PHYTOCHEMICAL STUDIES ON EXTRACTS OF SELECTED SOUTH AFRICAN INDIGENOUS MEDICINAL PLANTS

BY

Masande Nicholas Yalo

A thesis submitted in partial fulfilment of the requirement for the award of the degree of Doctor Philosophy in the Faculty of Natural Sciences University of the Western Cape

Department of Chemistry
Faculty of Natural Sciences
University of the Western Cape

Supervisor: Prof. Wilfred T. Mabusela

December 2021

ABSTRACT

Nature has been identified as a rich source of potentially useful chemicals. Throughout the years, phytochemical studies have led to the unearthing of a huge number of natural products, their chemical diversity is unique and many of them possess various biological activities.

South Africa is blessed with a rich plant biodiversity of +24,000 indigenous plants, representing about 10% of all higher plants on earth and with more than 50 % of such plant species found nowhere else in the world but in South Africa. However, only a few of the South African medicinal plants have been exploited to their full potential. Screening of more medicinal plants for biological activities and phytochemicals is important for finding potential new compounds for therapeutic uses.

As a contribution, this study attempts to relate specific constituents present in these plants with their widespread ethnomedicinal uses. The extractions were carried out using broad spectrum of solvents (hexane, dichloromethane, ethyl acetate, butanol, methanol and water). Fractionation was carried out using standard chromatographic techniques. A total of twenty-one compounds (**1-21**) were isolated from three of the investigated plants namely: *Asparagus lignosus, Cliffortia odorata*, and *Protea cynaroides*. These twenty-one isolated compounds were characterised and their structures were unambiguously established by spectroscopic methods including 1D and 2D NMR, FTIR, HRMS and UV. Four of the of the isolated compounds were reported for the first time from a natural source; 3,4-*bis*(4-hydroxybenzoyl)-1,5-anhydro-D-glucitol (**2**), 4-hydroxybenzoyl-1,5-anhydro-D-glucitol (**5**), 2-(hydroxymethyl)-4-oxo-4*H*-pyran-3-yl-6-*O*-benzoate-β-D-glucopyranoside (**7**) and 3-hydroxy-7,8-dihydro-β-ionone 3-*O*-β-D-glucopyranoside (**8**) (from *Protea cynaroides*).

Two compounds isolated from *Cliffortia odorata* demonstrated significant antidiabetic properties as compared to well known drugs acarbose (a known alpha-glucosidase and alpha-

amylase inhibitor), compounds **15** with an IC_{50} of 16.6 ± 1.02 µg/mL and **16** with IC_{50} values of 3.8 ± 0.32 µg/mL respectively while **20** isolated from *Apsaragus lignosus* demonstrated the weak *in vitro* alpha-glucosidase inhibitory activity with an IC_{50} value of 110.8 ± 2.52 µg/mL. For the alpha-amylase, none of the crudes and compounds showed any activity.

The extracts and isolated compounds were screened for their inhibitory activities on mushroom tyrosinase using L-tyrosine as substrate. The tyrosinase inhibitory activity of the isolated compounds showed the highest activity for **4** and **6** with IC_{50} values of 0.8776 ± 0.2 and 0.7771 ± 0.09 µg/mL respectively when compared with kojic acid (IC_{50} = 0,7062 µg/mL), a well-known tyrosinase inhibitor. Compounds **1**, **3**, **11**, **12**, and **20** showed weak inhibition with the IC_{50} values of 162.52 ± 1.21, 274.5 ± 2.12, 131.9 ± 1.05, 274.5 ± 1.26 and 128.9 ± 2.12 µg/mL respectively while the rest of the compounds showed lowest activity and some did not show at all.

This is the first scientific report to be carried out on the phytochemical and biological profiles of all three species as well as the isolation and characterization of compounds. The results suggest that these compounds might become natural candidates to inhibit alpha-glucosidase and tyrosinase.

KEYWORDS: *Asparagus lignosus*, *Cliffortia odorata*; *Protea cynaroides*, alpha-glucosidase, alpha-amylase, diabetes melitus, tyrosinase, chromatographic techniques.

DECLARATION

I, **Masande Nicholas Yalo** declare that the dissertation entitled **'Phytochemical studies on extracts of selected South African indigenous medicinal plants'**; has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own.

Signature:

Date: ……08/12/2021………

ACKNOWLEDGEMENTS

I exalt and bow down before the Creator, the most Merciful, the Gracious, the Compassionate, the Beneficent, the Sole source of Wisdom and knowledge for the abundant Blessings He bestowed upon me during the course of my thesis work. Glory indeed to Almighty God for helping me finish my doctoral studies.

I offer my gratitude to my research supervisor **Prof. W. T. Mabusela** for his guidance, support, patience, endless encouragement, positive criticism, discussion, editing and intellectual input.

I wish to express my profound sense of gratitude to **Prof. Ahmed Mohammed** from CPUT for his inspiration, constant guidance, valuable suggestions, sympathetic advice and unparalleled encouragement made throughout the final stage of my work. I am truly indebted to him.

