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FRACTIONATION AND VALORISATION OF BARK EXTRACTIVES FROM *EUCALYPTUS* SPECIES

by

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PREFACE

The research contained in this thesis was completed by the candidate for a master's degree while based in the Discipline of Chemical Engineering, School of Engineering, of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Howard College Campus, South Africa.

The contents of this work have not been submitted in any form to another university for a degree, and except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

As the candidate's supervisor,I approve this thesis for submission.

15 March 2019

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Signed: Prof. B. Sithole (Supervisor) Date:

“I dedicate this thesis to my late grandfather, Chapola Masetlwa”

DECLARATION

I, Jethro Masetlwa, declare that:

1. The research reported in this thesis, except where otherwise indicated, and is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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DECLARATION 2: PUBLICATIONS

List of Publications

Paper I: J. Masetlwa, B.B. Sithole, J. Andrew, Optimization of accelerated solvent extraction (ASE) of polyphenolic components from *Eucalyptus* tree barks (*E.grandis, E.smithii, E.nitens and E.dunnii*) [In Press]

Paper II: J. Masetlwa, B.B. Sithole, V. Chunilall, J. Andrew, Characterisation of antioxidant components from bark extracts of *E.grandis, E.smithii, E.nitens and E.dunnii* [In Press]

Other Related Publications

1. Fractionation and Valorisation of Bark Extractives from South African *E.grandis* and *E.nitens*
   J. Masetlwa, B.B. Sithole, J. Andrew
   2016 TAPPSA Conference and Exhibition, 21-22 September, UKZN, Durban, South Africa (2016). Poster presentation

2. *Fractionation and valorisation of bark extractives from Eucalyptus species*
   J. Masetlwa, B.B. Sithole, J. Andrew
   7th Forest Science Symposium, 18-20 June, Pietermaritzburg, South Africa (2017). Poster presentation

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Signed: Jethro Masetlwa

ABSTRACT

The notion of zero-waste in industrial production processes of widely used materials has gained momentum, as industries aim to gain revenue from waste materials which were previously considered as waste. Tree bark from wood obtained from sustainably managed plantations used in the production of timber and pulp industries is an underutilised waste that is mainly used for energy production in mills or left on plantations after debarking. *Eucalyptus* tree species are commonly used as raw-material in the pulp and paper industry throughout the world. In this thesis, the potential beneficiation of bark from South African planted *Eucalyptus* tree species (*Eucalyptus grandis, Eucalyptus dunnii, Eucalyptus smithii and Eucalyptus nitens*) as a source of valuable materials is investigated.

Secondary metabolites such as phenolic components, terpenes, steroids and alkaloids can extracted from *Eucalyptus* bark. In this study, accelerated solvent extraction was investigated for extraction of components in the bark and the components were characterised by a variety of analytical techniques. The investigation was undertaken by optimising the extraction process using the following parameters, temperature (80 to 160℃), number of static extraction cycles (1-3 cycles), solvent type (80% v/v of ethanol or 50% acetone v/v), *Eucalyptus* species, and particle size (850-500 µm, 500-375 µm and <375 µm).

The extraction process was optimised and response surface methodology (RSM) was used to model experimental data for statistical analysis of the Box-Beheknen design of the extraction process. A quadratic model was fitted, and optimum extraction parameters were a temperature of 117℃with greater than 2 number of static extraction cycles, and bark particles greater than 355 µm. The two target objectives were total phenolic content and total extractive content when using ethanol as the extraction solvent. The amount of phenolic components in the bark extracts was determined by the Folin–Ciocalteu method which using gallic acid as a calibration standard and detection on a UV-vis spectrophotometer. Amongst the four *Eucalyptus* bark species

studied, *Eucalyptus dunnii* contained the highest amount of phenolic components (5.52g/100g GAE)

In addition to using the Folin–Ciocalteu method, the chemical compositions of acetone and ethanol extracts of the bark samples were determined using Pyrolysis-Gas Chromography/Mass Spectrometry. The analysis showed that condensed tannins, with a building block of catechol units, were the most abundant phenolic components present in the bark extracts. Other components that were detected in high amounts were terpenes and terpenoids, and smaller amounts of monoterpenes and sesquiterpenes. Steroid components were also detected, with β-sitosterol being the most predominant one.

The extractive-free bark samples, remaining after removal of solvent extracts, was analysed using high pressure liquid chromatography to determine their chemical content of cellulose, hemicellulose and lignin.

ACKNOWLEDGEMENTS

First and foremost I would like to offer my deepest gratitude to my supervisor, Prof Bruce Sithole, for giving me an opportunity and unmeasurable resources to produce my thesis for myMaster's degree. Secondly, I offer my thanks to the staff at the Biorefinery Industry Development Facility (BIDF), Council for Scientific and Industrial Research (CSIR) in Durban, for allowing me to use their laboratory facilities to perform my experiments. Moreover, the CSIR also sponsored my undergraduate degree studies. It will be a mistake not to appreciate the sponsors of my research project, The Paper Manufacturers Association of South Africa (PAMSA) and SAPPI -their research funding deserves recognition. I thank the staff at SAPPI Shaw Research Centre in Howick (Mthokozisi Makhathini, Dr. Arnulf Kanzler and Wesley Naidoo) for assistance with supply of bark samples of the four *Eucalyptus* species used in this study.

I thank my colleagues at CSIR BIDF, for the memories we shared together, the food, laboratory and office space we shared. Mam Elsie Sibande for being a second mother, Dr Viren Chunilall for assistance with pyrolysis-gas chromatogram/mass spectrometry, Navan for the training of laboratory equipment and for debugging the accelerated solvent extractor during shut down periods, Pule Seemela for HPLC-HPAEC analysis. Moreover, all the research students that I got to know as my friends and family, Dr Atsile Ocwelwang, Dr Vezekile Zungu, Revelation Sebogodi and others. And lastly Dr. Tamrat Tesfaye for offering me advice on statistical experimental design of my experiments.

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CHAPTER 1

1. INTRODUCTION

1.1. Thesis: General Overview

In South Africa, the pulp and paper industry uses trees harvested from commercially sustainably managed plantations of about 762 000 hectares for production of pulp-based commodities. The sustainably managed plantations are vital to the South African economy as they contribute about 4.2% to the national manufacturing GDP (PAMSA 2016). Unfortunately, the pulp and paper industry, locally and worldwide experienced slow economic growth (about 1.4% rate) since the financial crisis of 2007–2008 (Carminati 2017). The markets have changed as new alternatives to traditional paper-based products are an adverse competition to the industry. An example of an alternative product is the rise of digital news that has rendered printed newspapers near to irrelevance (Bogdanski 2014, Bull and Kozak 2014). Thus biorefinery technologies have been advocated for as a way to revitalise the industry (Moshkelani *et al*. 2013, Rafione *et al*. 2014, Carminati 2017).

The purpose of this work is to assess biorefinery technologies that can be used to beneficiate bark from *Eucalyptus* wood used in South Africa. The objective is to assess the beneficiation by solvent extraction of high value chemicals (secondary metabolites) from bark. The target chemical molecules are polyphenolic compounds (hydrolyzable and condensed tannins) present in *Eucalyptus* bark (Vázquez *et al*. 2009). Thus solvent extracts of bark were characterised by analytical techniques to ascertain the presence of these compounds. The aim of the study was addressed by optimising and modelling the extraction process of phenolic compounds using accelerated-solvent extraction (pressurised-fluid extraction), followed by characterisation of the extracts using the Folin–Ciocalteu method and Pyrolysis-Gas Chromatography/Mass Spectrometry (Py-GC/MS).

1.2. Thesis: Research Contextss

About 600 million trees are planted in South Africa for the production of timber, pulp and paper products (PAMSA 2016). The pulp and paper industry is vital for economic transformation through employment during the production of forestry, timber, and pulp and paper (FTPP) products. The pulp and paper industry in South Africa is well managed from the plant nurseries, to the forests that are Forest Stewardship Council (FSC) certified and manufacturing mills that process wood to a wide range of pulp products (FSC 2009). However, the industry uses this resource inefficiently in that the majority of a harvested tree is lost as waste (Phillips and Sithole 2017). To sustain the economic relevance of the industry, new technologies and products should be developed to beneficiate the waste via biorefinery technologies.

Eucalyptus tree species are a vital feedstock that hasbeen used in the pulp and paper industry for decades. However, the industry only utilises about 40% of the tree throughout the whole production process generating tons of waste, such as bark, sawdust, blackliquor and paper-millsludge (Phillips and Sithole 2017). The waste generated can be exploited to generate new avenues that can result in high-profit margins. One of the forest biomass waste with a potential to be exploited is *Eucalyptus* bark (Harkin and Rowe 1971, Ogunwusi 2013). During the recovery of wood for FTPP operations, bark from *Eucalyptus* species is removed from the wood and the biomass is left in plantations or used in the mills for power generation in recovery boilers (Gavrilescu 2008). Value-added products can be obtained from a wide range of components found in the bark (Ogunwusi 2013).

Tree barkis considered as the outer part of the tree stem which includes every tissue (dead or alive) outside the vascular cambium on the stem. The stem of the tree consists of about 13-21% by weight of tree bark (Harkin and Rowe 1971). The debarking process is essential for pulping purposes for minimisation of detrimental effects such as reduced brightness and yellow spots on the paper caused by the presence of high extractives material from the bark (Gominho *et al*. 2014).

The high amount of bark available presents an opportunity for utilising this raw material to produce high-value products. The chemical components of the tree bark are not different from their parent wood, the shared main polymers in the bark and wood are cellulose, lignin, and hemicellulose (Harkin and Rowe 1971). Hardwood bark contains small amounts of extractive materials (secondary metabolites), which are a pool of low molecular weight chemical components present in the plant cells at different proportions and functions (Sebio-Puñal *et al*. 2012). The chemical difference between bark and wood chemically is that bark has a higher content of both hydrophilic and lipophilic extractives (Rowell *et al*. 2005). Hydrophilic extractives are composed of phenolic compounds such as condensed tannins that can be used for the production of adhesive resins, anti-inflammatory and anti-cancer drugs (Krogell *et al*. 2012). Efficient extraction of these materials from *Eucalyptus* bark can lead to economic beneficiation of the waste (Masoko and Eloff 2007).

This thesis entailed evaluation of the chemistry of secondary metabolites of bark from four *Eucalyptus* species and optimisation of their extraction process As well as analysis of the extractives-free bark to determine cellulose, lignin and hemicellulose contents. The optimisation process for the extraction of polyphenolic compounds was conducted using a statistically designed experiment, using independent process variables of the solvent extraction equipment. Finally, the chemical identity of the extractives was measured using Py-GC/MS and Folin–Ciocalteu method whereas the extractive-free components weremeasured by acid hydrolysis and HPLC. The results obtained for *Eucalyptus* bark were compared to those of black wattle bark, an industrial source of phenolic compounds.

1.3. Thesis: Research Questions

Main question: Can bark from South Africa *Eucalyptus* species be beneficiated into high value chemical materials?

The question above was answered by the following research objectives;

1. Evaluating, using an Accelerated Solvent Extractor, the optimum conditions for extraction of phenolic components from the bark. The variable parameters used to maximise the extraction process were extraction temperature, number of static extraction cycles, bark particle size, and the solvent used. The optimisation process was performed on four *Eucalyptus* species.

2. Determining mathematical models that best described the ASE extraction process when Response Surface Methodology was used through a Box-Behnken design on the bark from the *Eucalyptus* species.

3. Using Py-GC/MS to characterise the chemical composition of the extractives. Black wattle bark, a commercial source of tannin, was used as a comparison.

4. Studying the chemical composition of the extractive-free bark of the *Eucalyptus* species under study. This was done by measurement of carbohydrate, Klason lignin, and acid-soluble lignin contents of the samples. To determine the total carbohydrate content, a summative analysis of monomer sugars such as glucose, xylose, arabinose, galactose and mannose was performed using acid hydrolysis of the extracts.

1.4. Chapter Overviews

The thesis is divided into five chapters. The current chapter highlights the introductory concepts that deal with the perspective, significance and background of the experimental work performed and the research questions that the thesis aims to answer.

Chapter 2 is concerned with a literature review on extraction of phenolic compounds and the chemistry of bark. It contains literature on applications of ASE.

As this thesis is a journal article-based study, Chapter 3 highlights the first paper on the optimisation of extraction of phenolic compounds from bark of *Eucalyptus* species.

In Chapter 4, the second paper discusses applications of the optimised conditions obtained in Chapter 3 to extract phenolic compounds from bark samples and subsequent characterisation of the extracts using Py-GC/MS. The results obtained are discussed and compared to black wattle. The paper also deals with the determination of structural carbohydrates and lignin in the extracted bark samples.

Chapter 5 discusses the extent to which the results obtained answer the described research questions of the thesis and contextualises the results from the preceding two chapters. Also, the main conclusions of the thesis are highlighted, coupled with recommendations that may serve as a foundation for future work.

CHAPTER 2

2. LITERATURE REVIEW

2.1. *Eucalyptus* Species: Occurrence

The mean average rainfall in South Africa is about 560 mm, making it a water-scarce country with a diversified climate throughout the country (Albaugh *et al*. 2013). Most forest trees are planted in two provinces, Kwa-Zulu Natal and Mpumalanga that have average annual rainfall of 700mm and 500mm, respectively (Jones *et al*. 2015). Thus the country lacks trees that are suitable for use in the FTPP industries. Consequently, *Eucalyptus* trees were introduced in 1892-94 in order to establish the FTPP industries in South Africa (Lückhoff 1955). *Eucalyptus* species are drought-tolerant plant species with an ability to produce biomass and timber for the South African economy (Phiri *et al*. 2015). *Eucalyptus* plant species are hardwoods (or angiosperms) and their genus includes a variety of flowering trees and shrubs with about 700 known species along with their hybrids (Brooker 2002, Neiva *et al*. 2015). The origin of most of *Eucalyptus* species can be traced back to Australia, while a few originate from New Guinea and Indonesia (Neiva *et al*. 2015).

Eucalyptus trees attracted interest from global development researchers due to their inherent properties such as: adaptation to extreme temperate and tropical conditions; fast-growing sources of wood for FTPP production; and minor usages such as production of natural oils used for cleaning applications and natural sources of insecticides (Brooker 2002, Boland *et al*. 2006, Neiva *et al*. 2015). *Eucalyptus* species are planted in about 90 countries across temperate, subtropical and tropical climatic conditions covering about 18 million hectares combined (Rockwood *et al.* 2008). They have been the backbone of the FTPP industry for decades.

E.grandis and *E.globulus* are the most planted species in the world, but epidemical diseases have attacked these species and this has led to the development of research of

clones (hybrids) that are more tolerant to diseases (Van den Berg *et al*. 2018). Flooded gum or *E.grandis* grows to an average height of 45-55 m and has an average density of 545-955 kg/m^3; it originates from Australia in Newcastle and was discovered and reported by W. Hill in 1919 (Boland *et al*. 2006). The species was first introduced in South African plantations in 1932 in Berberton in Mpumalanga (Nel 1965). *E.globulus* is mainly planted in South American countries; in South Africa a close relative of *E.globulus* called shining gum (*E. nitens*) which is native to New South Wales and Victoria in Australia is planted for pulp production (Hunter *et al*. 2004, Boland *et al*. 2006). *E. Nitens* grows straight poles with average heights of 40-70m and have straw-coloured or pale pink heartwood with an average density of 530-750 kg/m^3 (Boland *et al*. 2006). Other *Eucalyptus* species planted in South Africa are Gully gum (*E. smithii*) with an average height of 40-45 m and Dunn's white gum (*E. dunnii) with* an average height of 30-35m (Boland *et al*. 2006, Maseko *et al*. 2007). The FTPP heavily relies on plantation of *Eucalyptus* species in South Africa, and the demand for *Eucalyptus* trees is likely increase as bioenergy resources are gaining interest in the world (Albaugh *et al*. 2013).

2.2. *Eucalyptus* species: Bark phenotype

Tree bark is the dark outermost layers of stems of woody plants existing outside the vascular cambium, as shown in Figure 2-1. It consists of two significant layers: outer bark and inner bark. The outer bark consists of mainly dead plant cells whereas the inner bark has cells that are biologically active. Dried bark is about 13-21% by weight and 9-15% by volume of a typical log (Harkin and Rowe, 1971). Bark of different *Eucalyptus* species are chemically and physically different from each other.

Boland *et al* (2006) outlined the phenotype of *Eucalyptus* bark as follows: the bark of *E. smithii* is moderately thick, has a rough texture throughout the trunk and is grey or dark brown in colour. At the top part of the tree, the bark sheds into long ribbons exposing a white smooth inner bark. The bark of Dunn's white gum is also rough and brownish in colour with a dense bark at the base from 1-4m. The top of the tree is smooth and decorticates into long ribbons (Boland *et al*. 2006). For *E. nitens*, the bark

is smooth throughout with a greyish colour and consists of a thin basal stocking of rough bark from the bottom of the stem a few metres up from the ground,and the bark sheds in long ribbons. And lastly, *E. grandis* contains a very short stocking greyish in colour and has a short black rough butt for the first 4m from the ground whereas the rest of the tree is smooth and powdery with colours ranging from greyish white to bluish grey (Boland *et al.* 2006).

