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PRESSURIZED HOT WATER EXTRACTION (PHWE) AND CHEMOMETRIC FINGERPRINTING OF PHYTOCHEMICALS FROM

*Bidens pilosa*

By

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A Dissertation submitted to the Faculty of Science,
University of Johannesburg,
South Africa

In partial fulfilment of the requirements for the award of a Master’s Degree in Technology: Food Technology

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Co-supervisor: Dr. N. E. Madala
Co-supervisor: Prof. P.A. Steenkamp

August, 2016
EXECUTIVE SUMMARY

Extraction plays a vital role in the study of phytochemicals as the quality of analytical results have often been directly linked to the efficacy of the extraction process. Conventional methods of phytochemical extraction utilize large quantities of organic solvents which stirs issues of safety and environmental health. Moreover, these methods are often labourious, time consuming and expensive. It was therefore imperative to look for an alternative method of phytochemical extraction that is efficient, cost-effective and more eco-friendly. Pressurized hot water extraction (PHWE) which is often referred to as subcritical water extraction (SWE) has promising prospects, and when properly harnessed (optimized) often deliver results that compare favourably to conventional methods. This current research work considered the application of PHWE together with front-line chromatographic and mass spectrometric analytical techniques, i.e. ultra-performance liquid chromatography coupled with a quadrupole time-of-flight mass spectrometry (UPLC-qToF-MS/MS) and multivariate chemometric analysis for investigating the phytochemical (flavonoids and di-acylated cinnamic acids) profile of *Bidens pilosa*, an indigenous and very potent medicinal plant.

The first phase of the study considered the extraction of flavonoid compounds from *B. pilosa* using PHWE at temperatures of 50, 100 and 150°C and subsequent analysis on UPLC-qToF-MS/MS. The findings revealed the possibility of extracting and characterizing 28 different molecules belonging to different classes of flavonoids, i.e. chalcones, flavonols and flavanones. These molecules have been reported to perform various important biological activities such as being anti-oxidative, hepatoprotective, anti-cancer and antibacterial properties. Further analysis of the data using chemometric principal component analysis (PCA) showed that the different temperatures studied yielded significantly different recovery patterns for the identified molecules. Generally, higher temperatures resulted in better extraction yields, as such, extracts obtained at 150°C resulted in the highest yields of the identified flavonoids.

In a subsequent experiment, PHWE was optimized using a cosolvent (methanol) for the extraction of di-acylated cinnamic acids, specifically, dicafeoylquinic acid (diCQA) and chicoric acid (CA) and their analogues, because it is common observation that these metabolites (particularly CA) are highly unstable during extraction and degrade at elevated temperatures. Further to this is the fact
that these metabolites are known to confer anti-HIV properties. Using different methanol compositions (i.e. 20, 40 and 60%) and a temperature profile of 50, 100, and 150°C, extracts were obtained from *B. pilosa* and analyzed using UPLC-qToF-MS/MS. It was also possible to identify different isomers (possibly cis-geometrical isomers) of these molecules. Essentially, significant yields were obtained even at low temperatures (50°C) using co-solvency, thus eliminating concerns of thermal degradation during PHWE at elevated temperatures. Statistical modeling of the results using response surface methodology (R² values ranging from 0.57 to 0.87) showed that extraction yields increased in proportion to methanol composition as well as temperature. However, methanol composition had a stronger effect on extraction yield than temperature.

In conclusion, our findings reveal that PHWE in combination with UPLC-qToF-MS/MS and chemometric analysis is suitable for the extraction and subsequent analysis of diverse flavonoids, hydrocinnamic acids and possibly other polyphenolic compounds from *B. pilosa*. Moreover, this technique can be optimized to ameliorate the effects of thermal degradation of thermolabile compounds at elevated temperatures. Prospects of this technique as an efficacious, cost effective, user-friendly, and greener alternative to traditional techniques of extraction of bioactive compounds from plant tissues is therefore highly promising.

**Key words:** Pressurized hot water, *Bidens pilosa*, flavonoids, di-acylated cinnamic acids, principal component analysis and response surface methodology.
DECLARATION

I, Sefater Gbashi, hereby declare that the composition of this dissertation and the work herein described was carried out entirely by myself unless otherwise cited or acknowledged. It has not been submitted for degree purposes at any other University or institution. All other sources used have been duly cited in text and acknowledged by complete references.

______________________
SEFATER GBASHI
DEDICATION

I dedicate this piece of work to God Almighty, the very essence of my existence and the sovereign ruler over the affairs of humanity. Also, to my family for their love and support.
ACKNOWLEDGEMENT

Foremost, I return all the praises to God almighty for His mercies and grace upon my life during the period of my study at the esteemed University of Johannesburg. These past two years of my life have been the best I can ever remember, all because of Divine providence.

Very few things in life cannot be expressed by words, one of which is my sincere and heartfelt gratitude to my supervisor Dr. P.B. Njobeh and my co-supervisors Dr. N.E. Madala and Prof. P.A. Steenkamp for their support and provision of a conducive atmosphere to do my research and write this dissertation. Truly, I enjoyed the best of guidance, motivation and knowledge, for which I could wish for nothing better.

Besides my supervisors, I wish to thank Mr. E. van Zyl (my HOD), Prof. I.A. Dubery, Dr. L.A. Piater, Dr. V. Mavumengwana, Dr. E. Kayitesi, Dr. J.Z. Phoku, Associate Prof. O.C. Nwinyi and Dr. O. Olurundare for their encouragement, support, insightful comments and mentorship. And to everyone who contributed in one way or the other towards the success of my research, I sincerely thank you.

I would like to render my profound appreciation to my lovely and wonderful friends and colleagues who always cheered me up and made my stay worthwhile: Mr. O.A. Adebo, Mrs. I.O. Adekoya, Miss J.A. Adebiyi, Miss A. Olugbile, Mr. E. Lulamba, Miss L. Motaung, Mrs. M.C. Temba, Mrs. M.O. Oladeji, Miss M. Olorunfemi, Miss K. Ndleve, Miss R. Changwa, Mrs. M. Bello, Mr. and Mrs. U. Okore-afor, Miss L.B. Mqhaba, Mr. Q. Mangena, Miss S. Moyo, Miss P. Mrwebi and Miss S. Mapala. And my colleagues at Biochemistry Department, Mr. B.S. Khoza, Mr. E. Ncube, Mrs. R.M. Ramabulana, Mr. M. Mhlongo, Miss K. Masike, Miss M. Makola and Miss P.S. Mudau, thank you for your assistance, companionship and guidance. God bless you richly.

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I wish to thank Prof E.H. Agba and Prof A.I. Ikeme for their immense support in making my study in South Africa a success. My heartfelt and unreserved gratitude goes to Prof M.A. Igyor who availed me the opportunity to study abroad; he so much believed in me much more than I believed in myself. Your support and words of encouragement will linger beyond time. I would also like to thank my parents Rev. (Pharm) and Pst. Mrs. D. Gbashi, my elder sister Mrs. M.N. Wombo, my elder brother Engr. S. Gbashi, my younger brother Mr. N.D. (Jr) Gbashi and my younger sister Miss Q.T. Gbashi. They were always encouraging and supporting me with their best wishes and prayers.

Last but not the least, I wish to appreciate the University of Johannesburg, through the Global Excellence and Stature (GES) Fellowship for providing the financial support for this study. The study was also supported via the Centre of Excellence in Food Security co-hosted by the University of Pretoria and University of the Western Cape, South Africa.
ARTICLES PUBLISHED OR WRITTEN FOR PUBLICATION


# TABLE OF CONTENTS

**EXECUTIVE SUMMARY** ........................................................................................................................................ i  
**DECLARATION**................................................................................................................................................... iii  
**DEDICATION**...................................................................................................................................................... iv  
**ACKNOWLEDGEMENT**.......................................................................................................................................... v  
**ARTICLES PUBLISHED OR WRITTEN FOR PUBLICATION** ........................................................................ vii  
**TABLE OF CONTENTS** ........................................................................................................................................ viii  
**LIST OF FIGURES** ............................................................................................................................................... xiii  
**LIST OF TABLES** ............................................................................................................................................... xvi  
**LIST OF ABBREVIATIONS** ................................................................................................................................. xvii  
**LIST OF SYMBOLS AND UNITS** ........................................................................................................................... xix  
**DISSERTATION OUTLINE** ................................................................................................................................... xxi  

**CHAPTER ONE** .................................................................................................................................................. 1  
1.0 **GENERAL INTRODUCTION** .......................................................................................................................... 1  
1.1 **BACKGROUND** ........................................................................................................................................... 1  
1.2 **PROBLEM STATEMENT** ............................................................................................................................. 1  
1.3 **HYPOTHESIS** .............................................................................................................................................. 2  
1.4 **AIM** ............................................................................................................................................................ 3  
1.5 **OBJECTIVES** .............................................................................................................................................. 3  

**CHAPTER TWO** ................................................................................................................................................ 4  
2.0 **LITERATURE REVIEW** ........................................................................................................................................ 4  
2.1 **BRIEF** ........................................................................................................................................................ 4
2.2 PHYTOCHEMICALS: OVERVIEW AND BIOLOGICAL IMPORTANCE........ 4

2.2.1 Phytochemicals and their significance ...................................................... 4

2.2.2 Classification and biological activities of important phytochemicals ............ 5

2.2.2.1 Flavonoids ............................................................................................... 7

2.2.2.2 Phenolic acids .......................................................................................... 8

2.3 Bidens pilosa ...................................................................................................... 11

2.3.1 Food uses and nutritional properties of B. pilosa ........................................ 12

2.3.2 Pharmacological importance and phytochemistry of B. pilosa ...................... 12

2.4 EXTRACTION OF PHYTOCHEMICALS ............................................................ 13

2.4.1 Conventional extraction methods for plant materials .................................. 14

2.4.1.1 Solvent extraction ..................................................................................... 14

2.4.1.2 Soxhlet extraction .................................................................................... 15

2.4.1.3 Microwave assisted extraction ................................................................. 15

2.4.1.4 Supercritical fluid extraction ..................................................................... 16

2.4.2 Other extraction methods ............................................................................. 17

2.5 PRESSURIZED HOT WATER EXTRACTION: CONCEPT AND APPLICATIONS ........................................................................................................... 18

2.5.1 Pressurized hot water .................................................................................. 18

2.5.2 Pressurized hot water extraction .................................................................. 19

2.5.3 Principle of pressurized hot water extraction ............................................... 20
2.5.4 Mechanism and instrumentation of pressurized hot water extraction ................................ 21

2.5.6 Factors that affect pressurized hot water extraction ......................................................... 23

2.5.5.1 Temperature ............................................................................................................... 23

2.5.5.2 Pressure .................................................................................................................... 23

2.5.5.3 Cosolvents and surfactants ......................................................................................... 24

2.5.7 Advantages and disadvantages of pressurized hot water extraction ................................ 24

2.5.7.1 Advantages ............................................................................................................... 24

2.5.7.2 Disadvantages of pressurized hot water extraction ..................................................... 25

2.5.8 Applications of pressurized hot water extraction ............................................................. 25

2.6 CHROMATOGRAPHIC SEPARATION, IDENTIFICATION AND CHEMOMETRIC ANALYSIS OF PHYTOCHEMICALS .................................................. 27

2.6.1 Chromatographic separation of phytochemicals ............................................................. 27

2.6.2 Identification and profiling of phytochemicals ............................................................... 28

2.6.3 Chemometric analysis of phytochemicals ...................................................................... 29

2.6.3.1 Principal component analysis .................................................................................... 30

2.6.3.2 Response surface methodology ............................................................................... 30

2.7 CONCLUSION .................................................................................................................. 31

REFERENCES ...................................................................................................................... 31

CHAPTER THREE ................................................................................................................. 55
PRESSURIZED HOT WATER EXTRACTION AND CHEMOMETRIC FINGERPRINTING OF FLAVONOIDS FROM *Bidens pilosa* BY UPLC-TANDEM MASS SPECTROMETRY 55
Abstract........................................................................................................................................55
Introduction ......................................................................................................................................56
Materials and methods..................................................................................................................58
Materials ..........................................................................................................................................58
Methods ...........................................................................................................................................58
Metabolite extraction.....................................................................................................................58
Chromatographic separation and mass spectrometry (UPLC-qToF-MS).................................58
Data analyses and identification of flavonoid compounds..........................................................59
Statistical analysis..........................................................................................................................60
Results and discussion..................................................................................................................60
Acknowledgement.........................................................................................................................73
References ........................................................................................................................................73
APPENDIX 3.0..................................................................................................................................82

CHAPTER FOUR.............................................................................................................................85
THE EFFECT OF TEMPERATURE AND METHANOL-WATER MIXTURE ON PRESSURIZED HOT WATER EXTRACTION (PHWE) OF ANTI-HIV ANALOGUES FROM Bidens pilosa........................................................................................................................85
Abstract........................................................................................................................................85
Background.....................................................................................................................................86
Experimental section......................................................................................................................87
Plant Materials and Metabolite Extraction.....................................................................................87
LIST OF FIGURES

Figure 2.1: Phytochemical classification ................................................................. 6

Figure 2.2: Image of B. pilosa plant (A), its flowers (B) and seeds (C) ...................... 11

Figure 2.3: Phase diagram of water as a function of temperature and pressure ......... 19

Figure 2.4: Dielectric constant of water as a function of temperature .................... 20

Figure 2.5: Simple laboratory setup of a PHWE unit ................................................... 22

Figure 2.6: Application of PHWE in different scientific fields from 1990-2014 (using ISI Web of Science database on 05-02-2015) ................................................................. 26

Figure 2.7: Summary of metabolite identification process ........................................ 29

Figure 3.1.1: Box-and-whiskers plots showing the yield distribution patterns for some of the identified flavonoids ................................................................. 67

Figure 3.1.2: Box-and-whiskers plots showing the yield distribution patterns for some of the identified flavonoids ................................................................. 68

Figure 3.2.1: PCA score plots based on UPLC tandem MS chromatograms from negative ionization spectra data showing various clustering patterns of B. pilosa extracted at different temperatures using PHWE ................................................................. 70

Figure 3.2.2: PCA loadings plots based on UPLC tandem MS chromatograms from negative ionization spectra data showing the dimensional subspatial orientation of identified flavonoid molecules relative to the different extraction temperatures during PHWE of B. pilosa ................................. 70

Figure 3.3: HCA dendrogram showing the degree of similarity/dissimilarity amongst extracts obtained at different extraction temperatures using the data's full dimensionality as obtained from the UPLC-qTOF-MS/MS chromatograms ................................................................. 71

Appendix 3.1: Representative single ion monitoring (SIM) chromatograms showing the elution of the different molecules that could not be defined after processing of the UPLC.
tandem MS data using MarkerLynx XS software: Molecule 17 (A), Molecule 21 (B), Molecule 24 (C), Molecule 25 (D) and Molecule 28 (E).

Appendix 3.2: OPLS-DA plots based on UPLC-qTOF-MS/MS chromatograms from negative ionization data showing the distribution of identified metabolites obtained at 50°C and 100°C.

Appendix 3.3: OPLS-DA plots based on UPLC-qTOF-MS/MS chromatograms from negative ionization data showing the distribution of identified metabolites obtained at 50°C and 150°C.

Appendix 3.4: OPLS-DA plots based on UPLC-qTOF-MS/MS chromatograms from negative ionization data showing the distribution of identified metabolites obtained at 100°C and 150°C.

Figure 4.1: Molecular structures of 3,4 diCQA (A), 3,5 diCQA (B), 4,5 diCQA (C), CA (D) and CA Isomer (E).

Figure 4.2: Box-and-whiskers plots showing the effect of temperature on the extractability of isomers of diCQA and CA using water-only PHWE: 3,4-diCQA (A), 3,5-diCQA (B), Cis-3,5-diCQA (C), 4,5-diCQA (D), CA (E) and CA isomer (F).

Figure 4.3.1: Representative UPLC tandem MS single ion monitoring (SIM) chromatograms for isomers of diCQA following PHWE of B. pilosa at 50°C using 60% MeOH (A), 40% MeOH (B), 20% MeOH (C) and 0% MeOH (water) (D).

Figure 4.3.2: Representative UPLC tandem MS single ion monitoring (SIM) chromatograms for chicoric acid and chicoric acid isomer following PHWE of B. pilosa at 50°C using: 60% MeOH (A), 40% MeOH (B), 20% MeOH (C) and 0% MeOH (water) (D).

Figure 4.4: Surface plots showing the effect of temperature and solvent composition on the extraction of diCQA and CA analogues: 3,4-diCQA (A), 3,5-diCQA (B), Cis-3,5-diCQA (C), 4,5-diCQA (D), CA (E) and CA isomer (F).
Figure 4.5: Pareto chart of standardized effects of temperature and solvent composition on the extraction of diCQA and CA analogues: 3,4-diCQA (A), 3,5-diCQA (B), Cis-3,5-diCQA (C), 4,5-diCQA (D), CA (E) and CA isomer (F).
LIST OF TABLES

Table 2.1: Basic structural configuration of flavonoids and the flavonols positional structural orientation ................................................................. 7

Table 2.2: Structural configuration of the major naturally occurring phenolic acids .......... 8

Table 2.3: Summary of some major phytochemicals and their biological activities .......... 10

Table 2.4: Nutritional profile of B. pilosa ................................................................. 12

Table 2.5: Organic solvents used for bioactive component extraction .......................... 14

Table 2.6: Important electro- and physicochemical properties of water that changes with increase in temperature and pressure ......................................................... 21

Table 3.1: Identified flavonoid metabolites extracted from B. pilosa using PHWE .......... 62

Table 4.1: Identified metabolites extracted from B. pilosa by PHWE ........................ 91

Table 4.2: Yield (mean relative peak intensity) of identified anti-HIV analogues (di-acylated cinnamic acids) extracted from B. pilosa using modified PHWE .......................... 96
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BPI</td>
<td>Base peak ion</td>
</tr>
<tr>
<td>CA</td>
<td>Chicoric acid</td>
</tr>
<tr>
<td>CCD</td>
<td>Central composite design</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>diCQA</td>
<td>Dicaffeoylquinic acid</td>
</tr>
<tr>
<td>DNP</td>
<td>Dictionary of natural products</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionization detectors</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>HCA</td>
<td>Hierarchical clustering analysis</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HLWE</td>
<td>Hot liquid water extraction</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>HWE</td>
<td>Hot water extraction</td>
</tr>
<tr>
<td>ID</td>
<td>Inner diameter</td>
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<tr>
<td>IT</td>
<td>Ion trap</td>
</tr>
<tr>
<td>KMI</td>
<td>KNApSAcK metabolite information</td>
</tr>
<tr>
<td>MAE</td>
<td>Microwave-assisted extraction</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MLS</td>
<td>Method of least square</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Two mass spectrometers in tandem</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MSCA</td>
<td>Multi-level simultaneous component analysis</td>
</tr>
<tr>
<td>MS\textsuperscript{n}</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>OD</td>
<td>Outer diameter</td>
</tr>
<tr>
<td>Pa</td>
<td>Pascal</td>
</tr>
<tr>
<td>PAHs</td>
<td>Polycyclic aromatic hydrocarbons</td>
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<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PCs</td>
<td>Principal components</td>
</tr>
<tr>
<td>PDA</td>
<td>Photodiode array</td>
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<tr>
<td>PHW</td>
<td>Pressurized hot water</td>
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<tr>
<td>PHWE</td>
<td>Pressurized hot water extraction</td>
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<tr>
<td>PLPWE</td>
<td>Pressurized low polarity water extraction</td>
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<tr>
<td>PLS-DA</td>
<td>Partial least square discriminate analysis</td>
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<tr>
<td>qToF</td>
<td>Quadrupole time-of-flight</td>
</tr>
<tr>
<td>R\textsuperscript{2}</td>
<td>Coefficient of determination</td>
</tr>
<tr>
<td>RSM</td>
<td>Response surface methodology</td>
</tr>
<tr>
<td>Rt.</td>
<td>Retention time</td>
</tr>
<tr>
<td>SFE</td>
<td>Supercritical fluid extraction</td>
</tr>
<tr>
<td>SIM</td>
<td>Single ion monitoring</td>
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<tr>
<td>SIMCA</td>
<td>Soft independent modelling of class analogy</td>
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<tr>
<td>SWE</td>
<td>Subcritical water extraction</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
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<tr>
<td>UPLC</td>
<td>Ultra-performance liquid chromatography</td>
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<td>UV</td>
<td>Ultraviolet</td>
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</table>
LIST OF SYMBOLS AND UNITS

%  Percentage
±  Plus or minus
≤  Less than or equal to
°C  Degree Celsius
µg  Microgram
µm  Micrometer
amu  Atomic mass unit
Bar  Bar
Da  Dalton
eV  Electron volts
ε  Dielectric constant
g  Gram(s)
h  Hour(s)
kV  Kilovolts
L/h  Liter per hour
m/z  Mass to charge ratio
mDa  Micro Dalton
Min  Minute(s)
mL  Milliliter
mL/min  Milliliter per minute
mm  Millimeter
ppm  Parts per million
Psi  Pounds per square inch
Sec  Second(s)
V  Volts

α  Probability significance

μg/mL  Microgram per milliliter
DISSERTATION OUTLINE

This dissertation covers studies on PHWE and chemometric fingerprinting of different phytochemicals from *B. pilosa* as analyzed on UPLC tandem mass spectrometry. A brief outline of the chapters presented in this dissertation is provided below.

