	-	-	-	-

UI = unidentified; E.O = oven dried ethanol extract; E.F = freeze-dried ethanol extract; M.O = oven-dried methanol extract; M.F = freeze-dried methanol extract; AM.O = oven-dried acidified methanol extract; AM.F = freeze-dried acidified methanol extract; AQ.O = oven-dried acidified water; AQ.F = freeze-dried acidified water; MAW.O = oven-dried methanol:acetone:water extract; MAW.F = freeze-dried methanol:acetone:water extract; AQ.OH = oven-dried/hydrolysed acidified water extract; AQ.FH = freeze-dried/hydrolysed acidified water extract; MAW.FH = freeze-dried/hydrolysed methanol:acetone:water extract; MAW.OH = oven-dried/hydrolysed methanol:acetone:water extract; MA = macromolecular antioxidant extract.

3.4. Conclusion

The phenolic, antioxidant and antimicrobial activities of extracts from freeze-dried *Opuntia ficus-indica* cladodes were significantly better than those of oven-dried fractions. MA and other hydrolysed extracts showed improved bioactivity profiles compared to the unhydrolysed/extractable phenol fractions. Besides the identified compounds, unidentified compounds also contribute to the biological activities of the extracts. Cladode extracts have potential economic and health benefits and could be utilised as nutraceuticals and food preservatives.

CHAPTER FOUR

Phenolic compound profile and biological activities of Southern African *Opuntia ficus-indica* fruit pulp and peels

Abstract

Opuntia species have been used in traditional medicine for decades and research on *Opuntia* plants and their by-product valorisation are also on the increase. This study investigated the phenolic compound profiles and biological activities of *Opuntia* prickly pear fruit and peel extracts and the effect of drying methods on extract yields and bioactivities. Ethanolic, methanolic and hexane extracts of freeze-dried *Opuntia* peels showed better *in vitro* antioxidant and antimicrobial activities compared to oven-dried peel and fruit pulp extracts. In most cases, freeze-dried samples also showed better extract yields. Extracts antibacterial activity were better against Gram-positive (inhibition zone range of 8.0-18.8 mm) compared to Gram-negative (inhibition zone range of 10.0-14.0 mm) bacterial strains. Minimum inhibitory concentrations (MICs) ranged from 2.50-18.75 mg/mL. The presence of pinellic acid is reported for the first time in *Opuntia* fruit extracts. Phenolic acids, flavonols and flavonoid derivatives were also identified. *Opuntia* peel and fruit pulp extracts were rich in biologically active phenolic compounds and could have potential nutraceutical and food industry applications.

4.1. Introduction

The safety concerns associated with synthetic antioxidants and the increasing global trend in antimicrobial resistance has necessitated investigations into new, but safe and natural bioactive molecules in plant extracts with antioxidant (Zrira *et al.*, 2016) and antimicrobial properties (Koubaa *et al.*, 2015b). *Opuntia* plant parts and by-products have recently been attracting a lot

of research interest and may be integral to the discovery of new and natural plant bioactive compounds. The prickly pear (*Opuntia* spp.) contains biologically active compounds and has found use in the management of non-communicable diseases such as diabetes (Tesoriere *et al*., 2005). Most investigations have focussed on the fruit pulp as a source of bioactive compounds (Zrira *et al*., 2016). The fruit peels have largely been ignored despite indications that they contain significant amounts of bioactive compounds (Milán-Noris *et al*., 2016). *Opuntia* peels make up 60% of the entire fruit but are usually not consumed by humans (Milán-Noris *et al*., 2016). Therefore, *Opuntia* peel by-products are often disposed of after fruit consumption (Ramadan and Mörsel, 2003a). The production of nutraceuticals from plant by-products such as *Opuntia* peels using food processing techniques will continue to expand as a cheap and cost-effective alternative (Shelembe *et al*., 2014; Torres *et al*., 2010). For example, *Opuntia* peels can be used as food or functional food supplements to enhance their benefits for human/animal health (Sepúlveda *et al*., 2018).

An important group of compounds associated with *O. ficus-indica* is polyphenols (Khatabi *et al*., 2011) which possess antioxidative and antimicrobial properties. Several reports have demonstrated a strong link between phenol content and antioxidant activity of extracts (Khatabi *et al*., 2011; Kuti, 2004). Although studies have reported antimicrobial and antioxidant activities of extractable polyphenol extracts from *Opuntia* spp. (Anwar and Sallam, 2016), scientific data supporting plant by-product valorisation are on the increase. Most research data on *Opuntia* fruits are generated from Mexico in the North American region (Castellanos-Santiago and Yahia, 2008; Milán-Noris *et al*., 2016), the temperate (Yeddes *et al*., 2013) and Mediterranean regions (Moussa-Ayoub *et al*., 2014; Zrira *et al*., 2016). In addition, most studies have shown that processing methods (Torres *et al*., 2010) and extraction solvents used (Abou-Elella and Ali, 2014) could affect yield, biological activity and phenolic compound profile of extracts.

The *Opuntia* plant is most commonly used as fodder in southern Africa (Snyman *et al*., 2007), and with regard to current knowledge as the time of this study, no data is available on the phenolic compound profile of southern African *Opuntia ficus-indica* fruit pulp and peel by-product extracts. Plant extract profiling increase the probability of new biomolecules discovery. Novel biomolecules could also serve as potential substrates for biotechnological applications (Kudanga *et al.*, 2017). However, bioactive molecule distribution, as well as biological properties of compounds in food materials such as *Opuntia* pear fruit and peels may be affected by cultivar/genetic factors, agronomic practices, environmental and climatic conditions (Moussa-Ayoub *et al*., 2014). Therefore, this study investigated the phenolic compound profile and *in vitro* biological activities of subtropical *Opuntia* fruit and peel extracts. In addition, the study reports the effect of drying methods and extraction solvent on the extract yield and biological activities and phenolic compound types identified.

4.2. Materials and methods

All standards and reagents used in this study were obtained from Sigma-Aldrich (South Africa). Other chemicals (and culture media) were obtained from Oxoid (UK) unless otherwise mentioned. All solvents and chemicals used were of analytical grade.

4.2.1. Culture and maintenance of microorganisms

The same pure cultures of Gram-positive and Gram-negative bacteria that were listed in Chapter three, subsection 3.2.2 were utilised. Bacterial stock cultures kept on agar slants and at 4°C were used to prepare overnight broth cultures (18-24 h) for use in experimental determinations (Sen and Batra, 2013).

4.2.2. Plant material collection and preparation

Opuntia fruits were manually harvested from a local orchard in Durban, Kwazulu-Natal province, South Africa. Fruits were washed in distilled water and sanitised with 80% ethanol. Fruit peels were removed, and pulp separated from seeds. Pulp and peels were cut into smaller pieces (2×0.5 cm) and oven-dried at 45°C for 24 h. Dehydrated peels and fruit pulp were dry milled in a blender and the powder (<2 mm particle size) stored in a closed container at 4°C. Separate wet milled pulp and peels were also freeze-dried for 24 h and thereafter stored in an airtight container at 4°C.

4.2.3. *Opuntia* fruit and peel phenols extraction

Peel and fruit extraction were carried out according to Abou-Elella and Ali (2014) with some modifications. Polar (ethanol, methanol, water) and non-polar (hexane) solvents were used. The polar solvents were used in acidified form to facilitate release of phenolic compounds (Shelembe *et al.*, 2014). Dried plant materials (5.0 g) were mixed with 50 mL of different extraction solvents [70% ethanol, acidified methanol, hexane, acidified water] in 250 mL flasks and shaken at 22-25°C for 18-24 h. The mashed mixtures were filtered with Whatman No.1 filter paper, and filtrate solvents were evaporated in a rotary evaporator at 45°C and 60 rpm (Abou-Elella and Ali, 2014). Extracts from acidified water were thereafter subjected to acid hydrolysis for 3 h using 5 mL of concentrated HCl. The resulting extracts were thereafter evaporated to dryness (oven dried). Acid hydrolysis was carried out to release the viscous/gel-like constituency of extracts for better determination of phenolic profile and biological activities. A total of sixteen extracts were obtained (four each from freeze-dried and oven-dried peels, freeze-dried and oven-dried fruit pulp). The yield (%, w/w) of each extract was calculated using the formula previously outlined in chapter three, subsection 3.2.4.

4.2.4. Total phenolic content (TPC) determinations

Phenolic content of *Opuntia* peel and pulp extracts were determined using the Folin-Ciocalteau method according to Milán-Noris *et al*. (2016). The method followed was the same as that described in chapter three, subsection 3.2.5. Total phenolic content of extracts expressed as milligram gallic acid equivalent per gram of extract (mg GAE/g).

4.2.5. Determination of antioxidant capacity

4.2.5.1. DPPH radical scavenging activity of extracts

In carrying out the DPPH assay, 50 µL of each extract (1-5 mg/mL) and 5 mL of 0.004% (w/v) solution of DPPH was mixed, vortexed and incubated in the dark for 30 min at room temperature. Then, absorbance readings were taken and EC_{50} values per sample calculated as described in chapter three, subsection 3.2.6.1.

4.2.5.2. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing power of derived peel and pulp extracts was determined as described in chapter three, subsection 3.2.6.2. The assay involved measurement of blue 2,4,6-tripyridyl-s-triazine complex formed at 593 nm which indicted reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions at low pH. FRAP determination was performed in triplicate. Results were expressed in μM (Fe (II)/g dry mass.

4.2.6. Antibacterial activity of fruit pulp and peel extracts

4.2.6.1. Agar well diffusion assay

The agar well diffusion method was used in antibacterial activity determination for pulp and peel extracts. A similar protocol described in chapter three, subsection 3.2.7.1 was followed. Respective extracts resuspension solvents were used as negative controls. After incubation, the diameter of the zone of inhibition around each well was measured to the nearest mm. The experiment was performed in triplicate.

4.2.6.2. Extracts MIC determination

MICs of extracts were determined using the broth microdilution method of Sen and Batra (2013) in sterile 96-well polystyrene U-shaped microtiter plates (Corning, Costar Cambridge, Mass., U.S.A.). The method followed was described in chapter three, subsection 3.2.7.2 with optical density (OD) readings taken at 600 nm alongside negative and positive controls in different wells. The experiment was replicated three times. The lowest concentration of the extract that inhibited growth was recorded as the MIC.

4.2.7. Extract compound profiling by LC-ESI-TOF/MS analysis

Extract samples were filtered through 0.2 mm syringe filters (VWR, Radnor, PA, USA), dried and resuspended in methanol for analysis. The method, chromatographic gradient/conditions and mobile phases used were described in chapter three, subsection 3.2.8. Individual compounds were identified based on retention time, UV spectra, and mass-to-charge ratio

(Yeddes *et al.*, 2013). Assignation of probable identity was done after cross reference with databases and literature consultation.

4.2.8. Statistical analysis

Results were reported as a mean ± standard deviation (SD). Data were subjected to one-way analysis of variance (ANOVA) and sample differences were determined by Duncan's Multiple Range test using SPSS version 16. P values < 0.05 were regarded as significant.

4.3. Results and discussion

4.3.1. *Opuntia* fruit pulp and extract yields

The yields of fruit pulp extracts were in the range 0.64-40.76%, while peel extracts yield ranged from 0.80-37.60%. Freeze-dried extract yields were higher and ranged from 0.64-40.76% compared to 0.34-32.40% from oven dried extracts (Table 4.1). For the same extraction solvent used, yields varied across plant material type and processing method applied. Ethanol (40.76%) and water (37.6%) extracts had the highest yields of freeze-dried samples, while hexane extracts generally had the lowest yields (0.34-0.8%).

Abou-Elella and Ali (2014) reported highest extract yield of 64.7% for *Opuntia ficus-indica* ethanolic peel extract, followed by 50.7% yield for methanol peel extracts, and less for water peel extract (36.12%). In this study, high polarity solvents showed high extract yields. A study has shown that extract yields significantly correlate with extraction solvent polarity such that high polarity solvents show better extraction yields (López-Vélez *et al.*, 2003). Extraction

solvents allow dissolution of different solutes. Polar compounds such as polyphenols are more likely to be expressed in solvents with high polarity index (PI) such as water, ethanol and methanol. Polarity index measures the relative ability of a solvent to allow the expression of polar solutes. In other words, a high yield/expression of polyphenols would be expected in a high PI solvent compared to a non-polar solvent with low PI (Abou-Elella and Ali, 2014). Phenols are usually better expressed in a mix of polar solvents such as aqueous ethanol and methanol (Abou-Elella and Ali, 2014). On the other hand, less polar compounds would be better expressed in low PI or non-polar solvents. Therefore, the solubility of phytochemical groups and solutes in different solvents depends on the solute selectivity power and polarity of the solvent (Abou-Elella and Ali, 2014). Hexane is a non-polar solvent used in the extraction of phytosterols. A significantly low yield was obtained probably due to the low phytosterol content of the *Opuntia* fruits, as well as the inability of hexane to allow expression of more polar solutes due to its low polarity index (Abou-Elella and Ali, 2014). Also, drying temperature and drying time affect extract yields (Torres *et al.*, 2010). Although in this study oven drying was carried out at relatively mild conditions of 45°C for 24 h which was not expected to significantly affect yields (Reyes *et al.*, 2011), yields were still lower than in freeze-dried samples. Freeze-drying and temperatures ≤50°C have been reported to minimise phytochemical losses and give better yields (Reyes *et al.*, 2011).

Table 4.1: Yield of *Opuntia ficus-indica* fruit and peel extracts. Values expressed as %, w/w.

Extracts	Peel/Freeze-dried	Peel/Oven-dried	Fruit pulp/Freeze-dried	Fruit pulp/Oven-dried
Ethanol	25.47±1.26^{c}	18.61±1.11^{d}	40.76±1.61^{a}	29.96±1.01^{b}
Acidified methanol	17.45±0.78^{c}	10.80±0.88^{d}	34.27±0.83^{a}	25.93±0.86^{b}
Hexane	0.80±0.34^{b}	1.14±0.62^{a}	0.64±0.22^{c}	0.34±0.08^{d}
Acidified water	37.60±2.51^{a}	32.40±3.44^{b}	25.10±1.52^{d}	27.00±1.34^{c}

Values are mean of three replicates ± standard deviation (n=3).
Values with different superscripts (a, b, c, d) in the same row are significantly different ($p<0.05$) as shown by Duncan's multiple range test.

4.3.2. TPC of *Opuntia* fruit pulp and peel extracts

The TPC of extracts generally decreased in the order ethanol > methanol > hexane > water. The least TPC was recorded for freeze- and oven-dried fruit pulp water extracts at 4.25 and 4.32 mg GAE/g, respectively. The highest TPC was recorded in oven-dried peel ethanol and freeze-dried peel methanol extracts at 17.59 and 16.51 mg GAE/g, respectively (Table 4.2).

O. ficus-indica fruits show significant variation in phenolic content. Variations in TPC of extracts may be linked to the phenolic component type present in extracts, as well as differences in processing methods and solvents used (Chavez-Santoscoy *et al.*, 2009). Fruit maturity, climate and quantification methodologies also affect TPC of plant extracts (Anwar and Sallam, 2016). Higher TPC were expressed with hydroalcoholic solvents possibly because hydroalcoholic solvents have high PI and allow the expression of polar and non-polar compounds (Abou-Elella and Ali, 2014).

4.3.3. Antioxidant activity of extracts

Hexane extracts generally showed the lowest EC_{50} of DPPH values (15.59-80.22 mg/mL) and had the highest antioxidant activity. Freeze and oven-dried peel extracts generally had lower EC_{50} values compared to freeze- and oven-dried fruit pulp extracts (Table 4.2). Freeze and oven-dried ethanol and methanol pulp extracts showed the least ability to quench the DPPH radical. These extracts showed the highest EC_{50} values ranging from 269.11-311.43 mg/mL (Table 4.2).

A decrease in FRAP of extracts was observed to follow the sequence, hexane > methanol > ethanol > water in most cases. Significant differences in antioxidant activities were observed across same solvent and plant material/processing method applied. Highest FRAP was observed

for hexane at 192.49 and 175.44 µmol Fe (II)/g for oven-dried and freeze-dried peel extracts, respectively, and least for water-extracted and oven-dried pulp extract at 15.49 µmol Fe (II)/g (Table 4.2).

Polyphenols in *Opuntia* fruit (Kim *et al.*, 2013) may have contributed to the antioxidant activity of extracts reported in this study. Phenolic antioxidants in extracts can block oxidative reactions caused by free radical generation through the ability to transfer hydrogen atoms or electrons, which may be detected in antioxidant assays (Kim *et al.*, 2013). Antioxidant activity values are also a function of phenol types and site and number of hydroxyl groups present. Usually the higher the number of hydroxyl groups, the better the antioxidant power (Gallegos-Infante *et al.*, 2009; Ravichandran *et al.*, 2012). Polyhydroxylated flavonoids and phenolic acids occurred frequently in our ethanolic and methanolic *Opuntia* extracts. However, the presence of flavonoid glycosides in ethanolic and methanolic pulp and peel extracts may have contributed to their reduced antioxidant activities (Table 4.2). Glycosylated polyphenols have reduced ability to donate hydrogen and are less effective as antioxidants compared to their free aglycone forms (Williams *et al.*, 2004). Flavonoids have also been previously reported in *Opuntia* peels (Koubaa *et al.* 2015a) and fruits (Hahm *et al.*, 2015).

On the other hand, high TPC does not always translate into high bioactivity. Mexican *Opuntia robusta* fruits with high phenol content showed low antioxidant activity, and yellow *O. albi-carpa* fruits with reduced TPC demonstrated weak antioxidant activity (Yahia and Mondragon-Jacobo, 2011). Extract biological activities may be attributed to a single extract component, or the synergistic effect of several extract components (Castellanos-Santiago and Yahia, 2008).

Table 4.2: Total phenolic content and antioxidant activities of *Opuntia ficus-indica* fruit and peel extracts.