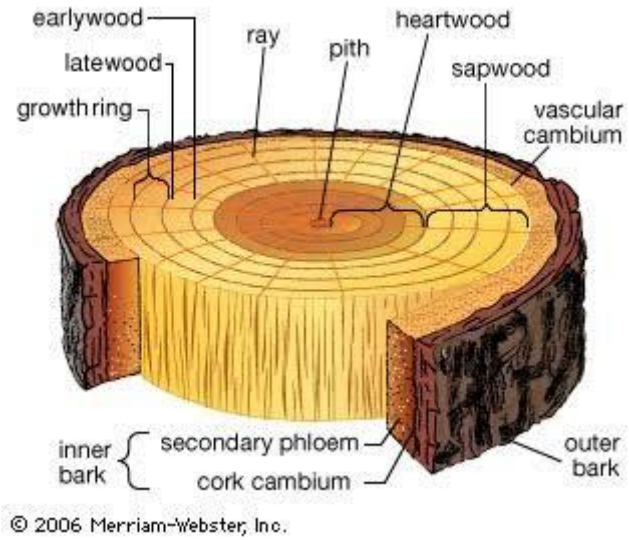

Figure 2-1 Cross-sectional cut of typical tree logs, showing outer and inner bark www.slideplayer.com/amp/4680799/

2.3. Chemical composition of tree bark

The anatomy, development and chemistry of the bark is different from other parts of the tree such as the wood, branches and leaves. Bark, like wood, is lignocellulosic fibrous material that is composed of three main polymers; holocellulose (cellulose and hemicellulose) and lignin (Harkin and Rowe 1971, Neiva *et al*. 2015). Cellulose is a linearised polymer with glucose as a building block; it is the most naturally occurring polymer on earth. The fundamental role of cellulose is to give the bark and wood structural strength as it is the main building material of plant cell walls (Rowell *et al*. 2005). Hemicellulose occurs alongside cellulose, and is a shorter chained heteropolymer made from glycosidic bonds of glucose, mannose, xylose and other monomeric sugars (Pettersen 1984, Rowell *et al*. 2005). Cellulose and hemicellulose

have lignocellulosic bonds with lignin, a heteropolymer that acts as a binding material in the cell wall forming strong and stable lignocellulosic trees (Pettersen 1984).

During mechanical or thermochemical pulping, hemicellulose and lignin are separated from the lignocellulosic matrix by the use of alkaline liquids such as sodium hydroxide and sodium bisulphite for Kraft pulping and revolving stones for mechanical pulping (Das and Houtman 2004). The bark of *Eucalyptus* contains a high amount of lignin, hemicellulose, ash and secondary metabolites (extractives) derived from plant cells. Bark is regarded as an unfavourable source of fibre to produce pulp due to its low pulp yield and the high cost associated with bleaching and cooking time in addition to undesirable deposits on the pulp (del Rıo *et al*. 1998). Table 2-1 shows the concentration of cellulose, hemicellulose and lignin from typical *Eucalyptus* species.

Table 2-1Polysaccharides and lignin composition of typical Eucalyptus species

	Cellulose (%)	**Hemicellulose (%)**	**Lignin (%)**
Eucalyptuscamaldulensis	50	17	29
Eucalyptuscloeziana	54	16	28
Eucalyptusgrandis	54	19	26
Eucalyptussaligna	50	15	27
Eucalyptusurophylla	53	19	24
Eucalyptus robusta	48	12	28
Eucalyptussphaericus	49	27	13

Adapted from (Pettersen, 1984)

2.4. Secondary metabolites: Bark extractives

The main three polymers (cellulose, hemicellulose and lignin) are not the only chemical components found in the bark. There also secondary metabolites (extractives), which are any chemical compounds produced from the plant cell apart from the primary three polymers discussed above. They include fats, sterols, sugars

waxes, tannins, waxes, suberins, lignans, flavonoids, alkaloids and many other classes (Yang and Jaakkola 2011). These compounds are usually classified as hydrophilic and lipophilic extractives; hydrophilic refers to polar compounds extracted using polar organic solvents such as water, ethanol, methanol and acetone (Theander 1985); whereas lipophilic refers to non-polar compounds soluble in solvents suchas cyclohexane, benzene and toluene (Theander 1985). In general, bark contains valuable extractive components that are mainly hydrophilic; they are 3-5 times more abundant as non-polar constituents which include waxes, fats, terpenes and steroids (Ogunwusi 2013). In the previous years, research has been conducted to valorise different barks by using different extraction techniques to target specific vital extractives. The following sections aim to discuss the chemistry of chemical classe of extractives that are mostly found in *Eucalyptus* trees, with emphasis on phenolic components (Eyles *et al*. 2003).

Phenolic components are a large class of extractives found in everyday natural plants. The chemical classification of polyphenolic compounds is the presence of a phenol unit or phenol derivatives chemically bonded to form polymers. To date, about 8000 of these polyphenolics have been identified in vegetables, fruits and liquids (Guo *et al*. 2009).They include simple phenolic acids, lignans, flavonoids, lignin and tannin components that are reported to originate from a classic biochemical skimmed acid pathway known as the phenylpropanoid pathway (Ferrer *et al*. 2008).

2.4.1. Phenolic Compounds: Chemistry of tannins

The first major phenolic compounds in the bark of hardwood species are tannins, which low molecular weight polyphenolic components ranging from 500 to 3000 Dalton (Gupta and Haslam 1979). In hardwood species, tannins are present in high amounts in *Eucalyptus*, acacia, quercus and betula (Hosseinihashemi 2016). They form strong complexes with proteins and are used in the production of leather (Falcão and Araújo 2011).

Tannins are categorised into two classes: condensed tannins and hydrolysable tannins. Hydrolysable tannins (shown in Figure 2-2) are classified as ellagitannins and gallotannins. Gallotannins dissociate into gallic acid **(1)** and its derivatives in an acidic environment and ellagitannins dissociate into ellagic acids **(3)** (Mueller-Harvey 2001). Gallotannins consist of a central core of polyol (especially glucose) esterified with gallic acid, whereas the sugar molecule for ellagitannins consists of polyols esterified with hexahydroxydiphenic acid. As shown in Figure 2-2, the compounds form oxidative linkages with a wide range of components (Koleckar *et al*. 2008). The amounts of hydrolysable tannins contained in tree bark are mostly lower than those of condensed tannins.

Condensed tannins or proanthocyanidin polymers are oligomers with the flavan-3-ol unit (**2**) as core monomer. The most abundant subgroups of condensed tannins found in hardwoods include procyanidins, prodelphinidins and others shown in Figure 2-3 (Koleckar *et al*. 2008). Procyanidins occur in high quantities compared to other proanthocyanidins; this class consists of catechol at the B-ring of a flavan-3-ol unit. The complexity and structural diversityarises from different stereochemistry and hydroxylation of the units because theflavan-3-ol unit structure has three stereocentres (Koleckar *et al*. 2008). Polymers and oligomersare formed from the B-ring linkages of flavan-3-ol unit monomers at C-4 at the top and the C-8 or C-6 of the B-ring unit at the bottom, as displayed in Figure (**3)** (Gupta and Haslam 1979, Koleckar *et al*. 2008). The A-ring linkagesare formed when C-2 of the upper unit of the flavan-1-ol is bonded with C-7 or the hydroxyl components of the lower unit, asshown in Figure 2-3(**4&5)** (Gupta and Haslam 1979). The degree of polymerisation of condensed tannins is 4-12 units of a flavonoid unit (Koleckar *et al*. 2008).

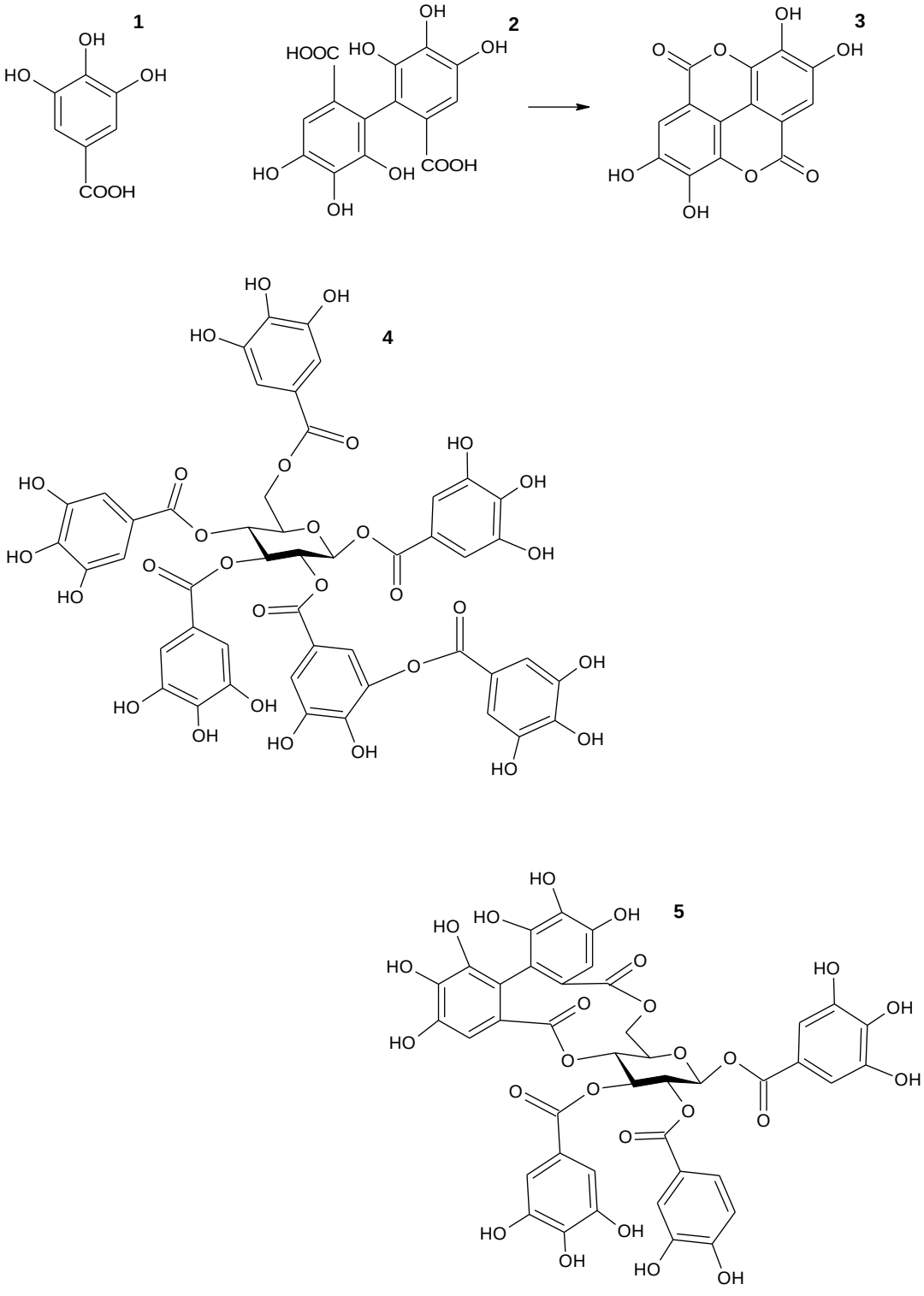

Figure 2-2 Chemical structures of hydrolysable tannins

Adapted from (Koleckar *et al.* 2008) 1. gallic acid, 2. hexahydroxydiphenic acid, 3. ellagic acid and 4 and 5 are examples of ellagitannin and gallotannin with glucopyranose as a core

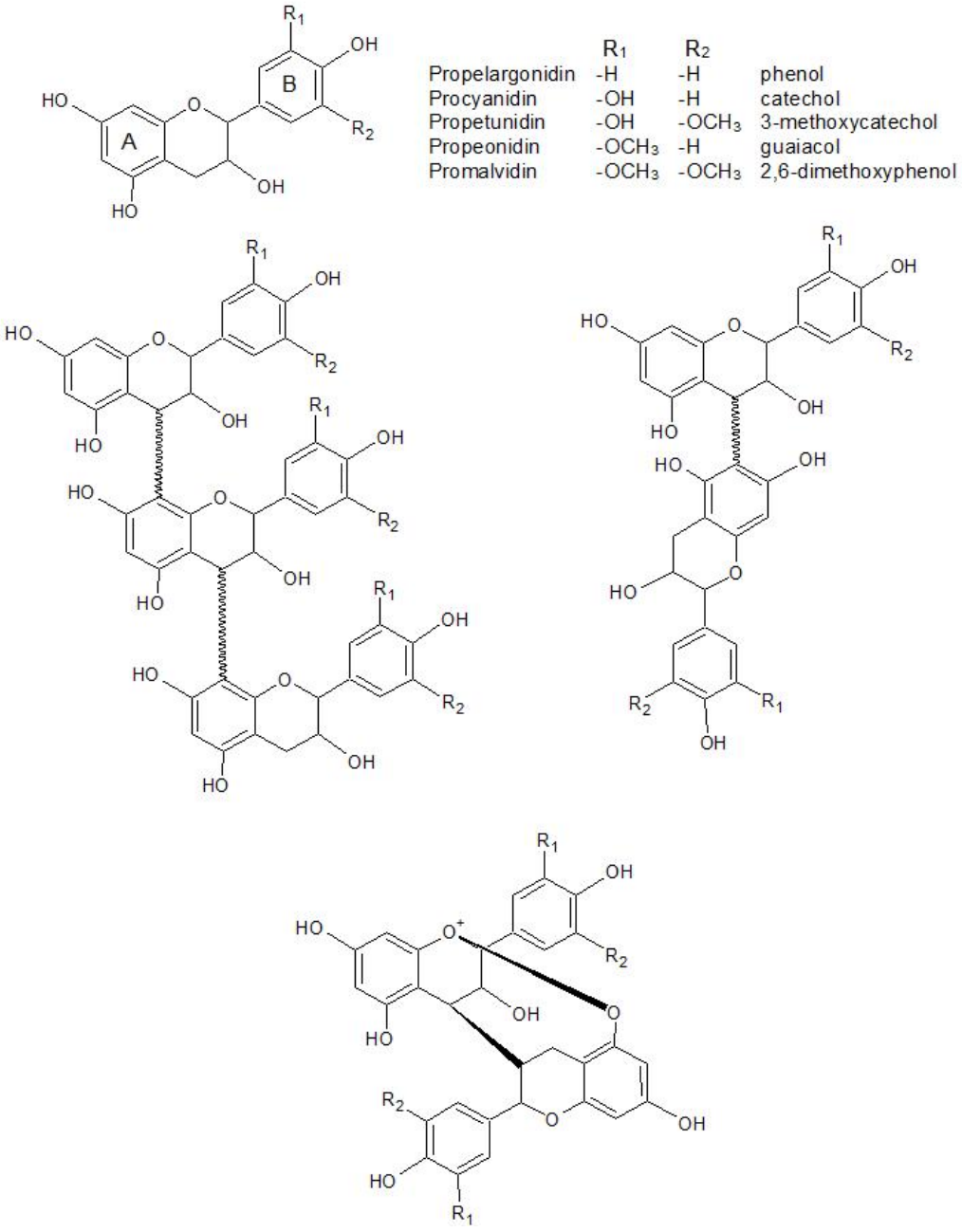

Figure 2-3 Types of condensed tannins with different structural configuration

Adapted from Koleckar *et al.* 2008

2.4.2. Phenolic Compounds: applications and valorisation of tannins

In industry tannins are generally referred to as vegetable tannins and are extracted from different plant sources. The two main sources of industrial condensed tannins are the wood quebracho and the bark of wattle; high amounts of condensed tannin can be extracted from these species (Roffael *et al*. 2000). Hydrolysable tannins are produced by different species such as chestnut, Aleppo oak, Chinese nutgall tree and Sicilian sumac (Kemppainen 2015).

An established market of utilisation tannin already exists, whereby the phenolic nature of the tannin is used to complex with proteins during the production of leather. It is stated that the total production of tannins ranges between 160-200 thousand tons per annum (Vieira *et al*. 2011). The majority of tannins produced are utilised to process raw hides for the production of high quality leather. Polyphenolic tannin complexes with collagen proteins in the hide resulting in colour changes coupled with improved strength and flexibility properties of the hide (Kemppainen 2015).

Another significant industry that uses the chemical nature of tannins is the wine industry. Tannins improve wine properties such as colour stability and minimisation of biological and chemical degradation due to their antioxidant, antiradical, and antibacterial properties (Kemppainen 2015, Silvateam 2015).

Tannins can be used as bio-adhesives formulations to replace synthetic phenol used in phenol-formaldehyde resins(Pizzi 2006). The bio-based tannin resins can be used in the manufacture of particle board and plywood to induce desirable properties such as high reactivity, low viscosity and low manufacturing costs at high volumes (Ebnesajjad 2010, Kemppainen 2015). Biofoam is another product that can be produced from tannins. Life Cycle Assessment showed that tannin-based biofoams from pine are environmentally friendly compared to petroleum based biofoams (González-García *et al*. 2016).

2.4.3. Terpenes and Terpenoids

Another major class of secondary metabolites that is available in tree bark is terpenes/terpenoids (Harkin and Rowe 1971). This class is comprised of low molecular weight components that have been isolated from a wide range of species. More than 30 000 molecules of terpenes and their derivative compounds have been identified in the plant kingdom (Zhang *et al*. 2011). The components are odoriferous, fragrant and flavouredand are thus mostly isolated for use in perfume production. Terpenes are biologically derived from isoprene (2-methyl butadiene) units. They are hydrocarbons whereasterpenoids are heterocarbons. The linkages of isoprene units for formation of terpenes follow the isoprene rule, whereby the tail(C-1) of the subsequent unit bonds to the head of the following unit(C-3) (Zhang *et al*. 2011, Kallassy 2017). The linkages result in cyclic or acyclic components; cyclisation removes a double bond from the unit (Kallassy 2017).

The nomenclature of terpenes is defined by the number isoprene units they contain: monoterpenes contain two-isoprene units sesquiterpenes contain three, diterpenes contain four; sesterterpenes contain five; and triterpenes contain six units (Kallassy 2017). Examples of cyclic monoterpenes with single rings include limonene, terpinolene and b-phellandrene (obtained from softwood species) are shown in Figure 2-4. Monoterpenes with two rings are widely distributed among the softwood plant kingdom. Examples include thujene, camphene, 3-carene and b-pinene and a-pinene. Sesquiterpenes are a complex class of terpenes that form many compounds with different skeletal structures (Kallassy 2017). Diterpenes are not widely distributed in hardwoods whereas sesquiterpenes have been identified from bark of *Eucalyptus* (Kallassy, 2017). Successful extraction of terpenes can result in high economic gains, as they have many industrial applications such production of flavours, high grade lubricants, fragrances and medicinal products (Jiang *et al*. 2016).