**Chapter One: General introduction**

This chapter gives a general overview of the research subject, providing relevant background information as well as describing problem under investigation. The chapter also highlights the assumptions (hypothesis), aim and objectives of the study.

**Chapter Two: Literature review**

This chapter presents an exhaustive appraisal of the research focus. It gives a description of phytochemicals and their significance, and also highlights the relevance of *B. pilosa*. The chapter further reviews various techniques for phytochemical extraction with emphasis on PHWE. Different approaches for chemometric analysis, particularly, principal component analysis were also appraised. Some aspects of this chapter titled ‘Subcritical water extraction in biological materials’ has been published in the journal - *Separation & Purification Reviews*.

**Chapter Three: Pressurized hot water extraction and chemometric fingerprinting of flavonoids from Bidens pilosa by UPLC-tandem mass spectrometry**

Chapter Three describes the extraction, characterization, and chemometric fingerprinting of flavonoids from *B. pilosa* using PHWE in combination with UPLC-qToF-MS/MS and PCA. The work described in this chapter has been re-submitted to the *CYTA – Journal of Food*, and accordingly presented herein following the specific guidelines of the journal and with due considerations of the comments and Suggestions by the reviewers.

**Chapter Four: The effect of temperature and methanol-water mixture on pressurized hot water extraction (PHWE) of anti-HIV analogues from Bidens pilosa**

Chapter Four describes the use of a cosolvent (methanol) at different temperatures for optimizing PHWE of different isomers of diCQA and CA from *B. pilosa* which are known to confer potent anti-HIV activities. This chapter is presented in the format of *Chemistry Central Journal*, and has
been re-submitted to the same journal for publication. Comments and suggestions provided by the reviewers on that manuscript have been considered in this chapter.

**Chapter Five: General discussion and conclusion**

This chapter reaffirms the research focus (i.e. problem statement and aim) of this dissertation, presents an overall discussion of the issues addressed in Chapters Three and Four and reaches a final judgment. Recommendations and prospects for future studies are also provided.
CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 BACKGROUND

Plants do not only provide vital nutrients needed for life, but also synthesize a wide variety of chemical compounds with some of them referred to as phytochemicals, known to perform important biological and therapeutic functions in humans (Atanasov et al., 2015; Essiett and Udo, 2015). As such, there has been an increased use of plants for addressing various health related issues, especially as herbal medicine for primary health care (Shen et al., 2012). This has fostered a resilient curiosity in understanding the chemical characteristics and phytochemical composition of these herbal plants. *Bidens pilosa*, an underutilized plant species is known to contain an enormous variety of interesting phytochemicals (Arthur et al., 2012; Bartolome et al., 2013). Generally regarded as a troublesome weed crop, it is widely distributed worldwide (Bartolome et al., 2013; El-Gawad et al., 2015). Despite that, it is consumed as a leafy vegetable and utilized for medicinal purposes, particularly in the tropical regions of the world (Bairwa et al., 2010; Bartolome et al., 2013). Understanding the phytochemical composition and diversity of this underutilized plant is therefore, a key to exploiting potentials embedded in it.

Extraction is a fundamental (and inevitable) step in the study of biomolecules from plants (Sasidharan et al., 2011; Islam, 2012). Conventional extraction techniques utilize large quantities of organic solvents, which stirs issues of human and environmental safety (Santana et al., 2009; Ismail and Hameed, 2013). Besides, these techniques are often tedious, time-consuming and expensive to employ (Choi et al., 2003; Teo et al., 2010). These mishaps have thus, propelled the quest for a greener route and more suitable technique for this vital step in the study of plant biomolecules. This has brought pressurized hot water extraction (PHWE) on the spotlight (Gbashi et al., 2016).

1.2 PROBLEM STATEMENT

Extraction is one of the most frequently performed and vital experimental procedure in the biochemical and bioanalytical sciences. More than a few techniques have been adopted for extracting phytochemicals from plant tissues, but issues such as environmental hazards and lengthy
procedures have significantly incited an apparent need for new extraction techniques that offer better results and advantages (Gbashi et al., 2016). Moreover, cost implications of these techniques are also often very high (Santana et al., 2009; Teo et al., 2010). Also worthy of note is the wide range of analytes with varying physicochemical characteristics present in plants that need to be extracted simultaneously. Pressurized hot water extraction is a promising and viable alternative to conventional extraction techniques (Aguilera-Luiz et al., 2011; Khoza et al., 2015; Matschediso et al., 2015; Plaza and Turner, 2015; Gbashi et al., 2016; Khoza et al., 2016). This is because, it is more environmentally friendly, cheap, fast, relatively easy to adopt, efficient and delivers results that compare favourably to conventional extraction methods (Bart, 2005; Gbashi et al., 2016).

A major advantage of PHWE is its utilization of water as the extraction solvent (Gbashi et al., 2016). Interestingly, extraction can be easily controlled to achieve selective a wide spectrum of extractable compounds by simply altering the temperature conditions (Liang and Fan, 2013). In this regard, sensitive and robust spectra procedures are ideal for subsequent analyses of the extracts. The combination of PHWE with advanced spectra analysis generates quality and high dimensional data sets that require specialized approaches (such as chemometrics) for their cogent interpretation (Khoza et al., 2015). This study set out to investigate the potential of combining PHWE with advanced chromatographic and spectrometric techniques in extracting and analyzing (using chemometrics) various phytochemicals from B. pilosa.

1.3 HYPOTHESIS

It was hypothesized in this research work that:

(i) Pressurized hot water extraction in combination with UPLC-qToF-MS/MS and chemometric models can be suitable for extraction and subsequent analysis of various phytochemicals in B. pilosa.

(ii) It is possible to minimize low recovery effects and thermal degradation of highly unstable and thermolabile phytochemical compounds through optimization using a cosolvent.
1.4 AIM

The aim of this research work was to utilize PHWE in combination with advanced chromatographic and spectrometric techniques for the extraction and chemometric analysis of phytochemicals from *B. pilosa*.

1.5 OBJECTIVES

To achieve the aim stated in Section 1.2, the following objectives were met:

- To extract and characterize different flavonoids from *B. pilosa* using PHWE in combination with UPLC-qToF-MS/MS.
- To optimize a PHWE method for the extraction of anti-HIV analogues (i.e. isomers of dicaffeyoylquinic acid and chicoric acid) from *B. pilosa*.
- To carry out a chemometric analysis of the extracted phytochemicals (flavonoids and isomers of dicaffeyoylquinic acid and chicoric acid) from *B. pilosa*. 
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 BRIEF

Phytochemicals are very important health agents present in plants, having been considered for their numerous beneficial properties. As such, an appraisal of phytochemicals and their significance is presented in this chapter. On the other hand, proper sample extraction constitutes an integral part and key component of phytochemical analysis and can largely affect analytical results. Hence, various techniques for phytochemical extraction, including their advantages and disadvantages are reviewed herein. In this chapter, specific focus on *B. pilosa* is also provided as it was the plant of interest in this study. Furthermore, different approaches for chemometric analyses, particularly, principal component analysis are also appraised herein.

2.2 PHYTOCHEMICALS: OVERVIEW AND BIOLOGICAL IMPORTANCE

2.2.1 Phytochemicals and their significance

Phytochemicals (or phytoconstituents or plant metabolites) are secondary bioactive chemical compounds found in plants (Enyiukwu *et al.*, 2014; Miresmailli and Isman, 2014). They are non-nutrients, but are needed for the purposes of disease and pathogen control (Liu, 2003; Doughari *et al.*, 2009; Sullivan, 2014). These group of compounds also perform a number of important biological functions among humans such as anticancer, antimicrobial, antioxidant, antidiarrheal activities amongst many others (Sasidharan *et al.*, 2011). At least 12,000 different phytochemicals from plants have been identified and characterized thus far, a number that is estimated to be less than 10% of the total (Lai and Roy, 2004; Tapsell *et al.*, 2006). Currently, a significant number of these phytochemicals are regard as safe and viable alternatives to synthetic drugs (Sasidharan *et al.*, 2011; Islam, 2012), with some already being clinically certified and commercialized (Rajani and Kanaki, 2008; Hirpara *et al.*, 2009). For example, Pycnogenol® (procyanidin), a complex mixture of phenolics extracted from *Pinus maritima*, has been commercialized for pharmacological use as an antioxidant (Dillard and German, 2000; Belcaro *et al.*, 2014).
These medicinal principles (phytochemicals) are present at varying proportions in different plant parts, i.e. leaves, stems, roots, flowers, fruits or seeds, but they are however more concentrated in the outer layers of plant tissues. Amounts vary amongst plants depending on the plant growth conditions, plant species (Saxena et al., 2013) as well as the age of the plant. Examples of plant families that reportedly possess higher contents of medicinally important phytochemicals include Asteraceae, Krameriaceae, Passifloraceae and many others (Thomas et al., 2009; Iwu, 2014).

### 2.2.2 Classification and biological activities of important phytochemicals

The exact classification of phytochemicals is tricky due to their high structural heterogeneity, diverse biochemical activity, vast numbers and distribution among plant species. Nevertheless, advances in analytical chemistry and biochemistry have made it possible to place most of the identified phytochemicals into fairly distinct groups (Figure 2.1) (Campos-Vega and Oomah, 2013; Saxena et al., 2013). Phenolics are the largest and most structurally diverse group of phytochemicals, and in this group are the flavonoids, which constitute the largest and most diverse class, accounting for about two-third of the dietary phenolics (Sudjaroen, 2009; Saxena et al., 2013). Coming next after the flavonoids are the phenolic acids, accounting for approximately all of the remaining third portion of phenolic compounds (Muñoz et al., 2011; Machu et al., 2015). There is a growing interest in the potential health benefits of phenolics (Shahidi and Ambigaipalan, 2015; Marhuenda et al., 2016). Other notable phytochemicals include lectins, glucosinolates, terpenes, polysaccharides, carotenoids, and others (Campos-Vega and Oomah, 2013). Specifically, the flavonoids and phenolic acids along with their respective health benefits will be discussed in the succeeding sections of this review.
Figure 2.1: Phytochemical classification (Adapted from Campos-Vega and Oomah, 2013).
2.2.2.1 Flavonoids

Flavonoids are a highly prevalent group of polyphenolic compounds in the plant kingdom with a benzo-γ-pyrene structural configuration (Kumar and Pandey, 2013). The structure can be described as consisting of 2 benzene rings (A and B), which are conjoined by a pyrene ring containing an oxygen atom (C) (Table 2.1) (Saxena et al., 2012). Flavonoids can be further subdivided into five categories, i.e., flavonols, flavones, flavonones, anthocyanidins and isoflavones (Ozcan et al., 2014). Though flavonoids are largely present in plants as glycosides, they also occur as aglycones, and methylated derivatives (Tapas et al., 2008; Kumar and Pandey, 2013). Various sugars (monosaccharides, disaccharides or trisaccharides) can bind to the flavonoid aglycone at different hydroxyl groups (Kim et al., 2015) and the most frequently occurring ones include L-rhamnose and D-glucose, arabinose, glucorhamnose or galactose (Kumar and Pandey, 2013).

Table 2.1: Basic structural configuration of flavonoids and the flavonols positional structural orientation (Adapted from Stalikas, 2007).

<table>
<thead>
<tr>
<th>Position/Name</th>
<th>5</th>
<th>7</th>
<th>3'</th>
<th>4'</th>
<th>5'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>-</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>OH</td>
<td>OH</td>
<td>-</td>
<td>OH</td>
<td>-</td>
</tr>
<tr>
<td>Galangin</td>
<td>OH</td>
<td>OH</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fisetin</td>
<td>-</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>-</td>
</tr>
<tr>
<td>Myricetin</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>-</td>
</tr>
</tbody>
</table>

Notable among these flavonoids is the frequently occurring quercetin, reported to have a protective effect against rat liver oxidative stress induced by sodium fluoride via its antioxidant activity (Nabavi et al., 2012). Kaempferol, another recurrent flavonoid has been implicated for potential
chemopreventive activity when it was observed to promote HepG2 cell apoptosis (Guo et al., 2016). Various chalcone derivatives have been observed to possess anti-microbial, anti-malaria, anti-HIV and anti-cancer activities (Lee et al., 2015). Kil et al. (2012) linked okanins to anti-inflammatory activities through the inhibition of nitric oxide production and regulation of inducible nitric oxide synthase expression. The anti-oxidative effects of various flavonols and their glycosides (including quercetin, kaempferol, myrecetin, rutin and morin) was investigated (Hou et al., 2004). The results indicated that these compounds are potent anti-oxidants against AAPH-[2,2'-Azobis(2-amidinopropane) dihydrochloride] and Cu$^{2+}$ induced by human low density lipoprotein peroxidation.

### 2.2.2.2 Phenolic acids

Phenolic acids are aromatic phytochemicals that have the distinctive carbon frameworks, i.e., the hydroxycinnamic and hydroxybenzoic structures (Stalikas, 2007; Khoddami et al., 2013). They are widely distributed throughout the plant kingdom (Ren et al., 2009). Although their basic structural orientation (skeleton) remains the same, the positions and numbers of the hydroxyl groups on the aromatic ring differentiate them and confer varying properties among them (Table 2.2) (Stalikas, 2007).

**Table 2.2: Structural configuration of the major naturally occurring phenolic acids**

(Adapted from Stalikas, 2007).

<table>
<thead>
<tr>
<th>Name</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinnamic acid</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>o-Coumaric acid</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>m-Coumaric acid</td>
<td>H</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>H</td>
<td>OCH$_3$</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>P-Hydroxybenzoic acid</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>H</td>
<td>OCH$_3$</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
</tr>
</tbody>
</table>
Phenolic acids have been extensively studied as antioxidant agents in humans (Piazzon et al., 2012; Saxena et al., 2012). In a study of the antioxidant activity of some plants in the Asteraceae family, Fraisse et al. (2011) found chicoric acid to contribute 68.96% of the antioxidant activity of *Taraxacum officinale* (34.08 g/kg), while 3,5-dicaffeoylquinic acid contributed 48.92% of the antioxidant activity of *Tanacetum parthenium* (30.08 g/kg). Caffeic acid, one of the predominant naturally occurring cinnamic acids, along with 19 of its derivatives have been linked to antihypertensive activity via dual renin–angiotensin–aldosterone system (RAAS) inhibition (Bhullar et al., 2014). Toxicity studies on WI-38 cells from humans revealed a non-toxic manifestation of the compounds in comparison to captopril, a clinically used drug. Analogues of caffeic acid such as chicoric acid and dicaffeoylquinic have also demonstrated anti-HIV activity (Stoszko et al., 2016). Elsewhere, chicoric acid exhibited antidiabetic and anti-inflammatory properties by improving insulin resistance, increasing glucose uptake, and attenuating glucosamine-induced inflammation (Zhu et al., 2015).

As stated earlier, phenolics constitutes a very significant percentage of phytochemicals and several of its subcategories have been directly associated with important therapeutic activities. Essentially, these therapeutic activities are structure dependent. A summary of the important classes of phenolics, their description and associated biological activities is presented in Table 2.3.
Table 2.3: Summary of some major phytochemicals and their biological activities.

<table>
<thead>
<tr>
<th>Phytochemical subgroup</th>
<th>Description</th>
<th>Examples</th>
<th>Biological activity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxybenzoic acids</td>
<td>Related derivatives of benzoic acid i.e., 2-hydroxybenzoic acid (o-hydroxybenzoic acid), 3-hydroxybenzoic acid (m-hydroxybenzoic acid), and 4-hydroxybenzoic acid (p-hydroxybenzoic acid)</td>
<td>Salicylic acid, p-hydroxybenzoic acid, vanillic acid, gentisic acid, syringic acid</td>
<td>4-Hydroxy benzoic acid is reported to have anti-bacterial, anti-fungal, anti-algal, anti-mutagenic, anti-sickling and estrogenic activity</td>
<td>(Manuja et al., 2013; Ozcan et al., 2014; Saxena et al., 2013; Stalikas, 2007)</td>
</tr>
<tr>
<td>Hydroxylcinnamic acids</td>
<td>C6-C3 skeleton. These compounds are hydroxy derivatives of cinnamic acid.</td>
<td>Cinnamic acid, chicoric acid, coumaric acid, caffeic acid</td>
<td>Anti-microbial activity, anti-ulcer, anti-inflammatory, anti-HIV, antioxidant, anti-tumor, anti-spasmodic and anti-depressant activities</td>
<td>(Ozcan et al., 2014; Mamta Saxena et al., 2013; Stalikas, 2007)</td>
</tr>
<tr>
<td>Flavonols</td>
<td>3-hydroxyflavone backbone (IUPAC name: 3-hydroxy-2-phenylchromen-4-one).</td>
<td>Kaempferol, myricetin, quercetin, isorhamnetin</td>
<td>Anti-bacterial, anti-cancer, hepatoprotective, and inhibition of oxidative stress</td>
<td>(Berger et al., 2013; Kumar and Pandey, 2013; Ozcan et al., 2014; Saxena et al., 2013)</td>
</tr>
<tr>
<td>Flavones</td>
<td>Backbone of 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one)</td>
<td>Apigenin, luteolin, tangeritin</td>
<td>Anti-bacterial activity, inhibitors of procarcinogen activation, anti-cancer and anti-tumour</td>
<td>(Kumar and Pandey, 2013; Ozcan et al., 2014; Saxena et al., 2013)</td>
</tr>
<tr>
<td>Flavanones</td>
<td>Generally glycosylated by a disaccharide at position seven to give flavanoneglycosides</td>
<td>Naringin, hesperetin, hesperidin, sterubin</td>
<td>Anti-inflammatory, analgesic effects, anti-cancer activity</td>
<td>(Kumar and Pandey, 2013; Ozcan et al., 2014; Saxena et al., 2013)</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Substituted derivatives of isoflavone, being related to the parent by the replacement of two or three hydrogen atoms with hydroxyl groups.</td>
<td>Genistein and daidzein</td>
<td>Anti-microbial, anti-cancer, estrogenic activity, reduction in cardiovascular diseases and effects on osteoporosis</td>
<td>(Kumar and Pandey, 2013; Ozcan et al., 2014; Suthar et al., 2001; Vitale et al., 2013)</td>
</tr>
<tr>
<td>Tannins</td>
<td>Typically, tannin molecules require at least 12 hydroxyl groups and at least five phenyl groups to function as protein binders</td>
<td>Ellagic acid, gallic acid, and pyrogallic acid</td>
<td>Anti-diabetic, anti-microbial, anti-inflammatory, and immune-regulating activities</td>
<td>(Ozcan et al., 2014; Saxena et al., 2013; Yang and Liu, 2014)</td>
</tr>
</tbody>
</table>
2.3 **Bidens pilosa**

*Bidens pilosa* is a herbaceous annual flowering herb originating from South America but particularly common in the tropical and subtropical regions of the world (Pozharitskaya *et al.*, 2010; Arthur *et al.*, 2012). It is an erect herb with a serrate, dissected or lobed form of green opposite leaves, yellow or white flowers and long narrow ribbed black seeds (Figure 2.2) (Yang, 2014). Taxonomically, *B. pilosa* belongs to the Asteraceae family and the *Bidens* genus (Bartolome *et al.*, 2013). Its common names are black-jack (South Africa), beggars tick (USA), cobblers peg, farmer's friend (Australia) and Spanish needle (Barbados) (Arthur *et al.*, 2012). *Bidens pilosa* is easy-to-grow, highly invasive and an adaptive herb that is listed as a weed hence, considered one of the most troublesome wild plants (Grombone-Guaratini *et al.*, 2005; Bartolome *et al.*, 2013).