Extracts	Peel/Freeze-dried	Pulp/Freeze-dried	Peel/Oven-dried	Pulp/Oven-dried
Total phenol content (mg GAE/g)				
Ethanol	15.13±0.01^{b}	14.83±0.10^{a}	17.59±0.02^{a}	14.39±0.02^{a}
Acidified methanol	16.51±0.01^{a}	9.94±0.01^{b}	13.36±0.03^{b}	9.22±0.02^{b}
Hexane	10.99±0.07^{c}	8.61±0.01^{c}	8.35±0.03^{c}	5.24±0.01^{c}
Acidified water	9.10±0.06^{d}	4.25±0.06^{d}	7.85±0.08^{d}	4.32±0.02^{d}
DPPH (EC_{50}, mg/mL) of extracts				
Ethanol	81.84±0.10^{b}	311.43±0.14^{a}	87.08±0.24^{b}	269.19±0.03^{b}
Acidified methanol	58.99±0.07^{c}	301.87±0.62^{b}	48.11±0.23^{c}	307.94±0.64^{a}
Hexane	16.89±0.01^{d}	80.22±0.17^{d}	15.59±0.40^{d}	60.97±0.13^{d}
Acidified water	145.14±0.32^{a}	132.28±0.10^{c}	131.05±0.07^{a}	131.26±0.12^{c}
Total antioxidant [FRAP, µmol Fe (II)/g] activity of extracts				
Ethanol	60.96±0.09^{c}	18.42±0.01^{d}	93.74±0.18^{c}	27.01±0.15^{c}
Acidified methanol	143.38±0.08^{b}	19.83±0.01^{c}	111.65±0.01^{b}	58.66±0.03^{b}
Hexane	175.44±0.07^{a}	137.65±0.02^{a}	192.49±0.44^{a}	71.24±0.33^{a}
Acidified water	58.70±0.09^{d}	22.95±0.12^{b}	40.68±0.09^{d}	15.49±0.18^{d}

Value are mean of three replicates ± standard deviation (n=3).
Values with different superscripts (a, b, c, d) in the same column are significantly different as shown by Duncan's multiple range test $p<0.05$.

4.3.4. Antimicrobial activity of pulp and peel extracts

The highest zone of inhibition (18.8 mm) was observed against Gram-positive MRSA for methanolic peel/freeze-dried extract (Table 4.3). *P. aeruginosa* was the most inhibited of the Gram-negative microorganisms with a 14.0 mm zone of inhibition observed when using aqueous oven-dried extract. Other strains were also inhibited to varying degrees by peel and pulp extracts (Table 4.3). Minimum inhibitory concentrations of the extracts were in the range of 2.50-18.75 mg/mL (Table 4.3).

In most cases in this study, freeze and oven-dried hydroalcoholic extracts showed better antimicrobial activity compared to aqueous extracts. This observation may be linked to the type

of compounds present as well as the type and polarity of solvents used (Abou-Elella and Ali, 2014). Ethanol and methanol allowed dissolution of an array of polyhydroxylated phenolic acids, pinellic acid, flavonoids and unidentified components. Water showed reduced antimicrobial activity compared to the alcoholic extracts. Usually, a good expression of phenols may increase extract TPC and affect antimicrobial activities. Nevertheless, comparable results showing that alcoholic extracts have the best antimicrobial activity have also been reported (Preethi *et al.*, 2010). This was mainly because aqueous alcoholic extracts contain polar and non-polar solutes due to their high PI. Aqueous extracts containing phenolic acids and unidentified compound species were effective in inhibiting Gram-negative *A. baumannii* and *P. aeruginosa* growth (Table 4.3). Compound structure, type and prevailing concentration around the cell may affect the ability to permeate microbial cells resulting in varied microbial response to extract components (Ciocan and Bara, 2007). However, acid hydrolysis may have released bound phenol compounds such as highly oxidised phenolic acids for better diffusion into the assay medium (Kurek *et al.*, 2011). Phenolic acids with high oxidation state have been reported to be more inhibitory and toxic to microorganisms (Ciocan and Bara, 2007). Antimicrobial toxicity may be attributed to the presence of piscidic, eucomic, caffeic, ferulic and other phenolics observed in water, alcohol and hexane extracts. Varying degrees of extracts biological activities were also observed in red beet extracts and were attributed to treatment processes which affected phenol content (Ravichandran *et al*., 2012).

Generally, more Gram-positive than Gram-negative microorganisms were susceptible to hydroalcoholic extracts. Gram-positive bacteria possess peptidoglycan cell wall which aid in cell wall penetration, while the lipopolysaccharide cell wall of Gram-negatives restrict transport across the membrane (Koubaa *et al*. 2015b). Broad-spectrum activity of *Opuntia* extracts has been reported (Koubaa *et al*. 2015b). Antimicrobial compounds are mostly aromatic organic molecules such as flavonoids and phenolic acids identified in our extracts. They act by

complexing with extracellular proteins, binding cell walls and inactivating enzymes (Ciocan and Bara, 2007).

Hexane extracts with the third highest TPC after ethanol and methanol extracts showed good antibacterial action in terms of MIC (Table 4.3). Studies have reported that TPC does not always correspond with antimicrobial activities (Chavez-Santoscoy *et al.*, 2009). The successful transport of non-polar hexane extract constituents across bacterial cell membranes may have contributed to the antibacterial activity. Extracts with good content of lipophilic compounds, pinellic acid, other fatty acids and tocopherols have shown high biological activities (Fernandes *et al.*, 2010). Similar observations were made in studies where hydrophobic compounds showed significant antioxidative and antimicrobial potentials (Celıkyurt, 2011).

Table 4.3: Antibacterial activity of *Opuntia ficus-indica* fruit and peel extracts.

Antibacterial activity			
Extracts	**Strain**	**Diameter of inhibition zone (mm)**	**MIC (mg/mL)**
Freeze-dried peel/ethanol	*Staphylococcus aureus* (G+)	15.1±0.1^{c}	9.38
	Methicillin resistant *Staph. aureus* (MRSA) (G+)	15.0±0.4^{c}	18.75
	Bacillus cereus (G+)	11.3±0.1^{g}	9.38
Oven-dried peel/ethanol	*Acinetobacter baumannii* (G-)	13.1±0.1^{e}	9.38
	MRSA	13.0±0.2^{e}	9.38
Freeze-dried peel/acidified methanol	MRSA (G+)	18.8±0.2^{a}	3.13
	S. aureus (G+)	15.3±0.1^{c}	3.13
	B. cereus (G+)	13.1±0.2^{e}	6.25
	Listeria monocytogenes (G+)	12.1±0.2^{f}	6.25
Oven-dried peel/ acidified methanol	*S. aureus* (G+)	15.2±0.1^{c}	9.38
	MRSA (G+)	16.3±0.3^{b}	9.38
	E. coli (G-)	12.2±0.1^{f}	18.75
	Pseudomonas aeruginosa (G-)	11.1±0.1^{g}	18.75
Freeze-dried peel/acidified water	*Acinetobacter baumannii* (G-)	12.1±0.1^{f}	6.25
Freeze-dried pulp/acidified water	*A. baumannii* (G-)	11.0±0.2^{g}	9.38
Oven-dried peel/acidified water	*P. aeruginosa* (G-)	14.0±0.1^{d}	12.5
	A. baumannii (G-)	11.1±0.1^{g}	12.5
Freeze-dried peel/Hexane	*B. cereus* (G+)	8.0±0.1^{i}	5.00
Oven-dried peel/Hexane	*Salmonella arizonae* (G-)	10.0±0.1^{h}	2.50
	S. aureus (G+)	10.2±0.0^{h}	2.50

Inhibition zones reported for hexane extracts at 20 mg/mL and other extracts at 300 mg/mL. G+ = Gram-positive; G- = Gram negative. Values are mean of three replicates ± standard deviation. Values with different superscripts (a-i) in the same column are significantly different as shown by Duncan's multiple range test.

4.3.5. Mass, UV properties and occurrence of phenols in extracts

Mass and UV properties of probable phenolic compounds identified were shown in Table 4.4. Compounds identified include phenolic acids, flavonoids and their derivatives (Tables 4.5 and 4.6). Piscidic acid had the highest percentage occurrence and relative occurrence of 93.75% and 8.43%, respectively. To our knowledge, pinellic acid with the second highest percentage and

relative occurrence of 75% and 6.75%, respectively, is reported in *Opuntia* fruits for the first time. The occurrence of all unidentified compounds was 42.13% compared to 57.87% for those identified (Table 4.5).

The biological activities herein reported for extracts may be attributed to the presence of identified and unidentified compounds. Phenolic acids and flavonoids have been reported in earlier studies on *Opuntia* peels (Koubaa *et al.*, 2015b) and nopals (Guevara-Figueroa *et al.*, 2010). Piscidic, eucomic, ferulic, coumaric and caffeic acids, as well as rutin, taxifolin, kaempferol, isorhamnetin and their glycosylated derivatives have been identified in *Opuntia* fruit pulp and peels as recently reviewed (chapter 2, subsections 2.6 and 2.7). Pinellic acid, which is reported for the first time, is linked with plant defence mechanisms and its antioxidant and antimicrobial properties (Aghofack-Nguemezi and Schwab, 2013) could be exploited in new functional food formulations following its purification from subtropical *Opuntia* extracts. Medical and other industrial applications are also possible. The compound is a secondary metabolite produced in plants in response to stressed environments. Levels of pinellic acid have also been shown to increase with fruit maturity (Aghofack-Nguemezi and Schwab, 2013). Peel extracts generally showed better *in vitro* bioactivities compared to pulp fractions which may be due to the array of phenolic compounds identified. Osorio-Esquivel *et al.* (2011) also showed that phenol compounds were present in significant amounts in peels compared to the fruit pulp. The phenolic compound profile was also affected by the extraction solvent used and plant material processing method applied. Flavonoids, phenolic acids and pinellic acid occurred frequently in freeze- and oven-dried samples (Table 4.6). The qualitative presence of phenolic compounds seemed not to be significantly affected by drying method. This comparable observation was possibly due to the fact that oven-drying was done at a less aggressive temperature of 45°C. Freeze-drying usually show better retention than oven drying especially when oven drying is carried out above 50°C (Reyes *et al.*, 2011; Torres *et al.*, 2010).

Table 4.4: Mass and spectral properties of compounds from *Opuntia* pulp and peel extracts.

Retention time (min)	M-H	M-H Formula	ppm error (<5ppm)	UV	Probable compound identity
7.23	609.50	$C_{27}H_{29}O_{16}$	0	265	Rutin
8.351	255.04	$C_{11}H_{11}O_7$	-2.4	275	Piscidic acid
11.25	355.06	$C_{15}H_{15}O_{10}$	-3.1	312	Caffeic acid 4-*O*-glucuronide
12.06	325.09	$C_{15}H_{17}O_8$	-1.5	282	p-Coumaric acid 4-*O*-glucoside
15.087	371.09	$C_{16}H_{19}O_{10}$	2.4	280	Dihydroferulic acid 4-*O*-glucuronide
16.967	739.20	$C_{33}H_{39}O_{19}$	-0.9	265, 342	Kaempferol 3-*O*-(2''-rhamnosyl-galactoside) 7-*O*-rhamnoside
17.12	739.20	$C_{33}H_{39}O_{19}$	0.3	265, 342	Kaempferol 3-*O*-rhamnosyl-rhamnosyl-glucoside
17.99	303.05	$C_{15}H_{11}O_7$	0	289	Taxifolin
19.53	623.16	$C_{28}H_{31}O_{16}$	-0.5	254, 268	Isorhamnetin 3-*O*-galactoside 7-*O*-rhamnoside
19.75	623.16	$C_{28}H_{31}O_{16}$	1.1	254, 352	Isorhamnetin 3-*O*-glucoside 7-*O*-rhamnoside
24.31	329.23	$C_{18}H_{33}O_5$	-1.2	367	Trihydroxy octadecenoic acid (pinellic acid)
12.20	239.05	$C_{11}H_{11}O_6$	-1.3	275	Eucomic acid
5.69	353.10	$C_{13}H_{21}O_{11}$	-3.1	265	Unidentified (not chlorogenic acid)
10.58	269.06	$C_{12}H_{13}O_7$	-0.4	275	Unidentified
14.06	351.12	$C_{14}H_{23}O_{10}$	-2.3	312	Unidentified
14.92	515.17	$C_{23}H_{31}O_{13}$	-0.2	266	Unidentified
17.56	769.21	$C_{34}H_{41}O_{20}$	-2.3	253, 354	Unidentified
20.04	553.32	$C_{26}H_{49}O_{12}$	0.7	270	Unidentified
20.24	363.07	$C_{17}H_{15}O_9$	-1.9	286	Unidentified
20.70	551.30	$C_{26}H_{47}O_{12}$	3.1	291	Unidentified
24.29	327.21	$C_{18}H_{31}O_5$	-1.8	268	Unidentified

Table 4.5: Pulp and peel extracts compounds and their occurrence (%)

Compounds	[a]Occurrence (%)	[b]Relative occurrence (%)
Rutin	25.00	2.25
Piscidic acid	93.75	8.43
Caffeic acid 4-*O*-glucuronide	37.50	3.37
p-Coumaric acid 4-*O*-glucoside	37.50	3.37
Dihydroferulic acid 4-*O*-glucuronide	50.00	4.49
Kaempferol 3-*O*-(2"-rhamnosyl-galactoside) 7-*O*-rhamnoside	56.25	5.07
Kaempferol 3-*O*-rhamnosyl-rhamnosyl-glucoside	50.00	4.49
Taxifolin	50.00	4.49
Isorhamnetin 3-*O*-galactoside 7-*O*-rhamnoside	43.75	3.93
Isorhamnetin 3-*O*-glucoside 7-*O*-rhamnoside	50.00	4.49
Trihydroxy octadecenoic acid (pinellic acid)	75.00	6.75
Eucomic acid	75.00	6.75
Total (identified)	-	**57.87**
*Unidentified (m/z 353.10)	25.00	2.25
*Unidentified (m/z 269.06)	75.00	6.75
*Unidentified (m/z 351.12)	50.00	4.49
*Unidentified (m/z 515.17)	50.00	4.49
*Unidentified (m/z 769.21)	50.00	4.49
*Unidentified (m/z 553.32)	50.00	4.49
*Unidentified (m/z 363.07)	43.75	3.93
*Unidentified (m/z 551.30)	50.00	4.49
*Unidentified (m/z 327.21)	75.00	6.75
Total % (unidentified)	-	**42.13**
Total % (all compounds)	-	**100**

[a]Number of times a compound was identified divided by total number of extracts, multiplied by 100.
[b]Number of times a compound was identified divided by number of times all compounds were identified across all extracts, multiplied by 100.

Table 4.6: Compounds identified in *Opuntia* pulp and peel extracts

Dried sample	**Freeze-dried peel**				**Freeze-dried pulp**				**Oven dried peel**				**Oven dried pulp**			
Extract/ Compound	**A1**	**A2**	**A3**	**A4**	**B1**	**B2**	**B3**	**B4**	**C1**	**C2**	**C3**	**C4**	**D1**	**D2**	**D3**	**D4**
Rutin	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-
Piscidic Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Caffeic acid 4-*O*-glucuronide	+	+	-	-	+	-	-	-	+	+	-	-	+	-	-	-
p-Coumaric acid 4-*O*-glucoside	+	+	-	-	-	-	-	-	+	+	-	-	+	+	-	-
Dihydroferulic acid 4-*O*-glucuronide	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-
Kaempferol 3-*O*-(2''-rhamnosyl-galactoside) 7-*O*-rhamnoside	+	+	+	-	+	+	-	-	+	+	-	-	+	+	-	-
Kaempferol 3-*O*-rhamnosyl-rhamnosyl-glucoside	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-
Taxifolin	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-
Isorhamnetin 3-*O*-galactoside 7-*O*-rhamnoside	+	+	-	-	+	-	-	-	+	+	-	-	+	+	-	-
Isorhamnetin 3-*O*-glucoside 7-*O*-rhamnoside	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-
Pinellic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Eucomic acid	+	+	-	+	+	+	-	+	+	+	-	+	+	+	-	+
Unidentified m/z	269, 327, 351, 363, 515, 551, 553, 769	327, 351, 363, 515, 551, 553, 769	327	269, 353	269, 327, 351, 515, 551, 553, 769	269, 327, 351, 363, 515, 551, 553, 769	327	269, 353	327, 351, 515, 551, 553, 769	327, 351, 363, 515, 551, 553, 769	327	269, 353	269, 327, 351, 363, 515, 551, 553, 769	269, 327, 351, 363, 515, 551, 553, 769	327	269, 353

A-D = dried powder sample used in extraction; A = Freeze-dried peel, B = Freeze-dried pulp, C = oven dried peel, D = oven dried pulp; 1-4 = extraction solvent used; 1 = ethanol extract, 2 = acidified methanol extract, 3 = hexane extract, 4 = acidified water extract.

4.4. Conclusion

The plant material, processing method and extraction solvent affect yield, biological activity and compound profile of *Opuntia* extracts. There was a wide variation in the occurrence of phenolic compounds in the investigated extracts. However, the frequent occurrence of pinellic acid is reported for the first time in *Opuntia* fruits and demonstrates potential for new compound(s) discovery. The fruit and peel extracts could be utilised as nutraceuticals and have potential for food and health applications. The unidentified compounds could also be contributing to the biological activities of the extracts. However, more sensitive methods are required in order to reduce the proportion of unidentified compounds.

CHAPTER FIVE

Enzymatic dimerisation of luteolin enhances antioxidant and antimicrobial activities

Abstract

Antimicrobial resistance and oxidative stress continue to be major challenges globally. The aim of the current study was to enzymatically transform luteolin for the improvement of antioxidant and antimicrobial activities, using the fungal laccase from *Trametes pubescens*. Laccase-catalysed homocoupling reactions were carried out in a monophasic system with ethanol as cosolvent. A new dimer (*m/z* 569) was produced and characterised for antioxidant and antibacterial activity. The dimer showed a modest but significant (1.1 – 1.2×) in antioxidant activity compared to luteolin as demonstrated by standard (DPPH, ABTS and FRAP) antioxidant assays. The antibacterial activity of the dimer also improved as the minimum inhibitory concentrations (MICs) recorded against *Escherichia coli* and methicillin resistant *Staphylococcus aureus* (MRSA), were substantially reduced (approximately halved), when the dimer was used instead of luteolin. A bactericidal mode of action (cell membrane disruption) was evident from the cell integrity and time-kill assays and scanning electron microscopy (SEM) images. These analyses showed loss of cell constituents, the formation of membrane pores as well as cell fragmentations. The novel dimer with its enhanced biological activity may be exploited in food, health and nutraceutical industries.

5.1. Introduction

There is an increasing trend of oxidative stress-related diseases as well as antibiotic resistance related to excessive use of antibiotics. Oxidative stress arises from the overproduction of free radicals and has been linked with non-communicable diseases (NCDs) such as cardiovascular disease, type 2 diabetes, chronic lung disease and cancer (Barnham *et al*., 2004; Hofman, 2014). Globally, there has been an upsurge in the incidence of NCDs (Mayosi *et al*., 2009). In southern Africa and other developing countries, it was predicted that seven out of every ten deaths will be attributed to NCDs by the year 2020 (UNDPI, 2011). Synthetic antioxidants have been used to manage NCDs, but most have been reported to have unwanted side effects such as causing sperm abnormalities, nausea, fatigue, cancer, asthma and degenerative diseases as result of their accumulation in biological systems over time (Wojcik *et al*., 2010).

Multidrug resistance (MDR) in microbial strains such as *Escherichia coli* and methicillin resistant *Staphylococcus aureus* (MRSA) occur because they are able to by-pass antimicrobial agents efficacies through evasive mechanisms such as enzyme inactivation and drug 'pump out' systems (Ali *et al*., 2018). Some associated problems related with antimicrobial resistance include malaise, diarrhoea, and disruption of the natural gut microflora (AHA, 2018), the re-emergence of, and/or inability to easily cure formerly treatable infectious diseases such as pneumonia, tuberculosis, salmonellosis, and other minor/self-limiting infections (Scarafile, 2016; Ventola, 2015). These problems lead to prolonged cost of treatments and hospital stays, and an increase in morbidity and mortality rates (WHO, 2019).