I am deeply indebted and very grateful with deep sincere appreciation to **Dr. Rajan Sharma** for his continuous help and guidance especially in the reviewing my work and giving constructive critics throughout the final stage of my work.

Mr Masixole "Arnold" Makhaba for his assistance with structural elucidation of some of the isolated compounds, I greatly appreciate his input. I thank the **National Research Foundation (NRF) of South Africa** for the financial support.

I would like to thank all my colleagues and friends for providing a stimulating and fun environment. Their support and colleagueship meant a lot. Through my time in the lab, I have had the greatest pleasure of working with them; **Mr. Ndikho "Legendary" Nako, Dr Mkhuseli "Main man" Koki, Mrs Olusola Watti, Miss Kelly Correia.** Also, from outside our lab, support and friendship came from **Prof Martin Onani, Dr Asanda Busa,**

Ultimately but not the least, none of this would have been possible without the love and patience of my family. I have been blessed with a very loving and supportive **family**. May **GOD** bless them in a mighty way in everything they do.

DEDICATION

I dedicate my dissertation work to my family for all of their continued love and support. A special feeling of gratitude to my loving parents, **Harrison Mbonelwa** and **Monica Nondwe Yalo** whose words of encouragement and push for tenacity ring in my ears till this day.
To My siblings **Nontobeko**, **Nwabisa**, **Sinalo** and **Sihle Yalo** who have been pillows, role models, catapults, cheerleading squad and sounding boards I have needed.

I also dedicate this dissertation to my three beautiful nieces **Inam, Lunje** and **Aluncedo Yalo** who have made my life brighter every day since they were born.
Finally, it is dedicated to my late grandparents, **Leonard Mntwabantu** and **Nofundile Yalo.** I wish that you could be here to celebrate with me.

LIST OF PUBLICATIONS

1. **Yalo, M.; Makhaba, M.; Hussein, A.A.; Sharma, R.; Koki, M.; Nako, N, and Mabusela, W.T**. Characterization of Four New Compounds from *Protea cynaroides* Leaves and Their Tyrosinase Inhibitory Potential. *Plants* **2022**, *11*, 1429. https://doi.org/10.3390/plants11101429

2. **Yalo, M.; Makhaba, M.; Sharma, R.; Sagbo, I.J., Maina, M.H.; Hussein, A.A and Mabusela, W.T**. A sulphated flavonoid and other phytoconstituents from the leaves of Cliffortia odorata and their alpha-glucosidase inhibitory activity. *Plants* **2022. (Manuscript under review).**

3. **Makhaba, M.; Yalo, M.; Pearce, K., Hussein, A.A and Mabusela, W.T.** Isolation and identification of pyrone-containing flavone from Helichrysum petiolare. *Plants* **2022. (Manuscript under review).**

TABLE OF CONTENTS

ABSTRACT .... **i**

DECLARATION .... **iii**

ACKNOWLEDGEMENTS .... **iv**

DEDICATION .... **vi**

LIST OF PUBLICATIONS .... **vii**

TABLE OF CONTENTS .... **viii**

LIST OF FIGURES .... **xiv**

LIST OF TABLES AND SCHEMES .... **xvi**

LIST OF ABBRIVIATIONS .... **xviii**

LIST OF ISOLATED COMPOUNDS .... **xx**

CHAPTER 1 .... **1**

INTRODUCTION **1**

1.1 General introduction to Natural products **1**

1.2 South African medicinal plants **2**

1.3 Diabetes Mellitus **4**

1.3.1 Type 1 diabetes 4

1.3.2 Type 2 diabetes 4

1.3.3 Symptoms and treatment of diabetes 5

1.4.1 Alpha-amylase 6

1.4.2 Alpha-glucosidase 6

1.5 Tyrosinase **8**

1.6 Problem statement **8**

1.7 Rationale of the study **9**

1.8 Justification **10**

1.9 Aim of the study **10**

1.10 Objectives **11**

1.10.1 General objectives 11

1.10.2 Specific objectives 11
1.11 Structure of the thesis 11
References 12
CHAPTER 2 22
LITERATURE REVIEW 22
2.1 Introduction 22
2.2 *Protea cynaroides* 22
2.2.1 Plant description 22
2.2.2. Distribution of *Protea cynaroides* 23
2.2.3 Ethnomedicinal use of *Protea cyanaroides* 24
2.2.4 Phytochemical studies 25
2.3 *Cliffortia odorata* 28
2.3.1 Plant description 28
2.3.2. Distribution of *Cliffortia odorata* 29
2.3.3 Ethnomedicinal use of *Cliffortia odorata* 30
2.3.4 Phytochemical studies 30
2.4 *Asparagus lignosus* 34
2.4.1 Plant description 34
2.4.2. Distribution of *Asparagus lignosus* 35
2.4.3 Ethnomedicinal use of *Asparagus lignosus* 36
2.4.4 Phytochemical studies 36
Conclusion 39
References 40
CHAPTER THREE 50
EXPERIMENTAL PROCEDURE 50
3.1 Materials and instrumentation 50
3.2. Reagents and solvents 50
3.3 Chromatography 50
3.3.1 Thin Layer Chromatography (TLC) 50