![Image of B. pilosa plant (A), its flowers (B) and seeds (C) (Adapted from Yang, 2014).]
2.3.1 Food uses and nutritional properties of \textit{B. pilosa}

\textit{Bidens pilosa} has a healthy nutritional profile (Table 2.4) and thus, considered a rich source of nutrients and medicine for humans and animals (Pozharitskaya \textit{et al.}, 2010). Many African countries such as South Africa, Nigeria, Kenya, Zambia, Mozambique, and Zimbabwe consume it as a vegetable or pot herb (Arthur \textit{et al.}, 2012). Its leaves and shoots (fresh or dried) are utilized as teas and in sauces (Chiang \textit{et al.}, 2003; Rybalchenko \textit{et al.}, 2010). The leaves have a resinous flavor and are added to salads or steamed and used in stews and soups (PFAF, 2012). Also, \textit{B. pilosa} has been used as a feedstuff for swine, and in South Africa and many other parts of Africa, livestock often grazes on the herb (Arthur \textit{et al.}, 2012).

| Table 2.4: Nutritional profile of \textit{B. pilosa} (Adapted from Yang, 2014). |
|---------------------------------|-------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Proximate composition          |                               |                 |                 |                 |                 |                 |                 |
| Plant (100 g)                  | Energy (kcal)                 | Moisture (%)    | Protein (g)     | Fat (g)         | Carbohydrate (g) | Fiber (g)  | Ash (g)        |
| Raw                            | 43                            | 85.1            | 3.8             | 0.5             | 8.4             | 3.9            | 2.2            |
| Dried                          | 33                            | 88.6            | 2.8             | 0.6             | 6               | 1.3            | 2              |
| Mineral content                |                               |                 |                 |                 |                 |                 |                 |
| Plant (100 g)                  | Calcium (mg)                  | Phosphorus (mg) | Iron (µg)       | Carotene equivalent (µg) | Thiamine (mg) |
| Raw                            | 340                           | 67              | 1800            | —               | —               |
| Dried                          | 111                           | 39              | 2.3             | —               | —               |

2.3.2 Pharmacological importance and phytochemistry of \textit{B. pilosa}

Apart from its use as food, \textit{B. pilosa} is also used in folklore medicine in the treatment of more than 40 diseases in man (Borges \textit{et al.}, 2013). All parts of this plant including the leaves, stem, roots, flowers and seeds, fresh or in dried form, are potent ingredients in traditional medicine (Oliveira \textit{et al.}, 2004; Yuan \textit{et al.}, 2008; Ashafa and Afolayan, 2009; Kviecinski \textit{et al.}, 2011). Moreover, \textit{B. pilosa} is often used alone or sometimes as an ingredient in herbal concoctions together with other medicinal plants (Yang, 2014). Some of its important biological activities include antimicrobial (Silva \textit{et al.}, 2014), anticancer and anti-pyretic activity (Sundararajan \textit{et al.}, 2006). Kumari and others (2009) investigated the anti-cancer activities of \textit{B. pilosa} leaves in a cytotoxicity-directed
fractionation strategy. They observed potent activities of a compound, phenyl-1,3,5-heptatriene, with IC$_{50}$ values of 8, 0.7, 0.49 and 10 µg/mL against human oral, colon, liver and breast cancer cell lines, respectively. *Bidens pilosa* has also been reported to possess anti-inflammatory and anti-allergic characteristics (Horiuchi and Seyama, 2008), anti-oxidative activity (Yang *et al*., 2006), anti-diabetic tendency (Lai *et al*., 2015) and several other beneficial properties have been reviewed in other studies (Bairwa *et al*., 2010; Bartolome *et al*., 2013).

The above-mentioned biological functions can be rationalized by the wide spectrum of phytochemicals detected in this plant (Silva *et al*., 2011; Bartolome *et al*., 2013). Heretofore, 201 compounds comprising flavonoids, aliphatics, terpenoids, aromatics, phenylpropanoids, porphyrins and other compounds such as caffeoylquinic acid derivatives and sesquiterpene have been identified from this plant (Chiang *et al*., 2004; Grombone-Guaratini *et al*., 2005; Silva *et al*., 2011). In a complementary study of the phenolic content and antioxidant activity of *B. pilosa* methanol extracts, Muchuweti and colleagues (2007) reported phenolic contents to the amount of 1102.8 mg/g. Flavonoids are however, the predominant class of phenolic phytochemicals in the *Bidens* genus (Chiang *et al*., 2004). A comprehensive list of identified phytochemicals from this plant have been compiled and provided by Silva *et al*. (2011).

### 2.4 EXTRACTION OF PHYTOCHEMICALS

Extraction is an initial, indispensable and vital step in the analyses of plant constituents. The entire analytical process could be jeopardized if the extraction method is not efficient (Pawliszyn, 2003; Smith, 2003). It is a physicochemical process that selectively dissolves one or more of the solutes into an appropriate solvent. Important features of any suitable extraction technique include safety, efficiency (yield), cost and availability of solvents and instrumentation, robustness and reproducibility, compatibility with various detection and quantification methods, conformity with the properties of the material to be extracted, simplicity and time-saving factors (Augusto *et al*., 2013; Gbashi *et al*., 2016).
2.4.1 Conventional extraction methods for plant materials

2.4.1.1 Solvent extraction

Solvent extraction also called liquid-liquid extraction is a frequently used extraction method in phytochemical analysis. It refers to a method to separate solutes based on their relative solubility in two different liquids that are immiscible (Sapkale et al., 2010). One or more solutes contained in a feed solution is transferred to another immiscible solvent, often by rigorously mixing the two immiscible phases, then allowing the two phases to separate (Sapkale et al., 2010; Zaiz et al., 2013). The enriched solvent is called the extract. Solvent extraction can also be used to describe the process of separating one or more soluble compounds from an insoluble compound or a complex matrix by preferentially dissolving that substance in a suitable solvent (Sapkale et al., 2010). Common solvents used for liquid-liquid extraction are presented in Table 2.5, although extensive reviews have been provided by several other authors (Mabiki et al., 2013; Ugochukwu et al., 2013; Phan and Nguyen, 2014).

Table 2.5: Organic solvents used for bioactive component extraction
(Adapted from Tiwari et al., 2011).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Phenol</th>
<th>Flavonols</th>
<th>Polyacetylenes</th>
<th>Flavonol</th>
<th>Terpenoids</th>
<th>Sterols</th>
<th>Alkaloids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>Tannins</td>
<td>Polyphenols</td>
<td>Coumarins</td>
<td>Fatty acids</td>
<td>Xanthoxylines</td>
<td>Totarol</td>
<td>Quassinoids</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Alkaloids</td>
<td>Terpenoids</td>
<td>Flavonoids</td>
<td>Tannins</td>
<td>Xanthoxylines</td>
<td>Quassinoids</td>
<td>Lectins</td>
</tr>
<tr>
<td>Ether</td>
<td>Chloroform</td>
<td>Flavonoids</td>
<td>Saponins</td>
<td>Tannins</td>
<td>Totarol</td>
<td>Lactones</td>
<td>Lectins</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Methanol</td>
<td>Terpenoids</td>
<td>Saponins</td>
<td>Tannins</td>
<td>Polypeptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>Water</td>
<td>Starches</td>
<td>Tannins</td>
<td>Saponins</td>
<td>Polypeptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>Anthocyanins</td>
<td>Starches</td>
<td>Tannins</td>
<td>Saponins</td>
<td>Lectins</td>
<td>Lactones</td>
<td>Lectins</td>
</tr>
<tr>
<td>Phenol</td>
<td>Tannins</td>
<td>Polyphenols</td>
<td>Coumarins</td>
<td>Fatty acids</td>
<td>Xanthoxylines</td>
<td>Totarol</td>
<td>Quassinoids</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Alkaloids</td>
<td>Terpenoids</td>
<td>Flavonoids</td>
<td>Tannins</td>
<td>Xanthoxylines</td>
<td>Totarol</td>
<td>Quassinoids</td>
</tr>
<tr>
<td>Ether</td>
<td>Chloroform</td>
<td>Flavonoids</td>
<td>Saponins</td>
<td>Tannins</td>
<td>Tannins</td>
<td>Tannins</td>
<td>Saponins</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Methanol</td>
<td>Terpenoids</td>
<td>Saponins</td>
<td>Tannins</td>
<td>Polypeptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>Water</td>
<td>Starches</td>
<td>Tannins</td>
<td>Saponins</td>
<td>Polypeptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>Anthocyanins</td>
<td>Starches</td>
<td>Tannins</td>
<td>Saponins</td>
<td>Lectins</td>
<td>Lactones</td>
<td>Lectins</td>
</tr>
<tr>
<td>Phenol</td>
<td>Tannins</td>
<td>Polyphenols</td>
<td>Coumarins</td>
<td>Fatty acids</td>
<td>Xanthoxylines</td>
<td>Totarol</td>
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Although this method of extraction is relatively easy and requires routine laboratory equipment, its use has been greatly limited because it enable the consumption of large quantities of organic solvents some of which are often not environmentally friendly (Santana et al., 2009; Tabaraki and Nateghi, 2011). Furthermore, solvent extraction often involve long extraction times, solvents of the required purity tend to be expensive and there are often additional costs with proper disposal of solvents/wastes after use (Sapkale et al., 2010; Tabaraki and Nateghi, 2011).

2.4.1.2 Soxhlet extraction

The Soxhlet extraction is a general and well-established technique for the extraction of phytochemicals, which is often used as a benchmark technique for evaluating the performance of other conventional solid-liquid extraction techniques (Wang and Weller, 2006; Lau et al., 2010; Sibiya et al., 2013). Amongst other conventional extraction methods, it is the most widely used (Tatke and Jaiswal, 2011). Essentially, Soxhlet extraction is employed when the target analyte has a limited solubility in a solvent, and the unwanted materials (or compounds) are insoluble in that solvent (Sarode et al., 2013). An elaborate overview of this extraction process and applications has been provided by Halder et al. (2012) and Luque de Castro and Priego-Capote (2010). The extraction technique has the disadvantages of involving enormously long extraction times and consumption of large volumes of organic solvents, which pose hazards to the environment. Solvent used can be up to 250 mL required to extract plant components from a mere 20 g of plant material, and the process of extraction extending for up to 24 h and more (Yadav and Agarwala, 2011). In addition, it emits toxic fumes (Ayre et al., 2013) and extraction is usually performed at boiling point of the extraction solvent, hence there is limited application for the extraction of thermolabile compounds (Wang and Weller, 2006; Tatke and Jaiswal, 2011).

2.4.1.3 Microwave assisted extraction

Microwave-assisted extraction (MAE) utilizes microwave energy to heat the sample matrix and the solvent in order to enhance the mass transfer rate of the analytes from the sample matrix into the solvent (Devgun et al., 2009). In the last decade, there has been increased popularity in the use of microwaves for the extraction of various constituents from plant materials and soil samples (Devgun et al., 2009; Sibiya et al., 2013), possibly due to the necessity to reduce the amount of organic solvent consumption during extraction. Fundamentally, MAE differs from other
conventional extraction methods because, the extraction process occurs as the result of alterations in the cell structure of the biomass matrix caused by electromagnetic waves (Veggi et al., 2013). Microwaving raises the temperature of the minute microscopic traces of moisture available in the plant cells, which results in evaporation, generating remarkably high pressure on the cell wall. The cell wall eventually raptures, hence increasing the mass transfer of the phytoconstituents (Tatke and Jaiswal, 2011). The principles, dynamics and applications of MAE have been extensively reviewed by Tatke and Jaiswal, (2011), Zhang et al., (2011), and Veggi et al., (2013).

Compared to other traditional extraction techniques, MAE enables reduced extraction time, faster extraction rates, increased yields and reduced solvent consumption (Doughari, 2012; Veggi et al., 2013). However, there are some limitations on its use, i.e., microwaves are ionizing electromagnetic waves that can initiate or catalyze chemical reactions or changes of some of the target metabolites (Ghani et al., 2008; Zhang et al., 2011), and the use of high temperatures that can lead to degradation of thermolabile metabolites (Wang, 2010; Zhang et al., 2011). The amount of sample used is limited to 1 g, which is insufficient for a fair homogenous analysis (Shu et al., 2000). Moreover, extraction yields can be low when the target compounds or extraction solvents are volatile or nonpolar. Also, subsequent filtration or centrifugation of the extracts is necessary to remove solid residues after extraction (Wang, 2010; Veggi et al., 2013).

2.4.1.4 Supercritical fluid extraction

Supercritical fluid extraction (SFE) represents a high diffusion fluid extraction technique (Co, 2010). Extraction involves the separation of components using supercritical fluids (most often CO2) as the extraction solvent (Sapkale et al., 2010). Supercritical fluids, i.e., fluids in a state above its critical point’s temperature and pressure, which are highly compressed gases exhibit properties of gases and liquids simultaneously in an interesting manner (Sharma, 2015). Supercritical fluids can diffuse through solid matrices like gases, and dissolve analytes like liquid solvents (Attawood and Florence, 2012; Sofi et al., 2013). They have zero surface tension and can easily be controlled by varying temperature and pressure conditions (Co, 2010), hence, they are good substitutes for organic solvents in various laboratory and industrial extraction processes (Sharma, 2015). Moreover, extraction using SFE is fast (process completed in 10 to 60 min) (Sapkale et al., 2010), automable and does not require large volumes of toxic solvents (Ayre et al., 2013). Another very
important benefit of SFE is its ability to leave no trace of the solvent in the extracts (Sapkale et al., 2010).

However, extraction using SFE suffers some major setbacks. For example, it is necessarily required that the target analyte be soluble in CO₂, otherwise extraction cannot occur (Co, 2010). Essentially, CO₂ itself is a non-polar solvent, hence, on its own it is not always suitable for the extraction of polar solutes (Shine et al., 2015). Moreover, the SFE equipment are quite expensive, and their operation requires technical skills as well as rigorous precautions (Bulgariu and Bulgariu, 2015; Shine et al., 2015). It is also highly energy-intensive (CO₂ compression and heating) when compared to traditional liquid extraction (Bulgariu and Bulgariu, 2015; Shine et al., 2015). Additional information on the principles and applications of SFE can be obtained from the literature (Reverchon and De Marco, 2006; Ayre et al., 2013; Capuzzo et al., 2013).

2.4.2 Other extraction methods

Aside from the extraction techniques discussed above, several other methods have been investigated for the extraction of various phytochemicals and bioactive components from plant tissues, some of which include accelerated solvent extraction (Sun et al., 2012), ultrasonic-assisted extraction (Vilkhu et al., 2008), solid-phase microextraction (Merkle et al., 2015), solid-phase extraction using molecular imprinted polymer (Pakade et al., 2013), magnetic solid-phase extraction (Ibarra et al., 2015), liquid-phase microextraction using hollow-fibre (Sibiya et al., 2013), QuEChERS extraction (Bruzzoniti et al., 2014) and many others that are comprehensively reviewed in literature (Jones and Kinghorn, 2006; Wang and Weller, 2006; Tiwari et al., 2011; Azmir et al., 2013; Doughari, 2012).

Although several techniques exist for the extraction of phytochemicals, shortcomings associated with these techniques are illustrious. As discussed previously in Section 2.4.1, these techniques are fundamentally limited by large volumes of organic solvents usage, some of which are well known for to be toxic and considered as environmental hazards, issues of low recovery efficiency, long and labourious procedures involved, loss in precision and high costs amongst others (Augusto et al., 2013). Hence, there is an eminent need for a greener approach (that eliminates or minimize usage of organic solvents), that promises improved recovery efficiency, while at the same time maintaining the credibility of the results, at reduce cost, and time (Augusto et al., 2013; Susanti et
Moreover, novel fields of biochemical research and subsequent applications are constantly emerging (e.g. “omics”), that ensure and more reliable extraction steps thus, limiting the application of conventional techniques in extracting active biomolecules from plant materials (Zhang et al., 2012; Augusto et al., 2013).

In addition, the adoption of an extraction method largely depends on the analytical objectives and the nature of the target bioactive compounds, and as such, plant materials selected on the basis of traditional uses need to be extracted by a technique that mimics as closely as possible, the traditional herbal drug preparation procedures (Sasidharan et al., 2011; Khoza et al., 2014). This has brought PHWE on a spotlight as an efficacious and highly promising alternative to traditional techniques of extraction. In the study of phytochemicals from medicinal plants such as B. pilosa, an added advantage to PHWE is its flexibility and broad spectrum of extractable compounds (Gbashi et al., 2016). This has fostered its widespread acceptance and adoption for various biochemical studies on metabolites (phytochemicals) and metabolomics (Liang and Fan, 2013; Khoza et al., 2015; Plaza and Turner, 2015; Khoza et al., 2016).

2.5 PRESSURIZED HOT WATER EXTRACTION: CONCEPT AND APPLICATIONS

2.5.1 Pressurized hot water

The term ‘pressurized hot water’ or ‘subcritical water’ refers to liquid water at temperatures between the boiling point and critical point temperature of water (100 – 374°C) (Asl and Khajenoori, 2013; Ravber et al., 2015). Water has many anomalous properties which are due to the very strong hydrogen bonding that exist in a water molecule (Aghamiri et al., 2001; Teixeira, 2008). For example, it is lightest in its gaseous state, but much denser (than predictable) as a liquid compared to its solid state (Brovchenko and Oleinikova, 2008). However, within subcritical and superheated temperature range (100 – 374°C), the hydrogen bond and other intermolecular forces are interrupted, making water a much more flexible and dynamic extraction solvent. Consequently, little changes in temperature can account for large changes in its solvation power, provided the pressure is sufficient to maintain it in a liquid state (Chaplin, 2008; Gbashi et al., 2016). The phase diagram of water presented in Figure 2.3, showing the various states of water under different conditions of temperature and pressure.
2.5.2 Pressurized hot water extraction

Pressurized hot water extraction has often been represented under different terminologies e.g. subcritical water extraction (SWE) (Asl and Khajenoori, 2013; Liang and Fan, 2013), hot water extraction (HWE) (Paredes et al., 2008), hot liquid water extraction (HLWE) (Wan and Li, 2011) and pressurized low polarity water extraction (PLPWE) (Cacace and Mazza, 2006; Guclu-Ustundag and Mazza, 2009). It is a green, cheap and easy-to-adopt extraction technique that utilizes water as the extraction solvent (Gbashi et al., 2016). The use of pressurized hot water (PHW) as an extraction solvent was first reported by Hawthorne et al. (1994), when it was observed that sub- and supercritical water can be used for the recovery of organic pollutants from soil samples. Since then, numerous scientific researches have been carried out towards exploiting this novel technology.
2.5.3 Principle of pressurized hot water extraction

The principle of PHWE is anchored on the molecular structure, physicochemical and thermodynamic properties of water. Water at atmospheric temperature and pressure has one of the highest dielectric constants (i.e. $\varepsilon=80$ at 25°C at 1 bar) amongst non-metallic liquids, which makes it a poor extraction solvent for low polarity compounds (Cabane and Vuilleumier, 2005; Kruse and Dinjus, 2007). However, as the temperature of water rises with sufficient pressure e.g. 250°C and 50 bar, its dielectric constant drops to within the ranges of organic solvents (i.e. from $\varepsilon=80$ to $\varepsilon=27$) such as ethanol ($\varepsilon=24$), methanol ($\varepsilon=33$), acetone ($\varepsilon=21$), and acetonitrile ($\varepsilon=37$) (Figure 2.4) (Alupului et al., 2012; Gbashi et al., 2016). As such, water will act like these organic solvents, dissolving a broad spectrum of low and medium polarity analytes (Gbashi et al., 2016). Table 2.6 shows how some important properties change when water is subjected to high temperatures and pressures.