Given the above global challenges, there is an increased world-wide demand for new, safe and natural antimicrobial and antioxidant compounds (Aremu *et al*., 2011). Plant bioactive compounds usually possess antioxidant (Zrira *et al*., 2016) and/or antimicrobial properties

(Koubaa *et al*., 2015b). Their addition to food and drug formulations would not only enhance benefits to human and animal health, but also increase consumer acceptance (Shlar *et al*., 2017). Recently studies have also focused on improving the biological activities of known plant phenolics. This is because new microbe-sourced antimicrobials or their chemosynthetic variants and new antioxidants are not being produced fast enough (Ahmed and Jambi, 2018; Aremu *et al*., 2011). Therefore, research involving the biocatalytic enhancement of plant-based phenolic compounds is increasing.

Laccase is one enzyme that is being investigated as a biocatalyst for the improvement of biological activities of phenolic compounds (Kudanga *et al.*, 2011). Laccase is a multi-copper oxidase enzyme that catalyse the oxidation of a suitable substrate to its radical form with concomitant reduction of molecular oxygen to water. The radicals formed can undergo several non-enzymatic reactions including coupling to form homomolecular or heteromolecular coupling products. The coupling reactions have found application in organic synthesis, particularly in the synthesis of new antioxidants (Nemadziva *et al.*, 2018) and other bioactive compounds (Kudanga *et al.*, 2011). The synthesised oligomeric products usually have improved biological activities (Zwane *et al.*, 2012). This is usually attributed to increase in the number of hydroxyl groups from which hydrogen atoms are abstracted, and/or an increase in electron-donating groups that help to stabilise the antioxidant after donating an electron (Kudanga *et al*., 2017). Laccase catalysis may contribute to the production of green, novel compounds which do not only have antioxidant properties but may also be active against drug resistant microbial pathogens. Therefore, research interest in laccases as catalysts in organic synthesis is increasing as summarised in a recent review article (Kudanga *et al*., 2017).

In the current study, we investigated laccase-catalysed biotransformation of luteolin, a bioactive compound found in many plants, including *Opuntia* species. Antioxidant and antibacterial

activities of the organically synthesised compound were compared to the starting substrate (luteolin) to evaluate enhancement of biological activities. In addition, the possible mode of antibacterial action of the synthesised product using *E. coli* and MRSA as model pathogens was investigated.

5.2. Materials and methods

5.2.1. Chemicals and reagents used

All chemicals used, including the solvents, were of analytical grade. Other chemicals (and culture media) unless otherwise mentioned were obtained from Oxoid (UK) and Biolab-Merck and Sigma-Aldrich (South Africa). The *Trametes pubescens* strain was obtained from the Cape Peninsula University of Technology, South Africa and is currently deposited in the stock culture collection in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa.

5.2.2. Laccase production from *T. pubescens*

Laccase enzyme production was carried out in a *Trametes*-defined medium (TDM) with copper induction on the third day of fermentation. The enzyme was harvested on the fifth day and partially purified using ammonium sulphate precipitation. This was followed by the determination of laccase activity (Childs and Bardsley, 1975) and protein concentration (Lowry *et al*., 1951).

5.2.3. Determination of laccase enzyme activity

Laccase enzyme activity was done according to Childs and Bardsley (1975). Laccase activity was determined spectrophotometrically by monitoring the oxidation of 2,2,-azinobis3-ethylbenzthiazoline-6-sulfonic acid (ABTS ε420 = 36,000 M^{-1cm-1}) as the substrate. The assay mixture contained 0.330 ml ABTS (5 mM), 2.5 ml 100 mM sodium acetate buffer pH 5.0, and 0.17 ml laccase extract. Laccase activity was determined by monitoring the oxidation of ABTS (1 mM) at 420 nm (ε = 36,000M^{-1} cm^{-1}) in 100 mM acetate buffer (pH 5.0). One unit of laccase activity was defined as the amount of enzyme required to oxidise 1 μmol of ABTS per minute at 25ºC.

5.2.4. Laccase protein concentration determination

Enzyme protein concentration was done using the Lowry assay (Lowry *et al.*, 1951). Bovine serum albumin (BSA - 1 mg/ml) solution was prepared by dissolving 10 mg BSA in 10 ml of distilled water. Using this as a stock, various concentrations of BSA were used to generate a standard curve (Appendix B). A 20 µl of aliquot sample was added in an Eppendorf tube followed by 180 µl of distilled water. This was mixed with 1 ml of Lowry's solution and incubated at room temperature for 15 min. Thereafter 100 µl of a 1.0 N Folin's phenol reagent was added and incubation in the dark for 30 min, after which absorbance was measured at 660 nm.

5.2.5. Oxidation of luteolin by laccase

Oxidation of luteolin by laccase was carried out according to a previous method by Zwane *et al.* (2012) with slight modifications. A monophasic system with ethanol as co-solvent was

employed in the biocatalytic oxidation reaction. The reaction mixture contained 50 mM substrate and was carried out in sodium acetate buffer (100 mM, pH 5.0) and 50% ethanol (v/v) at 37ºC, with orbital shaking at 180 rpm. The reaction was started by adding 3.3 U of laccase. An additional 3.3 U of the enzyme was added after 1 h to make up for enzyme inactivation by the solvent. The reaction was monitored hourly using high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC). Reaction reached completion after 3 h.

TLC analysis was performed on aluminium–backed silica gel 60 F_{254} (Merck) plates using toluene: ethyl acetate: formic acid (8:6:1, v/v/v) mobile phase. Reaction constituent profiles were visualised by exposure to ultraviolet (UV) light at 254 nm. In carrying out HPLC analysis, when miscible solvents were used, an equal volume of ice-cold absolute methanol was added to the reaction mixtures. This served to precipitate the protein. The mixture was then allowed to stand on ice for 20 min before centrifugation at 4ºC for 15 min at 14,000 ×g. Supernatants were then transferred into clean vials and analysed by HPLC. Reaction products separation was done on a reversed phase column (5µm, 4.6×150 mm) using a linear gradient of 0.1% v/v formic acid (solvent A) and acetonitrile (99.9%) (solvent B) at a flow rate of 0.5ml min^{-1}, an injection volume of 10µl and an oven temperature of 30ºC. The gradient set up was as follows: 98% A–0% A (20 min); 0% A–98% A (20–21 min); 98% A (21–23 min). Products were monitored and detected at 254 nm.

5.2.6. Purification of reaction product

Column chromatography and preparative TLC were used for purification of luteolin oxidation product using toluene: ethyl acetate: formic acid (8:6:0.5, v/v/v) as mobile phase. For TLC, the compounds were visualised by exposure to UV light at 254 nm. Product yield was calculated as a percentage of the initial amount of substrate (Adelakun *et al.*, 2012b).

5.2.7. Characterisation of luteolin product by LCMS

Mass spectra (MS) of product were acquired in the negative mode using a Shimadzu LCMS-2020 mass spectrometer (Shimadzu, Japan). An electrospray voltage was set to +3500V. Nebuliser gas pressure at 35 psi with gas flow of 9 L min^{-1} was used. A linear LC elution gradient of solvent A (0.1% formic acid) and solvent B (acetonitrile) was used for product separation. The gradient was set up as follows: 98% A to 0% A (20 min), 0% A to 98% A (20–21 min) and 98% A (21–23 min). Injection volume was 10 µL and flow rate set at 1 mL min-1 (Adelakun *et al.*, 2012).

5.2.8. Antioxidant activity of luteolin and its dimer

5.2.8.1. The DPPH (2,2-Diphenyl-picryhydrazyl) assay

The test antioxidant compound in methanol was added to the DPPH solution (100 µM in methanol). At 517 nm, the reduction in absorbance reading was monitored and measured. An 80% (v/v) methanol solution was used as blank, and DPPH in methanol was the negative control. The percentage DPPH inhibition was determined using:

$$I\ (\%) = [(A_0 - A_1)/A_0] * 100$$

where A_0 = absorbance of negative control, A_1 = absorbance of the compound/standard. The experiment was performed in triplicate. The compound concentration required to quench or scavenge 50% of the radical was determined and expressed as the EC_{50} value. Lower EC_{50} values were indicative of higher antioxidant capacity (Mahdi-Pour *et al.*, 2012).

5.2.8.2. ABTS [(2,2´-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid)] assay

An $ABTS^{•+}$ radical working solution was prepared 12-16 h prior to its use in the assay. The radical was produced by reacting 2.45 mM potassium persulfate and 7 mM of ABTS salt, and the mixture was stored in the dark until the assay was performed. The absorbance of the $ABTS^{•+}$ radical was adjusted with methanol to 0.70±0.002 at 734 nm. Test samples were prepared at different concentrations mixed with the $ABTS^{•+}$ and absorbances read after 30 min incubation at 25°C (Re *et al.*, 1999).

5.2.8.3. FRAP (ferric reducing antioxidant power) assay

A fresh FRAP reagent was made by mixing 2.5 mL of 20 mM ferric chloride ($FeCl_3$), 25 mL of 300 mM acetate buffer (pH 3.6) and 2.5 mL of 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl in a respective ratio of 1:10:1 (v/v/v). The mixture was incubated at 37°C for 15 min before use. Thereafter, 2.85 mL of the reagent and 150 µL of the test compound at 2 mM concentration were mixed. The mixture was incubated in the dark for 30 min and the absorbance of the coloured complex formed was measured at 593 nm (Prabhavathi *et al.*, 2016). The experimental determinations were replicated three times.

5.2.9. Determination of antibacterial activity and mode of action

5.2.9.1. Antibacterial assay using the disc diffusion method

Antibacterial activity determination was carried out according to Zhao *et al*. (2015) using the disc diffusion method due to small amounts of products purified and to optimize for antibacterial effect. Luteolin and its product were impregnated on sterile filter paper discs at 10

mM concentration. A 100µl of each of *E. coli* and MRSA broth (0.5 McFarland bacterial turbidity) was aseptically spread on petri dishes containing solidified Mueller Hinton agar. Plates were labelled accordingly for each bacterium and compound. Thereafter, discs impregnated with the test compounds were carefully placed on inoculated plates using sterile forceps and the edges gently pressed down against the solidified agar surface. Plates were then left for adequate diffusion, inverted and incubated at 37°C for 24 h. Thereafter, diameter of zones of inhibition were measured and recorded in mm.

5.2.9.2. Minimum inhibitory concentration (MIC) assays

MIC determination was done using the microbroth dilution technique in microtitre plate wells (Corning, Cambridge, U.S.A.) and 0.5 McFarland bacterial suspension standard (CLSI, 2000). Negative (suspension broth and compounds in solvent) and positive (bacterial broth suspensions) controls were included in separate wells. The experiment was replicated three times. The lowest concentration of the compounds required to visibly inhibit growth was taken as the MIC. Thereafter, an investigation of bacterial cell membrane integrity, time-kill assay and scanning electron microscopy (SEM) analyses of product-treated and untreated bacterial cells, were done to determine the potential mechanism of antibacterial action.

5.2.10. Bacterial cell wall integrity assay

The assessment of bacterial cell membrane integrity upon exposure to an antimicrobial compound/agent was measured according to Sadiq *et al*. (2017), with minor modifications. MRSA and *E. coli* cell cultures were centrifuged at 4000 x g for 15 min. Thereafter, a three times wash of sedimented bacterial cells in phosphate buffer solution (100 mM, pH 7.2) was done followed by the re-suspension of cells in the same phosphate buffer solution. Culture

suspensions in buffer were thereafter incubated with MIC and 2 x MIC concentrations of the product (controls were left untreated) for 6 h with orbital shaking at 37ºC. Cell suspensions were then collected and centrifuged at 11000 x g for 5 min. Supernatants were used for protein content determination using the Lowry assay method (Lowry *et al.*, 1951). The amount of cellular constituents in supernatants was measured at an absorbance of 260 nm using a UV-visible spectrophotometer (Biochrom LibraS21, England).

5.2.11. Bacterial time-kill assay

The time-kill curve analysis was used to determine the bacteriostatic or bactericidal effect of the luteolin oxidation product according to NCCLS macro-dilution guideline for the standardised time-kill assay (Sim *et al*., 2014). Microbial suspensions of *E. coli* and MRSA were prepared in the logarithmic growth phase and diluted to the final inoculum density required for the assay. A 0.5 mL inoculum density (approximately 10^6 - 10^8 cfu/mL) was added to 0.5 mL of test and control broth experiments to arrive at final concentrations of MIC and 2×MIC. The experimental set up was then orbitally shaken at 120 rpm and 37°C. An aliquot (100 µL) was taken at hourly intervals for the determination of colony forming unit per mL (cfu/mL) using the plate count method. If the original inoculum size was reduced by <3 log cfu/mL at the lowest concentration, the test compound was regarded as having a bacteriostatic effect. On the other hand, if the original inoculum size was reduced by ≥3 log cfu/mL at the lowest concentration, the test compounds was said to have a bactericidal effect. The experiment was performed in triplicate.

5.2.12. SEM observation of bacterial cells

The changes in morphology of bacterial cells treated with the oxidation product were observed by SEM microscopy. *E. coli* and MRSA were incubated in nutrient broth for 10 h at 37ºC, followed by cell treatment at the respective MIC and 2×MIC concentrations of the oxidation product (control cultures were left untreated). This was followed by incubation at 37ºC for 6 h. Thereafter, the cells were pelleted by centrifugation for 10 min at 1500 x g. Pelleted cells were then subjected to three washes in in 100 mM PBS (pH 7.2) and resuspended in the same buffer. A 40 μL aliquot of the bacterial cell suspension was then spread on a slide and allowed to dry. Dried cells on the slide were then embedded with gradient ethanol washes. Sputter coating of samples was done twice using gold particles in vacuum. Coating was followed by cell examination under Field Electron Gun-SEM (FEGSEM) (Hitachi S-3400N, Tokyo, Japan) (Sadiq *et al*. 2017).

5.2.13. Statistical analysis

Unless otherwise stated, experiments were carried out in triplicate and results expressed as a mean ± standard deviation (SD). Duncan's Multiple Range test in SPSS version 16 was used to determine differences between samples. *P* values less than 0.05 were considered as significant.

5.3. Results and discussion

5.3.1. Laccase production from *Trametes pubescens*

Ammonium sulphate precipitation was utilised in the partial purification of *T. pubescens* fungal laccase following separation of fungal biomass and other precipitates. Laccase activity of the

crude filtrate was carried out, as well as specific activity and protein content determination. The BSA standard curve for protein concentration determination showed good correlation (R^2 =0.9981) (Appendix B). The same curve was used to determine the protein concentration of partially purified (50% and 80%) fractions. As shown in Table 5.1, laccase enzyme recovery yield of only 4.74% was obtained with 50% ammonium sulphate precipitation compared to 21.45% yield from 80% ammonium sulphate purification. Increase in total activity (14.4U to 65.2U) and specific activity (25.71 U/mg to 40.75 U/mg) of the partially purified enzyme was also observed as ammonium sulphate salt concentration was increased from 50 to 80% (Table 5.1). Similar higher laccase enzyme recovery yield have been reported at higher stages (70-80%) of partial purification using ammonium sulphate salt. Adelakun *et al*. (2012a) recorded a 77.39% yield at 70% ammonium sulphate purification stage. Batch to batch variation is however common and widespread and enzyme recovery rates are dependent on various factors such as the strain used, presence or absence of inducers, content of fermentation medium, fermentation length and method used, and growth conditions during production (Kumar *et al*., 2016; Birhanli and Yeşilada, 2017).

Table 5.1: Partial purification table for laccase production

Purification Method	Vol. (ml)	Start activity (U)	Start protein (mg)	Pellet activity (U)	Pellet protein (mg)	Tot. activity (U)	Tot. protein (mg)	Specific Activity (U/mg)	Recovery (%)	Purification (fold)
Filtrate	1600	1.90	0.091	-	-	3040	14.3	21.11	80	1
50% Ammonium sulphate	10	1.9	0.091	1.44	0.056	14.4	0.56	25.71	4.74	1.22
80% Ammonium sulphate	10	19	0.091	6.52	0.160	65.2	1.60	40.75	21.45	1.93

5.3.2. Characterisation of oxidation product by TLC and LCMS

Laccase oxidation reactions using luteolin as the starting substrate yielded an oxidation product (Plate 5.1). The dimeric product retention factor (Rf) was lower (0.24) compared to that of the substrate (0.50) (Plate 5.1). This indicated that the product is more polar than the substrate because it is more tightly bound to the silica gel (Block *et al.*, 2016). An increase in the oxidation product polarity also suggested that an increase in hydroxylation in the dimerised product may have occurred. A compound's Rf value may vary and is dependent on factors such as the kind of solvent used in making the mobile phase, their mixing ratios and polarities, as well as nature of compounds being separated (Scientific, 2014). Laccase-catalysed oxidation of luteolin (tR=12.98 min) used as the starting substrate produced one major oxidation product (LP) (tR=9.13 min) (Fig. 5.1). Mass spectra of the major product showed m/z of 569 (M=570) in negative mode (Fig. 5.2) which indicates that a dimer was formed from luteolin (m/z 285).