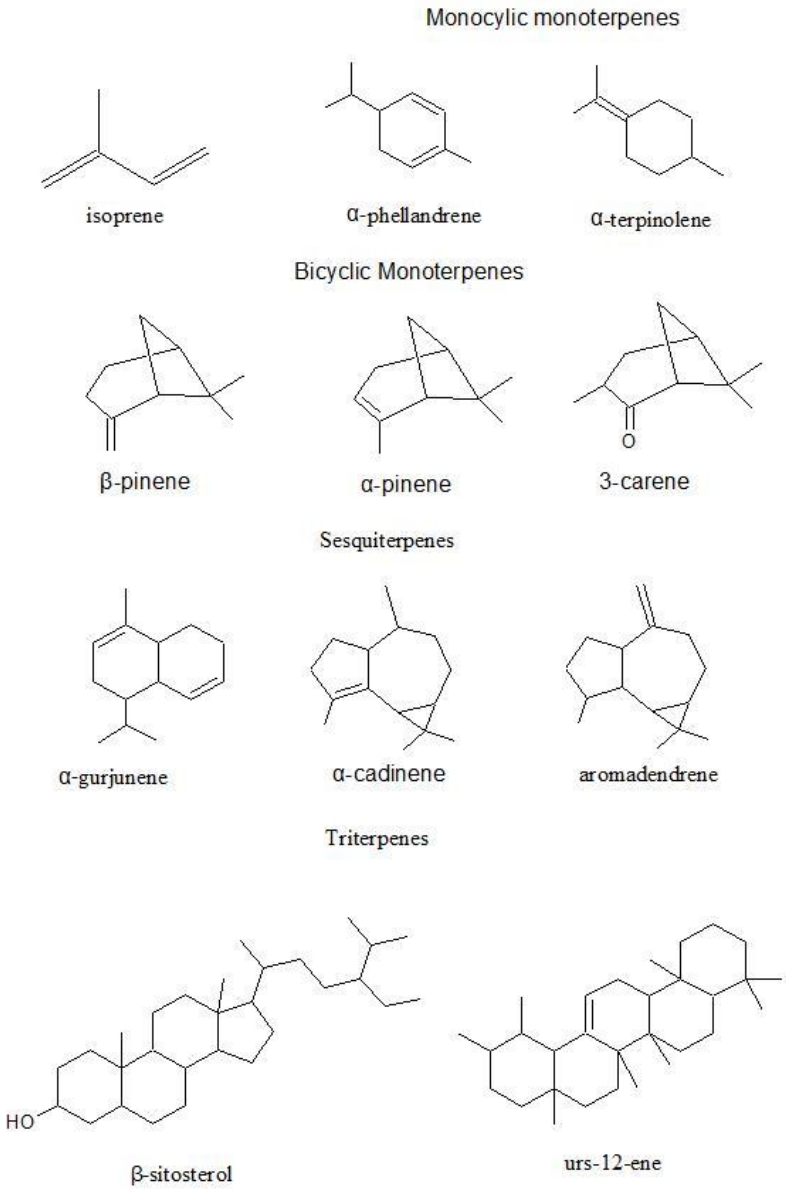

Figure 2-4 Examples of terpenes and terpenoids found in hardwoods

Adapted from Cho *et al.* (2017)

2.5. Solid-liquid extraction techniques

To recover extractive components from the bark, solvent extraction can be used. Organic solvents are mostly used to recover secondary metabolites from the lignocellulosic matrix; the polarity of the solvents is crucial as some extractives are only soluble in certain solvents. Lipophilic extractives such as terpenes, waxes and fats are extracted with non-polar solvents such as benzene, toluene and cyclohexanewhereas hydrophilic components such as phenolic compounds are extracted with polar solvents such as acetone, methanol and water (Theander 1985). Laboratory scale conventional extraction techniques that have been used for analytical purposes such as Soxhlet and percolation extractions, have limitations such as excessively long extraction periods, large amounts of solvents used, and low yields of solutes (Zakaria *et al*. 2010).

2.5.1. Accelerated Solvent Extraction (ASE)

Advanced analytical techniques have emerged to improve the extraction ability of solvents chosen to target specific solutes. One of these is accelerated solvent extraction (ASE). ASE or pressurised-fluid extraction uses low-boiling solvents at subcritical conditions which are elevated to temperatures up to 200℃ and pressures up to 3000 psi for the extraction of valuable chemical components in various biomass (Hossain *et al*. 2011). The high pressure enables solvents with relatively low boiling points to liquidify at low extraction temperatures (Kettle 2013). The extraction conditions are subcritical because the pressure is above the critical pressure point of extraction of the solvents and below their critical temperature point. The conditions also optimise the extraction process by lowering the viscosity and surface tension of the solvents and also solubilising target compounds by increasing the diffusion rate of the solvent and mass transfer (Richter *et al*. 1996, Hossain *et al*. 2011).

High temperatures increase the diffusion rate of analytes from the boundary layer by the surface of the sample particles to the bulk medium of the solvent, following Fick's first law of diffusion (Kaufmann and Christen, 2002). Operating at elevated

temperatures results in lower solvent viscosities that further result in an increased flowrate within the pores of the matrix (Richter *et al*. 1996). The degree of disruption of chemical interactions between the solute and matrix (van der Waals forces, hydrogen bond, dipole attractions) is increased. Summing up the effects outlined above, extraction at high temperatures occurs faster and utilises low volumes of the solvent (Hossain *et al.* 2011).

Figure 2-5 is a schematic diagram of the operation of an accelerated solvent extraction process. The extraction process as described by Richter *et al*. (1996) and Kettle (2013) starts by loading solid samples into a stainless cell with a frit and filter paper installed at the end caps of the cell. An automated arm grabs the cell containing the sample into an oven with a temperature control system. After the cell is inserted into the oven, the pump draws organic solvents into the sample cell. Solvents expand as they heat up during the pressurisation stage at pressures ranging between 500-3000 psi. The pressurisation occurs due to the expansion of the solvents. The addition of a fresh solvent in the operation of an ASE is similar to the solvent dripping from the condenser in a Soxhlet operation.

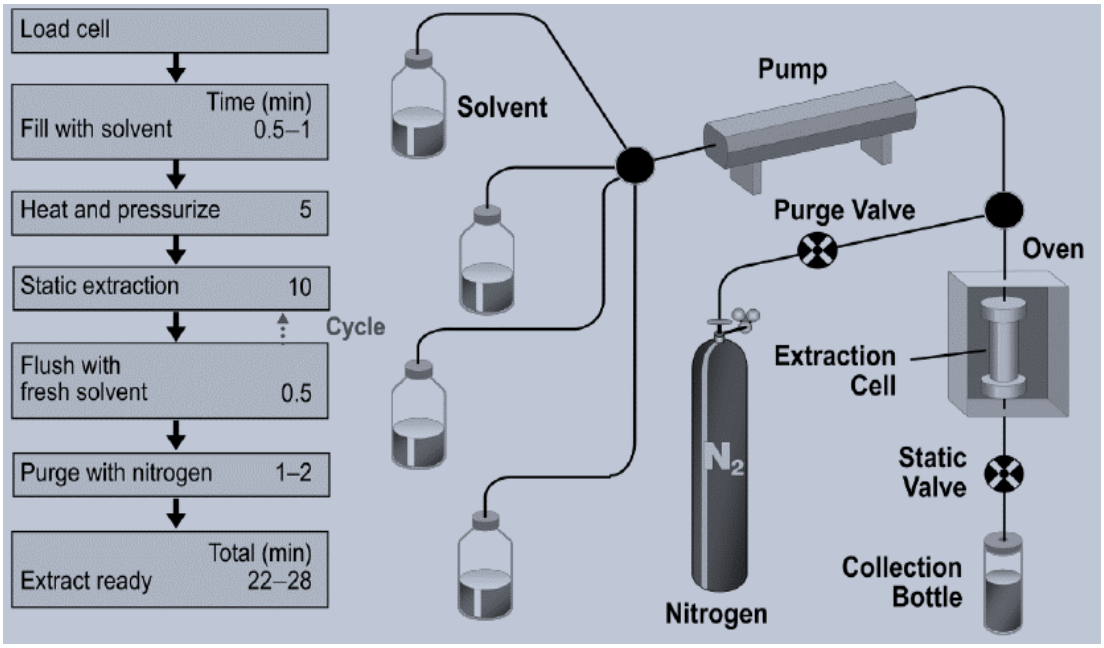

Figure 2-5 Schematic diagram of an ASE operation
(Kettle, 2013)

After the heat-up and pressurisation stage occurs, the extraction process enters a stage of static period. At this stage, a fresh batch of solvent is pumped into the cell to increase the extraction process by increasing the diffusion rates. After the static period, the cell is rinsed using nitrogen gas at 150 psi to purge the solvent out of the cell for a setperiod. Extracts are then collected in the collection vessels through a filter installed at the bottom of the cell and the solution is concentrated using either solid-phase separation or evaporation for trace analysis (Kaufmann and Christen, 2002).

2.5.2. Applications of ASE

Extraction of phenolic components from various biomass have been conducted. For example, Barros *et al*. (2013) extracted phenolic compounds from sorghum using ASE at 60, 120 and 150℃, with 50% and 70% ethanol/water (v/v). The study showed that phenolic content was 12% higher than in conventional extraction methods and extraction temperature was a leading factor during the extraction of phenolic extractives (Barros *et al*. 2013). Accelerated solvent extraction was applied on the bark of Spruce (*picea abies)* using different environmentally friendly solvents such as water, pentane, ethanol and ethyl acetate, and the results showed that using ethanol and water resulted in the highest antioxidant capacity of the extracts (Co *et al*. 2012). Another study on Spruce bark showed that elevated temperature above 140℃ resulted in degradation of extractives obtained using ASE when water was used as a solvent (Le Normand *et al*. 2012).

Several studies have optimised the extraction process of phenolic compounds by varying temperature, number of static cycles, concentration of the solvent, and other factors to allow for maximum extraction of polyphenolic extractives from various biomass (Hossain *et al*. 2011; Le Normand *et al*. 2012; Jablonsky *et al*. 2015). Jablonsky *et al.* (2015) optimised the extraction of bark of *Picea abies* using ethanol and determined that temperature had the greatest effect on the extraction yield of phenolic components. Extraction times and the number of extraction cycles were

investigated by Toubane *et al*. (2017) using different solvents such as ethanol, methanol and hexane on the roots of *Carthamus Caeruleus*. The results showed that increasing the temperature and extraction times when using ethanol resulted in higher yields of antioxidant material than when using the other solvents (Toubane *et al*. 2017). An experimental design that utilises response surface methodology such as Box-Benhken Design (BBD) and Central Composite Design (CCD)was used to optimise the extraction process of ASE on bark of different species (Le Normand *et al*. 2012).

2.5.3. Alternative solvent extraction techniques

Other extraction techniques such as supercritical fluid extraction (SFE) have been used to extract polyphenolic compounds. SFE uses supercritical CO_2 to extract phenolic compounds at conditions above the critical point of carbon dioxide (Vatai *et al*. 2009).Under these conditions the fluid penetrates the biomass matrix as a gas and dissolves solutes as a liquid. Addition of ethanol to the CO_2 changed the solvation properties of the gas and exhibited the highest total phenolic content for the bark of *Eucalyptus* globules (Santos *et al*. 2012). Other extraction techniques that have been used include Ultrasonic Assisted Extraction and Microwave-assisted extraction. Ultrasonic assisted extraction uses sonic waves to heat up and disrupt the biomass matrix while obtaining polyphenols - the technique was used on spruce bark immersed in ethanol and the results obtained showed that the extraction process was affected by the concentration of ethanol and temperature (Ghitescu *et al*. 2015).

2.6. Characterisation of extractives

2.6.1. Total phenolic content; Folin–Ciocalteu method

Phenolic compounds such as tannins, phenolic acids and flavonoids have diverse biological effects ranging from anti-carcinogenic to anti-inflammatory due to their inherent antioxidant capacity (Silvateam 2015). Different assay methods are used to estimate the concentration of phenolic (Kumar *et al*. 2014). One such method is the Folin-Ciocalteu method - a colorimetric methods that is often referred to as the Gallic Acid Equivalence method (GAE). It involves reacting the solution with phenolic

material with a Folin–Ciocalteu's phenol reagent. The reagent used is a combined solution of phosphotungstate and phosphomolybdate which react with phenolic components in the solution resulting in coloured components that can be detected using spectrophotometry (Ramirez-Sanchez *et al*. 2010). The selectivity is not entirely for phenolic and polyphenolics; it measures the reducing capacity in the sample since the majority of reducing substances react with the reagent (Everette *et al*. 2010). The majority of antioxidant components that undergo the reaction are phenolic in nature and thus the method is generally used to determine the total phenolic content (Everette *et al*. 2010). For example, the Folin–Ciocalteu was used for characterisation of total phenolic content in different studies that investigated the extraction of phenols from bark biomass (Hossain *et al*. 2011, Toubane *et al*. 2017).

2.6.2. Analysis of bark extracts using Pyrolysis-Gas Chromatography/Mass Spectrometry

More sophisticated and selective analytical techniques have been used to identify secondary metabolites in bark extracts. Pyrolysis-Gas Chromatography/Mass Spectrometry (Py-GC/MS) has proven to be useful in the characterisation of non-volatile biomass components that include lipophilic and hydrophilic extractives. The analysisis achieved by using a small amount of dried extracts which are heated at high temperatures above 550℃ to undergo fast pyrolysis where non-volatile polymers in the sample fragment into various monomers (Meier and Faix, 1992) (Sithole *et al.* 2012). The fragments are separated by gas chromatography by their affinity to the stationary phase such as silica, with a mobile phase of an inert gas such as helium or nitrogen in a column. As the fragments exit the column, a mass spectrometer is used to separate the fragments further using molecular mass, followed by their detection which results in a chromatogram that serves as a fingerprint of chemical components in the initial bark extracts. The benefits of Py-GC/MS is that it does not require extensive preparation compared to other chromatographic techniques such as HPLC (Meier and Faix 1992). Pyrolysis-Gas Chromatography/Mass Spectrometry requires a minimal amount of the sample, e.g. 1-100μg, and its analytical times are fast. Py-GC/MS was used to identify lipophilic and lignin extractives in E.*globulus, E.nitens,*

E,maiden, E.grandis and *E.dunnii* from the sample of *Eucalyptus* barks. Fragments of condensed tannins such as guaiacol derivatives were abundant during the analysis of lignin and carbohydrates in the bark extractives (Rencoret *et al.* 2007)

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CHAPTER 3 Paper I

3. Optimization of accelerated solvent extraction (ASE) of polyphenolic components from *Eucalyptus* tree bark (*E.grandis, E.smithii, E.nitens* and *E.dunnii*)

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Abstract

The purpose of this investigation was to establish optimum conditions for extraction of polyphenolic compounds from *Eucalyptus* bark. Accelerated solvent extraction was used on bark from four *Eucalyptus* species (*E.grandis, E.smithii, E.nitens and E.dunnii*) and the highest of polyphenolic extracts were ascertained by the Folin–Ciocalteu method and total extractive content of *smithii* the samples. The optimisation process was conducted using response surface methodology that utilised a Box-Behnken experimental design with 65 runs from four sets of extraction experiments. The variables investigated were temperature (80-160℃), three particle size classes (355, 500 and 850µm) and number of static extraction cycles (1-3). Optimum extraction of polyphenolic components occurred at a temperature of 117℃, with at least three static extraction cycles, bark particle size class of 500-850 µm. On the other hand, the optimum yield of total extracts from *Eucalyptus* bark was obtained at high temperatures, greater than 3 static extraction cycles and particle size class <355 µm.

3.1. Introduction

The beneficiation of waste materials as valuable feedstocks is becoming popular as the world is developing an ideology of using materials previously classified as waste to generate new revenue streams and shift from traditional synthetic substances derived from fossil fuel resources (Ferreira *et al.* 2016). The forestry, timber, pulp and paper industries generate large quantities bark as waste biomass. Bark is rich in polymers such as cellulose, hemicellulose and lignin, with small amounts of secondary metabolites (Yang *et al.* 2006, Croteau *et al.* 2000). Thus beneficiation of this waste biomass can generate valuable compounds. Thermo-chemical and biological techniques are some of the currently accepted technologies used to convert lignocellulosic polymers into valuable products such as biofuels from aerobic digestion, pyrolysis, and gasification technologies (Basu, 2013, Basu, 2010). The utilisation of secondary metabolites obtained from tree barks is minimal, but they do have potential applications in pharmaceutical, cosmetic, food and other manufacturing industries directly (Feng *et al.* 2013). Thus, beneficiation of bark biomass via extraction of these high value compounds could be an effective way for both waste minimisation and revenue generation.

Eucalyptus species have been the main source of hardwood pulp furnish in many countries such as Brazil that produces about 13 million tons of pulp per annum (Cebreiros *et al.* 2017). In South Africa, about 50% of 600 million trees are *Eucalyptus* and are planted on 720 000 hectares of land for the production of pulp (PAMSA, 2016).The bark produced by the FTPP industries is regarded as waste and is used for energy production by direct combustion because of its high calorific value and modest emissions (Ferreira *et al.* 2016).

As stated earlier *Eucalyptus* bark contains polyphenolic compounds such as tannins, lignans, flavonoids and phenolic acids which are important secondary metabolites that can be harnessed for various industrial applications. Tannins, for example, are bioactive with antioxidant properties derived from their inherent phenolic nature in the

chemical structure of both condensed and hydrolysable tannins (Parada and Fernández, 2017, Mota *et al.* 2013). The antioxidant properties of polyphenolic compounds have various health benefits such as reducing the risk of cardiovascular, viral and mutagenic diseases, minimising neurodegenerative disorders, cancer and inflammations (Lampe, 2003, Hossain *et al.* 2011). Another significant use is in the application of tannin-based adhesives which are binding substances used in the manufacturing of plywood boards (Yazaki, 2015). The challenge for valorisation of chemicals is that bark and other forest biomass are used for energy production in the mills, and chemical extraction could reduce the amount of forest biomass used in energy generation. However, chemical solvent extraction and energy production can be coupled together to increase the value obtained from a tree in the biorefinery concept.

Solvent extraction is an established technique used to extract these low molecular weight materials from lignocellulosic matrix. The shift from the use of traditional extraction techniques such as Soxhlet extraction to new techniques with greater selectivity has been adopted due to their successful application on various lignocellulosic biomass (Saha *et al.* 2015). These new techniques include accelerated solvent extraction (ASE), supercritical fluid extraction, and ultrasonic assisted extraction all of which have different advantages and disadvantages in their application (Ajila *et al.* 2011, Ghitescu *et al.* 2015, Talmaciu *et al.* 2015).