![Dielectric constant of water (50 bar)](image)

**Figure 2.4:** Dielectric constant of water as a function of temperature (Adapted from Gbashi et al., 2016).
Table 2.6: Important electro- and physicochemical properties of water that changes with increase in temperature and pressure (Adapted from Gbashi et al., 2016).

<table>
<thead>
<tr>
<th>S/No</th>
<th>Property</th>
<th>Status</th>
<th>Reference</th>
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<tbody>
<tr>
<td>1.</td>
<td>Adhesion and cohesion</td>
<td>Decreases</td>
<td>Chaplin (2008)</td>
</tr>
<tr>
<td>2.</td>
<td>Collision frequencies</td>
<td>Increases</td>
<td>Buhler et al. (2002); Kruse and Dinjus (2007)</td>
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<td>3.</td>
<td>Compressibility</td>
<td>Increases</td>
<td>Kruse and Dinjus (2007); Chaplin (2008)</td>
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<td>5.</td>
<td>Dielectric constant</td>
<td>Decreases</td>
<td>Kruse and Dinjus (2007); Chaplin (2008)</td>
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<tr>
<td>6.</td>
<td>Diffusivity</td>
<td>Increases</td>
<td>Kruse and Dinjus (2007); Teo et al. (2010)</td>
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<td>7.</td>
<td>Electrical conductivity</td>
<td>Increases</td>
<td>(Hawthorne, 2000)</td>
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<tr>
<td>8.</td>
<td>Extraction rates</td>
<td>Increases</td>
<td>Teo et al. (2010); Gupta et al. (2012)</td>
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<td>10.</td>
<td>Miscibility</td>
<td>Increases</td>
<td>(Weingartner and Franck, 2005)</td>
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<td>11.</td>
<td>Solubility</td>
<td>Increases</td>
<td>(Miller et al., 1998)</td>
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<td>12.</td>
<td>Surface tension</td>
<td>Decreases</td>
<td>(Chaplin, 2008)</td>
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<tr>
<td>13.</td>
<td>Viscosity</td>
<td>Decreases</td>
<td>(Kruse and Dinjus (2007); Teo et al., (2010)</td>
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</table>

2.5.4 Mechanism and instrumentation of pressurized hot water extraction

The extraction mechanism of PHWE is mainly a function of mass transfer effects brought about by processes of diffusion and convection (Ong et al., 2002) as follows: (1) there is a rapid entry of fluid (i.e., PHW) into matrix pores; (2) desorption of solutes from the matrix active sites; (3) diffusion of solutes through static fluid in porous materials; (4) dissolution of solutes in fluid; (5) diffusion of solutes through layer of stagnant fluid outside particles; and (6) elution of solutes through the bulk flowing of fluid (Asl and Khajenoori, 2013; Gbashi et al., 2016). In addition, the high temperatures enhance extraction by forcing the fluid into matrix areas (pores) where low pressure water may normally not reach (Gbashi et al., 2016).

The instrumental setup of a typical laboratory scale PHWE unit includes: a source of pure water, retention coil, a pump, an oven, an extraction cell, a backpressure valve and a cooling coil connected to the outlet (Figure 2.5). The sample matrix to be extracted is loaded unto the extraction cell which is located inside the oven. The oven (which usually has an automatic temperature control mechanism) is set to the desired temperature, the backpressure valve is locked and water
is pumped at a preset flow rate into the extraction cell through the retention coil. The retention coil which is also located inside the oven ensures that the water acquires enough thermal energy to attain the desired temperature at which the oven is operating. The extraction occurs in the extraction cell as the pressurized hot water flows through it. The backpressure valve (which is used to regulate the operating pressure) is gently released depending on the desired operating pressure and the hot water extract flows through the cooling coil and is collected at the outlet (Gbashi et al., 2016).

![Figure 2.5: Simple laboratory setup of a PHWE unit.](image)

### 2.5.5 Operational modes of pressurized hot water extraction

Extraction with PHW is performed in two basic modes, static (batch) mode or dynamic (flow-through) mode. In the static mode, the sample is retained in the extraction cell with pressurized hot water for short periods (but sufficient for an equilibrium to be reached between the solvent and analyte) after which the cell is purged and extract collected. The disadvantage of this mode is that the solvent might in a little while reach an equilibrium state with the analyte, hence no further extraction (diffusion or dissolution) of the analyte occurs irrespective of the retention time or temperature and pressure manipulation. Whereas, the dynamic mode allows for a steady flow of fresh solvent through the extraction cell which minimizes or eliminates solute-fluid equilibrium in a single operation when optimized. As such, extraction efficiency is higher in the dynamic mode.
(Teo et al., 2010), although, solvent consumption could be more, resulting in less energy efficiency compared to the static mode (Gbashi et al., 2016). This was also confirmed by Yang and Wyman (2004), where extraction in dynamic mode resulted in greater recovery of hemicelluloses and lignin from corn stover cellulose than static mode.

### 2.5.6 Factors that affect pressurized hot water extraction

A number of factors such as temperature, pressure, flow rate, particle size, co-solvents and surfactants affect the performance of PHWE. These are further described in the proceeding sections of this review.

#### 2.5.5.1 Temperature

Temperature has a very strong and decisive effect on the extraction efficiency of PHWE. Typically, extraction efficiency increases with increase in temperature. In the study of Yang et al. (2013), it was observed that the extraction yield of polysaccharides from *Grifola frondosa* positively correlated with increase in temperature, linearly. Accordingly, the extraction yields increased significantly from 6.9 to 32.8% with an increase in extraction temperature (from 100 to 230°C). Likewise, Ibañez et al. (2003) also observed that increasing the temperature clearly resulted in higher extraction yields of antioxidant compounds from rosemary plant. However, excess temperatures can result in thermal degradation of vital compounds, hence the need for optimization of the extraction process (Asl and Khajenoori, 2013; Khoza et al., 2015). Studies on the recovery of carvacrol and thymol from *Zataria multiflora* between 100 and 175°C showed that extraction efficiency increased steadily with increase in temperature up until 150°C, followed by a subsequent decrease with a noticeable burning smell (Khajenoori et al., 2009).

#### 2.5.5.2 Pressure

The direct effect of pressure on the extraction efficiency of PHWE has not been found to be significant. In a study on SWE (PHWE) extraction of caffeine from tea waste, Shalmashi et al. (2010) investigated the effect of pressures, 20, 30 and 40 bar on the recovery efficiency. The results indicated that pressure had no significant effect on the rate of caffeine. They attributed this to an insignificant pressure effect, which consequently could not cause any significant change on the density of water (water is fairly incompressible at temperatures below 300°C). As such, there was
no changes in the physicochemical properties of the water, as long as it was maintained in its liquid state. Similar observations were also made in other studies (Deng et al., 2004; Kronholm et al., 2004). Nevertheless, increased pressure can compromise matrix tissue membranes and forces the extraction fluid deep into matrix pores where water at lower pressure may not normally reach (Ong et al., 2006).

### 2.5.5.3 Cosolvents and surfactants

Modifiers such as cosolvents or surfactants are often used to enhance the extractability of PHWE. Cosolvents are secondary solvents added to enhance the efficiency of PHWE (Williams et al., 2013), while modifiers can alter important physicochemical properties of water (i.e. polarity, surface tension, hydrogen bonding strength and diffusivity) which results in an enhanced extractability (Curren and King, 2001; Teo et al., 2010). On the other hand, they can interact directly with the sample matrix, in a manner that reduces the activation energy required for analyte desorption and diffusion (Plaza and Turner, 2015; Gbashi et al., 2016). Arapitsas and Turner (2008) showed that it was possible to improve the extraction of anthocyanins from red cabbage by using 5% ethanol and 1% formic acid (Arapitsas and Turner, 2008). In another study, it was observed that the solubility of atrazine was doubled when urea was added to pressurized hot water, and when ethanol was used, the increase was over 10-folds (Curren and King, 2001). In addition to the above described factors, others that could affect the efficiency of PHWE includes solvent flow rate, nature of matrix and analyte, matrix particle size and geometry of extraction cell (Gbashi et al., 2016).

### 2.5.7 Advantages and disadvantages of pressurized hot water extraction

#### 2.5.7.1 Advantages

The major advantage of PHWE lies in its safety and environmental friendliness as the extraction solvent is water (which is non-toxic, non-flammable and renewable). More so, water is cheap, readily available and does not generate harmful by-products (Chemat et al., 2012; Liang and Fan, 2013). In comparison with traditional extraction techniques, PHWE is much user-friendly, involves few extraction steps and is not time-consuming and as such, human errors are greatly minimized. When put side-by-side with SFE, PHWE edges on the basis of being a less sophisticated technology, hence requiring much lower engineering and maintenance cost for
equipment (Bart, 2005; Gbashi et al., 2016). During extraction using PHWE, the fluid can be made to selectively extract a range of polar and moderately polar compounds by mere adjusting the temperature of the water, whereas SFE extracts only nonpolar or light-weight compounds (Curren and King, 2001; Liang and Fan, 2013). Moreover, PHWE is highly compatible with various analytical instrumentations (e.g. ToF-MS/MS), possibly because water is colourless and may not interfere with sorts of photodetection such as MS, ultraviolet detection or flame ionization detection (Gbashi et al., 2016; Khoza et al., 2016).

### 2.5.7.2 Disadvantages of pressurized hot water extraction

A profound drawback of PHWE is thermal degradation of some target analytes due to the elevated temperatures (Khoza et al., 2014). The recovery rate of polycyclic aromatic hydrocarbons was observed to decrease at temperatures exceeding 250°C due to thermal degradation (Moreno et al., 2007). Aside thermal degradation, performing PHWE at significantly high temperatures and pressures e.g. 374°C and 221 bar (critical temperature and pressure of water) could pose risk of reactivity of water (i.e. water could oxidize or catalyze hydrolysis of some compounds) (Teo et al., 2010). However, the adoption of a cosolvent or surfactant could eliminate the issues of thermal degradation or reactivity of water at high temperature, as satisfactory recovery rates could be achieved even at mild extraction conditions.

### 2.5.8 Applications of pressurized hot water extraction

In the last decade, PHWE has been extensively explored in the biochemical, pharmaceutical, environmental, food, and chemical industries (Figure 2.6). It has been successfully utilized for the extraction of nutritional constituents, pharmacoactive compounds and organic pollutants from vegetal tissues, food products, soil sediments and other ecological biomasses (Gbashi et al., 2016). In a recent study, Matshediso et al. (2015) optimized the extraction of three flavonols i.e. kaempferol, quercetin and myricetin and also investigated the total phenolic content in Moringa leaf powder using PHWE. Similar extraction method was also used for the isolation of pharmacologically important metabolites from Moringa oleifera leaves (Khoza et al., 2014), proanthocyanidins and catechins from grape seeds (García-Marino et al., 2006), antioxidants from microalga Spirulina platensis (Herrero et al., 2004), flavonoids from aspen knotwood (Hartonen et al., 2007) and proteins, carbohydrates and lignans from flaxseed meal (Ho et al., 2007). Fattah
et al. (2014) also extracted free fatty acids and oils from spent bleaching earth using a similar technique.

![Figure 2.6: Application of PHWE in different scientific fields from 1990-2014 (using ISI Web of Science database on 05-02-2015) (Adapted from Gbashi et al., 2016).](image)

Elsewhere, nifedipine was recovered from drug tablets using PHWE (SWE) at 150°C for pharmaceutical formulations (Richter et al., 2006). By means of PHWE, it was possible to recover important bioactive compounds such as ellagic acid, gallic acid, and corilagin from *Terminalia chebula Retz.* fruits (Rangsriwong et al., 2009). In an earlier study, Hawthorne et al. (2000) cross-examined extractions of polyaromatic hydrocarbons (PAHs) using SWE, soxhlet extraction, pressurized liquid extraction, and SFE. The results showed that extracts obtained by SWE were better, compared to those from the other techniques. Considering the wide literature attention PHWE has received, it is evident that it has been epic with far-reaching prospects and huge potential for various application due to the numerous advantages it offers (Teo et al., 2010; Shi et al., 2012; Asl and Khajenoori, 2013; Liang and Fan, 2013; Duy et al., 2015; Plaza and Turner, 2015; Gbashi et al., 2016).
2.6 CHROMATOGRAPHIC SEPARATION, IDENTIFICATION AND CHEMOMETRIC ANALYSIS OF PHYTOCHEMICALS

The success of a systematic phytochemical analysis further depends on the quality (robustness and sensitivity) of the analytical instruments used. A good chromatographic separation will offer better detection levels and improved mass spectrometry data quality (Castro-Perez et al., 2004). The enhancement of chromatographic resolution and sensitivity is especially important when analyzing complex samples such as plant extracts. Hence, an outline of different chromatographic separation techniques is presented in the proceeding sections of this chapter.

2.6.1 Chromatographic separation of phytochemicals

Analytical chromatography is employed to determine the presence and possibly also the concentration of analyte(s) in a sample. A range of analytical chromatographic platforms exist for metabolite fingerprinting of phytochemicals of which the most popular ones include thin layer chromatography (TLC), high performance liquid chromatography (HPLC), ultra-performance liquid chromatography (UPLC), and gas chromatography (GC) (Waksmundzka-Hajnos et al., 2008; Waksmundzka-Hajnos and Sherma, 2010; Pratima et al., 2013; Sheliya and Shah, 2013; Stashenko and Martinez, 2014). These chromatographic instruments are coupled to various detectors, responsible for converting physical or chemical attributes of the analyte into measurable signals corresponding to concentration, identity, and/or position (Snyder et al., 2010).

Commonly used detectors for phytochemical analysis include ultraviolet (UV) detectors, photodiode array detectors (PDA), flame ionization detectors (FID), Fourier transform infrared (FTIR) spectrometers, mass spectrometers (MS) and various tandem mass spectrometry systems (MS^n). The MS^n usually involves multiple steps of mass spectrometry selection, with some form of fragmentation occurring in between the stages, and different forms include time-of-flight mass spectrometry (qToF-MS/MS) and ion trap mass spectrometers (IT-ToF-MS/MS). Tandem or hybrid configuration of these detectors enhances metabolite identification by acquiring highly accurate and resolved MS/MS spectra, which are achieved by ion fragmentation through collision-induced dissociation (Xiao et al., 2012). Various features of chromatographic detectors have been reviewed by other authors (Swartz, 2010; Ramni et al., 2011) and reference could be made to these studies for further information.
The coupling of liquid chromatography to MS enables improved metabolite identification and quantitation by decreasing sample complexity and allowing metabolite separation prior to detection (Xiao et al., 2012). In particular, using UPLC coupled with qToF-MS/MS offers a new dimension to metabolite studies, facilitating attainment of better resolution and detection limits, improved throughput and enhanced data quality (Castro-Perez et al., 2004). Ultra-performance liquid chromatography (UPLC) is an advanced separation science that simulates (uses) the basic principles of HPLC increasing the overall combined elements of speed, sensitivity and resolution (Wu and Engen, 2006) during the process, while hybrid qToF-MS/MS offers an accurate mass measurement, high resolution, scan speed and generates information-rich datasets (Xie et al., 2012).

2.6.2 Identification and profiling of phytochemicals

Following chromatographic separation and spectrometric analysis, it becomes ultimately rational to identify key compounds responsible for important biological properties of a plant. Without this, the results of any spectrometric analysis are biochemically vague, uninterpretable and meaningless (Wishart, 2011). The most basic level of metabolite identification from mass spectrometric data is the molecular weight of the metabolite, matching the retention times and observing the corresponding fragmentation patterns on the mass spectrum (Figure 2.7) (Watson, 2013). Metabolites show symbolic fragmentation patterns that can be used to determine the structural information of the molecule. It is often possible to uniquely identify a compound from its spectrum by looking at the position of peaks and the distribution patterns of its ions by mass [more correctly: mass-to-charge ratio (m/z)]. The accurate mass obtained from high resolution MS can be assigned to a particular molecular formula (Watson, 2013). A formal guideline for confirmation of the precise elemental composition according to mass spectra of compounds has been defined by Kind and Fiehn (2007).
2.6.3 Chemometric analysis of phytochemicals

Chemometrics involve the application of statistical and mathematical methods that ensures the successful extraction of useful information and improve understanding of the chemical information contained in an analytical data (Bu, 2007; El-Gindy and Hadad, 2012). Fundamentally, spectrometric analysis of plants generates high-dimensional and complex data sets (metabolome) that defy visual inspection or conventional univariate statistical approaches. The utility of multivariate chemometric techniques enables maximum extraction of meaningful information from these enormous multidimensional data sets (Tugizimana, 2012). A number of multivariate chemometric techniques exist such as hierarchical clustering analysis (HCA), multi-level simultaneous component analysis (MSCA), principal component analysis (PCA), partial least square discriminate analysis (PLS-DA), response surface methodology (RSM) and soft independent modelling of class analogy (SIMCA). The detailed description and mathematical algorithms behind these techniques go beyond the scope of this review, but the following studies documented in the literature can be consulted for more detailed descriptions (Ballabio and Todeschini, 2009; Filzmoser et al., 2009; Khuri and Mukhopadhyay, 2010; Ceulemans et al., 2013; Dos Santos et al., 2013; Roussel et al., 2014). Nevertheless, a brief description of two of interest in this study (PCA and RSM) is presented in the next paragraph.
Chapter Two

2.6.3 Principal component analysis

Among the multivariate analysis techniques, PCA (Bro and Smilde, 2014) is the most effective and frequently used linear method for data compression (Daszykowski et al., 2003; Cordella, 2012). The major objective of PCA is to minimize the dimensionality of data while at the same time preserving variance in the data as much as possible (Cordella, 2012; Worley and Powers, 2013). Fundamentally, PCA uses an orthogonal transformation to condense high-dimension data into a set of a few uncorrelated latent variables, called Principal Components (PCs) (Daszykowski et al., 2007; Panigrahi, 2014). A key feature is that the transformation is modelled in such a manner that the first PC has the highest possible variance (i.e., explains as much of the variability in the data set as possible), and each subsequent component in turn has the largest variance possible under the constraint that it is uncorrelated to the preceding components (Panigrahi, 2014). This implies that the first few PCs are sufficient to adequately interpret the data structure (Daszykowski et al., 2007).

In phytochemical studies of plants, each metabolite in the complex spectrometric data set is a factor with its spectra and elution profile which are transformed by mathematical algorithms to the so called PCs. By means of PCA, interpretable graphical visualization of the original data set is afforded by way of two matrices, the PCA scores (which relate to the elution profiles in the case of chromatography) and PCA loadings (which relate to the spectra) (Brereton, 2003; Daszykowski et al., 2003; Tugizimana, 2012). The PCA scores maximizes the description of data variance, highlighting the similarities or differences in the data set. The PCA loadings further describes the contribution of separate variables to a given PC, thus providing information on sample clustering. Very interestingly, the elimination of insignificant PCs leads to a partial removal of data noise. All these features makes PCA very appropriate for multivariate data description, visualization and interpretation, and thus it remains the gold standard model to handle complex and high-dimensional data sets (Daszykowski et al., 2007, 2003; Yamamoto et al., 2009; Tugizimana, 2012).