The hydroxyl group on the benzene ring of the luteolin compound is *ortho*- or *para*- directing (Suparno *et al.*, 2005). The coupling of unpaired electrons may yield C-C linked dimers and/or ether linked dimers as proposed in Figs. 5.3 and 5.4, respectively. The respective reaction mechanisms are proposed as shown in Fig. 5.5 and 5.6. Molecules with a free C-5 position usually dimerise through 5−5 (C-C) and 4-O-5 (C-O-C) linkages (Boerjan *et al.*, 2003). However, ether linkages usually require higher heat of formation than C-C linkages (Valencia and Marinez, 2005) and they usually have lower activities (Matsuura and Ohkatsu, 2000) due to participation of the hydroxyl group in the linkage formation. Therefore, it is likely that the dimer formed was a C-C linked dimer as proposed in Fig. 5.3. The C-C dimer has also been the most frequently produced dimer in similar research (Gavezzotti *et al.*, 2014; Zwane *et al.*, 2012

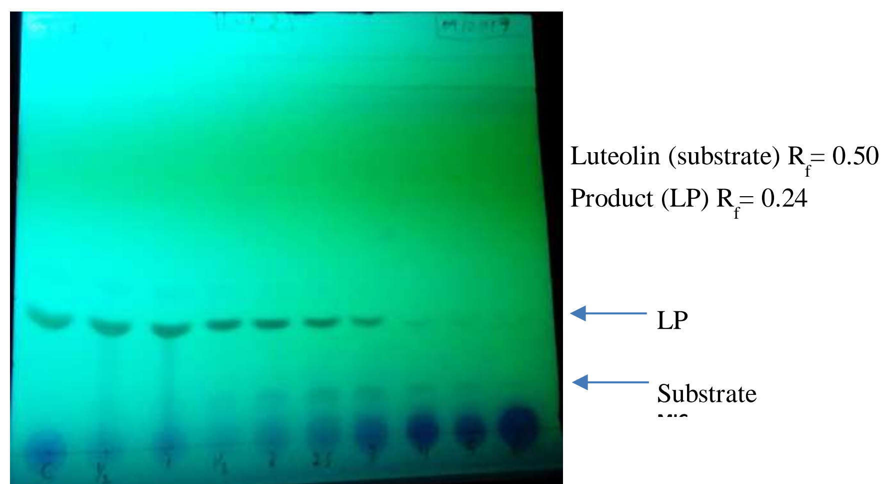

Plate 5.1: TLC plate showing luteolin product (LP) and substrate (S) lanes

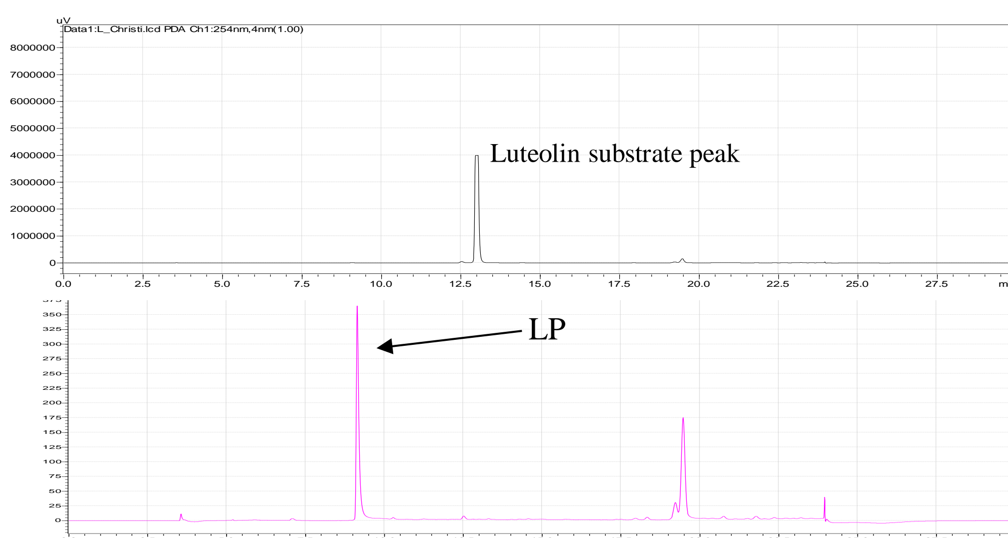

Figure 5.1: HPLC Chromatogram of luteolin substrate and its product (LP)

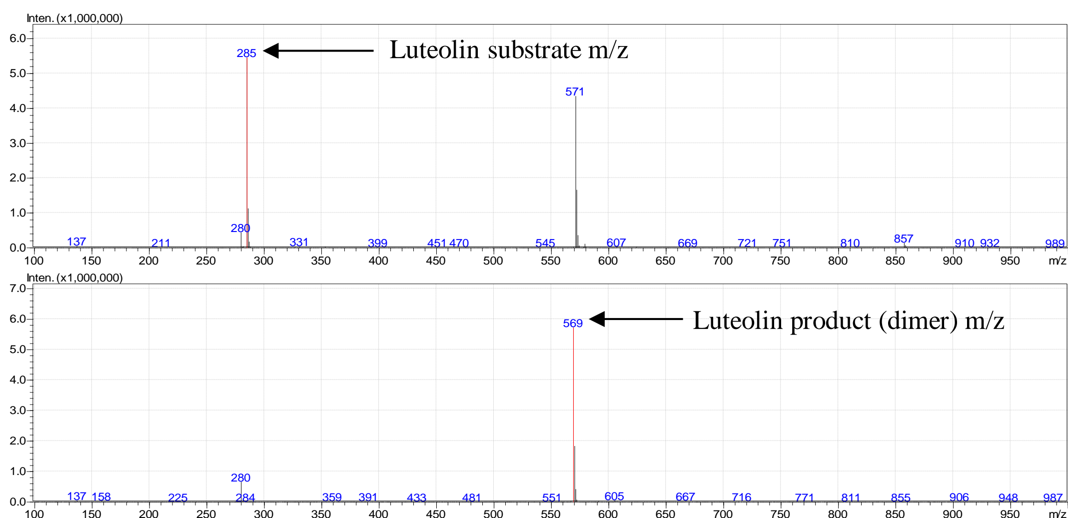

Figure 5.2: Mass spectrum of luteolin (*m/z* 285) and its dimeric product (*m/z* 569) in negative mode

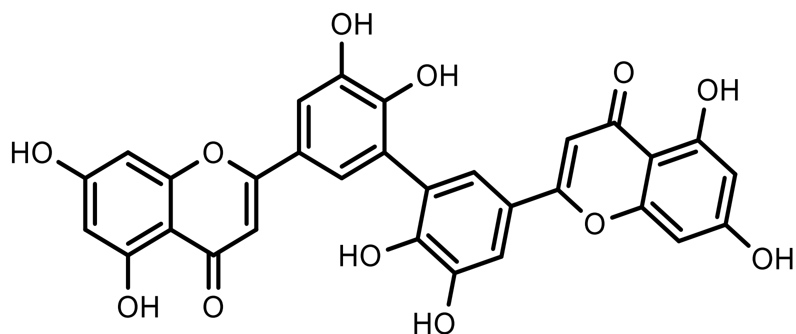

Figure 5.3: Proposed structure of the C-C linked luteolin dimer.

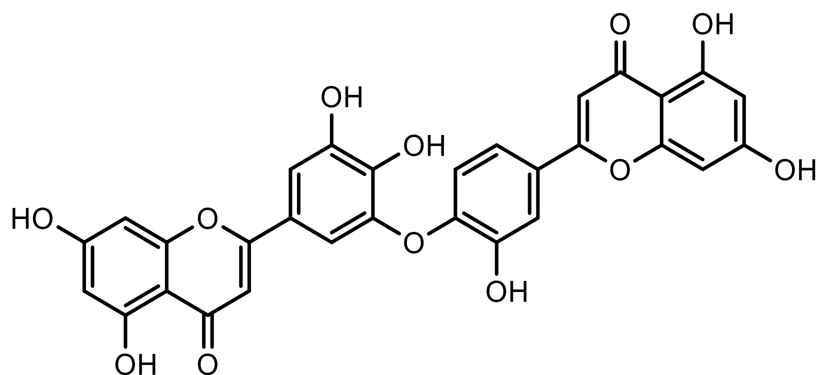

Figure 5.4: Proposed structure of the ether (C-O-C) linked luteolin dimer.

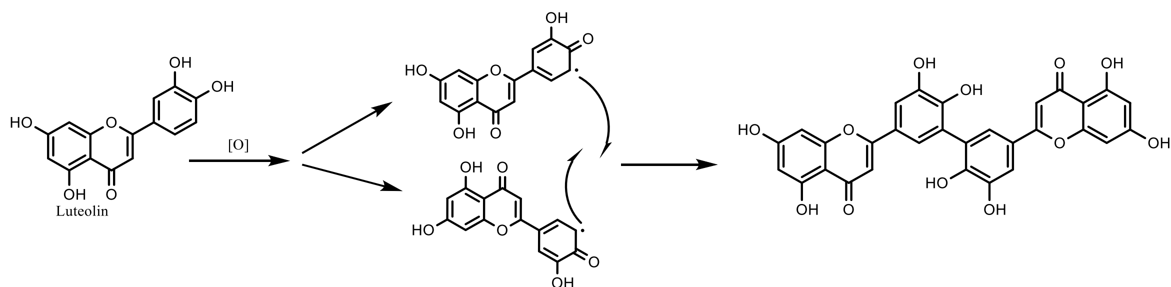

Figure 5.5: Proposed reaction pathway for the formation of the C-C linked luteolin dimer.

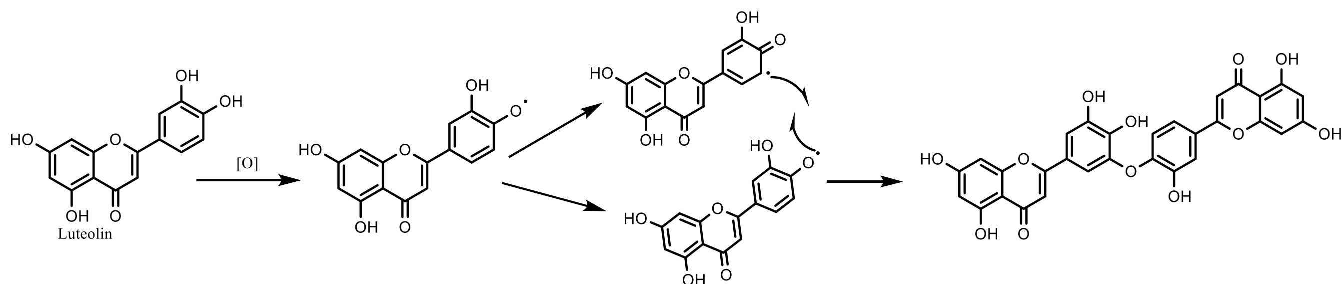

Figure 5.6: Proposed reaction pathway for the ether (C-O-C) linked luteolin dimer.

A low luteolin dimer yield was obtained (14.42±1.32%). This could be due to a combination of polymerisation reactions or non-specific side reactions which are usually associated with laccase catalysed radical-radical coupling reactions (Kudanga *et al.*, 2017). Variations in yield may also be linked to the kind of solvent or co-solvent used in biocatalysis (Adelakun *et al.*, 2012a). Other factors which affect product yield include the type of substrate and its structure, as well as the enzyme's affinity for the substrate (Kudanga *et al.*, 2017).

5.3.3. Antioxidant activity assays

Antioxidant activity of luteolin and its product are shown in Table 5.2. Product LP showed a modest but significant increase in hydrogen donating ability as measured by the DPPH and ABTS assays, compared to the substrate. Similarly, the ferric reducing antioxidant power improved 1.2-fold compared to substrate (Table 5.2). The dimerisation and/or oligomerisation of substrate molecules has almost always been associated with an enhancement of biological activity which is ascribed to the increase in number of hydroxyl (OH) groups in the new product (De Pinedo *et al.*, 2007; Adelakun *et al.* 2012b; Kudanga *et al.*, 2017).

Table 5.2: Antioxidant activity of luteolin and its products

Compound	Molecular weight (g/mol)	EC_{50}DPPH[c] (µM)	EC_{50}ABTS[c] (µM)	FRAP[d] (µM)
Luteolin	285	49.36±0.22[a]	15.69±0.23[a]	37.58±0.51[b]
Product (LP)	569	45.22±0.11[b]	14.90±0.07[b]	43.67±0.24[a]

Values with different superscripts (a, b) in the same column are significantly different ($p<0.05$). Results are mean ± standard deviation of three replicate determinations.

[c] EC_{50} value is defined as the concentration (µM) of substrate that results in 50% loss of DPPH• and ABTS• (Adelakun *et al.*, 2012a)

[d] Antioxidant ability to reduce Fe^{3+} complex to the ferrous Fe^{2+}form (Saeed *et al.*, 2007)

5.3.4. Antibacterial activity determination

The antibacterial activity of the luteolin dimer (LP) showed a 1.3-fold increase in the size of zone of inhibition against *E. coli* and only 1.1-fold increase against MRSA, compared to the substrate (Table 5.3). Similarly, a decrease in MICs against *E. coli* and MRSA strains, relative to the substrate, was also observed (Table 5.3). Increased zones of inhibition and reduced MICs for the product may also be a result of increased hydroxylation achieved during laccase-catalysed coupling reactions (Ciocan and Bara, 2007). Gram-positive MRSA showed higher susceptibility compared to Gram-negative *E. coli*. The presence of a peptidoglycan layer in Gram-positive cell membrane may facilitate uptake of the compounds (Zhao *et al*., 2015). On the other hand, the lipopolysaccharide layer in Gram-negative microorganisms obstructs compounds transport across the cell membrane (Zorofchian Moghadamtousi *et al*., 2014).

A previous study reported that the higher the antioxidant potential, the better the probability of a compound showing good antibacterial activity (Fazeli-Nasab *et al*., 2017). Polymeric phenol compounds with multiple number of hydroxyl groups have been reported to show superior antibacterial action (Ciocan and Bara, 2007; Hierholtzer *et al*., 2012). For example, Hierholtzer *et al*. (2012) observed an improvement in antibacterial activity of phlorotannin (polymerised form) compared to its monomeric phloroglucinol form. It is therefore not surprising that the dimer showed better antibacterial activity.

Table 5.3: Antibacterial activity of luteolin and laccase-catalysed oxidation product (LP)

	Luteolin		**Product (LP)**	
	Inhibition zone diameter (mm)	**MIC (mM)**	**Inhibition zone diameter (mm)**	**MIC (mM)**
Escherichia coli	5.25±0.25[b]	1.87	7.00±0.01[a]	0.94
methicillin resistant *Staphylococcus aureus* (MRSA)	7.00±0.01[a]	0.94	7.50±0.14[a]	0.47

Results are mean ± standard deviation of three replicate determinations.
Values with different superscripts (a, b) in the same row are significantly different ($p<0.05$).

5.3.5. Integrity of bacterial cell membrane

Bacterial cell membrane disruption, stimulation of cell apoptotic pathways, cell lysis and inhibition of cellular enzymes are some of the reported antimicrobial/bactericidal modes of action of luteolin (Joung *et al.*, 2016a,b; Tagousop *et al.*, 2018). Therefore, in addition to confirming the luteolin dimer antibacterial efficacy, we went further to determine the potential mechanism of action of the new product. Results showed greater leakage of proteins and cell constituents for MRSA compared to *E. coli* as compound concentration was increased (Table 5.4). This suggests damage to bacterial membranes after treatment with the dimer. The more significant leakage of cell constituents in MRSA may be attributed to the nature of the Gram-positive cell wall which facilitate better compound transport across the cell membrane (Zhao *et al.*, 2015). The presence of the lipopolysaccharide layer in Gram-negative *E. coli* cell wall may have contributed to the reduced levels in loss of cell constituents compared to MRSA. The lipopolysaccharide layer plays a major role in antimicrobial resistance in Gram-negative microorganisms (Zhao *et al.*, 2015; Sadiq *et al.*, 2017). Nevertheless, the dimer was able to compromise the structural integrity of the bacterial cell membranes and cause loss of cell proteins and nucleic acids. A previous study has shown that phenolic compounds are able to cause irreversible damage to microbial cell membranes (Borges *et al.*, 2013).

Hydroxyl groups in oligomeric and polyphenolic compounds are able to bind cell wall proteins and enzymes. This is achieved by the formation of unstable complexes that adversely affects the crosslinking stage of cell wall synthesis (Shi *et al*., 2016). Thereafter, cell membrane permeability increases due to disruption of cell wall synthesis. The increase in membrane permeability also causes structural changes in the microbial cell membrane and facilitates the leakage of cellular components (nucleic acid, glutamic acid) and influx of more of the antimicrobial compound (Wu *et al*., 2016) which could eventually lead to cell death. Phenolic compounds also act by binding to and inhibiting permease enzymes, denaturing bacterial proteins and causing cell membrane lysis in the process. This, however, occurs at high concentrations of the antimicrobial phenolic compound. The accumulation and increase of hydrophobic phenolic groups in the lipid bilayer causes increased hyperpolarisation of the cell membrane and may disrupt lipid–protein interaction and cell membrane potential (Wu *et al*., 2016).

Table 5.4: Effect of luteolin product (LP) on bacterial cell membrane integrity

		E. coli		**MRSA**	
		Protein (µg/ml)	**Cell Constituents (OD_{260})**	**Protein (µg/ml)**	**Cell Constituents (OD_{260})**
Luteolin product	2×MIC	2.32±0.04[a]	0.017	5.23±0.14[a]	0.116
	MIC	0.84±.0.11[b]	0.008	1.33±0.07[b]	0.053
	Control	0.04±.0.01[c]	0.002	0.01±0.01[c]	0.003

Results are means ± standard deviation of three replicate determinations.
Values with different superscripts (a, b, c) in the samecolumn are significantly different ($p<0.05$).

5.3.6. Bacterial rate of kill with time (time-kill assay)

The kill rates of treated and untreated (control) bacterial cells were plotted against time. A decrease in cfu/mL (8log to 3.2log) at MIC for *E. coli* (Fig. 5.7), and 8log to 3log reduction in

cfu/ml at MIC concentrations for MRSA (Fig. 5.8) was observed. The reduction in viable count was greater than the 3log cfu/mL cut-off which suggests bactericidal activity (Barry *et al.*, 1999). The luteolin dimer showed bactericidal effect at both concentrations (MIC and 2×MIC) and no viable cells were observed after 4 h. Cell growth in the untreated controls was increased for both strains during the assay time. The kill rate was more pronounced in MRSA (Fig. 5.8) compared to *E. coli* (Fig. 5.7) as more bacterial cells were killed within the first 1-3 h of exposure. The cell death may be due to disruption of cell membrane and leakage of cellular constituents as explained above or possibly inhibition of microbial enzymes (Joung *et al.*, 2016a,b). Differences in susceptibility of the strains could be due to differences in membrane structure as also previously explained. The bactericidal activity of the dimer could find application in food and medical industries.