3.3.2 Column chromatography (CC) 51
3.3.3.3 Electrospray ionization mass spectroscopy (ESI-MS) 51
3.4 Spectroscopy 51
3.4.1 Nuclear magnetic resonance (NMR) spectroscopy 51
3.4.2 Liquid chromatography mass spectrometry (LC-MS) 51
3.4.4 Ultra violet (UV) spectroscopy 52
3.5 Plant material 52
3.5.1 Collection and identification of plant material 52
3.5.2 Plant preparation 52
3.6 Extraction and fraction of plants 53
General sequential extraction 53
3.6.2. Extraction and isolation of compounds from BuOH extract of *Protea cynaroides* 54
3.6.3. Extraction and isolation of compounds from EtOAc extract of *Cliffortia odorata* 55
3.6.4. Extraction and isolation of compounds from BuOH extract of *Cliffortia odorata* 56
3.6.5. Extraction and isolation of compounds from BuOH extract of *Asparagus lignosus* 56
3.7 General experimental procedure for biological assays 57
3.7.1. Reagents 57
3.7.2. alpha-glucosidase inhibition activity 57
3.7.3. alpha-amylase inhibition activity 58
3.7.4. Tyrosinase inhibition assay 58
3.8 Statistical Analysis 59
References 60
CHAPTER 4 61
CHEMICAL CHARACTERIZATION AND BIOLOGICAL INVESTIGATION OF *PROTEA CYNAROIDES* 61
4.1 *Protea cynaroides* 62
4.1.1 Isolation of constituent of the ethyl acetate extract fraction 62
4.1.2 Isolation of constituent of the butanol extract 62
4:2 Structural elucidation and characterization of compounds 64
4.2.1: Structural elucidation of compound 1 64

4.2.2: Structural elucidation of compound 2 66
4.2.3: Structural elucidation of compound 3 72
4.2.4: Structural elucidation of compound 4 74
4.2.5: Structural elucidation of compound 5 76
4.2.6: Structural elucidation of compound 6 82
4.2.7: Structural elucidation of compound 7 86
4.2.8: Structural elucidation of compound 8 91
4.3 Physico-chemical data of isolated compounds from *P. cynaroides* 99
4.3.1: 4-hydroxybenzoic acid (1) 99
4.3.2: 3,4-bis(4-hydroxybenzoyl)-1,5-Anhydro-D-glucitol (2) New 99
4.3.3: 1,5-Anhydro-D-glucitol (3) 99
4.3.4: 3,4-dihydroxybenzoic acid (4) 99
4.3.5: 4-hydroxybenzoyl-1,5-Anhydro-D-glucitol (5) New 100
4.3.6: 3-hydroxykojic acid (6) 100
4.3.7: 2-(hydroxymethyl)-4-*oxo*-4*H*-pyran-3yl-6-*O*-benzoate-β-D-glucopyranoside (7) New .. 100
4.3.8: 3-Hydroxy-7,8-Dihydro-β-Ionone 3-*O*-β-D-glucopyranoside (8) New 100
4.4 Biological evaluation 101
4.4.1 Alpha-glucosidase and alpha amylase inhibition 101
4.4.2: Mushroom L-Tyrosinase assay 102
4.4.2.1: Anti-tyrosinase activity of various extracts from *P. cynaroides* 102
4.4.2.2: Anti-tyrosinase activity of isolated compounds from *P. cynaroides* 103
4.5: Conclusion 105
References 106
CHAPTER 5 109
5.1 *Cliffortia odorata* 109
5.1.1 Isolation and identification of the chemical constituents of the ethyl acetate fraction 110
5.1.2 Isolation and identification of the chemical constituents of the butanol fraction 110
5:2 Structural elucidation and characterization of compounds 112
5.2.1: Structural elucidation of compound 9 112

5.2.2: Structural elucidation of compound 10 116

5.2.3: Structural elucidation of compound 11 118

5.2.5: Structural elucidation of compound 13 121

5.3 Physico-chemical data of isolated compounds from *C. odorata* 141

5.3.1: 6-methoxychroman-4-one (9) 141

5.3.2: 2,4-dihydroxy-6-methoxyacetophenone (10) 141

5.3.3: Catechin (11) 141

5.3.4: *n*-butyl-alpha-D-fructofuranoside (12) 141

5.3.5: *n*-butyl -beta-D-fructofuranoside (13) 142

5.3.6: Isolariciresinol (14) 142

5.3.7: Kaempferol (15) 142

5.3.8: Kaempferol 3,7-*O*-disulphate (16) 142

5.3.9: Sucrose (17) 142

5.3.10: β-D-glucopyranose, α-D-glucopyranose, β-D-fructopyranose and β-D-fructofuranose (Honey). (18) 143