2.6.3.2 Response surface methodology

Response surface methodology (RSM) involves the use of mathematical and statistical tools in the development of an empirical and functional relationship between a response (output) variable and
a number of independent (control or input) variables (Khuri and Mukhopadhyay, 2010; Zulkiply, 2012). Besides, RSM is useful in concurrently studying the effects of different control variables on a response variable (Zulkiply, 2012). The mathematical principles of different approaches of RSM and the statistical implications thereof have been described in detail elsewhere (Bas and Boyaci, 2007; Bezerra et al., 2008; Azami, 2012). Amidst various approaches, the central composite design (CCD) is the most commonly used RSM technique as it offers the advantage of a reduced number of experimental runs for response optimization (i.e. it builds models without the need for a complete three-level factorial experiment) (Goncalves et al., 2006; Tamhane, 2014). This multivariate chemometric technique (CCD RSM) has been frequently used for optimization of different extraction processes for plant phytoconstituents (Nazari et al., 2007; Mirzajani et al., 2010; Shi et al., 2012).

2.7 CONCLUSION

From the literatures reviewed herein, it has been discerned that plants such as B. pilosa, though consumed for their nutritional benefits, also significantly contribute to health and wellbeing as they possess a wide variety of chemical compounds with vital biological functions (including medicinal properties). It was further shown that there is need for suitable analytical techniques to systematically study the phytochemical composition and characteristics of these plants to better exploit them. Extraction, which is a very important step in the study of phytochemicals has heretofore been deficient and constitutes a major area of research interest. Sequel to the challenges of conventional extraction techniques, inference from consulted literature indicate that PHWE is a viable alternative with results comparing favourably, and in some instances, better than other extraction techniques. It hence became necessary to look for ways of exploiting the numerous benefits of PHWE for the study of embedded phytochemicals in underexploited B. pilosa.

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Chapter Two


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CHAPTER THREE

PRESSURIZED HOT WATER EXTRACTION AND CHEMOMETRIC FINGERPRINTING OF FLAVONOIDS FROM Bidens pilosa BY UPLC-TANDEM MASS SPECTROMETRY

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Abstract

The need for greener extraction techniques that are quick and efficient has prompted the evolution of pressurized hot water extraction (PHWE). In the current study, the extraction of flavonoids from Bidens pilosa was demonstrated using PHWE at 50, 100 and 150°C. The extracts were analyzed on an ultra-performance liquid chromatography coupled with a quadrupole time-of-flight mass spectrometry (UPLC-qToF-MS/MS) and 28 different molecules belonging to different classes of flavonoids such as chalcones, flavonols and flavanones were identified. Our results clearly demonstrated that the extraction temperature of 150°C resulted in the highest yields of the identified molecules, amongst which include quercetin-3-O-glucuronide isomer, quercetin-3-hexose, okanin diacetylglucoside isomer, and okanin triacetylglucoside. Further analysis of the data using principal component analysis (PCA), a multivariate statistical model revealed differential distribution patterns of the identified molecules in extracts obtained at various temperatures. It was possible to clearly describe the distribution patterns of each molecule on the box-and-whiskers plot, which revealed best extraction conditions for each molecule. It was observed that the apparent differences in the distribution patterns of flavonoids was essentially a function of quantity rather than quality (i.e. selective extractability). In overall, the extraction yield increased proportionately with increasing temperature, hence 150°C was the optimal temperature for PHWE of flavonoids from B. pilosa. It
can thus be deduced that PHWE in combination with UPLC-qToF-MS/MS and pattern recognition chemometric models such as PCA is an excellent analytical approach for the extraction and analysis of flavonoids from plant tissues. This study also reiterates assertions that *B. pilosa* is a rich source of flavonoids.

**Key words:** Pressurized hot water, *Bidens pilosa*, flavonoids and principal component analysis.

**Introduction**

Plants have been known to be essential to life as they synthesize a wide variety of chemical compounds that perform important biological functions (Atanasov et al., 2015). *Bidens pilosa* is an underutilized plant species widely distributed all over the world (Grombone-Guaratini et al., 2005; Tereza, Mansanares, Semir, & Solferini, 2006). It is a rich source of food and medicine for humans and animals particularly in the tropics (Bairwa, Kumar, Sharma, & Roy, 2010; Bartolome, Villaseñor, & Yang, 2013). The plant contains a diversity of interesting metabolites, including hydroxycinnamic acids and flavonoids (Bartolome et al., 2013). *B. pilosa* is used in folklore medicine in the treatment of more than 40 diseases in man (Borges et al., 2013). Some of its important biological activities include antimicrobial (Silva et al., 2014), anticancer and anti-pyretic activity (Sundararajan et al., 2006), anti-oxidative activity (Yang et al., 2006), anti-inflammatory and anti-allergic characteristics (Horiuchi and Seyama, 2008), anti-diabetic tendency (Lai et al., 2015) and many other beneficial activities which have been reviewed in other studies (Bairwa et al., 2010; Bartolome et al., 2013).

The above-mentioned biological functions can be rationalized by the wide spectrum of metabolites detected in this plant (Silva et al., 2011; Bartolome et al., 2013). Heretofore, at least 201 compounds comprising flavonoids, aromatics, terpenoids and other compounds have been reported (Chiang et al., 2004; Grombone-Guaratini et al., 2005; Silva et al., 2011). A comprehensive list of identified metabolites from this plant have been compiled and provided by Silva et al. (2011). Flavonoids are however, the predominant class of phenolic metabolites in the *Bidens* genus (Chiang et al., 2004). They are quite a remarkable group of phytonutrients that are bioactive and play several different roles in the health of plants, animals and humans alike (Kris-Etherton et al., 2002; 2004). Understanding the metabolite composition of various underutilized plants is key to tapping
indigenous knowledge from them and exploiting their potential applications in pharmacology and folklore medicine.

Metabolite fingerprinting is a technological approach for providing information from chromatographic spectra of metabolites (Scholz, Gatzek, Sterling, Fiehn, & Selbig, 2004) that has been useful in biochemical and pharmaceutical studies of plants. Extraction is an empirical and important unit of operation in metabolite fingerprinting of plants as the quality of analytical results have often been directly linked to the extraction technique employed. Extraction has been shown to affect both quantitative and qualitative aspects of data generated (Song, Pranovich, & Holmbom, 2011; Khoza et al., 2014) as well as the reliability and consistency of the results (Tambellini, Zaremberg, Turner, & Weljie, 2013). Selection of an appropriate extraction technique is thus crucial particularly when considering the vast array of chemical species present in plants coupled with their individual physicochemical differences such as polarity and chemical stability.

Conventional extraction methods have been applied to extract plant metabolites for analysis. However, there are concerns on the potential health (human and environmental) implications associated with them. Essentially, these methods require large volumes of hazardous and environmentally unfriendly organic solvents (Vergara-Salinas et al., 2013) and, moreover, they are time consuming and laborious (Herrero, Cifuentes, & Ibañez, 2006). These shortcomings have propelled the evolution and adoption of greener and more efficacious techniques such as pressurized hot water extraction (PHWE) for the extraction of metabolites from plant tissues. At present, PHWE it is the most favored extraction technique with potentials to overcome these drawbacks (King, 2000). This technique is environmentally friendly and promises better selectivity as its solvation power can be manipulated over a wide spectrum of polarities. Moreover, it offers results that are comparable to (or even better than) those obtained when using conventional extraction methods, it is less expensive and requires shorter extraction times (Herrero et al., 2006). As an added advantage, PHWE utilizes water as the extraction solvent, which is readily available and compatible with most chromatographic instruments (Richter, Toral, & Toledo, 2006; Liang & Fan, 2013).

Pressurized hot water extraction is increasingly gaining attention in the biochemical and pharmaceutical industry, particularly for the extraction and analysis of plant metabolites (Mushtaq, Choi, Verpoorte, & Wilson, 2014). Heretofore, there have been no reports on the application of this technique for the extraction of flavonoids from B. pilosa. Interestingly, the phenomenon of PHWE
mimics those of traditional techniques for the preparation of food and herbal portions. In this study, a PHWE, an ecofriendly extraction technique was applied for the extraction and metabolite profiling of 28 flavonoids from *B. pilosa* using UPLC-qTof-MS/MS.

**Materials and methods**

**Materials**

Leaves and stems of *B. pilosa* plant used in this study were collected from different sites around the Venda region of Limpopo province (South Africa). Sample preparation followed procedures described by Khoza *et al.* (2014). The plant specimens were air-dried (< 10% moisture content) at ambient conditions in the dark for about 7 days and ground to powder (≤ 0.5 mm particle size) using a mortar and pestle. Solvents used in this study included UPLC/MS grade quality methanol and acetonitrile, purchased from Romil, MicroSep, South Africa. Ultrapure water was obtained from a Milli-Q Gradient A10 system (Millipore, Billerica, MA, USA), while analytical grade quality formic acid was purchased from Sigma Aldrich, Germany.

**Methods**

**Metabolite extraction**

Extraction of phytochemicals was achieved by a makeshift laboratory scale PHWE unit described in Khoza, Gbashi, Steenkamp, Njobeh, and Madala (2016). Pressurized hot water extraction of *B. pilosa* was conducted at temperatures of 50, 100 and 150°C, a pressure of 1000 ± 200 psi maintained using the back-pressure valve and extraction solvent (pure water) pumped at a flow rate of 5.0 mL/min for approximately 10 min. For the extraction, 4 g of homogenized ground plant materials was mixed with 2 g of diatomaceous earth (Sigma Aldrich, Germany), a dispersing agent and placed in an extraction cell located inside the oven with automatically regulated temperature (±1°C). The extracts were collected into sealed falcon tubes up to the 50 mL mark through the outlet coil immersed in a cooling water bath. The extracts were filtered using a 0.22 µm nylon syringe filter into a 2 mL HPLC vial and preserved at -20°C prior to analysis.

**Chromatographic separation and mass spectrometry (UPLC-qToF-MS)**

The chromatographic separation of compounds was performed on a UPLC hyphenated to a Synapt G1-qToF-MS instrument (Waters Corporation, Manchester, UK) equipped with a Waters Acquity

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HSS T3 C18 column (150 mm × 2.1 mm ID and of 1.8 µm particle size) and the column oven temperature maintained at 60°C. The mobile phases used were (A) 0.1% formic acid in deionized water; and (B) 0.1% formic acid in acetonitrile. The linear gradient program began with 2% A to 60% B for 24 min, ramped to 95% B at 25 min and kept constant for 2 min, then re-equilibrated at 5% B for 3 min. The total cycle runtime was 30 min with the mobile phases pumped at a flow rate of 0.4 mL/min.

Mass spectrometry was performed using a Waters qToF-MS instrument (Waters Corporation, Manchester, UK) fitted with electrospray ionization (ESI) source operated in both positive and negative ion electrospray modes. The m/z range was 100-1000 Da, scan time 0.2 sec, interscan delay 0.02 sec, with leucine encephalin (556.3 µg/mL) as a lock mass, standard flowrate 0.1 mL/min, and mass accuracy window of 5.0 mDa was used for MS data acquisition. Moreover, the instrument was operated on the following settings: collision energy of 3 eV, capillary voltage of 2.5 kV, sample cone voltage of 30V, detector voltage of 1650 V (1600 V in negative mode), source temperature at 120°C, cone gas flow at 50 (L/h), and desolvation gas flow at 550 (L/h). To achieve metabolite fragmentation patterns necessary for annotation or identification, the collision energy during MS acquisition was experimentally changed in the trap ion optics by acquiring data with various collision energy levels to generate typical MS<sup>E</sup> fragmentation patterns.

Data analyses and identification of flavonoid compounds

Raw data acquired from UPLC-qToF-MS/MS was entered into the MarkerLynx XS application software (Waters Corporation, Manchester, UK) for analysis and visualization. For maximum data output, the analysis was carried out using optimized parameters (Khoza et al., 2014). Here, only negative data were analyzed using similar optimized parameters, i.e., mass range 100-1000 with mass tolerance of 0.02 Da, retention time (Rt.) and Rt. window of 1-30 min and 0.2 min, respectively, whereas other parameters were automatically calculated.

Characterization of single components was performed via retention time and accurate molecular masses. Representative single ion monitoring (SIM) chromatograms for target molecules were generated using their m/z values. Moreover, various MS spectra for these molecules were obtained from the chromatograms, their fragmentation patterns observed, and molecular formulae calculated on the basis of a 5 ppm mass accuracy range. This information was used to confirm the identities of
these bio-markers following a search on the Dictionary of Natural Products (DNP) online database (dnp.chemnetbase.com/) and the KNAPSAcK metabolite information (KMI) database (http://kanaya.naist.jp/knapsack.jsp/top.html). Extraction yields for molecules identified represented the relative peak intensity figures of molecular peaks corresponding to the identified molecules. Relative peak intensity is a dimensionless quantity, and corresponded to the area-under-the-peak values obtained from the peak list. This data file (peak list) is the final output obtained after processing of the MS data using MarkerLynx software (Barbarini & Magni, 2010; Khoza et al., 2015).

**Statistical analysis**

The extraction yield patterns of each identified metabolite across the extraction temperature profile was graphically described by the Box-and-whiskers plots using IBM SPSS software version 22 (SPSS/IBM, Chicago, Illinois) (Khoza et al., 2015). A one-way analysis of variance (ANOVA) was performed to test for differences in the recovery patterns of identified metabolites across the different extraction temperatures using the above mentioned statistical software. Mean values of extraction yield were compared by Tukey’s post hoc test and means were deemed significantly different if the level of probability was ≤ 0.05 indicated on the Box-and-whisker plots (Khoza et al., 2015). In order to perform multivariate data analysis, i.e., principal component analysis (PCA), the subsequent data matrix obtained from MarkerLynx XS software was exported to SIMCA-P software version 12.0 (Umetrics, Sweden). Unless stated otherwise, all PCA models were Pareto scaled. From the PCA loadings plot, metabolites of which the levels were affected by temperature during extraction were also selected.

**Results and discussion**

Table 1 provides a list of flavonoids identified in extracts of *B. pilosa*. It was possible to identify 28 flavonoid metabolites including their respective positional glycosidic isomers. Characterization of the flavonoid metabolites was achieved using the MS fragmentation patterns and order of elution from the UHPLC chromatograms (Madala, Tugizimana, & Steenkamp, 2014). Their identities were confirmed using the DNP and KMI databases and various other literature reports in an approach previously reported (Madala, Steenkamp, Piater, & Dubery, 2013; Khoza et al., 2015). In view of

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that, Molecule (Mol.) 1 at Rt 15.13 min with $m/z$ 461.0698 [M-H]$^-$ and MS$^2$ fragment at $m/z$ 285.0370 obtained after loss of 176 amu (glucurone unit) (Table 1) was tentatively identified as kaempferol-3-O-glucuronide (Kajdžanoska, Gjamovski, & Stefova, 2010). Mol. 2 at Rt 15.18 min was tentatively identified as kaempferol-3-O-glucoside with $m/z$ 447.0927 [M-H]$^-$ and fragment at $m/z$ 285.0360 obtained due to loss of a hexose moiety (162 amu) (Kajdžanoska et al., 2010). Mols. 3 & 4 at Rt of 15.60 and 15.90 min, respectively, also showed similar fragmentation patterns as Mol. 2 and as such, these three molecules were identified as either geometrical or regional isomers of caftaric acid hexose. Mol. 5 at retention 16.47 min was identified to be kaempferol-3-acetyl-glycoside with $m/z$ 489.0989 [M-H]$^-$ and an MS spectrum showing product ion $m/z$ 285.0350 (after loss of 204 amu: acetyl-hexose) (Kajdžanoska et al., 2010; Khoza et al., 2015; Ramabulana et al., 2015). Mols. 6, 7 & 8 had the same fragmentation patterns as Mol. 5 and thus, they were identified as isomers.

Mol. 9 at Rt 11.36 had $m/z$ 653.0947 [M-H]$^-$ and fragment ions at $m/z$ 477.0663 (due to loss of a glucuronyl unit, 176 amu) and $m/z$ 301.0303, which indicates the aglycone quercetin and results from a further loss of 176 as indicative of a quercetin diglucuronide with the glucuronyl moieties attached at different positions on the flavonol ring. Furthermore, if the two glucuronyl moiety had been attached to the same position, the formation of an M-17 fragment at $m/z$ 477 would have been improbable, as it has been observed that when anthocyanin disaccharide conjugates fragment, they do so with the loss of an intact disaccharide unit (Giusti, Rodriguez-Saona, Griffin, & Wrolstad, 1999). Hence, the molecule was tentatively identified as quercetin 3,7-diglucuronide (Mullen, 2009).
### Table 3.1: Identified flavonoid metabolites extracted from *B. pilosa* using PHWE