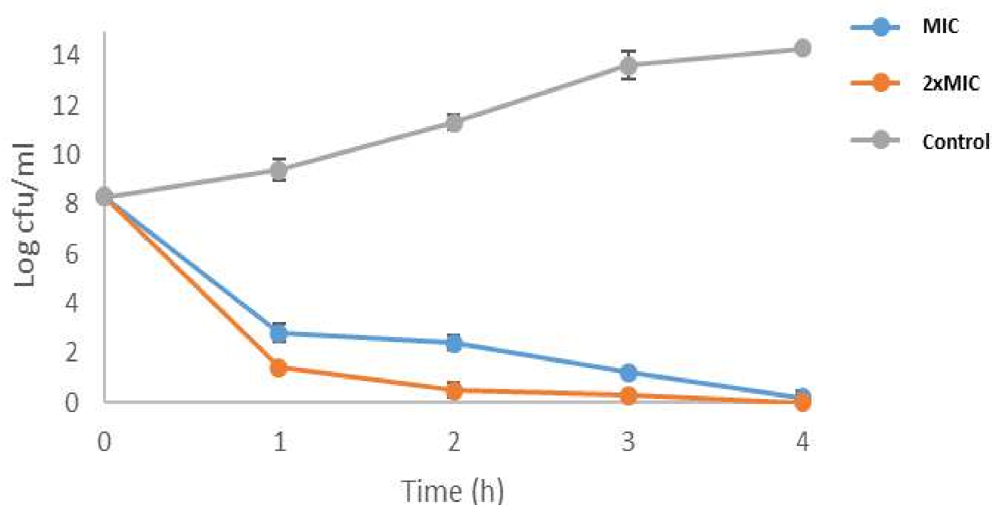

Figure 5.7: Time-kill curve for bactericidal action of dimer (LP) against *E. coli*
Note: Test compound is considered bactericidal if inoculum size was reduced by ≥3 Log cfu/mL (Barry *et al.*, 1999)

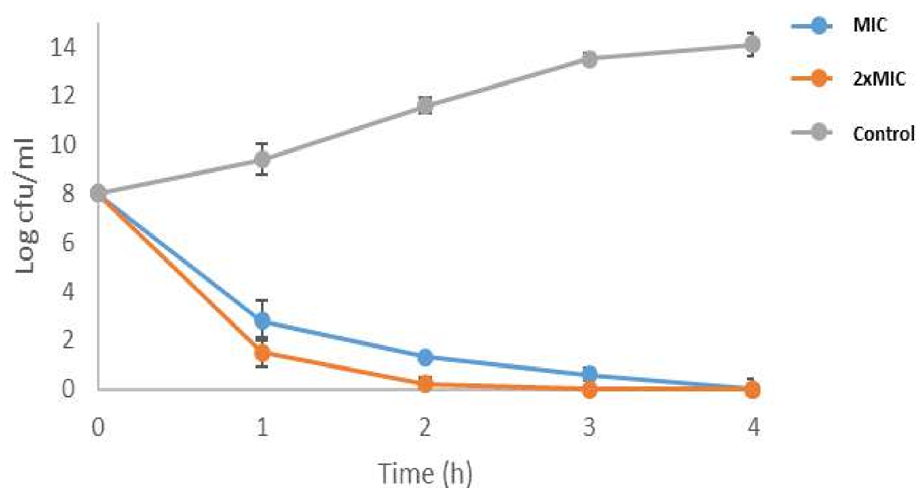

Figure 5.8: Time-kill curve for bactericidal action of dimer (LP) against MRSA
Note: Test compound is considered bactericidal if original inoculum was reduced by ≥3 Log cfu/mL (Barry *et al.*, 1999)**.**

5.3.7. SEM observations of cells

The antimicrobial mechanism of action of the luteolin dimer was further confirmed by SEM imaging of treated and untreated bacterial cells. Cell morphology and membrane integrity changes were pronounced in both *E. coli* and MRSA cells after treatment with the dimer (Fig. 5.9). The dimeric product showed a cell membrane disruptive effect on both tested pathogens. Many treated cells were deformed, pitted, broken or fragmented, which indicate strong bactericidal action. These observations support findings from cell integrity assays and may be responsible for the loss of proteins and other cell constituents. Some studies have also shown similar deformations and breakages in cell membrane after treatment with phenolic extracts or purified phenolic antimicrobial compounds (Diao *et al.*, 2014; Sadiq *et al.*, 2017). Resistant strains of *E. coli* and MRSA have food and medical relevance (Ali *et al*., 2018). Therefore, the luteolin dimer could have potential for application in food preservation, as well as in novel drug development.

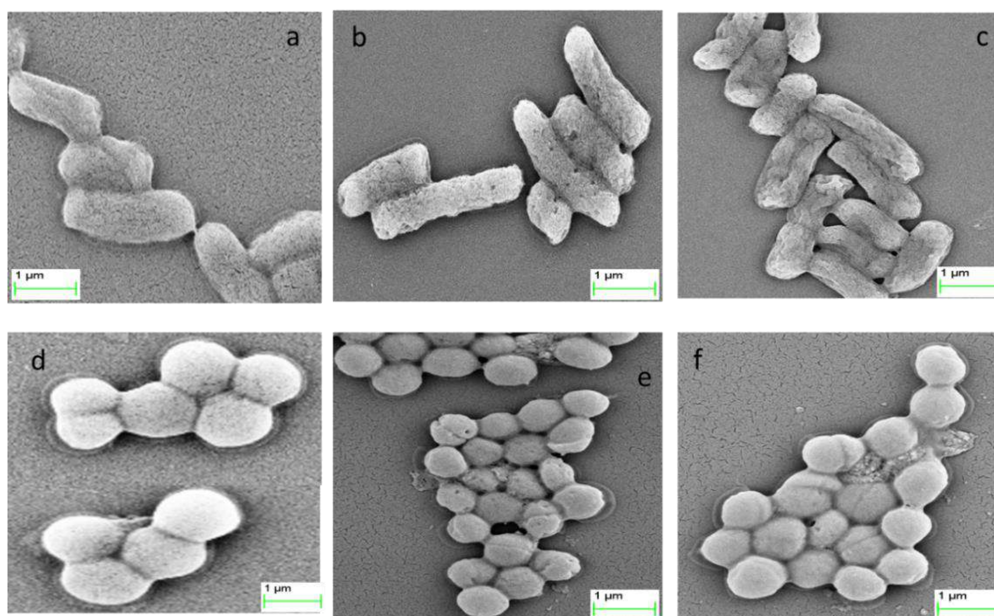

Figure 5.9: SEM images of bacterial cells treated with the luteolin dimer (LP). (a) untreated *E. coli* cells (control), (b) *E coli* cells treated with the MIC and (c) *E coli* cells treated with 2×MIC concentrations, d) untreated MRSA cells, (e) MRSA cells treated with MIC and (f) MRSA cells treated with 2×MIC.

5.4. Conclusion

This study reported for the first time the production of a new dimer from the biocatalytic action of laccase on luteolin. The generated dimer showed improved antioxidant and antimicrobial capacity compared to the starting substrate. A bactericidal mode of action of the dimer was demonstrated. The synthesised dimer may find application in food, pharmaceutical and medical industries.

CHAPTER SIX

Laccase-mediated modification of isorhamnetin improves antioxidant and antibacterial activities

Abstract

Enzymatic modification of phenolic molecules to higher molecular weight compounds is a promising approach for improving biological activity. In the current study the *Trametes pubescens* laccase was used to modify isorhamnetin (*m/z* 316) as a way of improving antioxidant and antibacterial activities. Using a monophasic system with ethanol as co-solvent, laccase-catalysed oxidation of isorhamnetin produced a dimer, IP1 (*m/z* 629, yield 28.8%) and an unidentified product, IP2 (*m/z* 457, yield 21.8%). The dimer showed ~2× increase in antioxidant capacity compared to isorhamnetin, as measured in the standard DPPH assay while the unidentified product IP2 was less active than isorhamnetin. *Listeria monocytogenes* and methicillin resistant *Staphylococcus aureus* (MRSA) showed susceptibility to IP1 (MIC was ~2× lower compared to isorhamnetin), while *Pseudomonas aeruginosa* was susceptible to IP2 (MIC ~2× lower compared to isorhamnetin). Both oxidation products showed a bactericidal mode of cation; membrane destruction was evidenced by loss of cell constituents in cell integrity assays, and by cell pores and cell fragmentations observed in scanning electron microscope images. Results indicated that the dimer had improved antioxidant and antibacterial activities and may be exploited for nutraceutical purposes and in other relevant industries. On the other hand, the product IP2 may be useful in the treatment of Gram-negative infections associated with *P. aeruginosa*.

6.1. Introduction

The threatening global challenge of antibiotic resistance and oxidative stress have necessitated an increase in research aimed at discovering novel bioactive compounds in plant extracts with antioxidant (Zrira *et al*., 2016) and antimicrobial properties (Koubaa *et al*., 2015b). Bioactive compounds which originate from plants are diverse, usually possess a broad spectrum of biological activities and enjoy high consumer acceptance (Shlar *et al*., 2017). Therefore, natural antimicrobial and antioxidant substances are in high demand globally (Aremu *et al*., 2011). Increase in demand for natural antioxidant compounds has also been necessitated by adverse effects attributed to the use of synthetic antioxidants such as allergenicity, carcinogenicity, sperm and DNA abnormalities (Wojcik *et al*., 2010). At the same time, the increasing trend in antimicrobial resistance (AMR) has led to an increase in the search for alternatives to existing antibiotics.

Interestingly, phenolic compounds which exhibit antimicrobial and antioxidant properties are abundant in plants/nature (Sofi *et al*., 2016). However, most monomeric phenols have low antioxidant capacity, with some having prooxidant activity (Matsuura and Ohkatsu, 2000), short half-life in the body (Kudanga *et al.*, 2011) and poor physico-chemical properties such as low stability and low solubility (Hierholtzer *et al.*, 2012). To overcome these shortcomings, researchers are investigating the possibility of oligomerisation of phenolic molecules to higher molecular weight compounds with improved biological activities.

Laccase is one enzyme that is being investigated as a biocatalyst for the synthesis of polymeric or oligomeric phenolic molecules. Laccases (EC 1.10.3.2) are generally regarded as green catalysts as they are capable of catalysing the oxidation of phenols and related compounds to form radicals and produce water as the only by-product. The coupling of the radicals formed

result in the formation of oligomeric bioactive compounds. The oligomeric compounds usually have higher activities (Kudanga *et al.*, 2011) and sometimes better physico-chemical properties such as improved solubility (Nemadziva *et al.*, 2018). The improvement in bioactivities is mainly attributed to the increase in the number of hydroxyl groups as an outcome of the repeated monomeric units. Although peroxidases were initially used for coupling reactions, in recent years, laccase have become the preferred catalyst. Unlike peroxidases, they do not require co-factors such as hydrogen peroxide. Therefore, research interest in laccases as catalysts for organic synthesis of bioactive compounds is increasing (Kudanga *et al.*, 2017). However, the multiplicity of phenolic compounds in nature means there is room for further investigations and the possibility of producing new bioactive compounds.

The current study reports the synthesis of new bioactive compounds through laccase-catalysed biotransformation of isorhamnetin. Antioxidant and antibacterial activities of the synthesised compounds were measured respective activities of the substrate for comparison purposes. In addition, an investigation on the possible mode of antibacterial action of the synthesised products using *L. monocytogenes* (ATCC 19111), MRSA (ATCC 33591) and *P. aeruginosa* (ATCC 27853) as model pathogens was carried out.

6.2. Materials and methods

Analytical grade chemicals and solvents were used in this study. Isorhamnetin was purchased from Sigma Aldrich (South Africa). Unless otherwise stated, all other chemicals (and culture media) were obtained from Oxoid (UK) and Biolab-Merck (Modderfontein, South Africa). The *Trametes pubescens* strain was obtained from the Cape Peninsula University of Technology, South Africa and is currently deposited in the stock culture collection in the Department of Biotechnology and Food Technology, Durban University of Technology.

6.2.1. Laccase production from *T. pubescens*

T. pubescens fungal laccase was produced and followed by laccase enzyme activity (Childs and Bardsley, 1975) and protein concentration (Lowry *et al*., 1951) determination. Protocols were the same as described in chapter five, subsections 5.2.2 to 5.2.4.

6.2.2. Oxidation of isorhamnetin by laccase

A monophasic system with ethanol as co-solvent was used for the enzyme modification reaction. A 50%, v/v buffer to solvent ratio was used. The reaction was carried out in sodium acetate buffer in a 1:1 (v/v) mixture of acetate buffer (100 mM, pH 5.0) and ethanol, at 37°C with orbital shaking at 180 rpm. The reaction was started by adding laccase (3.3 U). A further 3.3 U of the enzyme was added after the first and second hour to replenish the enzyme inactivated by the solvent. Monitoring protocols by TLC and HPLC were earlier described in chapter five, section 5.2.5. TLC analysis was performed on aluminium–backed silica gel 60 F_{254} (Merck) plates using toluene: dioxane: acetic acid (11:2.5:0.3, v/v/v) mobile phase, and HPLC using the gradient set-up described in section 5.2.5.

6.2.3. Reaction products purification

The products of isorhamnetin oxidation were purified by preparative TLC using toluene: dioxane: acetic acid (11:2.5:0.3, v/v/v) as mobile phase. In the purification by TLC, the compounds were visualised by exposure to UV light at 254 nm. Products yield were calculated as a percentage of the amount of initial substrate used in the reaction. The pure product fractions were dehydrated using a rotary evaporator and washed sequentially with acetone, methanol and

then acetone again to remove the acetic acid. Products yield were thereafter calculated as a percentage of the initial substrate mass which went into the reaction (Adelakun *et al*., 2012a).

6.2.4. Product characterisation by LCMS

The isorhamnetin oxidation products were characterised using the method described in chapter five, subsection 5.2.7. Liquid chromatogram and mass spectra (MS) characteristics of products were acquired in the negative mode using a Shimadzu LCMS-2020 mass spectrometer (Shimadzu, Japan).

6.2.5. Antioxidant activity assays

Antioxidant assays were carried out as described by Mahdi-Pour *et al.* (2012), Re *et al.* (1999) and Prabhavathi *et al.* (2016) with some modifications. The DPPH, ABTS and FRAP methods used were earlier described in chapter five, subsections 5.2.8.1, 5.2.8.2 and 5.2.8.3, respectively.

6.2.6. Determination of antibacterial activity

6.2.6.1. Antibacterial activity by disc diffusion assay

Antibacterial activity was assayed using the disc diffusion test followed by determination of the minimum inhibitory concentration (MIC) (Zhao *et al*., 2015). Isorhamnetin and its products were impregnated on sterile filter paper discs at 10 mM concentration. A 100 µl of each 0.5 McFarland bacterial turbidity of *L. monocytogenes* and MRSA (for isorhamnetin dimer product 1) and *P. aeruginosa* (for isorhamnetin product 2) was aseptically spread on petri dishes

containing solidified Mueller Hinton agar. The choice of microorganisms to use for each product was made after a susceptibility screening exercise across various bacteria. Plates were labelled accordingly for each bacterium and compound. Thereafter, discs impregnated with the test compounds were carefully placed on inoculated plates using sterile forceps. Plates were then left for adequate diffusion, inverted and incubated at 37°C for 24 h. Thereafter the diameter of zones of inhibition were measured and recorded in mm. A similar method was described in chapter five section 5.2.9, subsection 5.2.9.1.

6.2.6.2. Minimum inhibitory concentration (MIC) determination

MIC was determined by the microbroth dilution technique in microtitre plate wells (Corning, Costar, U.S.A.) using 0.5 McFarland bacterial suspension standards (CLSI, 2000). Cultures were incubated with the respective test compound and plates were incubated at 37ºC for 18-24 h. Negative (suspension broth and compounds in solvent) and positive (bacterial broth suspensions only) controls were included in separate wells. The experiment was done three times. The lowest concentration of the compounds required to visibly inhibit growth was taken as the MIC. A similar method was described in chapter five section 5.2.9, subsection 5.2.9.2. Following determination of antibacterial activity, integrity of bacterial cell membrane, time-kill assay and scanning electron microscopy (SEM) analyses of treated and untreated bacterial cells were carried out to determine the potential mechanism of antibacterial action of the oxidation products.

6.2.7. Determination of products antibacterial mechanism of action

The potential mechanism of action of the isorhamnetin oxidation products were determined using the bacterial cell membrane integrity assay (described in subsection 5.2.10), time-kill

assay (described in subsection 5.2.11), and SEM analysis of treated and untreated cells (described in subsection 5.2.12) of treated and untreated bacterial cells using antibiotic resistant typed strains of *L. moncytogenes,* MRSA and *P. aeruginosa*.

6.2.8. Statistical analysis

Unless otherwise stated, experiments were performed in triplicate and results were expressed as mean ± standard deviation (SD). Duncan's Multiple Range test in SPSS version 16 was used as a posthoc test to separate the mean values. *P* values < 0.05 were regarded as significant.

6.3. Results and discussion

6.3.1. Characterisation of oxidation products by TLC and LCMS

Isorhamnetin (used as the monomeric substrate), was successfully oxidised using *T. pubescens* laccase in 50% ethanol reaction with 100 mM sodium acetate buffer (pH 5) to yield two oxidation products (Plate 6.1). A 50% ethanol ratio to buffer was chosen based on an earlier study by Zwane *et al*. (2012). Product 1 (IP1) retention factor (Rf) was lower (0.16) compared to that of the substrate (0.36), and substrate Rf lower compared to product 2 (IP2) (0.59). This indicated that IP1 is more polar and more strongly interacted with the adsorbent (silica gel) probably due to an increase in number of hydroxyl groups compared to product 2 which is a less polar product (Block *et al*., 2016). A compound's Rf value may vary and is dependent on factors such as the type of mobile phase used, type of solvent used in making the mobile phase and their respective ratios and polarities, type and structure of compounds being separated, as well as a compound affinity for the adsorbent (Block *et al*., 2016).

HPLC analysis showed that laccase oxidation of isorhamnetin (tR=14.78) resulted in the production of two products IP1 (tR=10.67 min) and IP2 (tR=13.68 min) (Fig. 6.1). The retention times indicate that the products were more polar than the substrate possibly as a result of an increase in the number of hydroxyl groups as larger molecular weight compounds were formed (Block *et al.*, 2016). IP1 was more polar than IP2 indicating that it may be carrying more hydroxyl groups. LC-MS analysis in negative mode showed a *m/z* of 629 (M=630) for IP1 which indicated formation of a dimer isorhamnetin (*m/z* 315; M=316). On the other hand, a *m/z* of 457 was observed for IP2 (Fig. 6.2) indicating a lower molecular weight which supports the assumption that it may be carrying fewer hydroxyl groups (hence a lower polarity) compared to IP1).

The hydroxyl group on the aromatic ring is *ortho*- or *para* directing in the predicted IP1 dimer structures (Figs. 6.3 and 6.4) (Suparno *et al.*, 2005; Schultz *et al.*, 2001) and molecules with a free C-5 position usually dimerise through 5−5 (C-C) and 4-O-5 (C-O-C). Therefore, the proposed dimeric products of oxidative dimerisation of isorhamnetin are shown in Fig. 6.3 (C-C dimer) and Fig. 6.4 (C-O-C-linked dimer). The corresponding reaction mechanisms are proposed in Fig. 6.5 and 6.6, respectively. However, the C-C linkage has a lower heat for formation compared to the C-O-C linkage (Valencia and Marinez, 2005). It is therefore proposed that the dimer produce was the one linked through the more stable C-C bonds (del Río and Gutiérrez, 2008) (Fig. 6.3). This is also supported by the significant improvement in antioxidant activity; usually C-O-C linkages do not show a significant improvement in biological activity because of the participation of a hydroxyl group in the formation of the linkage. The second product, IP2, was probably formed through the coupling of an oxidative cleavage product and isorhamnetin monomer as proposed in Fig. 6.7. The corresponding reaction mechanism is proposed in Fig. 6.8. Therefore, there was no doubling of hydroxyl groups, and participation of a hydroxyl group in the formation of an ether linkage could have

resulted in low antioxidant activity, as will be discussed in the following section. Nevertheless, the presence of an added methoxy group and presence of unsaturation in an aromatic ring have been shown to enhance other biological activities such as antimicrobial activity more than antioxidant activity (Hierholtzer *et al.*, 2012).