Accelerated solvent extraction is a recent automated solvent extraction technique, which extracts solutes at subcritical conditions at high temperatures below the critical point temperature and pressures above the critical point pressure. Solvents that boil at low temperatures at atmospheric pressure retain their liquid state at subcritical conditions as the fluid penetrates the lignocellulosic matrix of woody biomass (Song *et al.* 2016, Hossain *et al.* 2011). The organic solvents produced by ASE result in higher extractive yields compared to traditional techniques because the process increases the solubility of the solute and improves its mass transfer and diffusion rate (Gomes *et al.* 2017). Furthermore, the solvent's ability to penetrate the biomass is increased as viscosity and surface tension are decreased (Song *et al.* 2016). Other benefits of ASE are associated with its quick extraction times, low quantity of solvents

used, and that the extraction process occurs in a light and oxygen-free environment. Oxygen-rich environments coupled with uncontrolled heat during the extraction processes degrade the chemical integrity of the secondary metabolites (Dawidowicz *et al.* 2006).

One of the factors that affect the selectivity of phenolic components is the solvent used during the extraction process. Some hydrophilic solvents that have previously been investigated by other researchers include methanol, ethanol and acetone in the extraction of antioxidant chemicals, for example, from the bark of rosemary and spruce trees (Frankó *et al.* 2017). Jablonsky *et al*. (2015) reported efficient ASE extraction of phenolic compounds from spruce bark using ethanol at temperatures ranging from 80 to 160℃. Ethanol and acetone are polar organic solvents reported as the most suitable mediums for the extraction of polyphenolic components from spruce and sorghum (Barros *et al.* 2013). To date, there is a lack of research on ASE optimum conditions for extracting polyphenolic components from bark of *Eucalyptus* species.

Response surface methodology has been a cornerstone concept for the optimisation of different extraction systems, where experimental designs such as the Box-Behnken and Central composite designs have been used to maximise the extraction process, and this has resulted in polynomial models obtained from regression analysis of the behaviour of extraction yields. However, the challenge with this is that these models only describe the response behaviour of one species at a time. This limitation can be minimised by performing an optimisation process that employs an experimental design that has the ability to describe the response behaviour of more than one species at a time. The resulting yield responses from each species can then be normalised and integrated into one optimisation system.

This study employed an accelerated solvent extraction process which used integrated response surface methodology as a guideline to investigate optimization of extraction of polyphenolic materials from the four *Eucalyptus* species under study. A Box-Behnken experimental design with centre points was used to investigate extraction factors such as temperature, particle size and number of static extraction cycles. The

aim of the study was to find the optimum processing conditions that result in maximum polyphenolic and extractive yields from *Eucalyptus* bark.

3.1. Materials and methods

3.1.1. Reagent and biomass collection

Ethanol (99.5%) and anhydrous sodium carbonate (99.5%) were obtained from Associated Chemical Enterprises, South Africa. Folin–Ciocalteu phenol reagent and gallic acid were purchased from Sigma Aldrich, USA, and acetone (99.5%) from Glassworld, South Africa. Bark sample were obtained from a plantation in the midlands of Kwa-Zulu Natal Province, South Africa. Fresh bark from four mature *Eucalyptus* tree species, namely: *E.grandis, E.nitens, E.dunnii and E.smithii,*were collected. During collection, three trees from each species were selected for the removal of bark strips. The strips came from 1.2 meters of the thick bark located at the bottom of the trunk, medium sized bark found in the middle of the trunk and the thin bark found at the top of the tree.

3.1.2. Biomass particle size preparation

The National Renewable Energy Laboratory (NREL) standard was used as a guideline for drying, chipping, milling and sieving the bark samples. The bark strips were first chipped and allowed to air dry to a moisture content of 10% (Hames *et al.* 2008). The moisture content was measured using a Kett 6-10 infrared moisture balance (Toyko, Japan). After that, a sequential process using a hammermill and a Wiley mill was used to grind the bark chips into smaller particles. A mechanical shaker with standardised sieves was used to screen the bark particles into different particle size classes. The particle size classes chosen were 850-500 µm, 500-375 µm and <375 µm.

3.1.3. Accelerated solvent extraction (ASE)

A Dionex ASE 350 (Dionex Corp, Sunnyvale, CA) was used to carry out the accelerated solvent extraction experiments. The automated extraction system involved placing the 4.00g of bark biomass samples into stainless steel cells the bottoms of which were fitted with frits and coupled with a glass fibre filters to prevent suspended particles from slipping into the collection vessels. The sequential extraction procedure entailed pumping the extraction solvents (80% v/v of ethanol or 50% acetone v/v) into the cells containing the biomass samples, pressurising the cell contents to 103.42 bar, and heating them to temperatures of 80, 120 and 160℃ for nine minutes. After this period, five minute static extraction cycles where 40% of the fresh solvent was pumped into the cells for further extraction were performed.

Finally, nitrogen gas was used to purge the solvent at 150 psi for 90 seconds. The extracts were collected and stored at 4℃ before analysis. The process factors investigated for the Box-Behnken experimental design were temperature (x_1), static cycles (x_2) and particle size (x_3). The minimum and maximum values used to generate coded variables were 80 and 160℃ for x_1, 1 and 3 static extraction cycles for x_2 and the particle size classes described in section 2.2 for x_3. In each size class, the middle size d_{50} was used to represent the size class during the regression analysis.

Table 3-1 Box-Behnken experimental design for accelerated solvent extraction of *Eucalyptus* bark.

Run	T (℃)	Static Cycles	Particle Size (µm)
1	160(+1)	2 (0)	500-850, 675 (+1)
2	160(+1)	1 (-1)	375-500, 427.5 (0)
3	80(-1)	2 (0)	<375, 177.5 (-1)
4	160(+1)	3(+1)	375-500, 427.5 (0)
5	160(+1)	2 (0)	<375, 177.5 (-1)
6	120(0)	2 (0)	375-500, 427.5 (0)
7	120(0)	3(+1)	500-850, 675 (+1)
8	120(0)	1(-1)	<375, 177.5 (-1)
9	80(-1)	2 (0)	500-850, 675 (+1)
10	120(0)	1(-1)	500-850, 675 (+1)
11	80(-1)	3(+1)	375-500, 427.5 (0)
12	120(0)	3(+1)	<375, 177.5 (-1)
13	120(0)	2 (0)	375-500, 427.5 (0)
14	120(0)	2 (0)	375-500, 427.5 (0)
15	80(-1)	1(-1)	375-500, 427.5 (0)
16	120(0)	2 (0)	375-500, 427.5 (0)
17	120(0)	2 (0)	375-500, 427.5 (0)

(+1) maximum, (0) mid-point) and (-1) minimum coded variables of the process independent variables

3.1.4. Total extractives content (TEC)

The ASE experimental extracts on from bark samples were collected in 250 ml vessels which were placed in 500 ml round bottom flasks and processed on a rotary evaporator to recover the solvent used during the extraction. The rotating speed of the round-bottom flask containing the solution was 30 rpm, and the solution was maintained at a temperature of 60 ℃ and a vacuum pressure of 250 mbar. Once most of the solvent was recovered, the samples were then fully dried in a vacuum oven set at 35℃ and 250 mbar. The total extractive contents was determined by taking the difference of the initial mass fed into the ASE cell and the mass of the dried extracts.

3.1.5. Total polyphenolic content

A modified method based on descriptions by Singleton and Rossi., (1965) and Georgé *et al.* (2005) was followed to determine total polyphenolic compounds. It involved dissolving 0.5 mg of the vacuum oven dried samples in 10 ml of 20% ethanol (v/v). Thereafter, 100 µl of the extracts were pipetted into a test tube, followed by the addition of 100 µl of Folin–Ciocalteu phenolic reagent. The mixture was allowed to react for 2 mins after which 800 µl of 20% sodium carbonate (v/v) was added to the mixture to stop the reaction. The solution was heated in a water bath at 40℃ for 20min, and then the absorbance was measured in a 1 cm cell at a wavelength of 750 µm. The total phenolic content was determined from a calibration curve constructed using gallic acid (0.2-10 mg GAE) as the standard. The calibration curve was prepared using the same solvent and process used for the samples.

3.1.6. Statistical experimental analysis

The optimisation approach used in this study aimed to maximise the total phenolic content (TPC) at a high total extractive content (TEC). The data obtained from the Box-Behnken experiments on the bark samples was processed using a response surface methodology analysis statistical software programme called Design-Expert, version 10.0.6 (Stat-Ease, Inc., Minneapolis, MN). The TPC and TEC data were normalised

using a standardisednormalising equation in y_{norm}, where the minimum and maximum values obtained from the responses were used to convert the responses of each species into a unitless range [0-1]:

$$y_{norm} = \frac{y_i - y_{min}}{y_{max} - y_{min}} \quad (1)$$

where y_{norm} is the normalised value of the measured response, y_i is the measured response, y_{min} and y_{max} are the minimum and maximum values obtained from the experimental data of TPC and TEC for each bark species. The normalised data was fitted in a second order polynomial equation Y_{pred}:

$$Y_{pred} = \alpha_0 + \sum_{i=1}^{2} \alpha_i x_i + \sum_{i=1}^{2} \alpha_{ii} {x_i}^2 + \sum_i \sum_{j=i+1} \alpha_{ij} x_i x_j \quad (2)$$

The predicted variable for both TPC and TEC is given by Y_{pred}; α_0 is a constant of the model and α_i is a constant that describes the linear behaviour of the model; α_{ii} is the quadratic coefficient; α_{ij} is the coefficient of interaction between the two independent variables x_i and x_j. The Analysis of Variance (ANOVA) table was generated by Design Expert software and it highlights the integrity of the fitted model on the normalized data, by displaying the lack of fit using Fisher's f-test and coefficient of regression (R^2 value). The significance of the independent variables and the model was measured by determining the 95% probability test ($p < 0.05$). Contour and 3-D surface plots of the model were plotted to depict visual behaviour of the effect of the two process variables against the predicted response. The three variables used for modelling were: temperature, number of static extraction cycles, and bark particle size.

3.2. Results and discussions

3.2.1. Total extractives and phenolic contents

Table 3-2 Minimum, maximum and average values of total extractives and polyphenolic content of *Eucalyptus* tree bark shows the minimum and maximum experimental values of TEC and TPC obtained from each set of accelerated solvent extraction runs of the different *Eucalyptus* species. *E.grandis* bark contained ethanol/water hydrophilic extracts that ranged from 6.60 to 20.58% with an average of

13.34% within the Box-Behnken experimental design performed in this study. The total extractives content of *E. nitens*, a close relative of *E. globulus*, ranged from 8.05 to 20.75% and averaged 12.76 %, whereas that of *E. dunnii* ranged from 8.69 to19.73% with an average of 13.34%. The species with the highest yield of extract was *E. smithii*, which the yield ranged between 12.91-33.19% averaged at 21.86%.

Table 3-2 Minimum, maximum and average values of total extractives and polyphenolic content of *Eucalyptus* tree bark

Species	**Total Extractives Content, %**		**Total Phenolic Content, g/100g GAE**	
	Min/Max	Average	Min/Max	Average
E.grandis	6.60-20.58	13.34	1.46-2.69	2.02
E.nitens	8.05-20.75	12.76	2.31-3.77	3.15
E.smithii	12.91-33.19	21.86	3.81-5.08	4.67
E.dunnii	8.69-19.73	13.34	3.98-4.53	4.24

E. grandis had the lowest amount of polyphenolic components that averaged at 2.02 g/100g GAE. TPC of *E. smithii*, *E.dunnii* and *E. nitens* were 4.67, 4.2, and 3.15 g/100g GAE, respectively. The minimum and maximum values obtained for TPC and TEC from each set of runs were normalised and integrated into one system. Feature scaling normalisation techniques were used to standardise the measured response range by rescaling to a range of [0, 1] for every measured response. The *Eucalyptus* species whose phenolic content has been analysed and reported the most is *E.globulus*. A value of 18.64 g/100g GAE was reported when 2.5% sodium sulphate was used during an extraction process using a Pyrex glass reactor (Vázquez *et al.* 2009). In another study a maximum value of 5.722 g/100g GAE was extracted from *E.globulus*. This value was obtained from a supercritical fluid extraction process using supercritical CO_2 modified with ethanol (de Melo *et al.* 2014).

3.2.2. Statistical analysis of the extraction process

The effects of bark particle size (x_3), static extraction cycles (x_2) and temperature (x_1) on TPC and TEC were evaluated. After feature scaling from four sets of experiments, a multiple regression analysis was performed. The independent variables were converted into coded variables that ranged from [-1, 1], that were then plotted against the TEC normalized data response of TPC ranging from [0, 1]. A total of 65 runs were analysed: they comprised 17 per specie each for *E. dunnii* and *E. smithii,* 15 for *E. nitens,* and 16 for *E. grandis*. The regression analysis resulted in a second order polynomial model equation which describes the predicted response of TPC and TEC in terms of the coded variables:

$$TEC_{norm} = 1.07 + 8.06\,x_1 - 1.09 \times 10^{-3}x_2 - 2.00 \times 10^{-3}x_3 + 1.62 \times 10^{-3}x_1x_2 - 7.03 \times 10^{-6}x_1x_3 + 6.06 \times 10^{-4}x_2x_3 - 2.77x_1^2 + 0.20x_2^2 + 1.58 \times 10^{-7}x_3^2$$

$$TPC_{norm} = -2.58 + 0.05\,x_1 + 0.11\,x_2 + 1.804\,x_3 - 9.79 \times 10^{-4}x_1x_2 - 1.17 \times 10^{-5}x_1x_3 + 1.31 \times 10^{-4}x_2x_3 - 178 \times 10^{-4}x_1^2 - 6.49\,x_2^2 - 6.21 \times 10^{-7}x_3^2$$

3.2.3. Optimization of extraction of polyphenolic components

Figure 3-1 to 3-3 show graphical three-dimensional surface plots of the model with their respective counter plots displayed underneath the surface. The surface plots show the 3-D behaviour of the response against the interaction between the two process variables. The contour plots indicate the significance of the ASE process variables against yield of TPC: the elliptical contour plots highlight greater significance whereas the circular plots highlight low significance. The first set of 3-D surface plots and contour plots in Figure 3-1 show the interaction of temperature and the number of static extraction cycles against yield of TPC. The surface plot in Figure 3-1 shows that from the quadratic model of the normalised results, the yield of polyphenolic components in *Eucalyptus* bark increases with temperature, reaching an optimum extraction at 117℃.

The model illustrates the significance of temperature on the extraction of polyphenic

material from *Eucalyptus* bark. Several studies have also shown that temperature has a great impact on TPC especially when the extraction process is performed using an ASE or techniques such as supercritical fluid extraction, ultrasonic assisted-extraction, and microwave-assisted extraction (Toubane *et al.* 2017, Ghitescu *et al.* 2015, Hossain *et al.* 2011). The optimum extraction temperature for most hardwood bark is generally reported to be around 120℃ for pressurised fluid extractions, for rosemary, marjoram, and oregano tree species the temperature was 129℃ (Hossain *et al.* 2011). The modelled surface plots show a decline in the amount of phenolic components above 120℃. This is in agreement with literature reports indicating that phenolic compounds are heat sensitive and high temperatures degrade the integrity of phenolic components(Casazza *et al.* 2012).

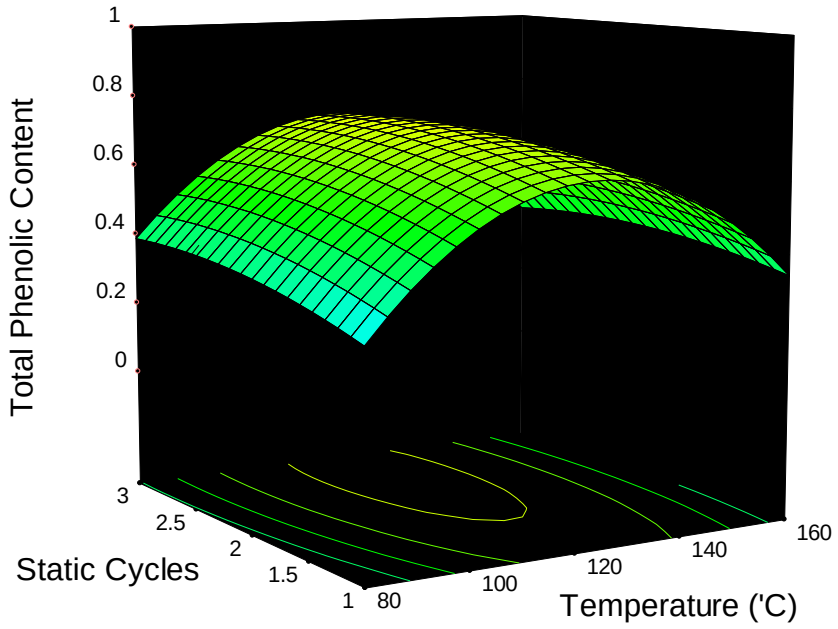

Figure 3-1 Surface and contour plots; Temperature and particle size against TPC

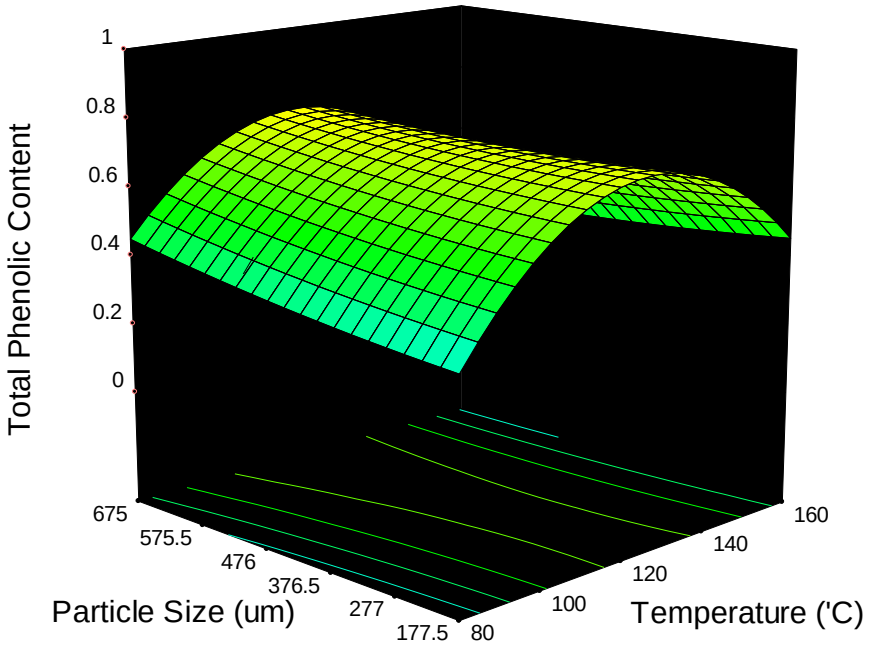

Figure 3-2 Surface and contour plots; Temperature and particle size against TPC

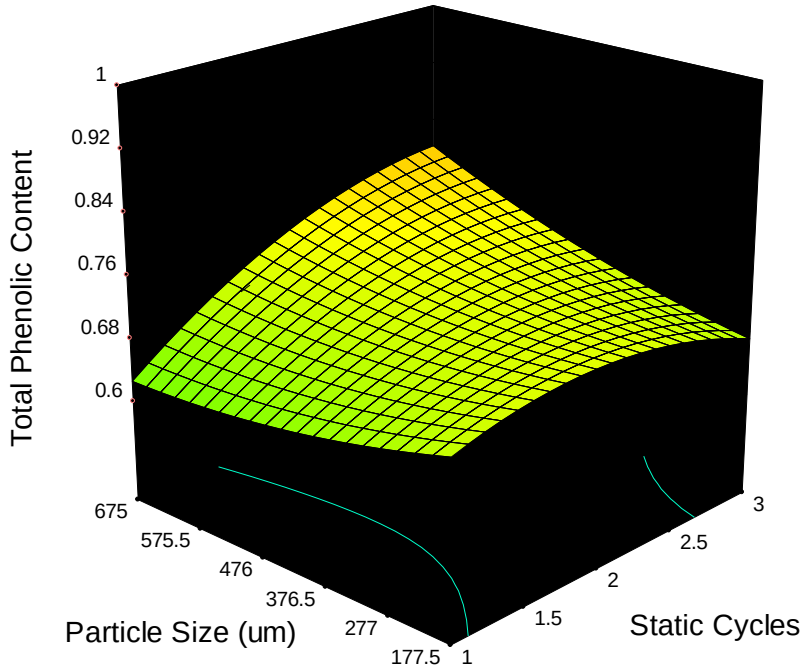

Figure 3-3 Surface and contour plots; Particle Size and number of static extraction cycles against TPC