<table>
<thead>
<tr>
<th>Mol. no</th>
<th>Compound name</th>
<th>Rt.</th>
<th>m/z</th>
<th>Molecular formula</th>
<th>Fragment ions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Kaempferol-3-O-glucuronide</td>
<td>15.13</td>
<td>461.0698</td>
<td>C_{21}H_{19}O_{12}</td>
<td>285.0370</td>
<td>Kajdžanoska, Gjamovski, and Stefova (2010)</td>
</tr>
<tr>
<td>2.</td>
<td>Kaempferol-3-O-glucoside</td>
<td>15.18</td>
<td>447.0927</td>
<td>C_{27}H_{30}O_{15}</td>
<td>285.0360</td>
<td>Kajdžanoska et al. (2010); Ramabulana et al. (2015)</td>
</tr>
<tr>
<td>3.</td>
<td>Kaempferol-3-O-glucoside isomer 1</td>
<td>15.60</td>
<td>447.0890</td>
<td>C_{27}H_{30}O_{15}</td>
<td>285.0324</td>
<td>Kajdžanoska et al. (2010); Ramabulana et al. (2015)</td>
</tr>
<tr>
<td>4.</td>
<td>Kaempferol-3-O-glucoside isomer 2</td>
<td>15.90</td>
<td>447.0907</td>
<td>C_{27}H_{30}O_{15}</td>
<td>285.0348</td>
<td>Kajdžanoska et al. (2010); Ramabulana et al. (2015)</td>
</tr>
<tr>
<td>5.</td>
<td>Kaempferol-3-acetyl-glycoside isomer 1</td>
<td>16.47</td>
<td>489.0989</td>
<td>C_{23}H_{22}O_{12}</td>
<td>285.0350</td>
<td>Ramabulana et al. (2015)</td>
</tr>
<tr>
<td>6.</td>
<td>Kaempferol-3-acetyl-glycoside isomer 2</td>
<td>16.54</td>
<td>489.0970</td>
<td>C_{23}H_{22}O_{12}</td>
<td>285.0356</td>
<td>Ramabulana et al. (2015)</td>
</tr>
<tr>
<td>7.</td>
<td>Kaempferol-3-acetyl-glycoside isomer 3</td>
<td>16.93</td>
<td>489.1066</td>
<td>C_{23}H_{22}O_{12}</td>
<td>285.0382</td>
<td>Ramabulana et al. (2015)</td>
</tr>
<tr>
<td>8.</td>
<td>Kaempferol-3-acetyl-glycoside isomer 4</td>
<td>17.15</td>
<td>489.1026</td>
<td>C_{23}H_{22}O_{12}</td>
<td>285.0356</td>
<td>Ramabulana et al. (2015)</td>
</tr>
<tr>
<td>9.</td>
<td>Quercetin 3,7-diglucuronide</td>
<td>11.36</td>
<td>653.0947</td>
<td>C_{27}H_{30}O_{19}</td>
<td>301.0307, 477.0663</td>
<td>Mullen (2009)</td>
</tr>
<tr>
<td>10.</td>
<td>Quercetin-3-rhamnosylhexoside isomer 1</td>
<td>14.59</td>
<td>609.1440</td>
<td>C_{27}H_{30}O_{16}</td>
<td>300.0235</td>
<td>Ramabulana et al. (2015)</td>
</tr>
<tr>
<td>11.</td>
<td>Quercetin-3-rhamnosylhexoside isomer 2</td>
<td>14.70</td>
<td>609.1426</td>
<td>C_{27}H_{30}O_{16}</td>
<td>300.0212</td>
<td>Ramabulana et al. (2015)</td>
</tr>
<tr>
<td>12.</td>
<td>Quercetin-3-O-gluconoride isomer 1</td>
<td>14.80</td>
<td>477.0657</td>
<td>C_{21}H_{15}O_{13}</td>
<td>301.0311</td>
<td>Mullen (2009)</td>
</tr>
<tr>
<td>13.</td>
<td>Quercetin-3-O-gluconoride isomer 2</td>
<td>14.93</td>
<td>477.0621</td>
<td>C_{21}H_{15}O_{13}</td>
<td>301.0362</td>
<td>Mullen (2009)</td>
</tr>
<tr>
<td>14.</td>
<td>Quercetin-3-glycoside isomer 1</td>
<td>14.88</td>
<td>463.0827</td>
<td>C_{21}H_{15}O_{12}</td>
<td>301.0327</td>
<td>Khoza et al. (2015)</td>
</tr>
<tr>
<td>15.</td>
<td>Quercetin-3-glycoside isomer 2</td>
<td>15.03</td>
<td>463.0847</td>
<td>C_{21}H_{15}O_{12}</td>
<td>300.0234</td>
<td>Khoza et al. (2015)</td>
</tr>
<tr>
<td>16.</td>
<td>Okanin triacetylglucoside isomer 1</td>
<td>18.85</td>
<td>575.1299</td>
<td>C_{27}H_{28}O_{14}</td>
<td>135.0397, 150.9949, 287.0512</td>
<td>KMI; Hoffmann and Hözl (1988); Harborne and Baxter (1999)</td>
</tr>
<tr>
<td>17.</td>
<td>Okanin triacetylglucoside isomer 2</td>
<td>19.13</td>
<td>575.1305</td>
<td>C_{27}H_{28}O_{14}</td>
<td>135.0408, 150.9975, 287.0493</td>
<td>KMI; Hoffmann and Hözl (1988); Harborne and Baxter (1999)</td>
</tr>
<tr>
<td>Mol. no</td>
<td>Compound name</td>
<td>Rt.</td>
<td>m/z</td>
<td>Molecular formula</td>
<td>Fragment ions</td>
<td>Reference</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------------------------------------</td>
<td>------</td>
<td>----------</td>
<td>-------------------</td>
<td>---------------------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>18.</td>
<td>Okanin triacetylglucoside isomer 3</td>
<td>19.24</td>
<td>575.1339</td>
<td>C_{27}H_{28}O_{14}</td>
<td>135.0394, 150.9981, 287.0530</td>
<td>KMI; Hoffmann and Hölzl (1988); Harborne and Baxter (1999)</td>
</tr>
<tr>
<td>19.</td>
<td>Tetrahydroxyflavanone triacetylglucoside isomer 1</td>
<td>19.92</td>
<td>575.1364</td>
<td>C_{27}H_{28}O_{14}</td>
<td>135.0399, 151.0002, 285.0342</td>
<td>KMI; Wang, Yang, Lin, and Sun (1997)</td>
</tr>
<tr>
<td>20.</td>
<td>Tetrahydroxyflavanone triacetylglucoside isomer 2</td>
<td>20.12</td>
<td>575.1345</td>
<td>C_{27}H_{28}O_{14}</td>
<td>135.0401, 150.9989, 285.0344</td>
<td>KMI; Wang et al. (1997)</td>
</tr>
<tr>
<td>21.</td>
<td>Tetrahydroxyflavanone triacetylglucoside isomer 3</td>
<td>20.25</td>
<td>575.1353</td>
<td>C_{27}H_{28}O_{14}</td>
<td>135.0413, 151.0007, 285.0439</td>
<td>KMI; Wang et al. (1997)</td>
</tr>
<tr>
<td>22.</td>
<td>Okanin diacetylglucoside isomer 1</td>
<td>16.88</td>
<td>533.1280</td>
<td>C_{25}H_{26}O_{13}</td>
<td>135.0409, 151.0061, 287.0524</td>
<td>KMI; Hoffmann and Hölzl (1988); Harborne and Baxter (1999)</td>
</tr>
<tr>
<td>23.</td>
<td>Okanin diacetylglucoside isomer 2</td>
<td>17.01</td>
<td>533.1307</td>
<td>C_{25}H_{26}O_{13}</td>
<td>135.0399, 150.9968, 287.0496</td>
<td>KMI; Hoffmann and Hölzl (1988); Harborne and Baxter (1999)</td>
</tr>
<tr>
<td>24.</td>
<td>Okanin diacetylglucoside isomer 3</td>
<td>17.10</td>
<td>533.1259</td>
<td>C_{25}H_{26}O_{13}</td>
<td>135.0377, 151.0032, 287.0507</td>
<td>KMI; Hoffmann and Hölzl (1988); Harborne and Baxter (1999)</td>
</tr>
<tr>
<td>25.</td>
<td>Okanin diacetylglucoside isomer 4</td>
<td>17.34</td>
<td>533.1229</td>
<td>C_{25}H_{26}O_{13}</td>
<td>135.0404, 150.9977, 287.0513</td>
<td>KMI; Hoffmann and Hölzl (1988); Harborne and Baxter (1999)</td>
</tr>
<tr>
<td>26.</td>
<td>Okanin diacetylglucoside isomer 5</td>
<td>17.45</td>
<td>533.1220</td>
<td>C_{25}H_{26}O_{13}</td>
<td>135.0399, 151.0006, 287.0488</td>
<td>KMI; Hoffmann and Hölzl (1988); Harborne and Baxter (1999)</td>
</tr>
<tr>
<td>27.</td>
<td>Tetrahydroxyflavanone diacetylglucopyranoside isomer 1</td>
<td>18.39</td>
<td>533.1205</td>
<td>C_{25}H_{26}O_{13}</td>
<td>135.0397, 150.9952, 287.0498</td>
<td>DNP; Li, Kuang, Okada, and Okuyama (2005); Yang et al. (2012)</td>
</tr>
<tr>
<td>28.</td>
<td>Tetrahydroxyflavanone diacetylglucopyranoside isomer 2</td>
<td>18.52</td>
<td>533.1263</td>
<td>C_{25}H_{26}O_{13}</td>
<td>135.0394, 150.9995, 287.0593</td>
<td>DNP; Li et al. (2005); Yang et al. (2012)</td>
</tr>
</tbody>
</table>

**Key:**  Mol. – Molecule; Rt. – Retention time; m/z – mass-to-charge ratio; KMI – KNApSAcK Metabolite Information; DNP – Dictionary of natural products.
Mols. 10 & 11 at Rt 14.59 and 14.70 min, respectively, were annotated as quercetin-3-rhamnosylhexoside with a precursor ion at m/z 609 [M-H]⁻ and a product ions at m/z 300.0235 and 300.0212 respectively, a quercetin aglycone [H-H-309] following the loss of a rutinodide sugar (Berger, Küchler, Maaßen, Busch-Stockfisch, & Steinhart, 2007; Abu-Reidah, Arráez-Román, Lozano-Sánchez, Segura-Carretero, & Fernández-Gutiérrez, 2013). To further deduce the sequence of the sugar, the positive ionization data was referenced since it provided results with more information. The MS spectrum in the positive mode showed a precursor ion at 611 [H+H]+ and fragment ions at 465 [H+H]+ and 303[H+H]+, and an adduct ion at m/z 633 [H+H]+ (Martucci, De Vos, Carollo, & Gobbo-Neto, 2014; Ramabulana et al., 2015). Mol. 12 at Rt 14.80 min was identified as quercetin monoglucuronide with a precursor ion at m/z 477.0621 [M-H]⁻ and a fragment ion at m/z 301.0311 (M-176 amu) (Mullen, 2009). Mol. 13 at Rt 14.93 had a similar fragmentation pattern as Mol. 12 and as such, they could be regarded as isomers.

Mol. 14 at Rt 14.88 was the parent compound quercetin-3-glycoside with a precursor ion at m/z 463.0847 [M-H]⁻ and MS² ion at m/z 301.0327 corresponding to the loss of a hexose molecule (Abu-Reidah et al., 2013; Khoza et al., 2015). Mol. 15 at Rt 15.03 min had a similar fragmentation pattern as Mol. 14 and as such it is considered an isomer of quercetin-3-glycoside. Mols. 16 to 21 were considered isobaric species as they contained a similar m/z value (575 [M-H]⁻), close retention times, and almost analogous fragmentation patterns (Table 1). However, due to the efficiency of our extraction method and its compatibility with highly advance chromatographic separation instrumentations (that have high MS¹ and MS² resolution precursor ion selection capabilities), it was possible to unambiguously distinguish between these molecules on single ion monitoring (SIM) chromatograms and subsequently annotate them accordingly. Consequently, Mols. 16, 17 & 18 at Rts 18.85, 19.13, 19.24, respectively, were considered isomers of okanin triacetylglucoside with a precursor ion at m/z 575 [M-H]⁻ and MS² ions at m/z 135, 150, and 287, respectively. These molecules have previously been isolated from B. pilosa (Hoffmann & Hölzl, 1988; Harborne & Baxter, 1999). Molecules 19, 20 & 21 at Rts 19.92, 20.12 and 20.25 min, respectively, had similar fragmentation patterns with m/z 575 [M-H]⁻ and product ions, respectively, at m/z 135, 150, and 285. As such, they were considered isomers of tetrahydroxyflavanone triacetylglucoside (Wang, Yang, Lin, & Sun, 1997).
Mols. 22 to 28 had the same \( m/z \) value of 533 [M-H]⁻ and similar fragmentation patterns with fragment ions at \( m/z \) 135, 150 and 287. A closer look at the single ion monitoring (SIM) chromatograms, indicated that these molecules could be isobaric species. Hence, Mols. 22 to 26 at Rts 16.88, 17.01, 17.10, 17.34 and 17.45 min, respectively, were identified as isomers of okanin- di-O-acetylglucoside. This molecule has been previously described in \( B. \) pilosa (Hoffmann & Hölzl, 1988; Harborne & Baxter, 1999). Mols. 27 and 28 at Rts 18.39 and 18.52 min, respectively, are considered isomers of tetrahydroxyflavanone diacetylglucopyranoside. This molecule have been previously isolated from a Bidens specie i.e. \( B. \) bipinnata Linn. (Li, Kuang, Okada, & Okuyama, 2005; Yang et al., 2012).

As can be seen from the fragmentation patterns of these compounds, they are structurally diverse with possibly different physicochemical properties. This reveals that PHWE is efficacious for extracting diverse flavonoids from \( B. \) pilosa which is in agreement with the data obtained by Khoza et al. (2014), demonstrating the successful extraction of flavonoids from \( M. \) foetida using PHWE. The polarity of pressurized hot water (PHW) can easily be manipulated to vary over an extended temperature range just by varying the temperature thereof (Chemat, Vian, & Cravotto, 2012). However, in order to ensure efficient extraction of the assorted flavonoids in \( B. \) pilosa using PHWE, a previously optimized temperature profile, i.e., range of 50 to 150°C was adopted (Khoza et al., 2014). Water at ambient temperature and pressure is more suitable in extracting polar compounds due to its relatively high dielectric constant (i.e. \( \varepsilon=80 \) at 25°C at \( 10^5 \) Pa) (Cabane & Vuilleumier, 2005; Kruse & Dinjus, 2007). However, as the temperature of water increases, its polarity which directly links to its dielectric constant decreases (from \( \varepsilon=53 \) at 110°C to \( \varepsilon=36.5 \) at 190°C) to the ranges of that of organic solvents such as methanol (\( \varepsilon = 32.6 \) at 25°C), thus dissolving a wide range of low and medium polarity analytes (Anekpankul, Goto, Sasaki, Pavasant, & Shotipruk, 2007; Teo et al., 2010). Moreover, the selective extractability of some of the flavonoid molecules during PHWE can be linked to their different structural and physicochemical properties. Elsewhere, it has been shown that there is a relationship between the structure of flavonoid molecules and temperature conditions during SWE (Ko, Cheigh, & Chung, 2014). Structural configuration such as the presence of double bonds, sugar, polarity of side and the number of carbon atoms in the
side groups can ultimately determine the extractability of PHWE (Carr, Mammucari, & Foster, 2011; Ko et al., 2014).

Figures 3.1.1 & 3.1.2 show the distribution patterns of some of the identified molecules on a box-and-whiskers plot. Although, all of these molecules were clearly identified by the peak-picking software, it became difficult to accurately annotate some of them (Mols. 17, 21, 24, 25 & 28) on the peak list obtained after processing the data matrix using MarkerLynx XS software, despite following previously optimized and established parameters (see Appendix 3.1). The reason for this phenomenon is unclear, however, it seems possible that our robust and sensitive tandem MS approach (UHPLC-qTOF-MS) is a step ahead when making use of our chemometric data analysis software responsible for processing (involving preprocessing, peak selection, peak deisotoping and deconvolution) the data matrix. However, some of the omitted ions share similar precursor ion and fragmentation patterns suggesting that they are isomers of one another.

In any case, following ANOVA results, it was possible to indicate on the plots temperature conditions that resulted in significantly different (p ≤ 0.05) yield patterns for each molecule. It can be seen that the yields of these molecules are strongly influenced by temperature conditions. Generally, flavonoid yield increased with increase in extraction temperature. The highest extraction yields were obtained at temperatures of 150°C and included the following molecules in decreasing order; Mol. 13 (Quercetin-3-O-gluconoride isomer 2), Mol. 15 (Quercetin-3-glycoside isomer 2), Mol. 27 (Tetrahydroxyflavanone diacetylglucoside isomer 1), Mol. 16 (Okanin triacetylglucoside) and Mol. 14 (Quercetin-3-glycoside). It was thus evident that quercetin and okanin were the most abundant among the identified aglycones in this plant. In a previous study by Ko, Cheigh, Cho, & Chung (2011), it was observed that the highest yield of flavonoids (quercetin) was obtained at a temperature of 165°C during PHWE otherwise called subcritical water extraction (SWE) of flavonoids from onion skin. Elsewhere, Cheigh, Chung, & Chung (2012) reported maximum yields of flavonoids (up to 99% of the total amount originally present) at an extraction temperature of 160°C during SWE of citrus peels.
Box-and-whiskers followed by different marker shape (and colour) are significantly different ($p \leq 0.05$).

Order of significance:  

Figure 3.1.1: Box-and-Whiskers plots showing the yield distribution patterns for some of the identified flavonoids.
Figure 3.1.2: Box-and-whiskers plots showing the yield distribution patterns for some of the identified flavonoids.
In order to further comprehend the effect of temperature on the relative flavonoid content of *B. pilosa* plant interpret the patterns within the data obtained from PHWE, we adopted a metabolite fingerprinting approach coupled to the chemometric principal component analysis (PCA). This technique is used to emphasize variations that bring out strong patterns in high dimensional dataset. It identifies patterns in the data and expresses the data by highlighting their similarities and differences (Jolliffe, 2002). Data generated in chemometric fingerprinting studies are usually large and highly dimensional, and since finding patterns in such data is hard using ordinary statistical models, PCA is an appropriate tool for analyzing this kind of data (Khoza et al., 2014; 2015). The PCA score plot indicates significantly different flavonoid distribution patterns amongst extracts based on extraction temperature profile (Figure 3.2.1), which reiterates prior observations that temperature is crucial during PHWE of flavonoids.

The PCA loadings plot (Figure 3.2.2) was used to identify the relationship between the temperature profile and the extracted metabolites in order to comprehend the tight grouping patterns observed in the PCA score plots. Hence, it was observed that the differential clustering into distinct groups on the PCA scores plot was due to the unique effect each extraction temperature had on the extractability of each molecule and that molecules with similar physicochemical characteristics had analogous distribution patterns. During PHWE of *Momordica foetida*, Khoza et al. (2015) also observed that different temperature conditions resulted in distinctive extraction patterns for different types of flavonoids. Overall, the PCA model provided symbolic representations from which the variations in metabolite profile due to temperature change could be conveniently visualized and interpreted.
Figure 3.2.1: PCA score plots based on UPLC tandem MS chromatograms from negative ionization spectra data showing various clustering patterns of *B. pilosa* extracted at different temperatures using PHWE.

Figure 3.2.2: PCA loadings plots based on UPLC tandem MS chromatograms from negative ionization spectra data showing the dimensional subspatial orientation of identified flavonoid molecules relative to the different extraction temperatures during PHWE of *B. pilosa*.
Furthermore, to visualize any potential outliers, hierarchical clustering analysis (HCA) was also generated. From the HCA dendrogram (Figure 3.3), it can be seen that at least one sample from 100°C extracts was an outlier, with characteristics similar to extracts obtained at both 50°C and 100°C. This could have been caused by the sample being the first one to be extracted just after extraction at 50°C when the temperature was still not sufficiently distributed across the entire heating system. Moreover, orthogonal partial least squares discriminant analysis (OPLS-DA) was also performed on the data set (Appendixes 3.2, 3.3 & 3.4), however, this added very little additional information but supported the clustering patterns as seen on the PCA score plot (Figure 3.2.1) and also re-affirms the distribution of some of the metabolites across extracts obtained by various temperatures, a phenomenon which is currently well depicted on the box-and-whiskers plots (Figure 3.1.1 & 3.1.2).

Figure 3.3: HCA dendrogram showing the degree of similarity/dissimilarity amongst extracts obtained at different extraction temperatures using the data's full dimensionality as obtained from the UPLC-qTOF-MS/MS chromatograms.

The dependency of flavonoid yield on temperature is due to their increased solubility in PHW as water temperature increases (Khoza et al., 2015). Moreover, the supplied heat energy increases the rate of diffusion of the molecules, as well as weakens the intermolecular forces within plant tissues, thus lowering the activation energy needed for the desorption process (Teo, Tan, Yong, Hew, & Ong, 2010). High pressures aid in extraction by disrupting tissue configuration and forcing water to
permeate matrix areas (pores) where water at lower pressures may not normally reach (Richter et al., 1996).

Though, extraction efficiency increases with an increase in temperature, extreme temperatures could result in degradation of flavonoids and similar polycyclic aromatic hydrocarbons (Andersson, Hartonen, Hyotylainen, & Riekkola, 2003; Yang & Hildebrand, 2006; Khoza et al., 2014) as well as possible oxidation of metabolites (Ko et al., 2014), hence temperature of 150°C was not exceeded. For example, we observed that 3 of the 28 identified metabolites (Molecules 1, 6, and 9) were best extracted at a temperature of 100°C rather than at 150°C. This could be as a result of thermal degradation of these metabolites. In a study on the effect of temperature on PHWE of pharmacologically important metabolites from *Moringa oleifera*, Khoza et al. (2014) observed that yields of some flavonoid molecules were adversely affected by increase in temperature, such that extraction temperature optimization was essential for efficiency and “pharmacological potency” of the extracts.

A number of studies have linked different flavonoids with various medicinal and pharmacological activities (Pandey, 2007; Kumar, Gupta, & Pandey, 2013; Kumar & Pandey, 2013). These activities have been suggested to be dependent on the structural configuration of the flavonoid molecule (Heim, Tagliaferro, & Bobilya, 2002; Kumar & Pandey, 2013). Interestingly, some of these flavonoids (*i.e.*, quercetin) have already been used in clinical trials (Hirpara, Aggarwal, Mukherjee, Joshi, & Burman, 2009). Quercetin, the most abundant flavonoid aglycone identified in our extracts have been suggested to be hapatoprotective (Tapas, Sakarkar, & Kakde, 2008; Kumar & Pandey, 2013) as well as regulate cell death pathways and proliferation of cancerous cells (Hirpara et al., 2009). Okanins, a dominant chalcone flavonoid in the genus *Bidens* has been reported to possess potent anti-cancer and antibacterial properties (Makita et al., 1996; Cushnie & Lamb, 2005). Various biological functions such as anti-cancer activity in several human cancer cell lines and inhibition of oxidative stress in animal and plants cells have been directly linked with kaempferol (Leung et al., 2007; Marfe et al., 2009; Berger et al., 2013). Essentially, we were able to extract various groups of important flavonoids from a highly potent medicinal plant (*B. pilosa*) using a green and efficient extraction method (PHWE). This flavonoid-rich herb has previously been linked with various folk
medications in Korea, China and Southern Africa (Kil et al., 2011; Arthur, Naidoo & Coopoosamy, 2012).