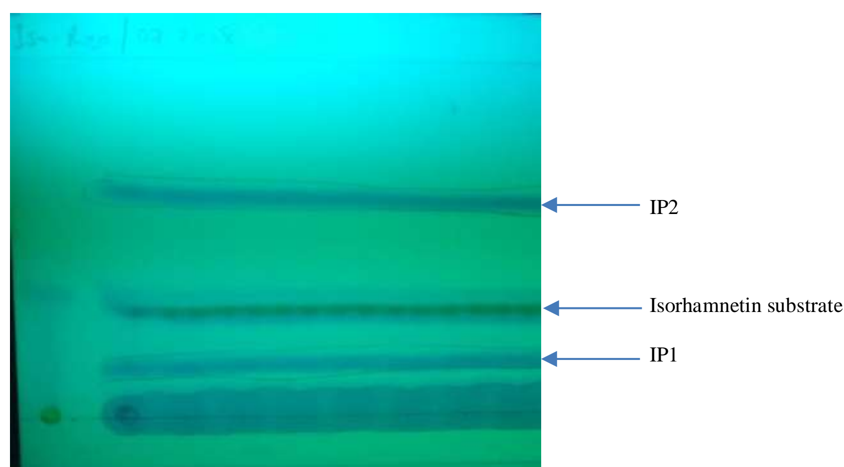

Plate 6.1: TLC plate showing products (IP1 and IP2) and substrate lanes

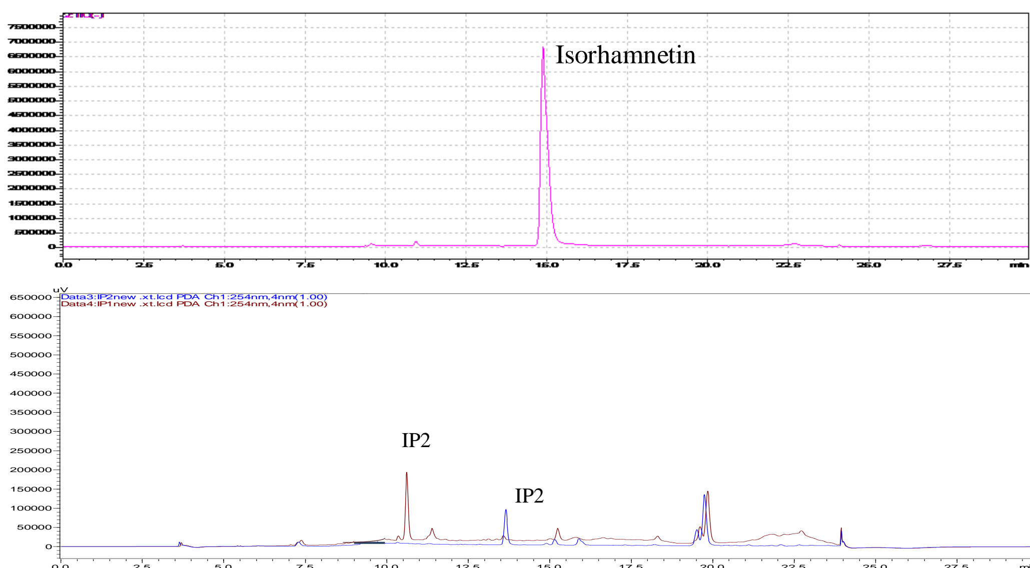

Figure 6.1: HPLC chromatogram of isorhamnetin and its oxidation products (IP1 and IP2)

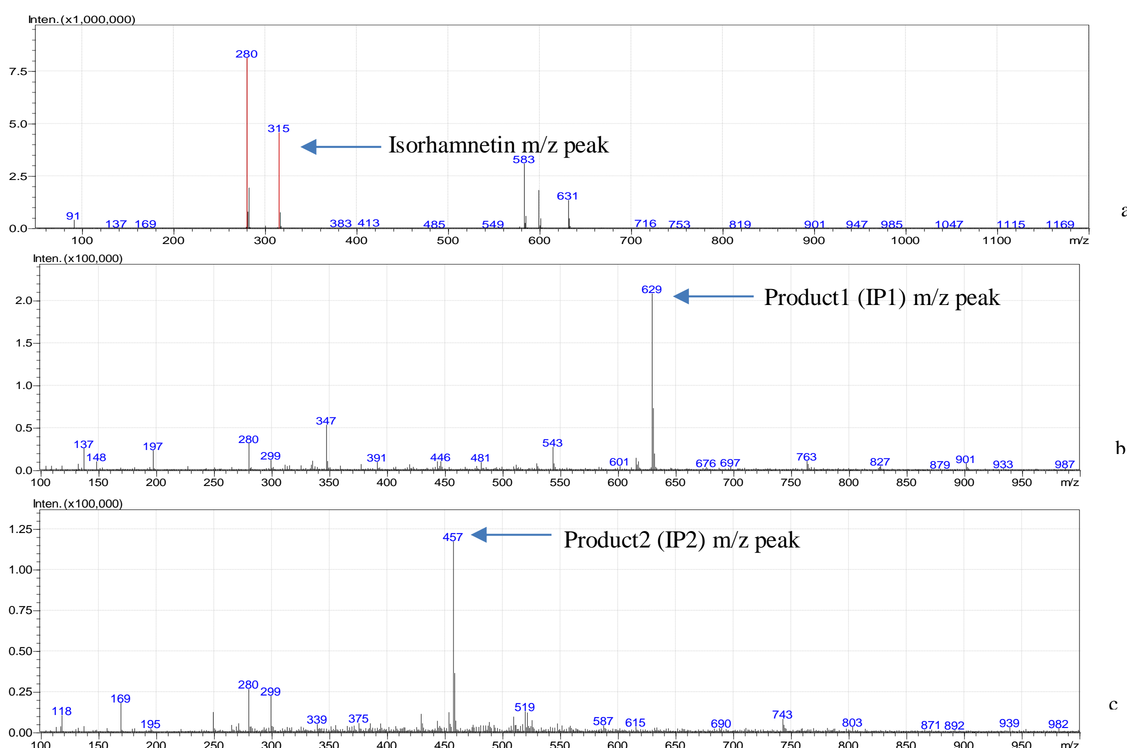

Figure 6.2: Mass spectra of (a) Isorhamnetin (m/z 315), (b) Product IP1 (dimer) (*m/z* 629) and (c) Product IP2 (*m/z* 457). The analysis was carried out in negative mode.

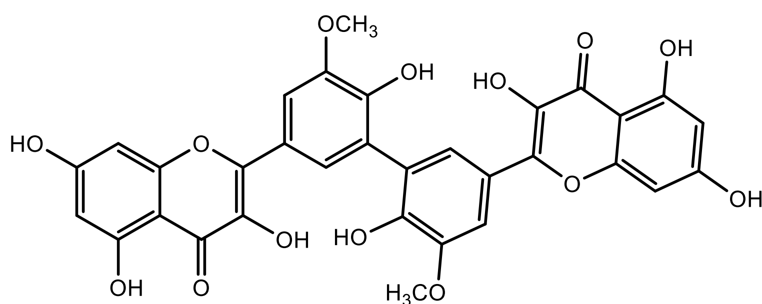

Figure 6.3: Proposed structure of the C-C linked dimer (IP1), m/z 629 (M=630).

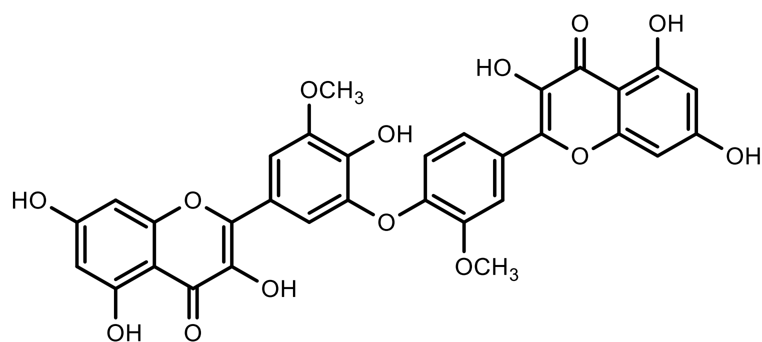

Figure 6.4: Proposed structure of C-O-C linked dimer (m/z 629, M=630).

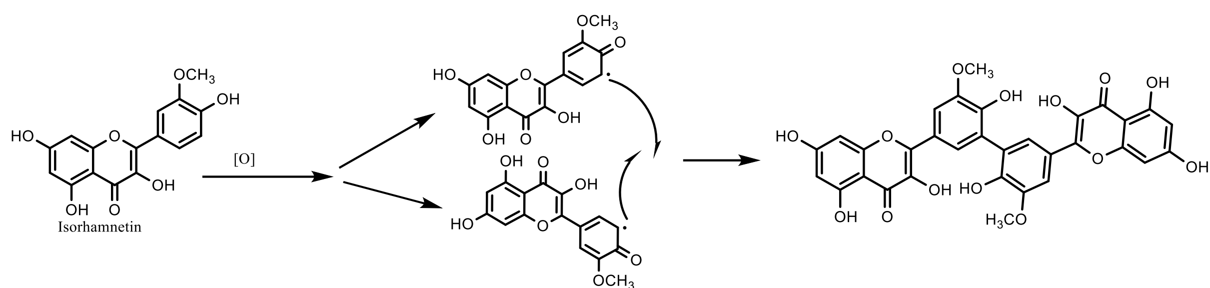

Figure 6.5: Proposed mechanistic pathway for the production of the C-C dimer through laccase catalysed dimerisation of isorhamnetin.

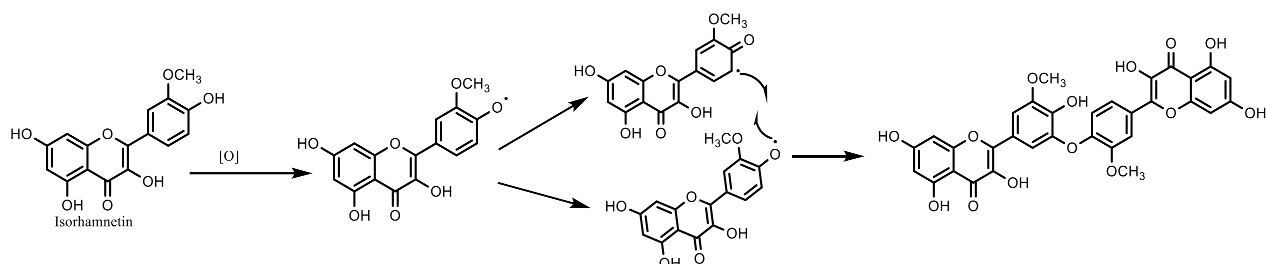

Figure 6.6: Proposed mechanistic pathway for the production of the ether linked dimer through laccase catalysed dimerisation of isorhamnetin.

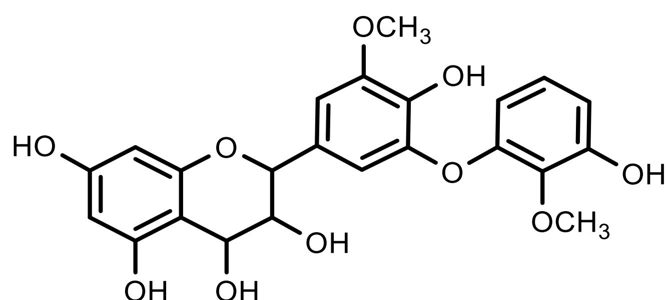

Figure 6.7: Proposed structure of IP2 (*m/z* 457, M = 458).

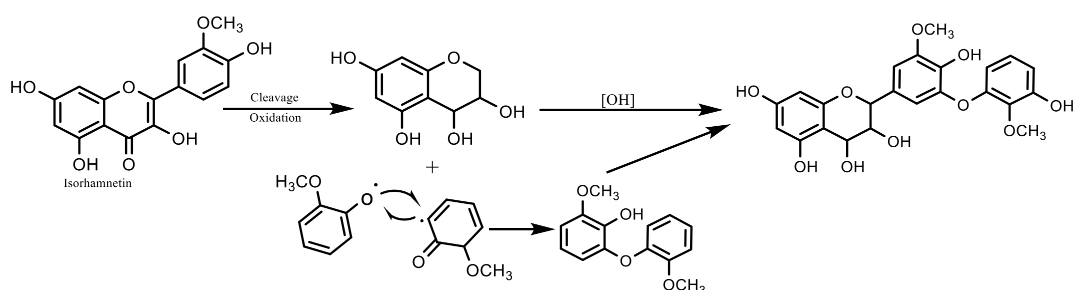

Figure 6.8: Proposed reaction mechanism for the laccase-catalysed production of IP2.

The yields of IP1 and IP2 were 28.8±1.20% and 21.8±0.91%, respectively (Table 6.1). Although the yields are comparable to other laccase-catalysed reactions (Adelakun *et al.*, 2012b), they are generally low. This may be attributed to non-specific side reactions, radical polymerisation instead of forming oligomers, and possibly inactivation of the enzyme by the solvent (Kudanga *et al.*, 2017). Yield may also be affected by the type and amount of solvent used, and the ease of oxidation of the substrate structure (Adelakun *et al.*, 2012a).

Table 6.1: Percentage yield of isorhamnetin products

Compounds	Derived product mass (mg)	Reaction mass (mg)	Yield (%)
Product 1 (IP1)	7.0	24.3	28.8±1.20[a]
Product 2 (IP2)	5.3	24.3	21.8±0.91[b]

Values with different superscripts (a, b) in the same column are significantly different ($p<0.05$). Results are means ± standard deviation of three replicate determinations.

6.3.2. Antioxidant activity of oxidation products

The results of antioxidant assays are shown in Table 6.2. The dimer IP1 showed a 2-fold significant increase in radical scavenging activity, compared to the substrate in the DPPH assay (Table 6.2). However, IP2 showed a lower scavenging activity compared to the substrate. This same trend in antioxidant activity was observed in the ABTS and FRAP assays (Table 6.2). The enhancement of hydrogen-donating ability or radical scavenging ability of the dimer (IP1) may be explained by the increase in hydroxylation after dimerisation (Zwane *et al.*, 2012).

Table 6.2: Antioxidant activity of isorhamnetin and its products

Compound	Molecular weight (g/mol)	EC_{50}DPPH[d] (µM)	EC_{50}ABTS[e] (µM)	FRAP[d] (µM)
Isorhamnetin	315	10.17±0.61[b]	11.05±1.01[b]	79.84±0.07[b]
Product 1 (IP1)	629	5.04±0.08[c]	9.63±0.44[c]	81.09±0.55[a]
Product 2 (IP2)	457	27.16±0.38[a]	18.21±0.56[a]	47.62±0.62[c]

Values with different superscripts (a, b, c) in the same column are significantly different ($p<0.05$). Results are means ± standard deviation of three replicate determinations.
[d] EC_{50} value is defined as the concentration (µM) of substrate that results in 50% loss of DPPH• and ABTS• (Adelakun *et al.*, 2012a)
[e] Antioxidant ability to reduce Fe^{3+} complex tothe ferrous Fe^{2+}form (Saeed *et al.*, 2007)

6.3.3. Antibacterial activity determination

With the dimer IP1, a 1.3- and 1.2-fold increase in the diameter of inhibition zones against *L. monocytogenes* and MRSA, respectively, was observed when compared to isorhamnetin (Table 6.3). However, only a modest 1.1-fold increase in zone of inhibition against *P. aeruginosa* was observed for IP2 when compared to isorhamnetin. It was also not active against *L. monocytogenes* and MRSA at the highest concentration (20 mM) used. IP1 was not effective against *P. aeruginosa* even at the highest concentration used. Reduced MICs for the products compared to the substrate may be due to increase in hydroxyl groups in IP1. Usually, a high number of hydroxyl groups or the presence of *ortho* hydroxylation increases the likelihood of a better antimicrobial effect. An improvement in antimicrobial action has been reported for polymeric phenol compounds that have several hydroxyl groups (Ciocan and Bara, 2007). For example, Hierholtzer *et al.* (2012) observed improved bactericidal activity in phlorotannin (polymerised form) compared to its monomeric phloroglucinol form. Enhancement of antioxidant and antimicrobial properties in dimeric/oligomeric products have also been attributed to an increase in electron-donating groups such hydroxy and methoxy (alkoxy) groups. The higher sensitive of the Gram-positive microbes to IP1 may be linked to the

peptidoglycan nature of their cell membrane which facilitate cell wall penetration (Zhao *et al*., 2015). On the other hand, Gram negative bacteria are usually more resistant because of the presence of lipopolysaccharides in the outer membrane which obstruct the transport of compounds across the cell membrane (Zorofchian Moghadamtousi *et al*., 2014). Nevertheless, the higher efficacy of IP2 (Fig. 6.7) on Gram-negative *P. aeruginosa* compared to other test bacteria was rather surprising. Gopalakrishnan *et al.* (2009) reported that at low concentrations, chemically synthesised antimicrobial compounds having methoxy and methyl functional groups at *para* positions of a phenyl ring showed strong antibacterial action on Gram-negative *P. aeruginosa* and *E. coli* compared to some Gram-positive test bacteria used. However, further research is necessary to fully understand the mechanism of action of IP2 and explain its effectiveness against the Gram-negative *P. aeruginosa*.

An antimicrobial compound's static or cidal action may be attributed to the binding of soluble and extracellular proteins and/or disruption of cell membrane integrity (Ciocan and Bara, 2007). Isorhamnetin brings about its bactericidal mode of action through the disruption of bacterial cell division, cell membrane and cell lysis and inhibition of DNA binding activity (Jin *et al*., 2013; Tagousop *et al*., 2018). Therefore, in addition to confirming the antibacterial efficacy of isorhamnetin products, we went further to carry out assays to determine the potential mechanism of action of the oxidation products.

Table 6.3: Antibacterial activity of isorhamnetin and its products

	Isorhamnetin		**Product IP1**		**Product IP2**	
	Inhibition zone diameter (mm) at 10mM	**MIC (mM)**	**Inhibition zone diameter (mm) at 10mM**	**MIC (mM)**	**Inhibition zone diameter (mm) at 10mM**	**MIC**
Pseudomonas aeruginosa	5.90±0.14[b]	1.25	0.00±0.00	-	6.45±0.07[a]	0.62
Listeria monocytogenes	5.25±0.31[b]	1.25	6.55±0.07[a]	0.62	0.00±0.00	-
methicillin resistant *Staphylococcus aureus* (MRSA)	5.90±0.14[b]	2.50	7.20±0.23[a]	1.25	0.00±0.00	-

Results are means ± standard deviation of three replicate determinations.
Values with different superscripts (a, b) in the same row are significantly different ($p<0.05$).
Note: Where a '-' is recorded there was no inhibition at the highest concentration (20 mM) used.