5.4 Biological evaluation 144

5.4.1 Alpha-glucosidase inhibition 144

5.4.2 Alpha-amylase inhibition 147

5.4.3: Mushroom L-tyrosinase assay 147

5.4.3.1: Anti-tyrosinase activity of various fractions and isolated compounds from *C. odorata* 147

5.5: Conclusion 149

References 150

CHAPTER 6 153

CHEMICAL CHARACTERIZATION AND BIOLOGICAL INVESTIGATION OF *ASPARAGUS LIGNOSUS* 153

6.1 *Asparagus lignosus* 153

6.1.1 Isolation of constituent of the BuOH extract fraction 153

6.2: Structural elucidation and characterization of compounds 155

6.2.1: Structural elucidation of compound 19 155

6.2.2: Structural elucidation of compound 20 158

6.2.3: Structural elucidation of compound 21 160

6.3. Physico-chemical data of isolated compounds from *A. lignosus* 164

6.3.1: 20-Hydroxyecdysone (19) 164

6.3.2: Quercetin (20) 164

6.3.3: Isorhamnetin-3-*O*-rutinoside (narcissin) (21) 164

6.4 Biological evaluation 165

6.4.1 Alpha-glucosidase inhibition 165

6.4.2 Alpha-amylase inhibition 166

6.4.3 Mushroom L-tyrosinase assay 167

6.5 Conclusion 169

6.6 References 170

CHAPTER 7 174

CONCLUSION AND RECOMMENDATIONS 174

Recommendations 176

Reference 177

NMR spectra of isolated compounds from *Protea cynaroides* 178

Appendix 1B 182

NMR spectra of isolated compounds from *Cliffortia odorata* 182

Appendix 1C 194

NMR spectra of isolated compounds from *Asparagus lignosus* 194

LIST OF FIGURES

FIGURE 1. 1: LIST OF ISOLATED COMPOUNDS FROM THE THREE PLANTS XX
FIGURE 1. 2: EXAMPLE OF DIFFERENT COMPOUNDS OF NATURAL PRODUCTS 2

FIGURE 2. 1: PHOTOGRAPH OF *PROTEA CYNAROIDES*...23
FIGURE 2. 2: DISTRIBUTION MAP *PROTEA CYNAROIDES* IN SOUTH AFRICA 24
FIGURE 2. 3: SOME PHENOLIC COMPOUNDS ISOLATED FROM *P. CYNAROIDES*.............................25
FIGURE 2. 4: PHOTOGRAPH OF *CLIFFORTIA ODORATA*. 29
FIGURE 2. 6: PHOTOGRAPH OF *ASPARAGUS LIGNOSUS* 35
FIGURE 2. 7: DISTRIBUTION MAP OF *ASPARAGUS LIGNOSUS* IN SOUTH AFRICA..................35

FIGURE 4. 1: CHEMICAL STRUCTURE OF COMPOUND 1 64
FIGURE 4. 2: CHEMICAL STRUCTURE OF COMPOUND **2** 66
FIGURE 4. 3: ^{1}H NMR SPECTRUM PROTON OF COMPOUND **2** IN DMSO-D_6 67
FIGURE 4. 4: ^{13}C NMR SPECTRUM OF COMPOUND **2** IN DMSO-D_6 68
FIGURE 4. 5: HSQC SPECTRA OF COMPOUND **2** 69
FIGURE 4. 6: HMBC SPECTRUM OF COMPOUND **2** 70
FIGURE 4. 7: KEY HMBC (→) AND COSY (▬) CORRELATIONS OF COMPOUND **2** 70
FIGURE 4. 8: CHEMICAL STRUCTURE OF COMPOUND **3** 72
FIGURE 4. 9: CHEMICAL STRUCTURE OF COMPOUND **4** 74
FIGURE 4. 10: CHEMICAL STRUCTURE OF COMPOUND **5** 76
FIGURE 4. 11: ^{1}H NMR SPECTRUM PROTON OF COMPOUND **5** IN CD_3OD.................................77
FIGURE 4. 12: ^{13}C NMR SPECTRUM OF COMPOUND **5**...78
FIGURE 4. 13: HSQC SPECTRA OF COMPOUND **5** 79
FIGURE 4. 14: HMBC SPECTRA OF COMPOUND **5** 80
FIGURE 4. 15: KEY HMBC (→) AND COSY (▬) CORRELATIONS OF COMPOUNDS **5** 80
FIGURE 4. 16: CHEMICAL STRUCTURE OF COMPOUND **6** 82
FIGURE 4. 17: ^{1}H NMR SPECTRUM PROTON OF COMPOUND **6** 83
FIGURE 4. 18: STACKED ^{13}C AND DEPT 135 SPECTRUM OF **6**..84
FIGURE 4. 19: HMBC SPECTRA OF COMPOUND **6** 85
FIGURE 4. 20: CHEMICAL STRUCTURE OF COMPOUND **7** 86
FIGURE 4. 21: ^{1}H NMR SPECTRUM OF COMPOUND **7** IN CD_3OD 87
FIGURE 4. 22: ^{13}C NMR SPECTRUM OF COMPOUND **7** IN CD_3OD 88
FIGURE 4. 23: HMBC SPECTRA OF COMPOUND **7** 89
FIGURE 4. 24: KEY HMBC (→) AND COSY (▬) CORRELATIONS OF COMPOUNDS **7** 89
FIGURE 4. 25: CHEMICAL STRUCTURE OF COMPOUND **8** 91
FIGURE 4. 26: ^{1}H NMR SPECTRUM OF COMPOUND **8** IN DMSO-D_6...92
FIGURE 4. 27: ^{13}C AND DEPT-135 NMR SPECTRA OF COMPOUND **8**...93
FIGURE 4. 28: ^{1}H-^{1}H COSY NMR SPECTRA OF COMPOUND **8**...94
FIGURE 4. 29: HSQC NMR SPECTRA OF COMPOUND **8** 95
FIGURE 4. 30: HMBC NMR SPECTRA OF COMPOUND **8** 96
FIGURE 4. 31: CHEMICAL STRUCTURES OF THE ISOLATED COMPOUNDS FROM *P. CYNAROIDES* ..98
FIGURE 4. 32: AVERAGE % INHIBITION OF ALPHA-GLUCOSIDASE OF THE PLANT EXTRACTS....102