Conclusion

This study demonstrated PHWE of 28 flavonoid molecules from B. pilosa, an underutilized herbal and food plant found abundantly in South Africa and some tropical regions of the world. The results reported herein show that PHWE is a feasible green technique for extracting different flavonoids with diverse structural and physicochemical properties. This simple technique is cheap, easy to adopt and utilizes water as the extraction solvent. Moreover, the dynamics of PHWE tends to parallel common food processing operations such as blanching, as well as other hydrothermal processes associated with preparation of herbal concoctions and food rations for consumption. The essence is to depict a true-as-possible reflection of the ethnopharmacological exposure of the layman (user of traditional medicine) who does not have access to the sophisticated methods for metabolite extraction scientists usually employ in the laboratory. Lastly, the results from this study reiterates B. pilosa as a rich source of flavonoids and that UPLC-qTOF-MS instrumentation, in combination with PCA, is a suitable omics profiling approach for the analysis of flavonoids and other metabolites in plants.

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Appendix 3.1: Representative single ion monitoring (SIM) chromatograms showing the elution of the different molecules that could not be defined after processing of the UPLC tandem MS data using MarkerLynx XS software: Molecule 17 (A), Molecule 21 (B), Molecule 24 (C), Molecule 25 (D) and Molecule 28 (E).
Appendix 3.2: OPLS-DA plots based on UPLC-qTOF-MS/MS chromatograms from negative ionization data showing the distribution of identified metabolites obtained at 50°C and 100°C.

Appendix 3.3: OPLS-DA plots based on UPLC-qTOF-MS/MS chromatograms from negative ionization data showing the distribution of identified metabolites obtained at 50°C and 150°C.
Appendix 3.4: OPLS-DA plots based on UPLC-qTOF-MS/MS chromatograms from negative ionization data showing the distribution of identified metabolites obtained at 100°C and 150°C.
CHAPTER FOUR

THE EFFECT OF TEMPERATURE AND METHANOL-WATER MIXTURE ON PRESSURIZED HOT WATER EXTRACTION (PHWE) OF ANTI-HIV ANALOGUES FROM Bidens pilosa

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Abstract

Background: Pressurized hot water extraction (PHWE) technique has recently gained much attention for the extraction of biologically active compounds from plant tissues for analytical purposes, due to its limited use of organic solvents, cost-effectiveness, ease-of-use and efficiency. An increase in temperature results in higher yields, however, issues with degradation of some metabolites (e.g. di-acylated cinnamic acid derivatives) when PHWE is conditioned at elevated temperatures has greatly limited its use. In this study, we considered possibilities of optimizing PHWE of some specific functional metabolites from Bidens pilosa using solvent compositions of 0, 20, 40, & 60% methanol and a temperature profile of 50, 100, and 150°C.

Results: The extracts obtained were analyzed using UPLC–qToF-MS/MS and the results showed that both temperature and solvent composition were critical for efficient recovery of di-acylated cinnamic acid derivatives, i.e., dicaffeoylquinic acid (diCQA) and chicoric acid (CA), which are known to possess anti-HIV properties. It was also possible to extract different isomers (possibly
cis-geometrical isomers) of these molecules. Significantly differential (p≤0.05) recovery patterns corresponding to the extraction conditions were observed as recovery increased with increase in methanol composition as well as temperature. The major compounds recovered in descending order were 3,5-diCQA with relative peak intensity of 204.23±3.16 extracted at 50°C and 60% methanol; chicoric acid (141.00±3.55) at 50°C and 60% methanol; 4,5-diCQA (108.05±4.76) at 150°C and 0% methanol; 3,4-diCQA (53.04±13.49) at 150°C and 0% methanol; chicoric acid isomer (40.01±1.14) at 150°C and 20% methanol; and cis-3,5-diCQA (12.07±5.54) at 100°C and 60% methanol. Fitting the central composite design response surface model to our data generated well-fitted models with R² values ranging from 0.57 to 0.87. Accordingly, it was possible to observe on the response surface plots the effects of temperature and solvent composition on the recovery patterns of these metabolites as well as to establish the optimum extraction conditions. Furthermore, the Pareto charts revealed that methanol composition had a stronger effect on extraction yield than temperature.

**Conclusion:** Using methanol as a cosolvent resulted in significantly higher (p≤0.05) even at temperatures as low as 50°C, thus undermining the limitation of thermal degradation at higher temperatures during PHWE.

**Keywords:** Pressurized hot water extraction, co-solvent, *Bidens pilosa*, di-acylated cinnamic acids, response surface modeling

**Background**

Plants constitute a vital part of the world’s primary health care [1]. *Bidens pilosa*, an underutilized plant species is a member of the Asteraceae family [2,3] widely distributed around the world [4]. It is rich in phenolic compounds that are of great medical significance [5,6]. More interestingly, *B. pilosa* has been shown to exhibit strong anti-HIV properties [7,8], a consequence of its rich composition of pharmacoactive metabolites, including various di-acylated cinnamic analogues [1,6-8]. As with other bioactive compounds, research is still ongoing to develop suitable techniques to extract these compounds from vegetal tissues. This continual quest for efficient and safe methods of extraction has propelled the evolution and adoption of pressurized hot water
Chapter Four

extraction (PHWE). Conventional organic solvent extraction techniques elicit issues of safety, they are labourious and also time-consuming [9,10]. Often referred to as subcritical water extraction [11], PHWE is an efficient and greener method for the extraction of bioactive compounds from plant materials [10,11]. It is particularly advantageous because water is cheap, readily available, and environmentally friendly [12]. Moreover, PHWE is a less sophisticated and an easy-to-use technology, requiring less time and expertise compared to conventional methods of extraction [13]. However, a major setback to this ingenious system has been the thermal degradation phenomenon observed at elevated temperatures for certain compounds [14–17], hence the need for optimization [18]. Amidst possible optimization approaches [19,20], the principle of co-solvency seems particularly promising in terms of enhanced extraction efficiency [21–24]. Accordingly, methanol has been recommended for pressurized liquid extraction [25]. It is 100% miscible with water and has a high solvation power for marker compounds compared to other solvents [26,27]. A study comparing the effectiveness of methanol and ethanol as cosolvents during supercritical fluid extraction have also reported the superior performance of methanol over ethanol [28]. This was also corroborated by Pinho and Macedo (2005) who observed that water-methanol mixture had a higher solvation power than its corresponding ethanol counterpart [29]. Furthermore, methanol is cheaper and readily available, thus could offer a good option as a cosolvent during PHWE. In this study, we investigated the effect of different compositions of methanol-water mixture and temperature conditions on PHWE of different isomers of diCQA and chicoric acid (CA) (anti-HIV analogues) from an underutilized plant, B. pilosa.

Experimental section

Plant Materials and Metabolite Extraction

*Bidens pilosa* plant materials were collected from the Venda region of Limpopo province (South Africa). Sample preparation and extraction followed procedures described by Khoza *et al.* [14]. The plant materials were air-dried (< 10% moisture content) at ambient conditions in a dark and well-ventilated room for 7 days after which, they were crushed to powder (≤ 0.5 mm) using a mortar and pestle. Extraction of phytochemicals was achieved by a makeshift laboratory scale PHWE unit [11]. The system consisted of a HPLC pump (Waters 6000 fluid controller, Waters
Corporation, Manchester, UK), stainless steel extraction cell (70 x 30 mm and approximately 20 mL) fitted with a metal frit i.e. filter (3/8 inches diameter, 1/32 inches thickness and 2.0 µm pore size), refurbished GC 600 Vega Series 2 oven (Carlo Erba Instruments, Italy) with an automatic temperature controllable unit, stainless tubing (1.58 mm in outer dimension (OD) and 0.18 mm inner dimension (ID), back-pressure valve (Swagelok, Johannesburg, South Africa), and a collection flask.

For the extraction, 4 g of ground leaves powder were mixed with 2 g of diatomaceous earth (Sigma, Aldrich, Germany), a dispersing agent and placed inside the extraction cell maintained at different oven temperatures of 50, 100 and 150°C. Extraction was performed in dynamic mode using different ratios of methanol-water mixture i.e. 0, 20, 40 and 60% composition of aqueous methanol (Romil Ltd, Waterbeach Cambridge). The solvent was delivered at a constant flow rate of 5 mL/min and a pressure of 1000±200 psi was maintained using the back-pressure valve. Extracts were collected in a falcon tube up to the 50 mL mark through an outlet coil immersed in a cooling water bath. Each extraction operation lasted for 10 minutes. The extracts were filtered using a 0.22 µm nylon syringe filter into a 2 mL HPLC capped vial and preserved at -20°C prior to analysis.

**Chromatographic Separation and Mass Spectrometry (UPLC-qToF-MS)**

The chromatographic separation was performed on a UPLC hyphenated to a Synapt G1 -qToF-MS instrument (Waters Corporation, Manchester, UK) equipped with a Waters Acquity HSS T3 C18 column (150 mm x 2.1 mm diameter; particle size 1.8 µm). The column oven temperature was maintained at 60°C. The mobile phases were (A) 0.1% formic acid in deionized water, and (B) mass spectrometry (MS)-grade acetonitrile with 0.1% formic acid. The linear gradient program began with 2% A to 60% B for 24 min, ramped to 95% B at 25 min and kept constant for 2 min, then re-equilibrated at 5% B for 3 min. The total cycle runtime was 30 min with a flow rate of 0.4 mL/min.

Mass spectrometry was performed using a Waters qToF-MS instrument (Waters Corporation, Manchester, UK) fitted with an electrospray ionization source (ESI) operating in both positive and negative ion electrospray modes. The m/z range was 100-1000, scan time 0.2 sec, interscan delay 0.02 sec, with leucine encephalin (556.3 µg/mL) as a lock mass, standard flowrate 0.1 mL/min,
and a mass accuracy window of 0.5 Da was used for MS data acquisition. Moreover, the instrument was operated on the following settings: collision energy of 3 eV, capillary voltage of 2.5 kV, sample cone voltage of 30V, detector voltage of 1650 V (1600 V in negative mode), source temperature at 120°C, cone gas flow at 50 (L/h), and desolvation gas flow at 550 (L/h). To achieve metabolite fragmentation patterns necessary for annotation or identification, the collision energy during MS acquisition was experimentally changed in the trap ion optics by acquiring data at 3, 10, 20, and 30 eV.

Data Analyses

Data acquired was analyzed and visualized using Markerlynx XS software (Waters Corporation, Manchester, UK). For maximum data output, the analysis was carried out using optimized parameters [14]. Here, only negative data were analyzed using similar optimized parameters, for reasons of better predictability without need for use of authentic standards [14,30]. Representative single ion monitoring (SIM) chromatograms for target molecules were generated using their m/z values. Moreover, various MS spectra for these molecules were obtained from the chromatograms, their fragmentation patterns observed, and molecular formulae calculated on the basis of a 5 ppm mass accuracy range. This information was used to confirm the identities of these bio-markers following a search of the Dictionary of Natural Products online database [31] in an approach previously reported [14].

Extraction yields for molecules identified represented the relative peak intensity figures of molecular peaks corresponding to the identified molecules. Relative peak intensity is a dimensionless quantity, and corresponded to the area-under-the-peak values obtained from the peak list. This data file (peak list) is the final output obtained after processing of the MS data using MarkerLynx software [32,33].

Statistical Analysis

A one-way analysis of variance (ANOVA) was performed on data obtained from Markerlynx XS software and the mass distribution patterns of the means graphically described by the Box-and-Whisker plots. Duncan’s multiple comparison test was performed using ANOVA to determine the differences between individual extraction conditions using IBM SPSS software version 22.
Mean values of extraction conditions were deemed to be different if the level of probability was ≤0.05.

The central composite design response surface model (CCD RSM) was fitted to experimental data in order to obtain the relationship between factors and optimize the response of Z (metabolite yield) in relation to X (solvent composition) and Y (extraction temperature) using Statistica rel 7 (StatSoft, USA) [37]. By using CCD, a total of 12 experimental runs (including 3 repetitions) were designed, 3 factor levels for temperature (50, 100, 150°C) conditions and 4 factor levels for solvent composition (0, 20, 40 and 60% methanol). In order to optimize the response, it was is essential for quadratic terms to be included in the polynomial function (i.e. a second-order polynomial model) represented by the form of equation 1:

\[ z(x, y) = c_{00} + c_{10}x + c_{20}x^2 + c_{01}y + c_{02}y^2 + c_{11}xy \] .................................(Equation 1)

In this case, Z was the dependent variable/predicted response factor, and X and Y the independent variables, \( c_{00} \) is a constant, \( c_{10} \) and \( c_{01} \) are the linear coefficients of X and Y, respectively, \( c_{20} \) and \( c_{02} \) are the quadratic coefficients of X and Y, respectively, and \( c_{11} \) is the interaction coefficient. Equation 1 was fitted to experimental data by using a statistical multiple regression approach called method of least square (MLS), which generates the lowest possible residual [38]. Model parameters and model significance were determined at \( p < 0.05 \). The fitness of the model was determined by evaluating the coefficient of regression (\( R^2 \)) obtained from the analysis of variance (ANOVA). The model fit generates the response surface that defines the behaviour of the response variable, which can be conveniently visualized on the surface plot and contour plot. By means of these plots, the optimized ranges for each factor (i.e. temperature and methanol composition) that leads to the highest response (metabolite yield) can be extracted [38,39].

Results and Discussion

* * pilosa* is rich in bioactive compounds that are of great medicinal significance [5,6]. In this study, we demonstrated the extraction of functional metabolites (specifically anti-HIV analogues) from this plant using a modified PHWE approach. The PHW was modified using different compositions of methanol-water mixture (0, 20, 40, & 60% methanol), and the effect of solvent composition and extraction temperature (50, 100 & 150°C) on the recovery of target metabolites was investigated.
Various isomers of diCQA and CA were successfully extracted. The identity of these metabolites in *B. pilosa* and closely related species has been reported in the literature [5,40]. Using a sensitive and robust tandem MS approach with settings presented elsewhere [41], it was possible to conveniently identify these molecules (Table 4.1).

<table>
<thead>
<tr>
<th>Mol. #</th>
<th>Mol. name</th>
<th>Rt</th>
<th>m/z</th>
<th>MS Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3,4-diCQA</td>
<td>15.53</td>
<td>515</td>
<td>353, 191, 173, 179, 135</td>
</tr>
<tr>
<td>2</td>
<td>3,5-diCQA</td>
<td>15.79</td>
<td>515</td>
<td>191, 179, 135</td>
</tr>
<tr>
<td>3</td>
<td>Cis-3,5-diCQA</td>
<td>15.98</td>
<td>515</td>
<td>191, 179, 135</td>
</tr>
<tr>
<td>4</td>
<td>4,5-diCQA</td>
<td>16.27</td>
<td>515</td>
<td>353, 191, 173, 179, 135</td>
</tr>
<tr>
<td>5</td>
<td>CA</td>
<td>16.20</td>
<td>473</td>
<td>311, 293, 179, 149, 135</td>
</tr>
<tr>
<td>6</td>
<td>CA Isomer</td>
<td>16.64</td>
<td>473</td>
<td>311, 293, 179, 149, 135</td>
</tr>
</tbody>
</table>

**Key:** Mol. # - moleculeumber; Rt - retention time; m/z - mass-to-charge ratio

In view of that, Molecules 1-4 were identified as isomers of dicaffeoylquinic acid (diCQA) i.e. 3,4-diCQA, 3,5-diCQA, cis-3,5-diCQA, and 4,5-diCQA, respectively, by their parent ion peak (in negative ionization mode) at m/z 515 with fragment ions at m/z 353, 191, 179, 173 and 135 [41,42]. These isomers were further distinguished by their order of elution and patterns of fragmentation as reported by these authors [43–45]. Molecules 5 and 6 were identified as chicoric acid (CA) and CA isomer, with a parent ion peak at m/z of 473, and MS² base peak ion at m/z of 311 (for di-caffeoyltartaric acid) due to the loss of a hexose (162 Da), and other fragments at m/z 179 (caffeic acid), and 149 (tartaric acid) [46,47]. The structural configurations of these metabolites are shown in Figure 4.1. These metabolites have widely been reported to exhibit high anti-HIV properties via the inhibition of HIV-1 integrase. Interestingly, these compounds have lethal doses that are multiple-times (at least 100-fold) above their antiviral concentrations [48].
Figure 4.1: Molecular structures of 3,4 diCQA (A), 3,5 diCQA (B), 4,5 diCQA (C), CA (D) and CA Isomer (E)

Figure 4.2 shows the box-and-whiskers plots of the effect of temperature on the extractability of target metabolites (Molecules 1-6) using non-modified (i.e. water only) PHWE. From these plots, it was clearly evident that PHWE was applicable for the extraction of diCQA and CA and their analogues, and that temperature played a key role in the recovery patterns of these molecules. It can be seen that extraction yield increased substantially with increase in temperature. 3,4-diCQA increased from 0.21 (50°C) to 53.04 (150°C), a 252-fold increase in recovery corresponding to a
100°C increase in temperature. Similarly, 3,5-diCQA and 4,5-diCQA increased by magnitudes of 33.72 and 54.03, respectively, following an increase in temperature from 50°C to 150°C.

Figure 4.2: Box-and-whiskers plots showing the effect of temperature on the extractability of isomers of diCQA and CA using water-only PHWE: 3,4-diCQA (A), 3,5-diCQA (B), Cis-3,5-diCQA (C), 4,5-diCQA (D), CA (E) and CA isomer (F).

The observed enhancement of recovery efficiency with increase in temperature can be attributed to the alteration of the properties of water at elevated temperatures. As the temperature of pressurized water increases its dielectric constant, viscosity and surface tension decreases, while its diffusivity increases [10,49]. Moreover, the thermal energy supplied can overcome cohesive (solute–solute) and adhesive (solute–matrix) interaction by decreasing the activation energy required for the desorption process [49]. Additionally, the high pressures involved in PHWE can facilitate extraction by forcing the fluid into areas of the sample matrix that would not normally be contacted by fluid under atmospheric pressure [50].

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Although temperature was found to be critical during PHWE of *B. pilosa*, the positive effect of temperature on the extractability of cis-3,5-diCQA, CA and CA isomer occurred only between temperatures of 50 and 100°C. At a temperature of 150°C there was a decrease in extraction yield for these molecules which can be attributed to thermal degradation. It is common knowledge that during PHWE, higher temperatures degrade some classes of plant metabolites [14,15]. This degradation phenomenon is a major limitation of PHWE. Moreover, it was apparent that target metabolites were only fairly soluble in low temperature water (50°C). Hence, it became necessary to optimize the PHWE method for a more efficient and safe recovery of these metabolites. In this regard, methanol was added as a cosolvent during PHWE of target metabolites from *B. pilosa*. Figures 4.3.1 & 4.3.2 show the extractability of target metabolites using (a) 0% methanol, (b) 20% methanol, (c) 40% methanol and (d) 60% methanol, at 50°C on single ion monitoring (SIM) chromatograms. From the visual evaluation of these chromatograms, it is clearly evident that incorporation of methanol significantly enhanced the recovery of diCQA, CA and their analogues during PHWE of *B. pilosa*.