6.3.4. Integrity of bacterial cell membrane

Leakage of proteins and cell constituents from *L. monocytogenes* and MRSA cells increased with an increase in the concentration of IP1 used to treat the cells (Table 6.4). This was also the case with IP2-treated *P. aeruginosa* cells (Table 6.4). Similar observations have been demonstrated in other studies (Sadiq *et al.,* 2017). Phenolic compounds (including high molecular weight polyphenols) functional groups bind to cell wall proteins and enzymes by forming unstable complexes, and inhibit the crosslinking stage of cell wall synthesis (Shi *et al.*, 2016). Inhibition of cell wall synthesis then results in increased cell membrane permeability. The increase in membrane permeability causes structural alterations in the microbial membrane, accelerates leakage of cellular components such as nucleic acid, glutamic acid and protein derivatives, and facilitate the influx of more of the antimicrobial compound (Wu *et al.*, 2016). Usually, at high concentrations, phenols ten to bind to and inhibit permeases thereby

denaturing bacterial proteins and causing cell membrane lysis (Shi *et al*., 2016). The accumulation and increase of hydrophobic phenolic groups in the lipid bilayer may also disrupt cell membrane potential and lipid–protein interaction due to increased hyperpolarisation (Wu *et al*., 2016).

Table 6.4: Effect of isorhamnetin products on bacterial cell membrane integrity

		Listeria monocytogenes		**MRSA**	
Isorhamnetin Products		**Protein (µg/ml)**	**Cell constituents (OD_{260})**	**Protein (µg/ml)**	**Cell constituents (OD_{260})**
	2xMIC	37.38±0.24[a]	0.072	32.94±0.12[a]	0.110
Product 1	MIC	11.73±0.11[b]	0.059	18.62±0.23[b]	0.090
	Control	3.08±0.02[c]	0.006	5.76±0.01[c]	0.002
		Pseudomonas aeruginosa			
		Protein (µg/ml)		**Cell constituents (OD_{260})**	
	2xMIC	13.68±0.04[a]		0.054	
Product 2	MIC	8.25±0.10[b]		0.041	
	Control	2.43±0.02[c]		0.006	

Results are means ± standard deviation of three replicate determinations.
Values with different superscripts (a, b, c) in the same column are significantly different ($p<0.05$).

6.3.5. Bacterial rate of kill

Time-kill assays showed more than 5 log reduction in colony forming units of MRSA (from 6 log cfu/mL to 0.1 log cfu/mL) and L. monocytogenes (from 6.3 log cfu/mL to 0.4 log cfu/mL) at respective MIC concentrations of IP1 within an hour (Fig. 6.9 and 6.10). IP2 reduced the original number of *P. aeruginosa* cells from 7 log cfu/mL to 1log cfu/mL within the first two hours of exposure (Fig. 6.11). This was a significant decrease in cell number, and for an opportunistic Gram-negative pathogen with well-known antibiotic resistance. Observed reductions from the original starting inoculum were greater than 3 log cfu/mL reduction benchmark for bactericidal activity. This suggests a bactericidal model of action. Cell growth

in the absence of the compound (control) was consistent and exponential for all strains during the assay time. The rate of kill was higher in *L. monocytogenes* than MRSA as almost all bacterial cells were killed within the first two hours of exposure to MIC concentration of the dimer IP1 (Fig. 6.9).

Oligomerisation and structural modification in our oxidation products could be responsible for their bactericidal activity over a short period of time in time-kill assay (Olajuyigbe and Afolayan, 2012). Bacterial growth inhibition and death may have been achieved by microbial enzyme inhibition and cytoplasmic membrane destabilisation (Shi *et al*., 2016; Wu *et al*., 2016). The variation in the compound killing kinetics for the different strains was expected. Although both test strains were susceptible to IP1 and are Gram positive microbes with different cellular morphologies (*L. monocytogenes* is a coccobacillus, while MRSA is a coccoid shaped bacterium) (Lee and Kim, 2018), more important are the possible presence or absence of intrinsic cellular resistance factors which may impact the killing rate in both microbes at varying degrees (Kaur and Peterson, 2018). These mechanisms may be better developed in MRSA than in *L. monocytogenes*. Several genes (mobile genetic elements such as *mecA* and *aac*6' gene in MRSA, *gyrA* in *L. monocytogenes* and a variety of other genes) contribute to the innate resistance in all microorganisms mediated by the cell membrane and efflux systems (Kaur and Peterson, 2018; Kumar and Schweizer, 2005). *Listeria monocytogenes* possesses a modified antibiotic target (penicillin-binding proteins - PBP) which constitutes part of its intrinsic resistance mechanisms to antimicrobials, but PBP bind poorly to antimicrobial compounds (Cox and Wright, 2013). Microbes may also be able to modify antibiotic compounds through the addition of acetyl, phosphate, or adenyl groups to make them less harmful (Kaur and Peterson, 2018). Antibiotic degradation may also take place through enzymes that facilitate the hydrolysis of the antimicrobial compounds, for example, β-lactamases in MRSA. Efflux pumps remove antimicrobial compounds from within the microbial cell using energy from adenosine

triphosphate (ATP) (Kumar and Schweizer, 2005). The effect of antimicrobial agents on cells may also be by-passed by cells producing additional antibiotic targets or subunits which mimic the real targets and these targets are prone to being bound by the antibiotic agents instead (Kaur and Peterson, 2018).

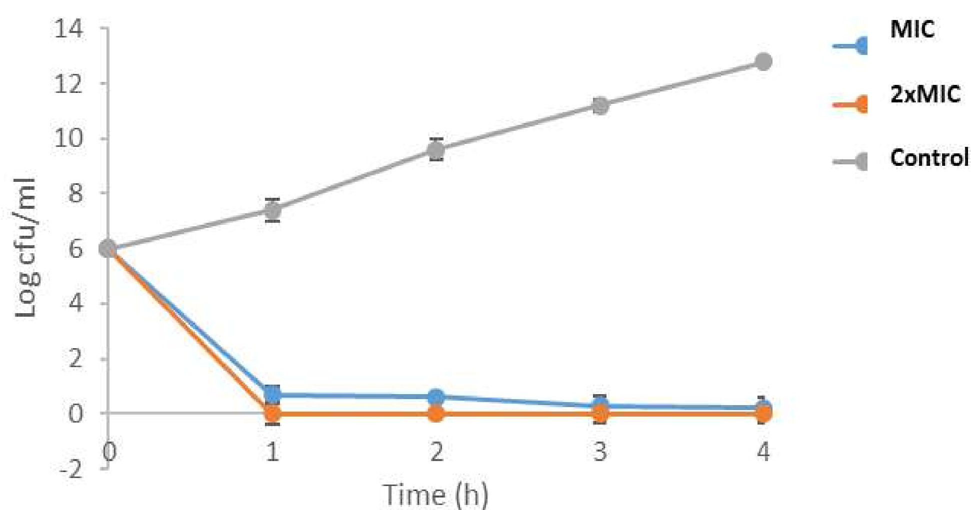

Figure 6.9: Time-kill curve for bactericidal action of IP1 against MRSA
Note: Test compound was considered bactericidal if original inoculum was reduced by ≥3 Log cfu/mL (Barry *et al.,* 1999).

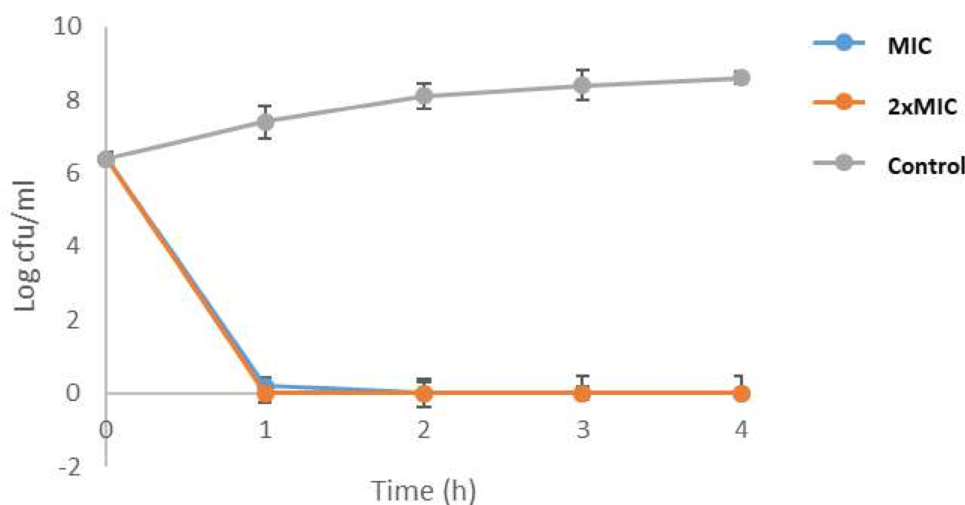

Figure 6.10: Time-kill curve for bactericidal action of IP1 against *L. monocytogenes*
Note: Test compound was considered bactericidal if original inoculum was reduced by ≥3 Log cfu/mL (Barry *et al.,* 1999).

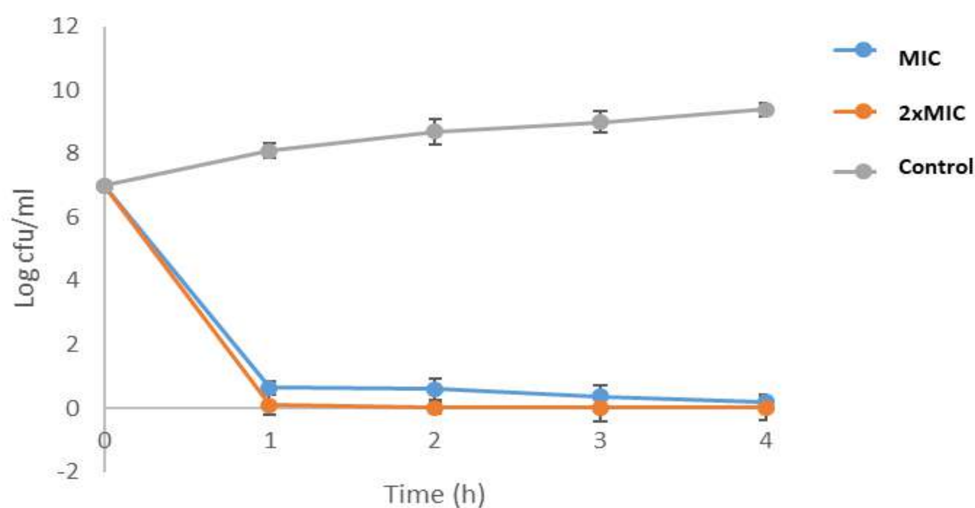

Figure 6.11: Time-kill curve for bactericidal action of IP2 against *P. aeruginosa*
Note: Test compound was considered bactericidal if inoculum was reduced by ≥3 Log cfu/mL (Barry *et al.,* 1999).

6.3.6. SEM observation of bacterial cells

Following the initial antibacterial, cell integrity and time-kill assays, we went further to confirm the potential antibacterial mechanism of action of our isorhamnetin novel products by SEM visualisation of treated and untreated bacterial cells. Bases on the antimicrobial activities, changes in cell surface morphology in MRSA and *L. monocytogenes* were assessed after treatment with the dimerised product (IP1) (Fig. 6.12). Similarly, changes in cell morphology of *P. aeruginosa* were evaluated after treatment with IP2 (Fig. 6.13). The changes were compared with untreated (control) cells. Results showed disruption of cell membrane surface, dips/pores, deformation, cell fragmentations (cell breakage leading to irregularity in cell lengths) and loss of cell constituents (Figs. 6.12 and 6.13). Findings were consistent with results of cell integrity assays. This suggests that the loss of cell constituents could have been a result of successful penetration and transport of products across the cell membrane and into the cells. Deformations and other changes in cell morphology and membrane integrity have been reported upon exposure of bacterial cells to phenolic extracts (Zhao *et al.*, 2015) and phenolic compounds (Sim *et al.*, 2014) with antimicrobial activity. The bactericidal effect of both

products may be attributed to their ability to circumvent resistant mechanisms such as the presence of resistance genes and the pump-out efflux system resulting in penetration of the bacterial cell wall and cell membrane (Wu *et al.*, 2016).

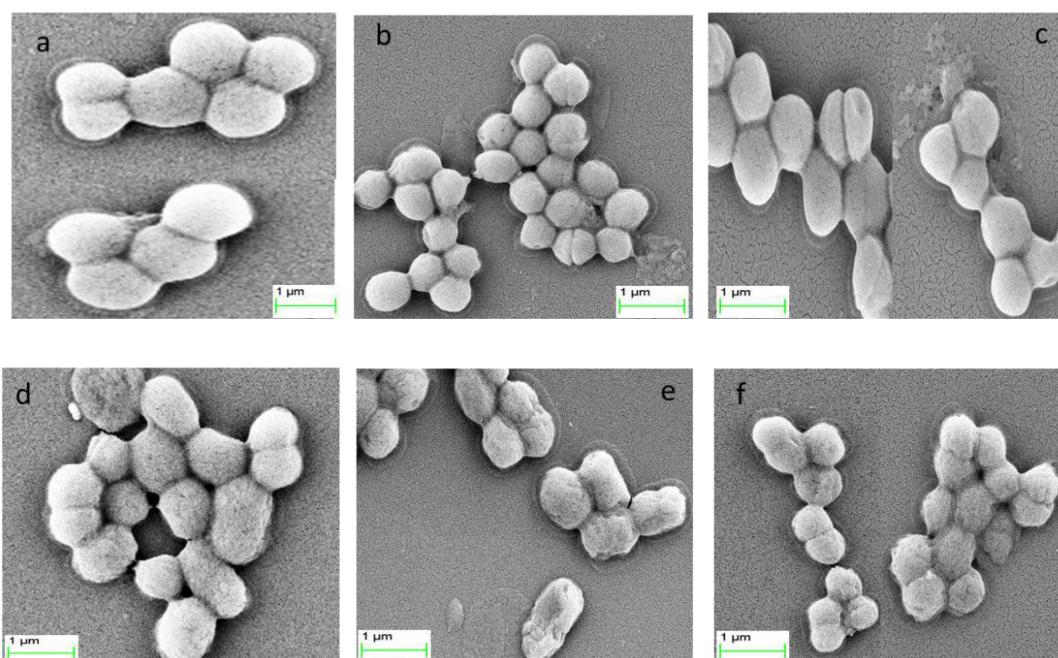

Figure 6.12: SEM images of bacterial cells treated with the dimer (IP1) (a) untreated MRSA cells (control), (b) MRSA cells treated with MIC and (c) MRSA cells treated with 2xMIC concentrations, (d) untreated *L. monocytogenes* cells (control), (e) *L. monocytogenes* cells treated with MIC and (f) *L. monocytogenes* cells treated with 2×MIC.

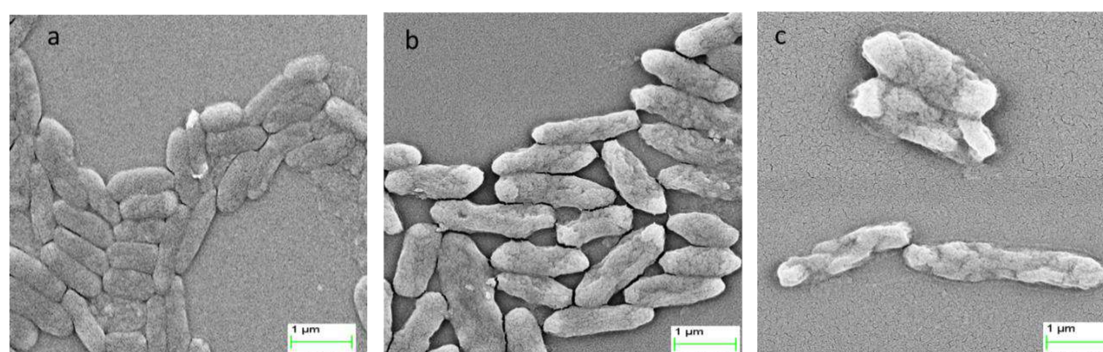

Figure 6.13: SEM images of *P. aeruginosa* cells treated with product (IP2) (a) untreated *P. aeruginosa* cells (control), (b) *P. aeruginosa* cells treated with MIC and (c) *P. aeruginosa* cells treated with 2×MIC.

6.4. Conclusion

The biocatalytic oxidation of isorhamnetin by the fungal laccase from *T. pubescens* resulted in the production of two main products IP1 (a dimer) and IP2 (unidentified). The dimeric product showed improved antioxidant and antimicrobial capacities compared to the starting substrate. However, antimicrobial activity varied with microorganisms tested in the case of IP2. The product IP2 was more active against Gram negative *P. aeruginosa* than the IP1 dimer. A bactericidal mode of action of both products was demonstrated. Biologically synthesised products may find application in the food industry, as well as in the pharmaceutical and medical industries.

CHAPTER SEVEN

GENERAL DISCUSSION

In this thesis, *Opuntia* cladode, fruit pulp and peel extract profiling was used to determine phenolic compound types in different extracts. The effect of extraction solvents and plant processing methods such as freeze-drying and oven-drying, on phenolic composition, antioxidant and antibacterial activities was also studied. Thereafter, laccase was used to modify two selected *Opuntia*-related phenolic substrates after initial screening to determine their suitability as good laccase substrates. The first part of this section discusses the effect of processing methods on yield, phenolic profile, antioxidant and antibacterial activities of *Opuntia* cladode extracts (Chapter 3). This was followed by a focus on the effect of plant material dehydration methods on yield, phenolic profile, antioxidant and antibacterial activities of *Opuntia* fruit pulp and peel extracts (Chapter 4). A third research chapter focuses on laccase application in the enzymatic modification of luteolin, an *Opuntia*-related flavonoid compound, for improving biological activities (Chapter 5). The final experimental chapter reported laccase catalysis in homocoupling reactions using isorhamnetin, another selected *Opuntia* phenolic compound, as a substrate (Chapter 6).

The phenolic compositions of *Opuntia* cladode extracts (Chapter 3) and *Opuntia* fruits and by-products (Chapter 4) were determined as potential sources of new and natural phenolic compounds. Extract yields were affected by plant material, drying method applied and extraction solvent used. In most cases, freeze-dried extracts produced better phenolic content and activities than oven-dried fractions (Torres *et al*., 2010). Generally, extracts demonstrated varying degrees of antioxidant and antibacterial activities depending on their phenolic composition. However, in a few cases phenolic content did not directly correlate with biological

activities. Macromolecular antioxidant (MA) extracts, however, showed the best biological activity profile, followed by the hydroalcoholic and hexane fractions (Chapters 3 and 4). Sample hydrolysis and use of alcoholic solvents allowed good expression of phenolic plant constituents. Eucomic acid, coumaric acid, piscidic acid, quercetin, isorhamnetin and kaempferol, were identified in the cladodes (Chapter 3), while rutin and taxifolin flavonoids, caffeic acid, piscidic acid, coumaric acid, ferulic acid and eucomic acid were identified in the fruits (Chapter 4). The relevance of extract profiling cannot be overemphasised in the discovery of novel and natural bioactive compounds. *Opuntia* extract profiling in this study led to the first-time report of isovitexin 7-*O*-xyloside-2"-*O*-glucoside, polyhydroxypregnane glycoside and neohancoside C in *Opuntia* cladodes (Chapter 3), and pinellic acid in *Opuntia* fruits (Chapter 4). The observations agreed with the first research hypothesis which stated that southern African *Opuntia* cladodes and fruits would show a different phytochemical profile, and presence of new bioactive compounds as nutritional/phytochemical profiles have been shown to vary with differences in geographical location and climatic conditions (Lallouche *et al*., 2015). The new compounds could serve as fingerprint molecules for the identification and taxonomic classification of southern African *Opuntia* cladodes and fruits.