Figure 4. 33: Average % inhibition of tyrosinase by the extracts from *P. cynaroides* 103
Figure 4. 34: IC_{50} plot of compound 4 and 6 from *P. cynaroides* against kojic acid .. 104
Figure 4. 35: IC_{50} plot of compound 1 and 3 from *P. cynaroides* 104

Figure 5. 1: Chemical structure of compound **9** 112
Figure 5. 2: ^{1}H NMR spectrum of compound **9** in $CDCl_3$ 113
Figure 5. 3: HMBC spectra of compound **9** 114
Figure 5. 4 : HMBC and COSY correlations of compound **9** 114
Figure 5. 5: Chemical structure of compound **10** 116
Figure 5. 6: Chemical structure of compound **11** 118
Figure 5. 7: Chemical structure of compound **12** 120
Figure 5. 8: Chemical structure of compound **13** 121
Figure 5. 9: Chemical structure of compound **14** 123
Figure 5. 10: ^{1}H NMR spectrum of compound **14** in CD_3OD 124
Figure 5. 11: Stacked ^{13}C and DEPT 135 spectrum of **14** in CD_3OD 125
Figure 5. 12: COSY spectrum of compound **14** in CD_3OD 126
Figure 5. 13: HSQC spectrum of compound **14** in CD_3OD 127
Figure 5. 14: HMBC spectrum of compound **14** in CD_3OD 128
Figure 5. 15: Key HMBC and COSY correlations of **14** 128
Figure 5. 16: Chemical structure of compound **15** 130
Figure 5. 17: Chemical structure of compound **16** 131
Figure 5. 18: ^{1}H NMR spectrum of compound **16** in DMSO-d_6 132
Figure 5. 19: ^{13}C NMR spectrum of compound 16 in DMSO-d_6 133
Figure 5. 20: Chemical structure of compound **17** 135
Figure 5. 21: Chemical structures of compound **18** 137
Figure 5. 22: ^{1}H NMR spectrum of compound **18** in D_2O 138
Figure 5. 23: Stacked ^{1}H NMR spectra of compound **18**, sucrose, glucose, and fructose in D_2O. 139
Figure 5. 24: Chemical structures of the isolated compounds from *C. odorata* 140
Figure 5. 25: Average % inhibition of alpha-glucosidase of the plant extracts in triplicates 144
Figure 5. 26: alpha-glucosidase inhibitory activity estimation of IC_{50} values isolated compounds **15** and **16** 146
Figure 5. 27: Average % inhibition of Tyrosinase of the plant extracts 148
Figure 5. 28: IC_{50} plot of compound **11** and **12** 149

Figure 6. 1: Chemical structure of compound **19** 155
Figure 6. 2: Chemical structure of compound **20** 158
Figure 6. 3: Chemical structure of compound **21** 160
Figure 6. 4: Key HMBC (→) correlations of compounds **21** 161
Figure 6. 5: Chemical structures of the isolated compounds from *A. lignosus* 163
Figure 6. 6: Average % inhibition of alpha-glucosidase of the plant extracts 165
Figure 6. 7: % of Tyrosinase inhibition by A. lignosus extracts 167
Figure 6. 8: IC_{50} plot of compound **20** 168

LIST OF TABLES AND SCHEMES

TABLES

TABLE 1. 1: SOME MEDICINAL PLANTS USED BY THE MAJORITY OF SOUTH AFRICANS 3
TABLE 1. 2: MOST STUDIED AND COMMONLY USED ANTIDIABETIC MEDICINAL PLANTS 7