![Figure 4.3.1: Representative UPLC tandem MS single ion monitoring (SIM) chromatograms for isomers of diCQA following PHWE of *B. pilosa* at 50°C using 60% MeOH (A), 40% MeOH (B), 20% MeOH (C) and 0% MeOH (water) (D).](image-url)
The enhancement in extraction efficiency was both qualitative (number of components) and quantitative, and also in proportions to the percentage of methanol composition as was apparent from the base peak ion (BPI) chromatograms (not shown) and from the intensity of colour of the extracts (not shown). Table 4.2 presents the extraction yields obtained at various extraction conditions of temperature and solvent composition. These results indicate that extraction conditions (temperature and solvent composition) resulted in significantly different (p≤0.05) recovery patterns for each metabolite. It was also possible to show the main compounds recovered and in descending order of yield, they include 3,5-diCQA with a yield of 204.23±3.16 extracted at 50°C and 60% methanol; chicoric acid (141.00±3.55) at 50°C and 60% methanol; 4,5-diCQA (108.05±4.76) at 150°C and 0% methanol; 3,4-diCQA (53.04±13.49) at 150°C and 0% methanol; chicoric acid isomer (40.01±1.14) obtained at 150°C and 20% methanol; and cis-3,5-diCQA (12.07±5.54) obtained at 100°C and 60% methanol.
Table 4.2: Yield (mean relative peak intensity) of identified anti-HIV analogues (diacylated cinnamic acids) extracted from *B. pilosa* using modified PHWE.

<table>
<thead>
<tr>
<th>Extraction conditions</th>
<th>3,4-diCQA</th>
<th>3,5-diCQA</th>
<th>Cis-3,5-diCQA</th>
<th>4,5-diCQA</th>
<th>CA</th>
<th>CA isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₅₀C₀</td>
<td>0.21±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.70±0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.00±0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.82±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>T₅₀C₂₀</td>
<td>1.91±0.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.30±8.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.36±0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.21±0.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.85±8.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.27±1.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>T₅₀C₄₀</td>
<td>37.12±9.42&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>188.24±2.48&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.64±4.86&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>85.13±2.21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>131.47±3.65&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>36.27±1.97&lt;sup&gt;bcd&lt;/sup&gt;</td>
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<tr>
<td>T₅₀C₆₀</td>
<td>32.54±10.41&lt;sup&gt;bc&lt;/sup&gt;</td>
<td><strong>204.23±3.16&lt;sup&gt;c&lt;/sup&gt;</strong></td>
<td>4.01±3.67&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>91.71±2.10&lt;sup&gt;cd&lt;/sup&gt;</td>
<td><strong>141.00±3.55&lt;sup&gt;d&lt;/sup&gt;</strong></td>
<td>30.13±0.55&lt;sup&gt;bc&lt;/sup&gt;</td>
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<tr>
<td>T₁₀₀C₀</td>
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<td>34.36±2.62&lt;sup&gt;bcd&lt;/sup&gt;</td>
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<tr>
<td>T₁₀₀C₂₀</td>
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<td>150.46±16.03&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>0.10±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>84.22±1.31&lt;sup&gt;c&lt;/sup&gt;</td>
<td>105.23±18.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.77±5.67&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>T₁₀₀C₄₀</td>
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<td>186.70±4.43&lt;sup&gt;e&lt;/sup&gt;</td>
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<td><strong>12.07±5.54&lt;sup&gt;c&lt;/sup&gt;</strong></td>
<td>96.93±2.81&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>T₁₅₀C₀</td>
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<td><strong>124.75±15.97&lt;sup&gt;c&lt;/sup&gt;</strong></td>
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<td><strong>108.05±4.76&lt;sup&gt;de&lt;/sup&gt;</strong></td>
<td>121.66±2.86&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>30.19±0.60&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>T₁₅₀C₂₀</td>
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<td>93.67±3.40&lt;sup&gt;d&lt;/sup&gt;</td>
<td>126.17±2.23&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td><strong>40.01±1.14&lt;sup&gt;d&lt;/sup&gt;</strong></td>
</tr>
<tr>
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<td>181.25±4.98&lt;sup&gt;de&lt;/sup&gt;</td>
<td>0.76±0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>98.48±2.55&lt;sup&gt;d&lt;/sup&gt;</td>
<td>128.33±2.51&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>38.03±0.59&lt;sup&gt;cd&lt;/sup&gt;</td>
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<tr>
<td>T₁₅₀C₆₀</td>
<td>41.54±10.46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>175.11±2.64&lt;sup&gt;de&lt;/sup&gt;</td>
<td>0.07±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>99.66±2.21&lt;sup&gt;de&lt;/sup&gt;</td>
<td>123.48±2.28&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>37.37±0.55&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Level of significance**

| *** | *** | ** | *** | *** | *** |

**Key:** Values represent means of triplicate extraction yield ± SEM (standard error of the mean). Values within the same column followed by different superscripts are significantly different (P < 0.05). Level of significance *** p<0.001, and ** p<0.01. Values in bold (within a column) represent the highest extraction yields for the molecule.

T₅₀C₀ – extraction at 50°C and 0% methanol; T₅₀C₂₀ – extraction at 50°C and 20% methanol; T₅₀C₄₀ – extraction at 50°C and 40% methanol; T₁₀₀C₀ – extraction at 50°C and 60% methanol; T₁₀₀C₂₀ – extraction at 100°C and 0% methanol; T₁₀₀C₄₀ – extraction at 100°C and 20% methanol; T₁₀₀C₆₀ – extraction at 50°C and 40% methanol; T₁₅₀C₀ – extraction at 50°C and 60% methanol; T₁₅₀C₂₀ – extraction at 50°C and 20% methanol; T₁₅₀C₄₀ – extraction at 50°C and 40% methanol; T₁₅₀C₆₀ – extraction at 50°C and 60% methanol

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Essentially, the adoption of methanol as a cosolvent during PHWE made it possible to achieve significantly ($p \leq 0.05$) higher extraction yields even at a low temperature of $50^\circ C$, which was heretofore, unachievable using water only even when temperatures were raised to $150^\circ C$. For example, at a constant temperature of $50^\circ C$, the extraction yield of 3,5-diCQA increased by a factor of 55.2 as methanol composition rose from 0% methanol (water only) to 60% methanol. Likewise, CA increased by a factor of 50 from 0% methanol to 60% methanol, under similar temperature conditions. This is in agreement with the earlier report of Ong et al. [51] who observed that at constant temperature, a better extraction efficiency could be achieved by increasing the amount of ethanol added in the water (0–30%), during the pressurized liquid extraction of tanshinone IIA in *Salvia miltiorrhiza*.

Particularly, the efficient recovery of CA at low temperatures is very interesting and desirable because, this compound is known to be highly unstable and degrade rapidly during the extraction process [9,52,53]. This metabolite has been proposed as an indicator compound for quality control due to its instability and rapid degradation when compared to other secondary metabolites within plant materials [9,18]. Enhancement due to the incorporation of methanol as a cosolvent during PHWE can be associated with interactions based on polarity. As organic compounds diCQA and CA are highly soluble in organic solvents such as methanol, and were favoured by higher percentages of methanol. The presence of methanol in water greatly reduced the polarity of water without a need for increasing the temperature. Moreover, as compared to pure water, water-methanol mix is a less dense solvent mixture which has lower surface tension, lower hydrogen bonding strength between water molecules and higher diffusivity [10]. As such during extraction, there was a higher permeability into the cellular structures of the matrix, which resulted in better extractability.

Also, it was observed that the gradient increase in extraction yield due to incorporation of methanol as a cosolvent during PHWE was more steep (rapid) at low temperatures compared to higher temperatures. For example, when comparing the rate of increase of methanol use from 0 to 60%, 4,5-diCQA increased by a factor of 45.86 at $50^\circ C$, 4.71 at $100^\circ C$, and 0.92 at $150^\circ C$ (Table 4.2). Moreover, at higher temperatures ($150^\circ C$), there was a slight decrease in recovery efficiency as methanol composition increased. We saw that for all extractions obtained at $150^\circ C$, the highest
Chapter Four

Yields were obtained at 40% methanol rather than the expected 60% methanol. To give an instance, the recovery of CA rather decreased by 3.8% when methanol composition was increased from 40 to 60% during extraction at 150°C. This could be as a result of evaporation of methanol, which has a boiling point of 64.7°C. The reason for this phenomenon is unclear and requires further investigation.

In order to better interpret and describe the patterns in our data set, we adopted the central composite design response surface methodology (CCD RSM) statistical approach. Response surface methodology is an ideal statistical approach to employ when a response or a group of responses of interest are influenced by more than one variable [54]. In our case, extraction yield was influenced by temperature and solvent composition. Accordingly, the CCD RSM was fitted to the experimental data with R² values ranging from 0.57 to 0.87, implying that the fit explains 57 to 87% variability in the response variable. Coefficient of determination (R²) values above 0.70 indicates a model that fits the data well. Three dimensional surface plots were generated from the model fit in order to conveniently visualize the interrelationship of the levels of factors and the recovery patterns of target metabolites (Figure 4.4). From these plots again, it was visibly evident that temperature and more profoundly methanol composition were critical for the efficient extraction of different isomers of diCQA and CA. The colour bands on the smooth surface corresponds to the response of the dependent variable relative to the levels of the independent variables such that, regions with dark green colour represent low extraction yields, while those regions with dark red colour represent high extraction yield. Hence, it was possible to determine regions with the most efficient performance of the system through visual inspection of the surfaces. Equations 2-7 represent the response surface equations for Molecules 1-6 in that order.
Figure 4.4: Surface plots showing the effect of temperature and solvent composition on the extraction of diCQA and CA analogues: 3,4-diCQA (A), 3,5-diCQA (B), Cis-3,5-diCQA (C), 4,5-diCQA (D), CA (E) and CA isomer (F).
Chapter Four

\[ z = -19.92745 + 0.24828x + 0.00133x^2 + 1.42157y - 0.00639y^2 - 0.00771xy \ .......... \text{(Equation 2)} \]
\[ z = -147.33873 + 3.16447x - 0.00914x^2 + 6.03789y - 0.01645y^2 - 0.02835xy \ .......... \text{(Equation 3)} \]
\[ z = -9.48606 + 0.24191x - 0.00122x^2 + 0.14846y + 0.00073y^2 - 0.00103xy \ .......... \text{(Equation 4)} \]
\[ z = -78.08366 + 1.31680x - 0.00108x^2 + 3.38805y - 0.00953y^2 - 0.01857xy \ .......... \text{(Equation 5)} \]
\[ z = -114.32891 + 2.83421x - 0.00773x^2 + 3.62055y - 0.00286y^2 - 0.02593xy \ .......... \text{(Equation 6)} \]
\[ z = -31.73426 + 0.75804x - 0.00204x^2 + 0.95422y - 0.00333y^2 - 0.00524xy \ .......... \text{(Equation 7)} \]

Where
\[ x = \text{methanol composition}; \ y = \text{temperature}; \ z = \text{extraction yield} \]

Furthermore, the model fit afforded insights on the patterns of distinct variable effects and pairwise (mutual) variables interactive effects on the response variable (Figure 4.5). Figures 4.4 & 4.5 show the Pareto charts of standardized factor effects from which the magnitude and importance of each effect (p≤0.05) can be envisaged. The reference line indicated on the chart (α = 0.05) distinguishes between significant and insignificant effects, such that any effect that extends beyond this reference line is significant [55]. As such, the linear effect of temperature had the highest impact on extraction yield for 3,4-diCQA, followed by the interactive effect of temperature and solvent composition, the linear effect of solvent composition, the quadratic effect of solvent composition, and the quadratic effect of temperature. Linear effect of a variable means that the variable correlates directly proportional to the response variable, whereas the quadratic effect of a variable implies that the response variable is correlated with the square of that variable.

A strong quadratic effect of a variable (p<0.05) implies that the optimal levels of the response falls within the range of the experimental values for that variable, and vice versa. From the fitted models, none of the quadratic effects was significant (p≤0.05), implying that all optimal extraction conditions fall outside the experimental domain. 3,5-diCQA had the weakest quadratic effect on solvent composition, meaning that this molecule has the highest solubility in methanol (an indication of high polarity). Moreover, it can also be seen that temperature and solvent composition had a significant (p≤0.05) synergistic effect on the recovery patterns of 3,5-diCQA, 4,5-diCQA, and CA (Figure 4.5). In general, solvent composition had a higher impact on the recovery efficiency of target metabolites than temperature.
Figure 4.5: Pareto chart of standardized effects of temperature and solvent composition on the extraction of diCQA and CA analogues: 3,4-diCQA (A), 3,5-diCQA (B), Cis-3,5-diCQA (C), 4,5-diCQA (D), CA (E) and CA isomer (F).
Conclusion

Using a modified PHWE approach, we demonstrated the extraction of pharmacologically relevant metabolites, diCQA and CA and their analogues from *B. pilosa*, metabolites known to possess anti-HIV properties. It was observed that although temperature was an important factor to effectively extract these metabolites, extraction efficiency of PHWE can be greatly enhanced by introducing an auxiliary solvent (in this case, methanol). Essentially, it was possible to extract significant amounts of highly unstable metabolites, which is an indicative of an effective extraction method for recovering thermo-labile compounds from plant materials. It was further statistically deduced that solvent composition was a stronger factor that influenced extractability of the target metabolites when compared to temperature. In comparison to the conventional methods of extraction, our modified PHWE method was less time consuming (the total time of extraction being approximately 15 min, whereas solvent extraction takes about 2 h). Moreover, the use of organic solvents was also substantially reduced. The ease and simplicity of the method developed herein is also worthy of note. Once more, the efficacy and applicability of PHWE for the extraction of functional metabolites from plant tissues is reaffirmed, while reiterating the importance of *B. pilosa* and its associated metabolites. Further research could be done using other solvents as alternatives to methanol. Additionally, the synergistic effect of co-solvency and other parameters such as pH on the recovery pattern of metabolites could also be investigated.

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References


CHAPTER FIVE

5.0 GENERAL DISCUSSION AND CONCLUSION

5.1 General discussion

There has been a major resurgence in interest in phytochemicals from medicinal plants, because of numerous evidences that link them to various protective health benefits (Dias, 2012; Doughari, 2012; Saxena et al., 2013). *B. pilosa* is a very potent herbal plant and rich in various pharmacoactive phytochemicals (Silva et al., 2011; Arthur et al., 2012; Bartolome et al., 2013; Khoza et al., 2016). Though, indigenous to South Africa and abundantly available, *B. pilosa* has been neglected and underutilized as a bush weed, which makes it potentially vulnerable to genetic erosion and extinction. On the other hand, improvement in extraction (an integral part of phytochemical analysis) has been a recurrent topic in current research in bioanalytical sciences (Augusto et al., 2013). The fundamental objectives of this vital step have been to adopt cost-effective, fast and reliable techniques that offer better sensitivity and shifts towards green chemistry. Pressurized hot water extraction has been a well-favoured extraction technique in this regard (Gbashi et al., 2016). Besides, subsequent analytical approaches such as UPLC-qToF-MS/MS and chemometric analysis have also been associated research interests alongside developments in extraction techniques (Khoza et al., 2014; Madala et al., 2014; Khoza et al., 2015, Ramabulana et al., 2015; Khoza et al., 2016).

In the present study, PHWE in combination with UPLC-qToF-MS/MS and chemometric analysis was used to explore the phytochemical profile of *B. pilosa*. Results obtained from the first part of the study strongly corroborates the theory that PHWE could extract phytochemical compounds of various physicochemical characteristics. Accordingly, various positional glycosidic isomers of 28 flavonoid molecules belonging to different classes of flavonoids (flavonols, chalcones and flavanones) were identified. Further chemometric analysis (PCA) revealed that temperature greatly affected the recovery rates of these molecules, moreover, significantly differential distribution patterns were observed at different temperature conditions. Although, for most of the molecules, extraction yields were higher at a temperature of 150°C, there was eminent indications of thermal
degradation for some of the identified molecules at temperatures above 100°C. Similar observations have been reported elsewhere for PHWE of *M. oleifera* plant (Khoza et al., 2014).

Furthermore, results from this study, showed that it was possible to greatly undermine the effects of thermal degradation during PHWE at elevated temperatures by means of methanol as a cosolvent. Target analytes for optimization which included different isomers of diCQA and CA (in particular CA) are known to be highly unstable during PHWE. In our previous study on the same plant, it was practically impossible to detect chicoric acid using PHWE at 100 and 150°C (Khoza et al., 2016). However, when methanol was introduced as a cosolvent during PHWE, significantly (p≤0.05) higher extraction yields of these compounds were achieved at a relatively lower temperature (50°C). By means of statistical modelling [CCD RSM (R² ranging from 0.57 to 0.87)] it was deduced that the enhancement in extraction yield was due to increase in temperature and percentage methanol composition used. However, the effect of methanol composition on extraction yield was much more prominent and the effect of temperature on was limited.

### 5.2 Conclusion

Inference from the results obtained in this study confirm the first hypothesis that PHWE in combination with UPLC-qToF-MS/MS and chemometric models can be a suitable analytical approach for the screening of different classes of phytochemicals in *B. pilosa* as stated previously in Section 1.3 of this dissertation. Besides, the results also establishes the second hypothesis that it is be possible to minimize low recovery effects and thermal degradation of highly unstable and thermolabile phytochemical compounds through optimization using a cosolvent. In addition, this study further emphasizes the relevance of *B. pilosa* as a rich source of important functional metabolites. It is thus recommended that further studies be done to characterize other biologically relevant compounds that could be present in *B. pilosa* using PHWE. Essentially, the technique (PHWE) is relatively cheap, fast, simple to perform and offers quality results while greatly minimizing (or eliminating) the need for organic solvents. The observations herein could further stimulate interest and propel the adoption of PHWE in many other applications beyond the scope covered in this study as well as scale-up for industrial use.
References


ADDENDUM

IDENTIFICATION OF HYDROXYLCINNAMOYL TARTARIC ACID ESTERS IN BIDENS PILOSA BY UPLC-TANDEM MASS SPECTROMETRY

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Abstract

\textit{Bidens pilosa} is a medicinal plant used for the treatment of several physiological illnesses. In South Africa, as the case may be for other African countries, this plant is equally consumed as a vegetable. In the current study, pressurized hot water extraction (PHWE) technology was employed for the extraction of polyphenolic compounds from leaves of \textit{B. pilosa} under two different temperature conditions (100 and 150 \textdegree C). Accordingly, extraction of these compounds was made possible at 150 \textdegree C and analysis of these extracts using UPLC-qToF-MS/MS revealed the presence of several hydroxylcinnamoyl tartaric acids. Here, different isomers of coutaric-, caftaric-, fertaric-, chicoric acid and caftaric acid glycosides were detected. The contribution of mass spectrometry fragmentation towards the characterization of these molecules is also presented. To the best of our knowledge, this is the first report of these molecules in \textit{B. pilosa}.

Keywords: \textit{Bidens pilosa}, UPLC-qToF-MS/MS, hydroxylcinnamoyl tartaric acids, cinnamic acid derivatives.
ADDENDUM 2

SUBCRITICAL WATER EXTRACTION IN BIOLOGICAL MATERIALS

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Abstract

Extraction is a vital prerequisite in most scientific studies involving the isolation and analysis of compounds from biological/environmental systems. The use of large quantities of organic solvents in performing both conventional and modern methods of extraction of these substances stirs issues of safety, environmental health and cost-effectiveness. Subcritical water extraction (SWE) offers a suitable, safe, cost-effective and environmentally safe alternative compared to other methods as it takes advantage of the special properties of water (such as lower dielectric constant, lower hydrogen bonding strength and decreased surface tension) under high temperature and pressure conditions (100 - 374°C, >50 bar) to extract non-polar analytes. This review paper presents a critical appraisal of the principles and dynamics of SWE, and the current applications as a viable tool in the extraction of compounds from various biological matrices. Although further research needs to be performed to improve on its application, the adoption of SWE in the extraction of phytochemicals as well as other bioactive molecules including mycotoxins from both plant and animal components seems promising and needs to be properly exploited.

Keywords: Subcritical water extraction, phytochemicals, temperature and biological materials.