Given the increased consumer awareness of adverse effects attributed to the use of synthetic antioxidants and antimicrobials in various industries, there is an increase in global demand for health promoting natural components/additives/supplements in food and other industries (Aremu *et al*., 2011). New microbial sources of antimicrobials are not readily available, and synthetic antioxidants cause adverse health effects (Wojcik *et al*., 2010). Therefore, the utilisation of biotechnological tools and systems to produce novel molecules with improved biological capabilities from known natural compounds is a current trend in research. It is also worthy noting the reported application of crosslinking enzymes such as laccase for improving

activities of monomeric phenols through coupling reactions (Nemadziva *et al*., 2018; Kudanga *et al*., 2017).

In the light of the foregoing, the ability of laccase to form novel products from phenoxy radicals generated from oxidation of *Opuntia*-related monomeric substrates in optimised reaction systems was investigated (Chapters 5 and 6). Laccases are known to oxidise a wide array of phenolic and non-phenolic compounds (Riva, 2006; Kudanga *et al.*, 2017). Thus, they are important biocatalysts in organic synthesis. Although yields obtained in this study (luteolin dimer 14.42±1.32%, chapter 5; isorhamnetin dimer 28.8±1.20%, chapter 6, and unidentified product IP2, 21.8±0.91%, chapter 6), were comparative to other studies (Adelakun *et al.*, 2012a,b; Gavezzotti *et al*., 2014), the yields were still low. The non-specificity of laccase oxidation reactions may cause low product yields. Free radical generation is important and must occur prior to their crosslinking or coupling. The oxidation reaction stage is difficult to control and usually result in a mixture of products (Kudanga *et al*., 2017). Uncontrolled polymerisation also results in a loss of target dimeric or oligomeric products in organic synthesis. Although the use of organic co-solvents reduces polymerisation, it is not completely eliminated. The use of organic solvents also compromises enzyme activity (Klibanov, 2001). A major limitation of organic solvents arises from the fact that water is fundamentally required by enzymes for conformational flexibility and catalysis activity. Therefore, the loss of this essential water that is bound to the surface of the enzyme in organic solvent systems results in lessened enzyme activity and conformational flexibility (Saha *et al.*, 2010; Klibanov, 2001).

The enhancement of antioxidant activity of molecules is usually the main aim when laccase coupling reactions are carried out. In this study, the possibility of improving antibacterial activity and potential antimicrobial mechanism of action of the new oxidation products were also investigated. The luteolin and isorhamnetin dimers showed improved antioxidant and

antimicrobial activities compared to their corresponding substrates. The antioxidant and/or antimicrobial activity of molecules has been reported to improve with an increase in the number of hydroxyl groups after oligomerisation (Cai *et al*., 2006; Kudanga *et al*., 2017). Dimerised products showed improved antioxidant and antibacterial activity probably due to the doubling of hydroxyl groups in the C-C coupled products. The enhancement in antioxidant and antimicrobial activities in the dimeric products indicated that the products were most likely formed through a C-C linkage of the monomeric phenolic compounds. According to earlier research, C-C linked dimers/products show better improvement in biological activity compared to ether (C-O-C) linked products (Adelakun *et al.*, 2012a,b). In ether linkages, some hydroxyl groups are lost as they participate in ether linkage formation. The predicted structures showing the doubling of hydroxyl groups which is consistent with the improvement of activities were shown in chapters 5 and 6.

The mode of antimicrobial action was elucidated using scanning electron microscopy (SEM) observation, bacterial cell integrity and time-kill assays. Bactericidal action was determined based on a ≥3Log reduction in cfu/mL of initial inoculum size used at beginning of time-kill assay (Barry *et al*., 1999). Furthermore, cell integrity tests showed loss of cell constituents with increasing product concentration, while SEM observations showed formation of dips/pores/pits on the cellular membrane surfaces and also showed bacterial cell fragmentations. Cell integrity assays and SEM observations indicated that oxidation products were active in the destruction of bacterial cell membranes. The improvement in biological activities in the synthesised oligomers/products indicated that laccase-catalysed modification of phenolics could be a viable option for improving activities. The enzymatic biotransformation of readily available and natural plant compounds to derive new molecules with enhanced biological capabilities has great prospects for industries such as food, medical and pharmaceutical industries (Pezzella *et al*., 2015).

CHAPTER EIGHT

CONCLUSION AND RECOMMENDATIONS

8.1. General conclusion

There has been an increase in research activities on the profiling of phenolic extracts for potential discovery of new and natural bioactive compounds. This is due to the increase of incidences of multidrug resistant microbial strains, and adverse effects associated with the use of synthetic antioxidants. Natural molecules are believed to be better absorbed *in vivo*, and research has also been channelled towards improving the biological activities of natural molecules. This study investigated the compound profile of southern African *Opuntia* cladodes, fruit pulp and peel. In addition, the *T. pubescens* laccase was used to modify selected compounds (luteolin and isorhamnetin) as a way of improving biological activities of the compounds.

Below is a brief summary of the key findings.

The *Opuntia* plant consists of a wide variety of phenolic compounds, most of which are yet to be reported. The type of compounds expressed was dependent on the choice of extraction solvent. Phenolic compound profile, antioxidant and antibacterial activities were better in macromolecular antioxidant (MA) and hydroalcoholic extracts of cladodes, fruits and peels compared to aqueous fractions. Higher biological activities were recorded for MA and other hydrolysed extracts compared to the unhydrolysed/extractable polyphenol (EP) fractions. Both EP and MA extracts showed the presence of compounds (isovitexin 7-*O*-xyloside-2"-*O*-glucoside, polyhydroxypregnane glycoside, neohancoside C and pinellic acid) that had not been

previously reported to be associated with the *Opuntia* plant. Also, freeze-dried *Opuntia* extracts demonstrated better total phenolic content and bioactivities than oven-dried extracts. However, in some cases extracts phenolic content did not directly correlate with biological activities.

T. pubescens laccase crosslinked phenolics in homocoupling biocatalytic reactions with relatively good yields. The laccase-mediated oxidation of luteolin and isorhamnetin resulted mainly in dimeric products of the oxidised substrates. The dimers showed improved antioxidant and antibacterial activities, compared to the substrates. However, the antioxidant activity of the unidientified isorhamnetin product 2 (IP2 at *m/z* 457, M=458) was lower compared to the substrate. All three synthesised products showed bactericidal activity as demonstrated by time kill assays and SEM images of treated bacterial cells. While studies have proposed the use of flavonoid compounds in combination therapy for reducing antimicrobial resistance, the use of biotechnological enzymes in the modification of flavonoids and other compounds for improvement of antibacterial activities is hereby proposed in addition.

Overall, this study has demonstrated that *Opuntia* extract phenolic compound profiling can lead to the discovery of new compounds especially in environments that have not been previously investigated. The study has also shown that laccase modification of *Opuntia* phenolics can lead to the production of new products with improved biological activities. This approach could be a viable alternative for the production of potent, novel bioactive compounds from known natural bioactive molecules.

8.2. Recommendations

Future work may look at further characterisation of extracts using more sophisticated and sensitive methods since many extract compounds remained unidentified. The new *Opuntia*

compounds identified for the first time may be subjected to preliminary small-scale laccase oxidation reactions to determine their viability as good laccase substrates. Laccase heterocoupling of some of these natural compounds may also be attempted to produce hybrid antioxidants and antimicrobials. Future studies may also be focused on gaining a better understanding of reaction engineering protocols to improve yields through elucidation of thermodynamic mechanisms.

Additional work on other potential biological activities of the laccase modified products may give interesting findings. The use of molecular docking techniques as well as structure-activity analysis of new antimicrobial compounds/drugs may also produce interesting findings on antimicrobial mechanisms of action. The monitoring of intracellular pathways could also be studied to add to current knowledge on resistance and susceptibility patterns and mechanisms. The stepwise combination of tyrosinase, another biotechnological enzyme, with laccase may also yield new phenolic compounds with new structures that contribute to enhancement of biological activities. Finally, nuclear magnetic resosnance (NMR) analysis could be further attempted to confirm or otherwise, the predicted structures.

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APPENDIX A: Published papers

Food Research International 112 (2018) 328–344

Review

Opuntia (Cactaceae) plant compounds, biological activities and prospects – A comprehensive review

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ARTICLE INFO

Keywords:
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Macromolecular antioxidants
Biological activities
Nutraceuticals

ABSTRACT

Opuntia species are utilized as local medicinal interventions for chronic diseases and as food sources mainly because they possess nutritional properties and biological activities. The *Opuntia* plant is distributed worldwide and has great economic potential. Differences in *Opuntia* species phytochemical composition exist between wild and domesticated species, and within species. *Opuntia* aerial and underground parts exhibit beneficial properties due to their phenolic content, other antioxidants (for example ascorbate), pigments (carotenoids, betalains), and other unidentified components. This work comprehensively reviews the phytochemical composition of the different aerial and underground plant parts of *Opuntia* species. The applications of *Opuntia* compounds and their biological activities are also discussed. Other topical aspects covered include *Opuntia* spp. taurine composition, *Opuntia* side effects, *Opuntia* by-products valorisation and the role of *Opuntia* spp. in tackling antimicrobial resistance. Although biological activities have been extensively researched, much less information is available on reaction mechanisms, herbal mixtures toxicology and commercialisation prospects – aspects which should be considered for future research in this area.

LWT - Food Science and Technology 111 (2019) 337–344

Phenolic compound profile and biological activities of Southern African *Opuntia ficus-indica* fruit pulp and peels

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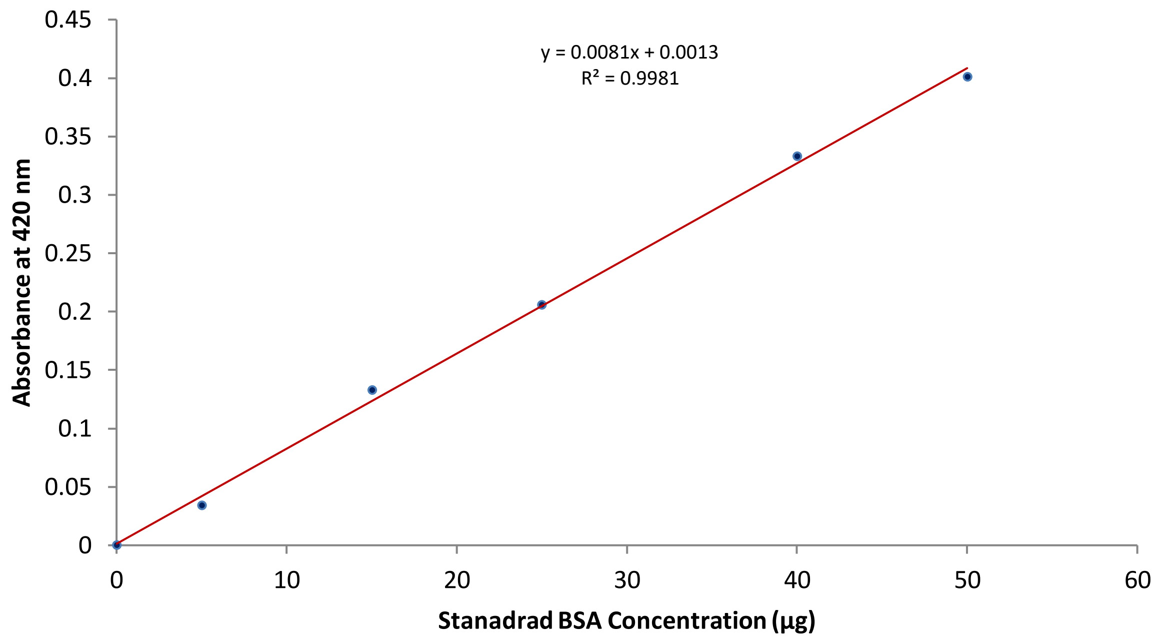
ARTICLE INFO

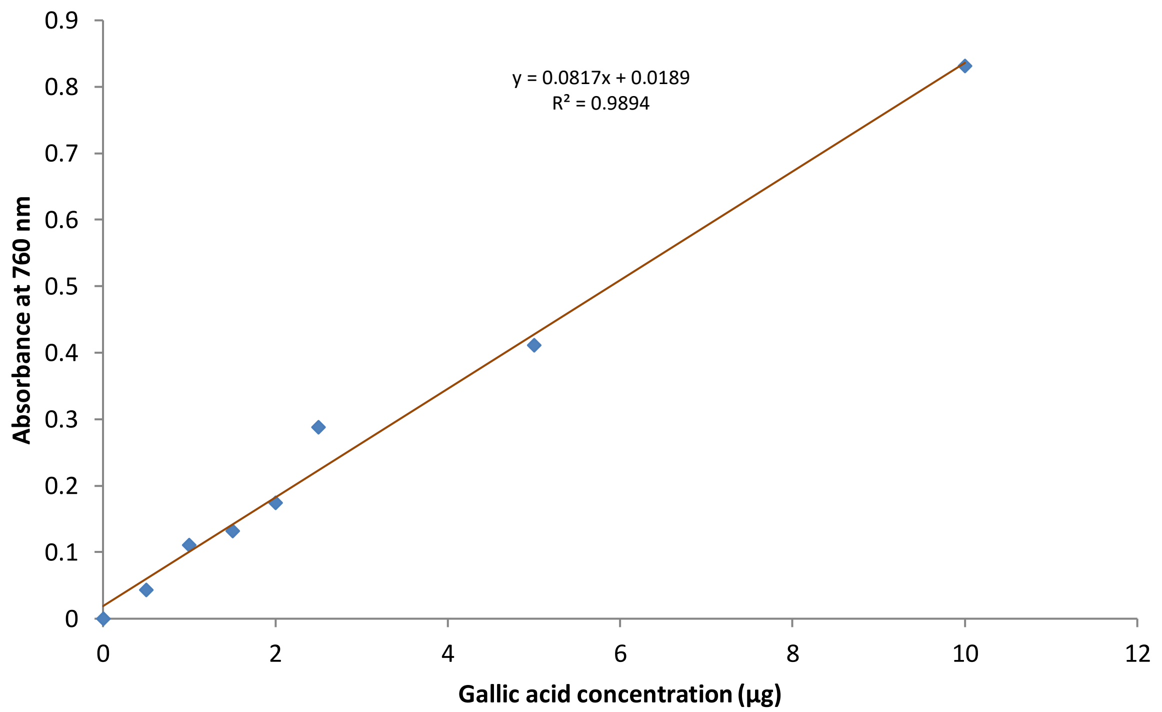
Keywords:
Opuntia
Phenolic compound profile
Antioxidant activity
Antimicrobial activity

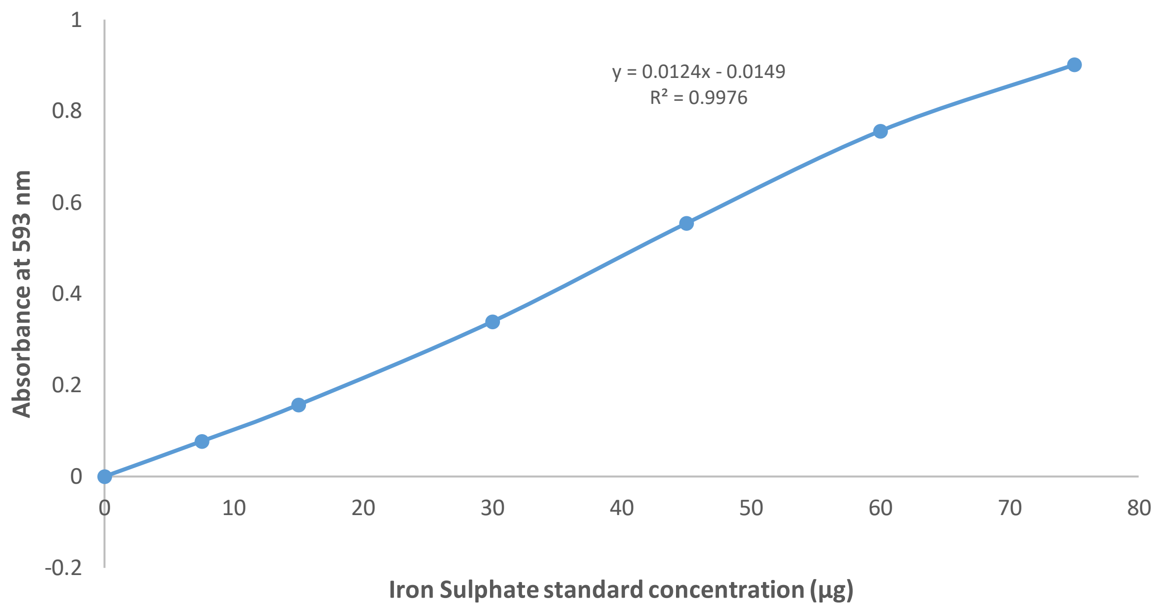
ABSTRACT

Opuntia species have been used in traditional medicine for decades and research on *Opuntia* plants and their by-product valorisation are also on the increase. This study investigated the phenolic compound profiles and biological activities of *Opuntia* prickly pear fruit and peel extracts and the effect of drying methods on extract yields and bioactivities. Ethanolic, methanolic and hexane extracts of freeze-dried *Opuntia* peels showed better *in vitro* antioxidant and antimicrobial activities compared to oven-dried peel and fruit pulp extracts. In most cases, freeze-dried samples also showed better extract yields. Antibacterial activities of the extracts were better against Gram-positive (inhibition zone range of 8.0–18.8 mm) compared to Gram-negative (inhibition zone range of 10.0–14.0 mm) bacterial strains. Minimum inhibitory concentrations (MICs) ranged from 2.50 to 18.75 mg/mL. The presence of pinellic acid is reported for the first time in *Opuntia* fruit extracts. Phenolic acids, flavonols and flavonoid derivatives were also identified. *Opuntia* peel and fruit pulp extracts were rich in biologically active phenolic compounds and could have potential nutraceutical and food industry applications.

APPENDIX B






y = 0.0124x - 0.0149
R² = 0.9976
Absorbance at 593 nm
Iron Sulphate standard concentration (µg)
1
0.8
0.6
0.4
0.2
0
-0.2
0
10
20
30
40
50
60
70
80