TABLE 2. 1: SOME ISOLATED COMPOUNDS OF *PROTEA* GENERA AND THEIR BIOLOGICAL ACTIVITIES. 26
TABLE 2. 2: SOME ISOLATED COMPOUNDS AND ACTIVITIES OF *CLIFFORTIA* GENERA 31
TABLE 2. 3: SOME ISOLATED COMPOUNDS AND ACTIVITIES OF *ASPARAGUS* GENERA 37

TABLE 4. 1: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUNDS 1 65
TABLE 4. 2. ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUNDS 2 IN DMSO-D_6...................71
TABLE 4. 3: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUNDS 3 IN D_2O. 73
TABLE 4. 4: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUND 4 IN PYRIDINE D_5.................75
TABLE 4. 5: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUNDS 5 IN CD_3OD. 81
TABLE 4. 6: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUNDS 6 IN DMSO-D_6 84
TABLE 4. 7: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUNDS 7 IN CD_3OD......................90
TABLE 4. 8: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUNDS 8 IN DMSO-D_6..................97
TABLE 4. 9: IC_{50} OF ISOLATED COMPOUNDS AND ACARBOSE ON ALPHA-GLUCOSIDASE AND ALPHA-AMYLASE 102
TABLE 4. 10: % INHIBITION OF TYROSINASE AND IC_{50} OF COMPOUND 4, 6 AND KOIC ACID 104
TABLE 4. 11: IC_{50} VALUES OF ISOLATED COMPOUNDS OF *P. CYNAROIDE* 105

TABLE 5. 1: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUNDS 9......................................115
TABLE 5. 2: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUNDS 10 117
TABLE 5. 3: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUND 11....................................119
TABLE 5. 4: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUND 12 AND 13........................122
TABLE 5. 5: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUND 14 129
TABLE 5. 6: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUND 14 AND 15........................134
TABLE 5. 7: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUND 17....................................136
TABLE 5. 8: INHIBITORY ACTIVITY (%) OF *C. ODORATA* EXTRACTS AGAINST ALPHA-GLUCOSIDASE ENZYME 145
TABLE 5. 9: INHIBITORY ACTIVITY (%) OF *C. ODORATA* COMPOUNDS AGAINST ALPHA-GLUCOSIDASE ENZYME 145
TABLE 5. 10: IC_{50} VALUES OF TESTED COMPOUNDS ON ALPHA-GLUCOSIDASE 146
TABLE 5. 11: IC_{50} VALUES OF ISOLATED COMPOUNDS FROM ETOAC EXTRACT OF *C. ODORATA* 148

TABLE 6. 1: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUND 19 IN CD_3OD 156
TABLE 6. 2: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUND 20 IN CD_3OD 159

TABLE 6. 3: ^{1}H AND ^{13}C NMR SPECTRAL DATA OF COMPOUND 21 IN CD_3OD 162
TABLE 6. 4: INHIBITORY ACTIVITY (%) OF *C. ODORATA* EXTRACTS AGAINST ALPHA-GLUCOSIDASE ENZYME 165
TABLE 6. 5: INHIBITORY ACTIVITY (%) OF *A. LIGNOSUS* COMPOUNDS AGAINST ALPHA-GLUCOSIDASE ENZYME 166
TABLE 6. 6: IC_{50} VALUES OF ISOLATED COMPOUNDS FROM BUOH EXTRACT OF *A. LIGNOSUS* . 168

SCHEMES

SCHEME 4. 1: A SCHEME OF EXPERIMENTAL PROCEDURE FOR THE ISOLATION OF COMPOUNDS FROM *P. CYNAROIDE* 63

SCHEME 5. 1: A SCHEME OF EXPERIMENTAL PROCEDURE FOR THE ISOLATION OF COMPOUNDS FROM *C. ODORATA* 111

SCHEME 6. 1: A SCHEME OF EXPERIMENTAL PROCEDURE FOR THE ISOLATION OF COMPOUNDS FROM *A. LIGNOSUS* 154

LIST OF ABBRIVIATIONS

1D NMR	one-dimensional nuclear magnetic resonance
2D NMR	two-dimensional nuclear magnetic resonance
^{1}H NMR	proton nuclear magnetic resonance
^{13}C NMR	carbon 13 nuclear magnetic resonance
br	broad signal
$CDCl_3$	deuterated chloroform
$CHCl_3$	chloroform
CD_3OD	deuterated methanol
COSY	correlation spectroscopy
d	doublet
DCM	dichloromethane
dd	doublet of doublets
DEPT	distortionless enhancement by polarization transfer
DMSO	dimethyl sulfoxide
ESI-MS	electrospray ionization mass spectrometry
et al.	*et altera* (and others)
EtOAc	ethyl acetate
FTIR	Fourier-transform infrared spectroscopy
g	gram
GC-MS	gas chromatography-mass spectrometry
HMBC	heteronuclear multiplebond connectivity
HSQC	heteronuclear single quantum coherence
Hz	herz
IC_{50}	Half maximal inhibitory concentration