The surface plots displayed in Figure 3-2 and Figure 3-3 illustrate that the number of static extraction cycles have minimum impact on the amount of polyphenolic compounds extracted unlike temperature. However, as the number of static extraction cycles increases, more polyphenolic compounds can be extracted because the increased number of static extraction cycles enhances the interactions between the extraction solvent and the solutes in the lignocellulosic matrix and as a result, the rate of diffusion increases, further resulting in more solubilisation of polyphenolic components (Toubane *et al.* 2017). The particle size of the bark biomass also has minimal impact on the response as illustrated in Figure 3-2 and Figure 3-3. The yield of TPC only increased with increasing particle size when the temperature was also increased. Small sized particles clogged the frit and filter paper at the bottom of the stainless steel cell; this may have been the reason why the quantity of solvent pumped into the collection vessel was so low. The experimental conditions that resulted in optimum extraction of TPC are a temperature of 117.19℃, at >3 static extraction cycles, and bark particle size class of 500-850 μm.

3.2.4. Optimization of extraction of total extractives content

As previously been mentioned in Chapter xx, industrial applications of extractives in bark include tannin-based adhesives, binding agents, and antioxidant products. Thus large quantities of phenolic components are in demand. Hence efficient extraction of the compounds in maximum yield from bark biomass is important. Figure 3-4, Figure 3-5 and Figure 3-6 are 3-D surface plots that show the behaviour of the total TPC yield as a function of extraction parameters. Figure 3-4 and Figure 3-5 illustrate models which resulted in high extractive yields at temperatures reaching the maximum boundary of 160℃. Finally, Figure 3-5 and Figure 3-6 highlight that high quantities of extracts are obtained from smaller bark sized particles only when the number of static extraction cycles approached 3.

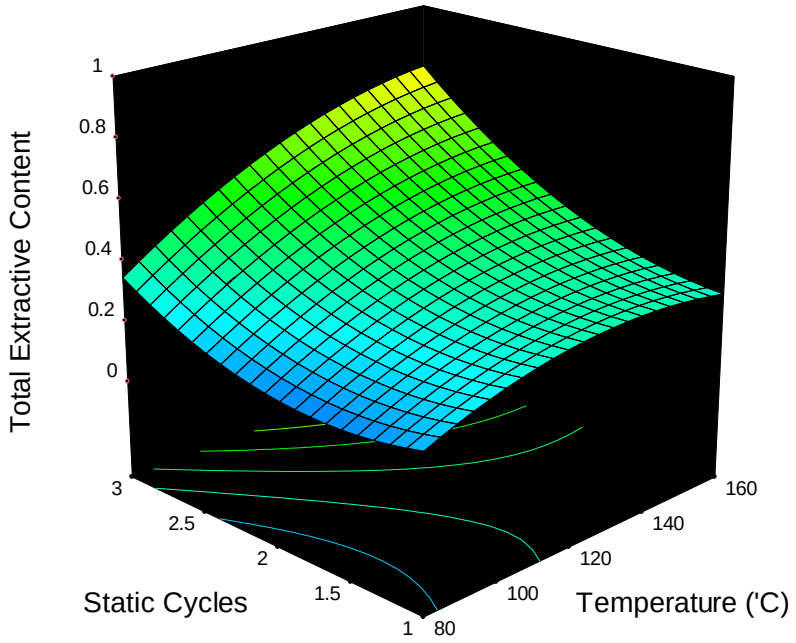

Figure 3-4 Surface and contour plots; Temperature and number of static extraction cycles against TEC

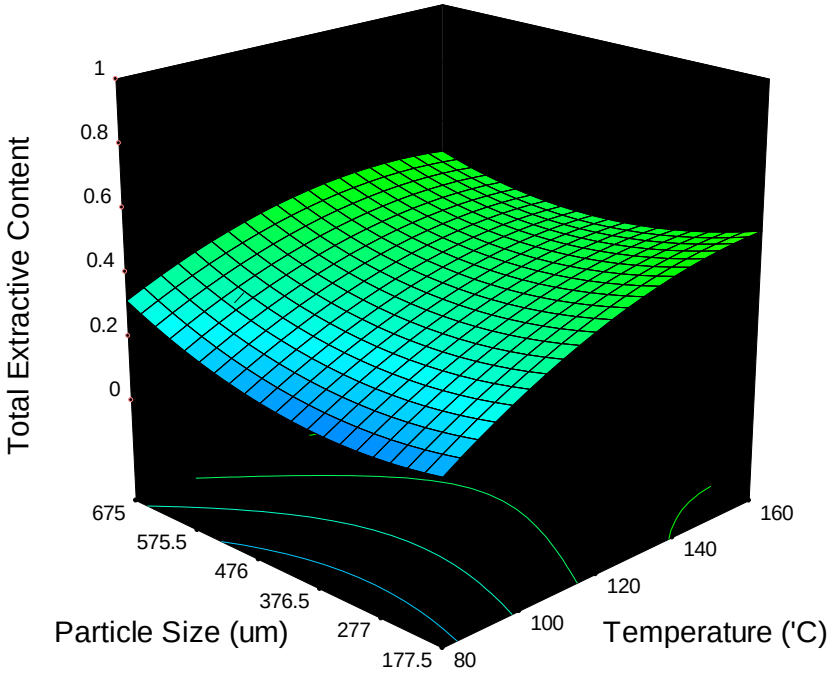

Figure 3-5 Surface and contour plots; Temperature and particle size against TEC

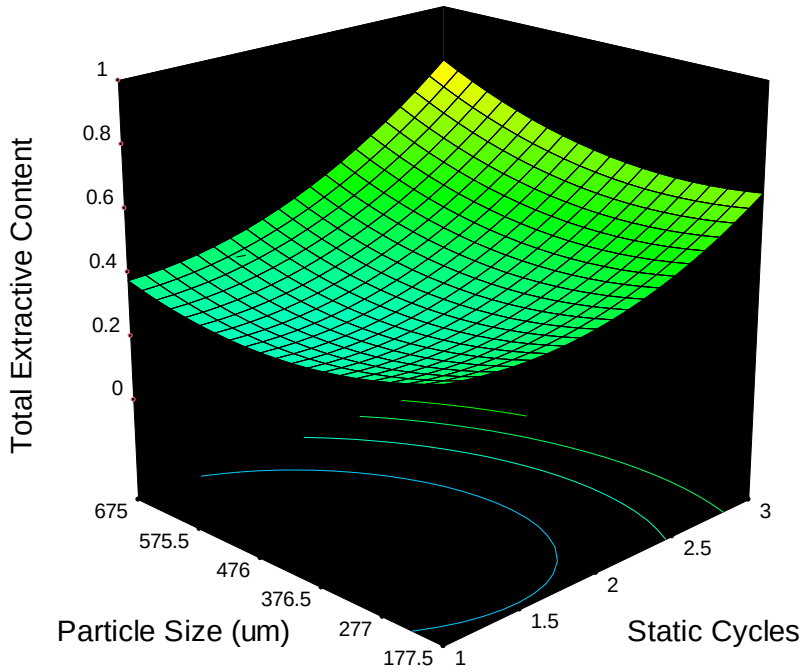

Figure 3-6 Surface and contour plots; Particle size and static extraction cycles against TEC

3.2.5. Verification and optimisation of the predicted model

The predicted model was investigated to check its validity and reliability before making any recommendations on optimum extraction conditions. Figure 3-7 and Figure 3-8 show a graph of the actual experimental data against the data obtained from the predicted model. An important point of note is that the predicted and actual values of both TPC and TEC are slightly different.

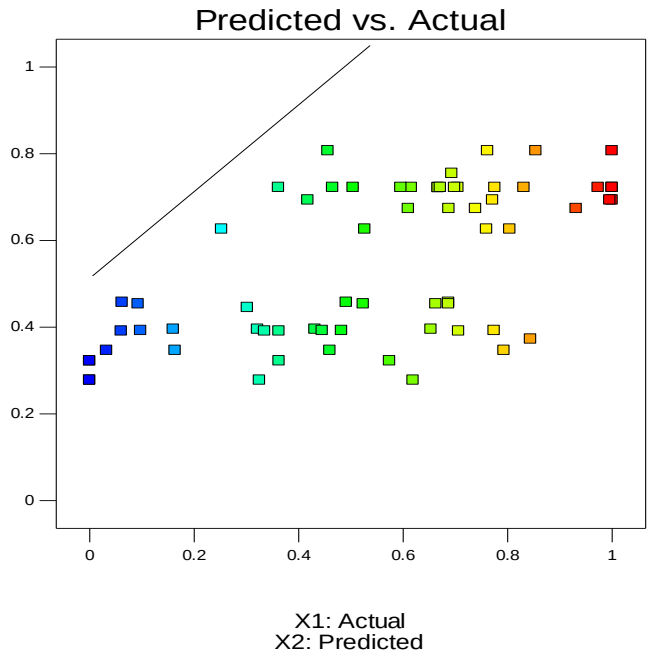

Figure 3-7 Correlation of the actual experimental values and models predicted values of TPC

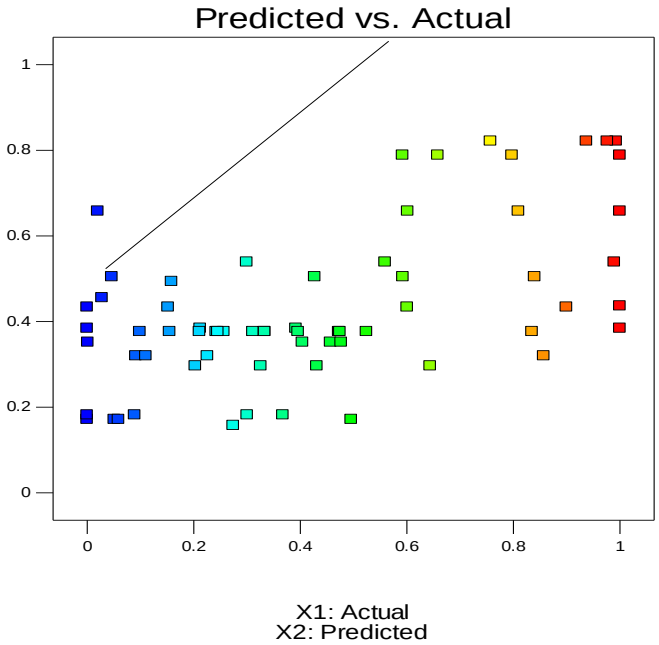

Figure 3-8 Correlation of the actual experimental values and predicted model values of TEC

The integrity of the model was assessed by performing an analysis of variance

(ANOVA). ANOVA results of a fitted quadratic model passed the F-test confidence level, as the p-value was 0.0054 for TEC and 0.0014 for TPC ($p < 0.05$). The drawback of the model is that the value of the determination coefficient for both responses was low, 0.33 for TEC and 0.37 for TPC. The adjusted determination coefficient (R^2_{adj}) which indicates the goodness-of-fit of the regression procedure undertaken is also low, 22.04 % and 26.00 %. Figure 3-7 and Figure 3-8 show that the goodness-of-fit between the predicted values and the actual values from the experiments is low. Finally, the values of 65.60 and 44.22 % obtained from the Coefficient of Variance showed a low confidence in the behaviour of the model.

3.3. Concluding remarks

The objective of this study was to determine the conditions that result in optimal extraction of total phenolic components at the highest total extractives content. Data on three variables, namely: temperature, number of static extraction cycles and bark particle size was collected for four *Eucalyptus* bark species. Using response surface methodology in the form of a Box-Behnken design showed that ASE at 117℃ with > 3 static extraction cycles, and bark particle size class of 500-850µm resulted in optimal extraction of the Total Polyphenolic Content of the four *Eucalyptus* species (*E.grandis, E.smithii, E.nitens* and *E.dunnii*).

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CHAPTER 4 Paper II

4. Characterisation of antioxidant components in bark extracts of *E.grandis*, *E.smithii*, *E.nitens* and *E.dunnii*

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Abstract

The forest industries generate large amounts of waste biomass bark that is largely combusted for energy production or disposed of by landfilling. With the advent of biorefinery technologies beneficiation of waste biomass is an important tenet of biorefinery technologies. This study is aimed at ascertaining if *Eucalyptus* can be better beneficiated by extraction of valuable compounds from the biomass. Thus chemical compositions of bark samples from *E.grandis, E.smithii, E.nitens* and *E.dunnii* were studied to determine their amounts and chemical composition and use the information to ascertain if the bark could be beneficiated via extraction of any potentially high value components from the bark. The bark samples were processed to recover hydrophilic extracts and the residual fibres were analysed for lignin and cellulose content. The extractives recovered were compared with those of *Acacia mearnsii*, commonly known as Black Wattle, a commercial source of tannins. The extraction process was performed using Accelerated Solvent Extraction and the total polyphenolic content was measured using the Folin–Ciocalteu method. Among the *Eucalyptus* bark samples studied, *E.dunnii* had the highest content of phenolic compounds (5.52g/100g GAE) versus 8.95 g/100g GAE in Black Wattle. Pyrolysis-Gas Chromatography/Mass Spectrometry was used to characterise the chemical nature of the bark extracts. All bark samples showed high amounts of phenolic components in extracts performed with 50% acetone. Major components detected were catechol, guaiacol and phenol derivatives from the B-ring of a flavan-3-ol unit of condensed tannins. Analysis of the extractives-free bark showed that *E.nitens* had the highest glucose content whereas *E.dunnii* had the highest Klason lignin content. Various valuable chemical components were detected in the bark, about a one third of the

composition of the bark biomass can be utilised can be extracted prior to energy generation.

4.1. Introduction

Finding alternative sources of valuable chemicals from renewable sources such as the waste derived from the forestry industry is a major tenet of biorefinery technologies. Wood from *Eucalyptus* species is widely used for timber, pole, pulp and paper production due to its fast seasonal growth, high pulp yield, high strength and track record in the forest industries, especially in South Africa, Brazil, and Australia(Bison *et al.* 2006;Neiva *et al.* 2015; Rockwood *et al.* 2008; Santos *et al.* 2017). This processing results in generation of large amounts of waste bark biomass. Currently, this waste is used for energy production or is disposed of by landfilling. Are there more potentially valuable uses of this waste biomass?

The bark of *Eucalyptus* species contains a diverse conglomeration of components produced by the plant cell. The components have various industrial applications such as tanning agents in the leather industry, antioxidants in medicinal applications, binders in adhesive industries (Miranda *et al.* 2016;González *et al.* 2017; Diouf *et al.* 2013). Polyphenolic components such as condensed and hydrolysable tannins can be extracted from *Eucalyptus* barks using hydrophilic solvents such as ethanol, methanol and acetone. The adequate extraction and valorisation of polyphenolic components such as condensed tannins could increase the value of *Eucalyptus* bark by enabling its use as industrial feedstock (Diouf *et al.* 2013).

The polyphenolic components in *Eucalyptus* bark are predominantly condensed tannins (also known as proanthocyanidins), i.e. polymers formed from covalent bonds of flavan-3-ol derivatives (Figure 4-1). The most abundant condensed tannin is of the procyanidins type which contains catechol and has its derivative on the B-ring of the flavan-3-ol unit. Figure 4-1 shows some representative important condensed tannins derived from phenol, guaiacol, and mequinol (Ohara *et al.* 2003, Diouf *et al.* 2013). Polyphenolic components have a wide range of applications across different industries

such as cosmetic, pharmaceutical and adhesive industries (Aires *et al.* 2016). Polyphenolic components have antioxidant, anti-inflammatory and anti-carcinogenic properties, these properties lead to the production of healthcare products that have the ability to reduce and negate the effects of cancer among other benefits (Pérez *et al.* 2014).