J	coupling constant
L	liter
LC	liquid chromatography
LC/MS	liquid chromatography-mass spectrometery
m	multiplet
MeOH	methanol
mg	milligram
mL	millilitre
*m*M	millimolar
MS	mass spectrometry
m/z	mass per charge
μg	microgram
μL	microliter
NMR	nuclear magnetic resonance
ppm	parts per million
q	quartet
s	singlet
t	triplet
TLC	thin layer chromatography
UV	ultra-violet

LIST OF ISOLATED COMPOUNDS

Figure 1. 1: List of isolated compounds from the three plants

CHAPTER 1

INTRODUCTION

1.1 General introduction to Natural products

The use of medicinal plants in Southern Africa is widespread with many South African households using medicinal plants for the treatment of various ailments (Atawodi, 2005). These medicinal plants are a rich source of phenols which are essential to the human diet for optimum health (Rice-Evans, et al., 1996). A number of the different classes of these phenols are biologically active and responsible for the anti-oxidant, anti-inflammatory, chemopreventive, and, neuroprotective activities (Atawodi, 2005; Long, et al., 2009). Natural product chemistry is a discipline in which investigation of the chemical composition of medicinal plants is undertaken towards the identification of the various classes of organic compounds present in medicinal plants, which are responsible for their biological activity. The plant kingdom is rich and has abundant sources of remedies for the prevention and cure of various human ailments, as well as maintenance of a healthy and normal physiological state (Goswan, 2002).

Plants that have medicinal activities are known to have the advantage of possessing chemical diversities of secondary metabolites. Such medicinal properties are often of superior, and if not, of similar activity as modern synthetic drugs. A wide range of phytochemicals that includes flavonoids, anthocyanins, alkaloids, and, coumarins among others, has been discovered (Jassim and Naji, 2003). In Africa, people's reliance on herbal medicine is very high, as indicated by the fact that 70% of the population depends on traditional medicines for their primary health care needs, despite the lack of scientific information on efficacy and side effects (Kokwaro, 1996). This is normally due to limited access to modern health facilities. Poverty further aggravates the situation as sometimes the available medicine is unaffordable.

Depending on their chemical composition, the natural products may be grouped according to a recurring structural feature, or they may be grouped according to the genus of their plant source,

or by their physiological effects (antimicrobials, antibiotics, analgesics), or by similarities in the route by which they are synthesized by the organism (biosynthesis) and these groups are classified as phenols, terpenes, triterpenoids, alkaloids, coumarins, fatty acids as well as tannins, etc (Cannell, 1998; Mann et al., 1994; Rahman, 2012). This chemical diversity is a result of secondary metabolism which occurs within the living organism (plant) from which the natural products are derived (Caturla, et al., 2005).

vanillin limonene squalene

caffeine coumarin caprylic acid glucogallin

Figure 1. 2: Example of different compounds of natural products

1.2 South African medicinal plants

South Africa is a country that has a strong history of traditional healing, and hosts wide biodiversity of approximately 30,000 flowering plant species, accounting for almost 10% of the world's higher plant species (Louw et al., 2002). Approximately 80% of black South Africans still depend on using plants for medicinal purposes because they are cheap, safe, easily accessible, culturally acceptable, better tolerated by patients, and at times the only therapy that subsists (Shaikh, 2011). Statistically, about 25% of the world's plants are found in South Africa and about 3000 medicinal plants are often used for traditional medicine in South Africa with 2942 used for human administration (Van Wyk, 2008). The various uses of plants include

treatment of numerous diseases and life-threatening conditions such as viral diseases, diabetes, and cancer (Street and Prinsloo, 2013).

Table 1. 1: **Most commonly used medicinal plants by the majority of South Africans**

Plant name	**Part used**	**Uses**	**Reference**
Aloe forex	roots, stems, and leaves	for arthritis, conjunctivitis, toothaches, sinusitis, stomach pains, burns, insect bites, sores, and sunburn, eczema	Crouch et al., 2006; Watt and Breyer-Brandwijk, 1962; Bruce, 1975.
artemisia afra	roots, stems, and leaves	coughs, colds, fever, loss of appetite, colic, headache, earache, intestinal worms, and malaria	Van Wyk et al. 2005.
Aspalathus linearis	leaves and twigs	pregnancy to relieve heartburn and nausea, colic relief, and, as a milk substitute for infants	Gruenwald, 2009.
Helichrysum	leaves and twigs	coughs, colds, fever, infection, menstrual pain, headaches, insomnia, hypertension, allergies, and diabetes.	Van der Walt, 2008; Van Staden, 2009.
Hypoxis hemerocallidea	roots and corm	cold, flu, hypertension, adult-onset diabetes, psoriasis, urinary infections, testicular tumors, prostate hypertrophy, HIV/AIDS and some central nervous system disorders, anticancer, antidiabetic, antimicrobial, antioxidant, and anti-inflammatory	Hutchings et al., 1996; Laporta et al., 2007; Ojewole, 2006; Steenkamp et al., 2006; Mills et al., 2005.
Merwilla natalensis (Scilla)	bulb	gastrointestinal ailments, sprains and fractures, tumors, menstrual pains, infertility, and to induce labor and delivery in pregnancy	Van Wyk et al. 1997; Watt and Breyer-Brandwijk, 1962; Sparg et al., 2002; Moodley et al., 2004
Sutherlandia frutescens	shoots	treatment of cancer, gastric ailments, gynecological problems, rheumatism, edema and fevers and also as a bitter tonic or blood purifier, colds, influenza, chicken-pox, diabetes, varicose veins, piles, inflammation, liver problems, backache, and rheumatism	Van Wyk and Albrecht, 2008; Drewes et al., 2006; Crouch et al., 2006.