Since bark contains these potentially high value compounds, it is worthwhile ascertaining if it would be beneficial to use the waste bark biomass to extract these compounds instead of combusting it for energy purposes. Solvent extraction techniques such as assisted solvent extraction; microwave and ultrasonic assisted extraction have been successfully used by several researchers to extract polyphenolic components from different *Eucalyptus* species (Ghitescu *et al.* 2015, Hou *et al.* 2016, Ilghami *et al.* 2015).

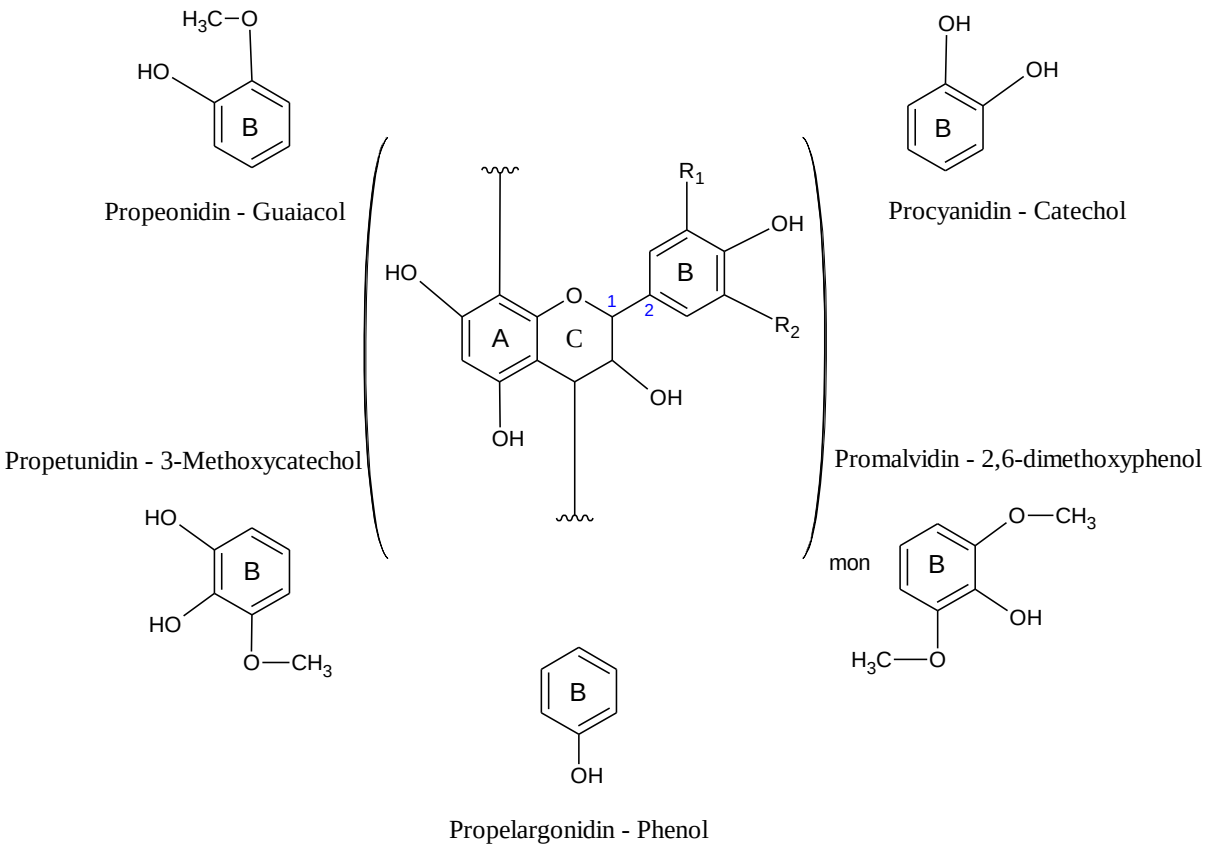


Figure 4-1 Different types of condensed tannin derived from the flavan-3-ol monomer unit

The diversity of polyphenolic components within the same family, genus and species of hardwood is wide. However, various analytical techniques such as electrospray ionisation mass spectrometry; size–exclusion chromatography; gas chromatography/mass spectrometry; and NMR analysis can be applied to understand their different chemistries (Diouf *et al.* 2013, Keskes *et al.* 2017). These techniques are disadvantageous because they require pre-treatment of the sample before its analysis. For this reason, Py-GC/MS is the most useful analytical technique: it has been used for decades to characterise polyphenolic components (Galletti *et al.* 1995, Ohara *et al.* 2003). The technique's advantage is that it requires small quantities of sample with minimal or no pre-treatment (Sithole *et al.* 2012).

This study entailed extraction of polyphenolic compounds from the bark of four *Eucalyptus* species (*E.grandis, E.smithii*, *E.nitens* and *E.dunnii*) using accelerated solvent extraction where ethanol and acetone were used as extraction solvents. After extraction, the remaining biomass was hydrolysed with acid and then analysed by for Klason lignin, acid soluble lignin, and carbohydrate content.

4.1. Materials and Methods

4.1.1. Reagents and biomass preparation

Bark from twelve year old *Eucalyptus* species, namely, *E.grandis, E.nitens, E.dunnii* and *E.smithii,* were collected from commercial plantations situated in the KwaZulu-Natal midlands, a subtropical area located on the east coast of South Africa. The region has an annual average rainfall of 843mm and average daily temperatures ranging from 18.9℃ in winter to 25.8℃ in summer. Three logs were harvested from each tree and debarked, and both the inner and outer bark were collected. The first was from the bottom of the trunk (1.2m from the ground); the second from the midpoint of the trunk; and the last one from the top of the tree stem. The bark strips were chipped, sorted and reduced in size by grinding with a hammermill and Wiley mill. The ground samples were fractionated using mechanical sieves with a mesh size of 375μm(Hames *et al.* 2008).

The chemical reagents used were ethanol (99.5% purity) and sodium carbonate anhydrous (99.5% purity), both purchased from Associated Chemical Enterprises (South Africa). Folin–Ciocalteu phenol reagent and gallic acid were purchased from (Sigma Aldrich, USA) and acetone (99.5% purity) was procured from Glassworld (South Africa).

4.1.2. Accelerated solvent extraction (ASE)

Approximately 4.0 g of the inner and outer bark samples were extracted with 80% Ethanol/water v/v and 50% acetone/water v/v using an automated extraction technique, i.e., the Dionex ASE 350 (Dionex Corp, Sunnyvale, CA). Conditions for the extraction process were: extraction for 9 minutes at 120℃; operating pressure of 1500 psi; and 5 min static extraction cycles. The extraction process variables were obtained from the optimum conditions found in section 3.2.3 and 3.2.4. The extracts were reduced to small volumes (~10 mL) using a rotary evaporator operating at 60 ℃ and pressurised at 250 mbar. The samples were then dried in a vacuum oven at 35 ℃ for 12h. A total of 16 runs, including duplicates experiments for each solvent and each *Eucalyptus* bark species, were run. The amounts extracted were determined gravimetrically.

4.1.3. Total polyphenolic content

A modified Folin–Ciocalteu method described by Singleton and Rossi (1965) and George (2005) was used to determine the total phenolic content of vacuum oven-dried extracts. Known amounts (~0.5 mg) of bark extracts were dissolved in 10 ml of 20% ethanol (v/v). Thereafter, 100μl of the solubilized extracts and 100μl of Folin-Cateliu phenolic reagent were mixed for 2 min, and the reaction was stopped by addition of 800 μl of 20% sodium carbonate (v/v) into the mixture. Absorbance of the solution was measured at 750 μm using an Agilent Cary UV-Vis spectrophotometer. Total phenolic content was determined by from a calibration curve prepared from a gallic acid standard (0.2-10 mg/GAE) processed similarly as the samples.

4.1.4. Chemical characterization of *Eucalyptus* barks samples

The bark samples were analysed for the presence of structural carbohydrates and lignin using a National Renewable Energy Laboratory (NREL) standard as reported by Sluiter *et al.* (2008). The extractives-free bark samples obtained after accelerated solvent extraction were hydrolysed with dilute (32% and 4%) H_2SO_4 to separate lignin and polysaccharides from the lignocellulosic matrix. The hydrolysed extracts were analysed by High-Performance Anion-Exchange Chromatography coupled with Pulsed Electrochemical Detection(HPAEC-PAD) using a Dionex ICS-5000 system equipped with a CarboPac PA20 20 cm column, a strong anion exchange column (Dionex Corp, Sunnyvale, CA). A 2 M NaOH was used as mobile phase at a flowrate of 1 ml/min on 20μL injections of extracts. Glucose, xylose, mannose, arabinose and galactose were used as standards for monomeric sugars.

Klason lignin was determined by gravimetric analysis of the residue filter cake from the autoclaved hydrolysis solution of the biomass. The filter cake on a previously weighed filter paper was air dried in petri dishes overnight. The weight of the insoluble lignin was calculated by subtracting the weight of the filter paper after drying. Acid soluble lignin was determined by using the hydrolysis liquor aliquot obtained after filtration. The aliquot was diluted to an absorbance 0.7-1.0 using 4% sulfuric acid as a solvent and also as a background solution. The absorbance was measured using Agilent Cary UV-Vis spectrophotometer.

4.1.5. Py-GC/MS analysis

The ethanol and acetone extracts were analysed by Py-GC/MS using a multi-shot pyrolyser, EGA/PY-3030D (Frontier Laboratories, Fukushima, Japan) attached to a Shimadzu gas chromatograph/ mass spectrometer (QP2010 SE). Approximately 0.1μg of each extract was accurately weighed in a stainless steel cup and pyrolysed at 550℃ for 10 seconds. The interface temperature to the analytical column was set at 350 ℃

and the chromatographic separation of the pyrolysis products was performed using an ultra-alloy capillary column with the following dimensions, 30 m x 0.25 mm with 0.25 µm film. The injection port was set at 250 °C and the column flow rate was set at 1.0 mL/min with helium as the carrier gas. The split injection ratio was 70:1. The gas chromatography temperature programme used was: (i) set at 50 °C for 2 min; (ii) increased from 50 °C to 300 °C at a rate of 10 °C /min; (iii) then kept at the maximum temperature for a further 5 min. The ion source temperature and interface temperature in the mass spectrometer were set at 200 °C and 300 °C, respectively. The scan range used for the mass selective detector was from 40to 650m/z. The pyrolysis products were identified by comparing their mass spectra with the mass spectra of the Wiley and National Institute of Standards and Technology libraries.

4.2. Results and Discussions

4.2.1. Accelerated Solvent Extraction

Table 4-1 Total Phenolic Content and Total Extractives Content of bark samples after extraction with different solvents shows a comparison of the amount of extractives obtained at 120°Cbetween extraction processes conducted with 80% ethanol and 50% acetone. Acetone removes 4mg/g more extracts on average for *E.grandis*, *E.nitens* and *E.dunnii* compared to ethanol. There is no significant difference in the amount of extracts obtained between 50% acetone and 80% ethanol for *E.smithii* and wattle. The average amount of extracts obtained for *E.smithii* for both solvents is 98% while that of wattle is 35.74% for ethanol and 33.94% for acetone. Amongst the four *Eucalyptus* bark species analysed, *E.smithii* has the highest amount of total extractive components, 33.25% for ethanol and 35.11% for acetone. *E.grandis* has the lowest amount of extracts amongst the four *Eucalyptus* bark species. This means that *E.smithii* is the best *Eucalyptus* species for the application of biorefinery technologies because of its potential to produce extractive components in high yields.

4.2.2. Total Polyphenolic Components

Several assay methods are used to indicate the antioxidant ability of bark extracts, this study used the Folin–Ciocalteu method. Table 4-1 compares the amounts of Total Phenolic Content and Total Extractive Content obtained between acetone and ethanol for the four *Eucalyptus* species and wattle.

Table 4-1 Total Phenolic Content and Total Extractives Content of bark samples after extraction with different solvents

Bark sample	Solvent	Total Extractives Content, %	Total Polyphenolic Content, g/100g GAE	TPC/TEC
E.grandis	80% Ethanol	13.39	2.18	16,28%
E.grandis	50 % Acetone	20.46	2.55	12,46%
E.nitens	80% Ethanol	15.23	3.85	25,28%
E.nitens	50 % Acetone	19.40	4.54	23,40%
E.dunnii	80% Ethanol	18.51	4.22	22,80%
E.dunnii	50 % Acetone	21.51	5.52	25,66%
E.smithii	80% Ethanol	33.25	3.46	10,41%
E.smithii	50 % Acetone	35.11	3.80	10,82%
Wattle	80% Ethanol	43.20	6.87	15,90%
Wattle	50 % Acetone	48.22	8.94	18,54%

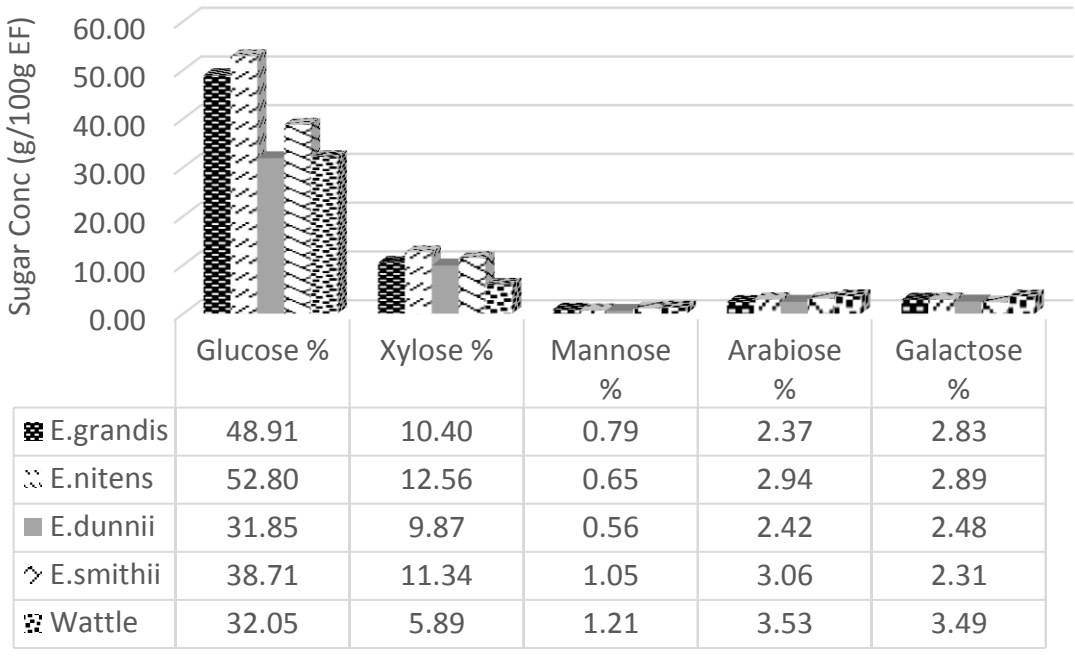
Higher amounts of total extracts from eucalyptus bark are obtained when 50% acetone/water is used. *E.grandis* had the lowest amounts of TEC (13.39%) when ethanol was used and 20.46% for acetone. A trend of an increase in TEC is observed across all eucalyptus species when acetone is used as an extracting solvent. An average increase of 4.22% TEC is observed when acetone replaces ethanol as an extraction solvent. *E.grandis* had the highest increase of TEC compared to all the *Eucalyptus* species for acetone. However, the selectivity of total phenolic content (TPC/TEC) of *E.grandis* decreased, this shows that acetone extract other components other than phenolic components from the bark. As the amount of TEC increases amongst the bark of *Eucalyptus* the selectivity decreases, this was shown by the bark of *E.dunnii* with TEC of 33.51% for ethanol and 35.51% for acetone with selectivity as low as 10.41% and 10.82% respectively. Organic polymers might degrade into different chemical derivatives that are soluble in organic solvents such as ethanol and acetone, leading to low selectivity of phenolic components. *E.nitens* had the highest content of phenolic components amongst the eucalyptus species for both acetone and ethanol, with the highest selectivity for phenolic components.

Comparing the accelerated solvent extraction of phenolic components of the bark of *Eucalyptus* with a bark of Black wattle, a commercial source of tannins, TPC of wattle are higher than those of *Eucalyptus* bark. However, the selectivity for both acetone and ethanol are lower than that of *E.nitens* under the same extraction conditions.

4.2.3. Carbohydrates and lignin content

After extraction, 60%-90% of the bark biomass used remained. This residue is comprised of macromolecules that are not soluble in acetone and ethanol. To maintain the idea of minimum waste, the chemistry of the residue needs to be investigated for beneficiation of its lignocellulosic content. Figure 4.2 shows the carbohydrate content (represented as monomeric sugars) in in ethanol-extracted bark samples. Wattle, *E.dunnii* and *E.smithii* have the smallest quantities of total monomeric sugars, at 47.18%, 46.17% and 56.47%, respectively. *E.grandis* and *E.nitens* had the highest

quantities of total monomeric sugars with high glucose content, of 48.91% and 52.80% respectively.



	Glucose %	Xylose %	Mannose %	Arabiose %	Galactose %
E.grandis	48.91	10.40	0.79	2.37	2.83
E.nitens	52.80	12.56	0.65	2.94	2.89
E.dunnii	31.85	9.87	0.56	2.42	2.48
E.smithii	38.71	11.34	1.05	3.06	2.31
Wattle	32.05	5.89	1.21	3.53	3.49

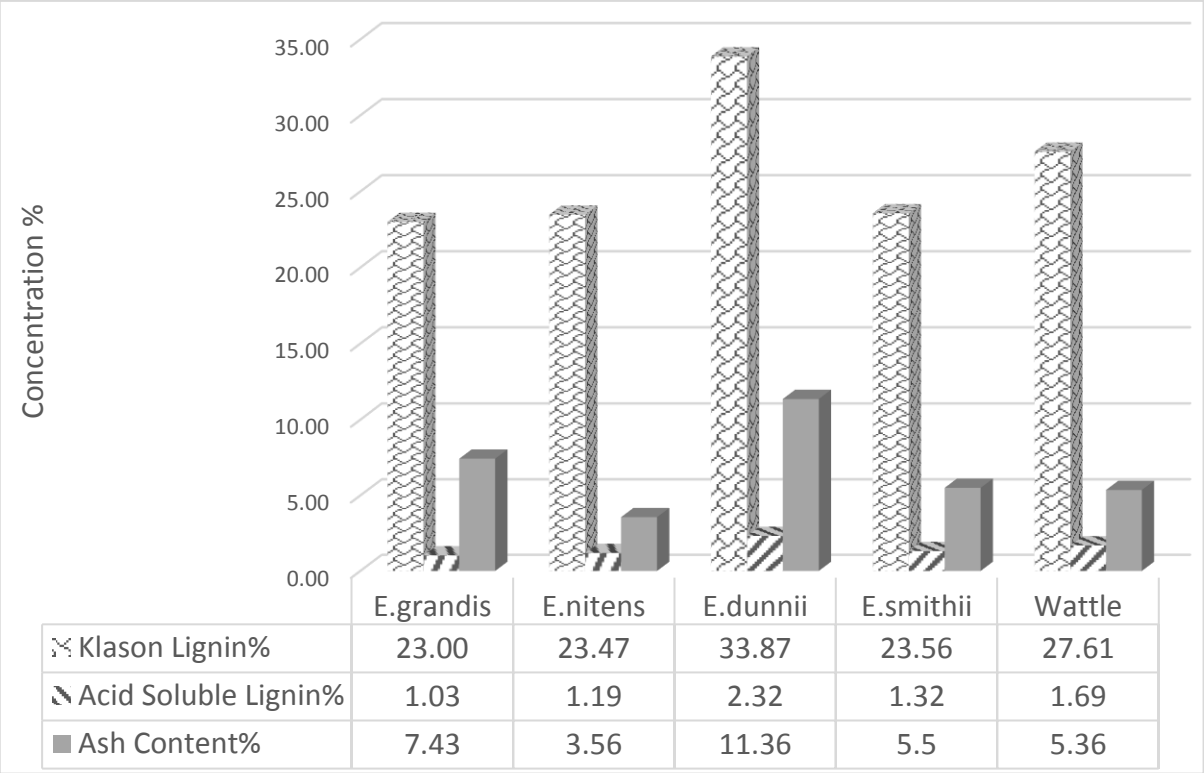
Figure 4-2 Type and concentration sugars in extractives-free bark

Thus, the residual biomass left after extraction for *E.grandis* and *E.nitens* are suitable for biorefinery technologies like enzymatic and aerobic digestion which require high quantities of glucose concentrations. All *Eucalyptus* bark species have an average of 11.01% for xylose content, a useful monomer used in the production of high value products such as xylitol and furfural. Other monomeric sugars such as mannose, arabinose and galactose are present in smaller quantities compared to xylose and glucose, thus, the potential for utilisation in biorefinery is negligible. The content of arabinose and galactose averages 2.83% in the extractive-free bark of *Eucalyptus* species. Wattle bark has a low quantity of monomeric sugars but has a high quantity of ethanol and acetone soluble extractives as shown by Table 4.1 and Figure 4.2. This proves that polysaccharides were extracted during the accelerated solvent extraction.

Figure 4-2 shows the lignin and ash contents of the four *Eucalyptus* species and wattle. Lignin is a phenolic polymer formed when monolignols coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol undergoes radical polymerisation. Klason lignin

(insoluble residue left after filtration of monomeric sugars) and acid soluble lignin (measured from the filtrate used for HPLC analysis) are used to estimate the total lignin in various biomass. The estimation of lignin serves as a source of information for technologies that either require low or high amounts of lignin in the composition of the biomass. Such as enzymatic digestion and fermentation whereby the lignin content in the cell wall negatively impacts cell wall by enzymes such as methyltrasferase and L-phenylalanine (Sewalt *et al.* 1998). Lignin content is inversely proportional to biological kinetics of biotechnological process that valorise bark. *E.dunnii* had the highest amount of total lignin while *E.grandis, E.nitens* and *E.smithii* have relatively similar concentrations of Klason lignin which averages 23.34% of the total dry bark. The bark of *E.dunnii* is not an adequate candidate for biotechnological processes.

High concentration of inorganic components in the bark measured through the determination of ash content is a negative challenge for 100% utilisation of *Eucalyptus* bark. During extraction of high value components from the bark, inorganic materials are also recovered, they reduce the purity of the target organic chemical components. The sources of high amounts of inorganic materials in barks are silica materials from the soil that are carried to the trunk of the tree by environmental factors such as winds and rains. *Eucalyptus* bark analysed in this study have high ash content as shown by Figure 4.2. *E.dunnii* has the highest ash content of 11.36% of the dry bark.



	E.grandis	E.nitens	E.dunnii	E.smithii	Wattle
Klason Lignin%	23.00	23.47	33.87	23.56	27.61
Acid Soluble Lignin%	1.03	1.19	2.32	1.32	1.69
Ash Content%	7.43	3.56	11.36	5.5	5.36

Figure 4-2 Klason lignin, insoluble lignin and ash content

4.2.4. Py-GC/MS analysis

Figure 4-3 and Figure 4-4 highlight an example of pyrograms obtained in this study after Py-GC/MS analysis of extracts of *E.dunnii* and black wattle recovered using acetone as a solvent. While Table 4-2 Chemical composition of Eucalyptus bark *extracts:* from *Py-GC/*MS analysis. The relative abundances of the components was calculated using peak areas of components that were detected in the pyrograms. Identities of components were based on their mass spectral patterns and only those that 85% or more fit with spectra in the libraries were considered genuine. From this peaks with similar chemistries and functional groups were grouped into groups, namely, mainly phenolics, terpene derivatives, steroids (including sterols), ketones, fatty alcohols, and acids as shown in Table 4-2.

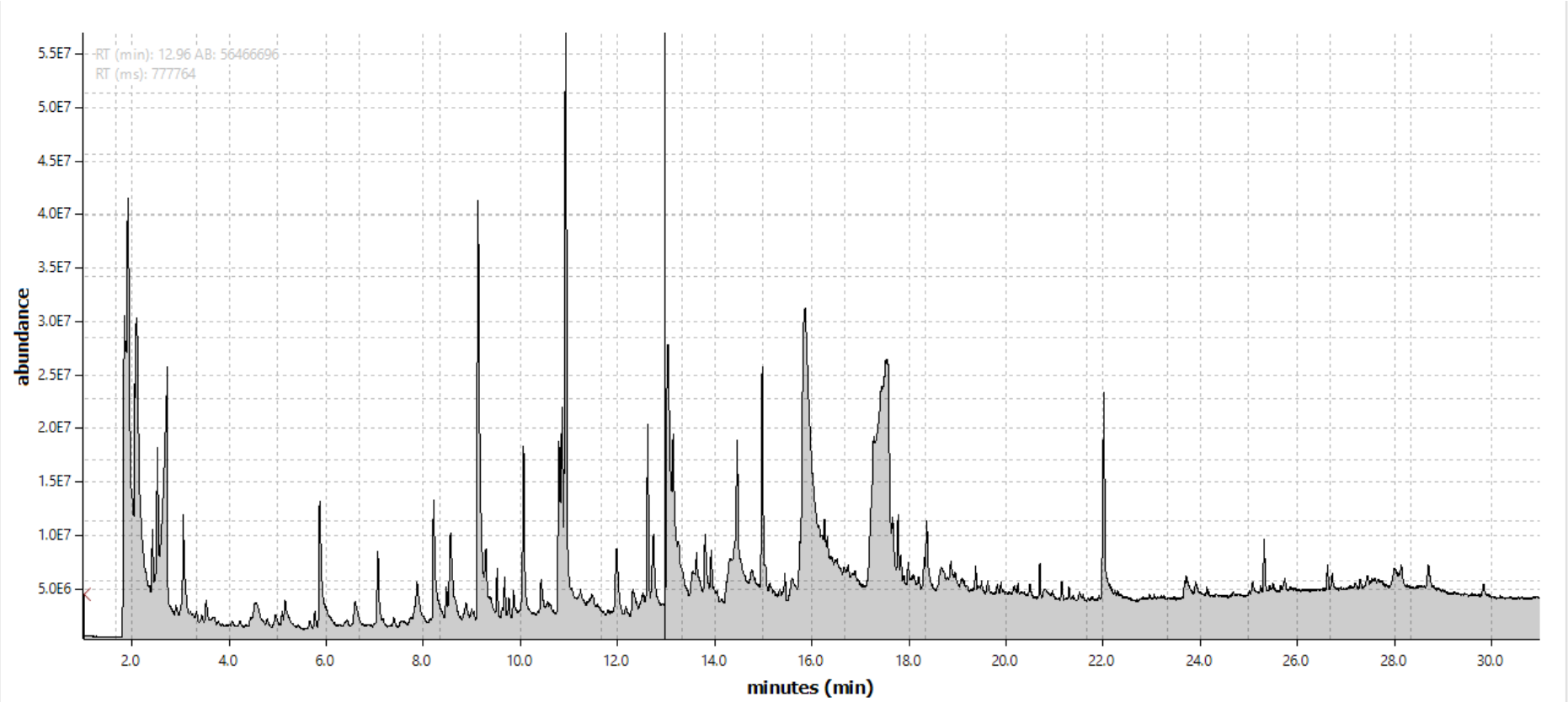


Figure 4-3 Pyrogram of acetone extracts of Eucalyptus dunnii

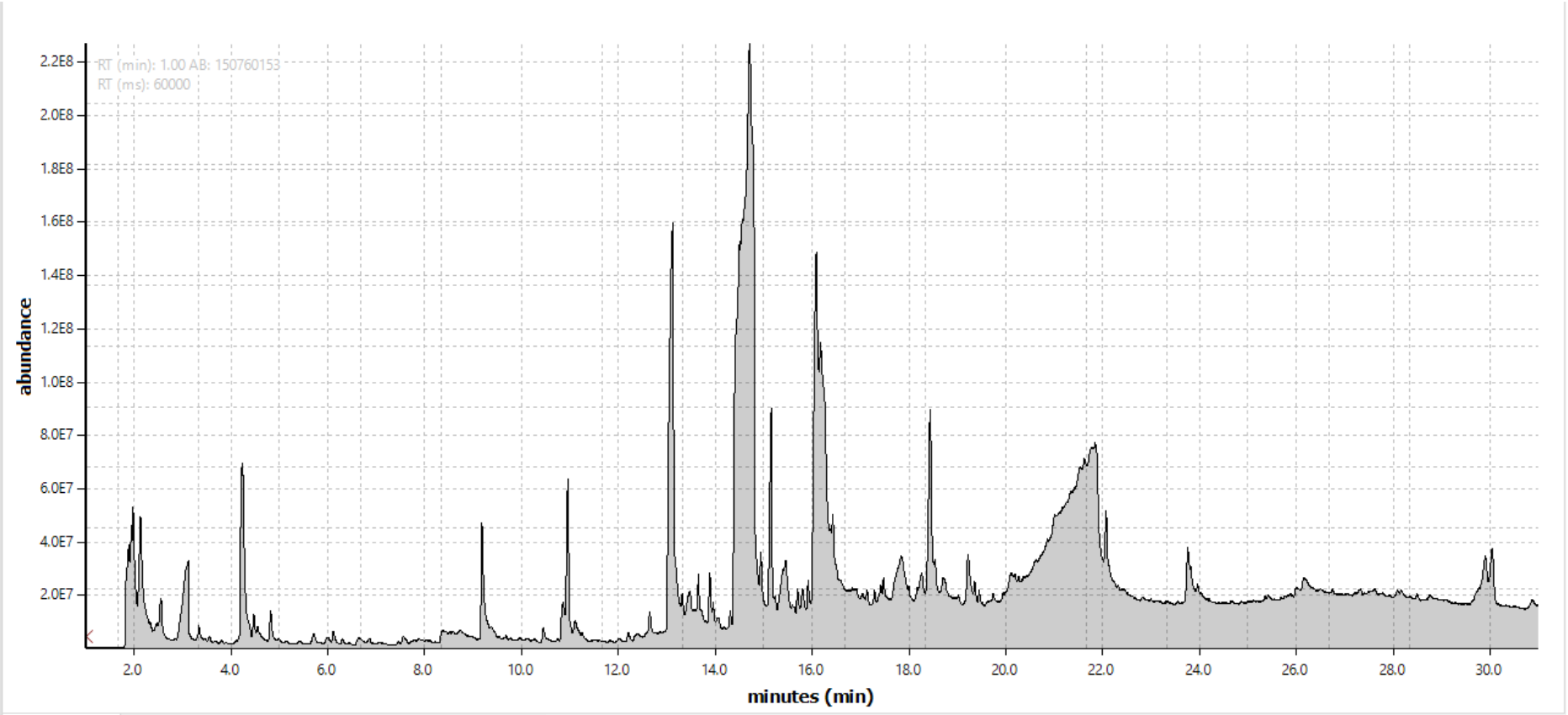


Figure 4-4 : Pyrogram of acetone extracts of black wattle

Table 4-2 Chemical composition of Eucalyptus bark extracts: from Py-GC/MS analyses

Peak area %										
	E dunnii		*E grandis*		*E smithii*		*E nitens*		*Wattle*	
	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone
Phenolic Compounds	18.78	36.37	10.89	13.67	11.43	37.28	9.69	17.15	12.99	14.63
Phenol	2.57	3.9	3.1	4.03	0	4.21	0	0	0	1.1
o-Cresol	0.31	1.71	0	0.57	0.33	1.49	0.2	0	1.13	0.54
Ethylphenol	0.21	0.33	0	0.28	0	0	0	0	0	0.1
Carvacrol	0.47	0	0.08	0.12	2.47	0	1.66	0	0	0
Thymol	0.17	0	0.16	0.11	0.22	0	0.46	0.35	0	0
Mequinol	0	5.71	0	0.15	0	4.63	0	2.82	0.19	1.36
Guaiacol	1.84	0.11	0.93	2.03	0.64	0	0	0	1.34	0
Creosol(methylguaicol)	0.59	1.35	0.34	0.33	0.24	0.28	1.66	0	0.22	0.26
P-Ethylguaiacol	0.61	0.74	0.94	0.52	0.48	0.64	0	0.26	0.24	0
p-vinylguaiacol	1.11	2.56	0.46	0.33	0	0.37	0.84	1.13	0	1.26
4-Allylguiacol	0	0.43	0.26	0.33	2.08	0	0	4.54	0.13	0
Catechol	7.35	3.39	1.75	3.1	3.77	5.87	3.3	0	0	5.18
3-methoxycatechol	1	0.79	1.36	0.72	0.51	0.75	0.24	0.38	0.26	0.51
Pyrogallol derivatives	1.32	11.94	0.95	0.52	0	13.42	1.33	4.7	1.4	0

Pyrogallol	0	10.09	0	0	0	11.69	0.9	3.54	0	0
Syringol	1.32	1.85	0.95	0.52	0	1.73	0.43	1.16	1.4	0
Resorcinol derivatives	0	0	0	0	0	0	0	0	2.82	2.83
Resorcinol, 2-methyl	0	0	0	0	0	0	0	0	2.65	0.85
4-ethylresorcinol	0	0	0	0	0	0	0	0	0.17	1.98
Other phenolic	1.23	3.41	0.56	0.53	0.69	5.62	0	2.97	5.26	1.49
	E dunnii		*E grandis*		*E smithii*		*E nitens*		*Wattle*	
	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone
Furan and Pyran derivatives	18.25	10.52	21.59	17.9	7.12	12.13	1.62	3.44	6.66	7.7
Methylfuran	2.73	1.34	2.48	2.51	1.4	1.41	1.04	1.22	0.32	0.38
Furan, 2-ethyl-	0.16	0	0.23	0	0	0	0	0	0	0
Furan, 2,5-dimethyl-	0.35	0	0.42	0.44	0	0.14	0	0	0	0.03
FURFURAL	3.41	1.35	3.16	3.23	1.33	1.49	0.21	0.61	0	0
Citraconic anhydride	0.1	1.33	0.44	0	0	0.3	0	0	0	0.54
5-Methylfurfural	0	1.22	1.26	0	0	1.33	0	0	0	0
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	0	1.17	1.5	1.25	0.84	0.79	0	0.18	0	0.05
5-Hydroxymaltol	0	0.92	0.67	0	0	1.18	0	0	0	0
5-Hydroxymethylfurfural	9.24	0	8.93	5.82	3.07	3.66	0	0	5.19	4.77
Other furans and pyran	2.26	3.19	2.5	4.65	0.48	1.83	0.37	1.43	1.15	1.93
terpene and terpenoids	17.42	1.89	8.05	3.17	46.57	1.13	37.48	32.81	2.23	0.22
Terpinolene	1.72	1.52	0.67	0.6	4.84	0.06	9.94	2.25	0	0

Gurjunene	0.27	0	0	0	5.94	0	3.07	1.86	0	0
Aromadendrene	0.96	0	0	0	7.83	0	3.85	0	0	0
Pinene	0	0	0.2	0	0	0	4.32	0.4	0	0
Menthatriene	0	0	0	0	5.91	0	1.02	3.41	0	0
Phellandrene	2.15	0	1.66	0	4.54	0	0.77	0	0	0
Fenchene	0	0	0	0	1.87	0	0	6.49	0	0
Menthene	0.48	0.16	1	0	1.69	0	0.68	0	0	0
Cymenene	2.87	0	1	1.36	0	0	3.04	3.76	0	0
Cadinene	0	0	0	0	0.48	0	0	0.19	0	0
Thujone	2.31	0	0	0.33		0	0	2.37	0	0
Other terpene and terpenoids	6.66	0.21	3.52	0.88	13.47	1.07	10.79	12.08	2.23	0.22
	E dunnii		*E grandis*		*E smithii*		*E nitens*		*Wattle*	
	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone
Aromatic derivatives	4.45	7.8	2.65	3.81	3.31	3.09	2.12	3.01	4.81	0.3
Organic Ketones	8.92	9.23	9.32	7.06	1.9	3.25	1.09	2.29	0.71	1.42
Steroids	0.43	0.29	0	13.21	0.98	5.86	2.69	0.83	0	3.57
Fatty aliphatic compounds	0.97	2.92	3.86	14.93	6.34	4.59	4.94	0.79	17.34	6.54
Aliphatic compounds	2.52	1.51	21.71	3.76	1.9	6.91	5.39	3.31	5.27	9.84
Amino derivatives	0.31	0.7	8.13	0.21	3.03	0.61	7.04	12.81	39.29	32.01
Dissolved Sugars	3.2	10.25	4.13	1.46	0	13.62	0	1.84	5.39	19.15

4.2.5. Phenolic Components

Several phenolic components were identified that eluted between 7.5 - 19.5 min for *Eucalyptus*, as shown by Figure 4-4. *E.smithii* has the highest quantity of phenolic components, 37.28%as shown by the sum of the peaks for its acetone extracts in Table 4-2. In general, amongst the *Eucalyptus* bark species analysed, acetone extracts exhibited higher area percentages of phenolic components detected than ethanol extracts. The composition of the chemical components in the phenolic extracts Show that the dominant chemical compounds are phenol, pyrogallol, catechol and mequinol. Pyrogallol is a pyrolysis derivative of gallic acid, *i.e.* a monomer of hydrolyzable tannins. The *Eucalyptus* species with the highest quantity of hydrolysable tannins is *E. dunnii*. The mechanism of pyrogallol formation from gallic acid was first reported by Scheele in 1786; the high interest in this molecule lies in its capacityas an antioxidant capacity that can be exploited in the production of medicinal derivatives such as anti-HIV (Kratz *et al.* 2008), anti-ulcerogenic (Jung *et al.* 2013), anti-inflammatory (Couto *et al.* 2013), antimicrobial (Kubo *et al.* 2003) and antifungal products (Kubo *et al.* 2001)

Polymers with flavan-3-ol (C6-C3-C6 flavonoid unit) skeletal unit are called catechols; they are derivatives of proanthocyanidins, also known as condensed tannins. Figure 4-1shows two benzene rings bridged with a pyrononeto form a flavonoid unit. Condensed tannins are differentiated from one another by the distribution of hydroxyl groups substituted on their B-rings. The most common tannins are procyanidins which have two hydroxyl groups substituted at position (1,4) of the B-ring. Other types include pelargonidin, which has phenol at the B-ring, peonidin, petunidin and llayidin (Figure 4-1). Pyrolysis cleaves the B-ring from the C-ring (benzopyran), resulting in the fragmentation of catechol, guaiacol and phenolfrom the flavonoid unit. A summary of the area percentages of these peaks is shown in Table 4-2.

4.2.6. Terpenes and terpenoids

The second class of significant compounds detected in *Eucalyptus* bark extracts is terpenes and terpenoids. The chemical structure of these compounds is distinguished by the presence of repeating isoprene units in their structure. *E.smithii* has the highest percentage of terpenes (hydrocarbon isoprene units) and terpenoids (isoprene units attached to another functional group), with the main species being α-terpinolene (4.84%), p-menthatriene (5.91%), aromadendrene (6.33%), α-phellandrene (4.54%), terpenoids viridiflorol (2.59%) and camphoric anhydride (2.93%) (Enzell and Wahlberg, 1980). This is followed by *E.nitens*, which has monoterpenes and sesquiterpenes as the dominant terpenes: monoterpenes have one isoprene unit and sesquiterpenes have two repeating isoprene units. The ethanol extracts of *E.nitens* exhibited high area percentages of α-fenchene (6.49%), p-cymenene (3.76%), α-cadinene, terpinolene (2.01%), α-gurjunene (2.19%) and other terpenes and terpernoids (37.48%). The main terpenes and terpenoids extracted with acetone were terpinolene (9.75%), β-pinene (3.15%), gurjunene (3.07%) and p-cymenene (3.04%).

Ethanol extracts contained higher amounts of terpenes and terpenoids than acetone extracts as can be seen in Table 2. The data is supported by studies conducted Scortichini and Rossi, (1991) and Péres *et al.* (2006) who used ethanol to obtain crude extracts of terpenes from different kinds of biomass. In general, extraction of *Eucalyptus* bark with ethanol or acetone results in low yields of terpenes and terpenoids.

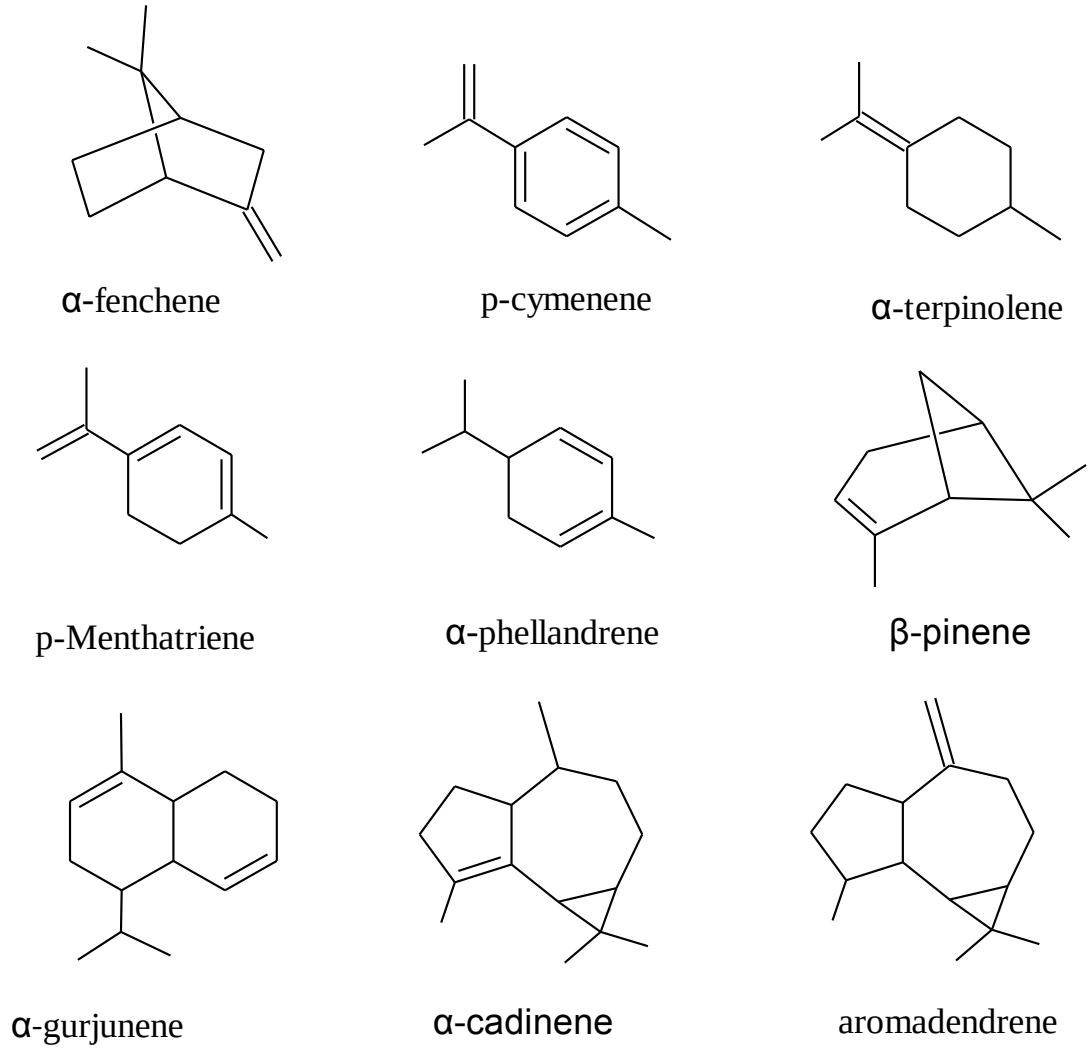


Figure 4-5 Significant terpene compounds detected in Eucalyptus bark extracts

4.2.7. Comparison of chemical components in bark from Black Wattle and *Eucalyptus*

Py-GC/MS shows that the chemistry of bark extracts from *Eucalyptus* bark is different from that of black wattle (Figure 4-3 and Figure 4-4). The amount of phenolic components detected from black wattle bark ethanol extracts was 12.99% and 14.63% for acetone extracts. Similarly to *Eucalyptus* bark, analysis by Py-GC/MS showed the same condensed tannin components, namely, catechol, phenol and guaiacol derivatives. Resorcinol derivatives such as methyl resorcinol and ethyl resorcinol were present in significant quantities in both ethanol and acetone extracts for Black wattle

and not detected in *Eucalyptus* bark. Alkaloids, mainly mome-inositol and 2-furanethanamine, were the highest chemical components detected in black wattle bark extracts with a peak areas of 16.76% and 21.27% respectively for acetone extracts, and 3.85% and 21.51% respectively for the ethanol extracts. Dissolved polysaccharide sugar derivatives were the higher in black wattle than in *Eucalyptus* bark.

4.3. Conclusions

Eucalyptus bark permit the successful extraction of secondary metabolites using accelerated solvent extraction. Acetone recovered more total phenolic components and total extractive material in *Eucalyptus* bark with ethanol having more selectivity than acetone. *E.dunnii* and *E.smithii* are the *Eucalyptus* species with the highest quantity of polyphenolic content recovered. Analysis of chemical distribution of ethanol and acetone extracts using Py-GC/MS showed a high presence of phenolic components, terpenes, and terpenoids. Condensed tannins are the dominant secondary metabolites of phenolic in nature detected. *Eucalyptus* species and black wattle showed were highly distinctive as black wattle had a high content of sugar derivatives and alkanoids. *E.grandis* and *E.nitens* had the highest amount of structural carbohydrates in the extractive-free bark.

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CHAPTER 5

5. Summary, conclusions and recommendations

5.1. Summary and discussion of papers

The following section aims to highlight the importance of the results obtained from the two papers discussed in the previous chapters. The significance is discussed by showing the relevance of the results within the concept of biorefinery. Beneficiation and valorisation of secondary metabolites from *Eucalyptus* bark depends on the degree of efficiency of the solvent recovery processes. The process depends on several experimental processes such as temperature, solvent type, pressure, mass-to-liquid ratio, particle size and the type of extraction. One of the significant factors used for extraction of target molecules is the type of solvent used in the recovery process. Utilisation of extracted secondary metabolites using organic solvents is limited by the selectivity and affinity of secondary metabolites to the solvent used to recover the target molecules. Hydrophilic extractives dissolve in polar solvents whereas lipophilic metabolites are obtained from non-polar solvents. The following section aim to provide information on whether *Eucalyptus* bark contains valuable components that can provide financial gains through solvent extraction.

5.1.1. Paper I-Summative Discussion

The target chemical materials are tannins which are phenolic components found in the bark of hardwoods, especially wattle. Paper I highlight the optimisation of the extraction of phenolic components using accelerated solvent extraction. The process is a sequential method whereby pressurised organic solvent penetrate plant cell walls to recover target molecules at set operating conditions. The study showed that *Eucalyptus* bark can be successfully used for the extraction of phenolic components using an ASE when dried to a moisture content of approximately 10%. The optimisation process was conducted using response surface methodology, where a Box-Beheknen design was used as the experimental guideline.

Experimental runs were performed on each of the four individual *Eucalyptus* bark species using ethanol as a solvent. The data was normalised to generate an integrated model with 65 runs, whereby process variables, temperature, particle size and static extraction cycles were used to generate a polymeric that described the extraction process. Graphical optimisation tools using Design Expert™ 10.0.1 were used to plot 3-D dimensional surface plots from regression analysis of the normalised data. The target responses were total phenolic components, measured using UV-vis spectrophotometry and Folin–Ciocalteu method. The model showed that operating conditions for ASE of tannins were obtained at 117℃, static extraction cycles greater than 3 and bark particle size class of 500-850μm. Temperature effects were significant in the extraction process. The total extractive content of all soluble extracts averaged 15.325% for *Eucalyptus* species and total phenolic content averaged 3.52g/100g GAE among the *Eucalyptus* bark species used. The total amount of extractives includes the total weight of all ethanol soluble extractives (such as terpenes, tannin, fatty acids). Twenty-three percent (22.97%) of the extracts recovered from *Eucalyptus* barks are phenolic components.

The above results are averages, individual values for each of the *Eucalyptus* bark species is discussed extensively in section 3.3.1. The bark species with the highest amount of tannin components is *E.smithii*, while *E.grandis* had the lowest amounts. Thus, *E.smithii* bark is the favourable species; it has the potential to recover tannin molecules at maximum yields.

Above results show that tannins from *Eucalyptus* bark can be recovered using pressurised fluid extraction. The market value of tannin is estimated to be around $ 3.39 billion by 2025, therefore using the optimised extraction conditions, adequate separation and purification method (such a liquid-liquid extraction and evaporation), tannin can be a valuable component with economical potential to be exploited.

5.2. Paper II-Summative discussion

In the first paper, ethanol soluble metabolites were recovered during the optimisation pressurised fluid extraction of phenolic components. The second paper conducted a comparison between ethanol soluble extracts with those of acetone. Acetone has higher dipole moment than ethanol; this means that acetone is more polar than ethanol. It was found that acetone is more efficient in recovering tannins, this is supported by the fact that an average of 24.12% of total extracts were obtained from the *Eucalyptus* bark species with an average of 4.10% for total phenolic components. However, acetone extract more tannins alongside other acetone soluble secondary metabolites, leading to more dissolved contamination that would increase production costs. Ethanol is more selective than acetone in recovering phenolic components.

The characterisation of hydrophilic extractive components which is the main objective of the second paper was obtained using the operating conditions optimised in paper I. The characterisation was obtained using Py-GC/MS on vacuum dried extracts. Analytical pyrolysis produces pyrograms that fingerprint the chemical components that were fragmented from the dried extracts at 550℃ (examples of these pyrograms are shown in Figure 4-3). The pyrograms were compared to those found in Wiley and NIST libraries. Peak areas are used as concentration indicators of the relative abundance of the detected components. The results showed that phenolic components were predominant as they are pyrolysis fragments of tannin molecules and their derivatives. The phenolic components were catechol, Guaiacol and phenols. The pyrolysis results confirmed the UV-vis results in section 4.2.2 that acetone extract more phenolic components than ethanol. As acetone extracts had more concentration of phenolic components than ethanol, the selectivity of the extraction decreases from acetone to ethanol. Paper II also compared the chemistry of tannins obtained from black wattle with those of *Eucalyptus* bark. Condensed tannins of resorcinol in nature were observed in the bark of black wattle which are chemically different from that of *Eucalyptus* barks.

However, several studies showed that phenolic components founds in *Eucalyptus* bark such as catechol and guaiacol are the main monomers in tannin used for commercial purposes.

Other secondary metabolites were detected in the bark extracts, making separation of tannin molecules from the total solvent extractives important for beneficiation of *Eucalyptus* bark. Other extractives that lowers the purity of tannins include hydrophilic and lipophilic extractives that are soluble in ethanol and acetone include terpenes, steroids, alkaloids, monomeric sugars, fatty acids and simple aliphatic compounds. Terpene molecules were the most abundant metabolites detected, and separation of this components from tannins is significant for valorisation purposes

5.3. Conclusions

From this thesis it can be concluded that:

- Optimisation of the extraction of phenolic components (tannins) using accelerated solvent extraction for *Eucalyptus* barks of *E.grandis*, *E.dunnii*, *E.nitens* and *E.smithii* was successfully performed. A quadratic polymeric equation can describe the behaviour of the extraction process.

- Temperature is the significant factor for extraction of phenolic components and the optimum temperature of 117℃ was obtained. The number of static extraction cycles and particle size class has minimal effects on the total phenolic content.

- Condensed tannins can be extracted using 50% acetone v/v and 80% ethanol v/v, acetone results with higher total phenolic content with ethanol having the highest selectivity for phenolic components.

- *E.smithii* and *E.dunnii* have more phenolic components compared to *E.nitens* and *E.grandis*. Phenolic components in *Eucalyptus* bark are lower than those of commercial source of Black tannin.

- Condensed tannins of catechol, guaiacol and phenol can be detected at a temperature of 550℃ using Py-GC/MS analysis for *Eucalyptus* bark. This shows that *Eucalyptus* bark has potential for tannin production.

- Terpenes and terpenoids are some of the other secondary metabolites abundant in *Eucalyptus* barks when the extraction is performed with 80% ethanol v/v. Terpenes and terpenoids decrease the purity of tannins extracted and subsequent purification steps are required prior to utilisation of tannin.

- When structural carbohydrates were measured from the *Eucalyptus* bark sample, *E.nitens* and *E.grandis* showed more glucose content compared to *E.smithii* and *E.dunnii*. This data showed that *Eucalyptus* bark can provide polysaccharide for other technologies, such as furfural and xylitol production.

5.4. Outlook and future work

The two papers discussed in this study only investigated minimum avenues needed for efficient utilisation of *Eucalyptus* bark within the biorefinery concept. Other factors are needed to establish the proposed concept of extracting secondary metabolites from the bark. Purification of tannins is one of avenues that needs further investigation, the optimised process reported in this study mostly deals with the recovery of maximum yield. Identification of processes that enable the separation of tannins from the total extracted material is crucial for its utilisation.

Drawing from Paper I, several experimental factors that were not investigated in this study can be exploited to optimise the recovery of total phenolic content. The experimental factors that can be exploited during ASE include, total extraction time,

solid-to-liquid ratio, solvent type, concentration of the solvent and other process variables. Other solvent extraction technologies that may be considered for recovering tannins include ultrasonic-assisted extraction, microwave-assisted extraction and supercritical fluid extraction.

Extraction experiments were performed on individual *Eucalyptus* species. A mixture of different *Eucalyptus* species and other commercially planted species can be used to determine the extent which tannin components can be extracted. Natural *Eucalyptus* are threatened by pathological deterioration and climate change, the solution to these negative effects is to plant hybrids derived from merging species that are tolerant to harsh conditions with natural *Eucalyptus* species. Performing extractions on emerging hybrids is important for the future in the event naturally occurring species become uneconomical to plant.

Paper II, showed an indication of the chemical distribution of components in the bark extractives. It is recommended that tannin standards be used during the Py-GC/MS analysis to narrow the concentration of tannins in *Eucalyptus* species. Other chemical components were identified from the pyrolysis analysis, namely: terpenes, steroids, alkaloids and fatty acids should be exploited to match economical technologies that can utilise them as feedstocks. Economical evaluation of the scale of the process could be exploited to assess the possibility of the beneficiation of tannins derived from *Eucalyptus* bark using pressurised fluid extractions. Lastly, extractive-free bark which is the solid material left after the extraction process could also be evaluated to assess the ability of the biomass to become feedstock in beneficiation technologies such as sugar extraction, fermentation and paper making.