1.3 Diabetes Mellitus

Diabetes mellitus is a group of metabolic diseases in which the person has high blood glucose (blood sugar) either because insulin production is inadequate, or the body's cells do not respond properly to insulin, or both (Alberti et al., 1998; WHO, 1999; Khan et al., 2009). Diabetes can be classified into type 1, type 2, and gestational diabetes. In type 1, the body does not produce insulin and approximately 10% of all diabetic patients fall into this group (Daneman, 2006). In type 2 diabetes, the body does not produce enough insulin for proper function and it is estimated that 90% of all diabetic cases worldwide are type 2 (WHO, 1999). In addition, the prevalence of the disease is increasing rapidly with around 392 million people diagnosed with type 2 diabetes in 2015 compared with 30 million in 1985 (Vos et al., 2016; Smyth and Heron, 2006). Gestational diabetes is defined as a condition in which your blood sugar levels become high during pregnancy (WHO, 2013) and, may improve or disappear after delivery. Even though it may be transient, gestational diabetes may damage the health of the fetus or mother, and about 40% of women with gestational diabetes develop type 2 diabetes later in life (Mayfield, 1998).

1.3.1 Type 1 diabetes

Type 1 diabetes is sometimes known as insulin-dependent diabetes mellitus or juvenile-onset diabetes because its clinical manifestations occur before the age of 40. In this group, patients need to take insulin for the rest of their lives. However, individuals with type 1 diabetes are recommended to follow a healthy eating plan, do adequate exercises to lead a normal life (WHO, 2016).

1.3.2 Type 2 diabetes

Overweight and obese people have a much higher risk of developing type 2 diabetes compared to their healthy counterparts. Furthermore, being overweight, physically inactive, and eating

wrong foods all contribute to the risk of developing type 2 diabetes. Aging also contributes to the risk of developing type 2 diabetes (Jain and Saraf, 2010). This is attributable to the fact that with age, people tend to put on weight due to being less physically active. It is a general recommendation that people with type 2 diabetes ought to eat healthily, be physically active, and test their blood glucose. They may also need to take oral medication and/or insulin to control blood glucose levels. The risk of cardiovascular disease is much higher for diabetic cases. It is therefore crucial that blood pressure and cholesterol levels are monitored regularly. Factors that put patients with diabetes at risk include smoking, inactivity, family history, high blood pressure and abnormal cholesterol and triglyceride levels. It is therefore prohibited to smoke as smoking is thought to have serious effects on cardiovascular health. Other factors that have a bad effect on diabetic patients include hypoglycemia (low blood glucose) and hyperglycemia (when blood glucose is too high) (WHO, 2006).

1.3.3 Symptoms and treatment of diabetes

Patients with high blood sugar typically experience polyuria (frequent urination), polydipsia (increasingly thirsty) and polyphagia (hungry), unusual weight loss, fatigue, cuts and bruises that do not heal, numbness and tingling in hands and feet. Patients with type 1 diabetes are treated with insulin injection as well as special diet and exercise whereas those with type 2 are usually treated with oral medication eg. metformin but sometimes insulin injections are also required (Jain and Saraf, 2010; Maruthur et al., 2016).

Treatment of type 2 diabetes has often ranged from allopathic medicine to traditional medicine, in the latter medicinal plants (ethnobotanicals) have been used. In allopathic medicine, other drugs are used in addition to metformin including sulfonylureas which help the body to make more insulin, meglitinides which stimulates the pancreas to release insulin, thiazolidinediones which makes the body more sensitive to insulin and dipeptidyl peptidase-IV inhibitors which help reduce blood sugar levels. Each of these medications has side effects as result, the

prevalence of the disease is increasing rapidly with around 392 million people diagnosed with type 2 diabetes in 2015 compared with 30 million in 1985 (Vos et al., 2016; Smyth and Heron, 2006).

1.4 Enzyme inhibitors

Alpha glucosidase and alpha amylase are the important enzymes involved in the digestion of carbohydrates. Alpha Amylase is involved in the breakdown of long chain carbohydrates and alpha glucosidase breaks down starch and disaccharides to glucose. They serve as the major digestive enzymes and help in intestinal absorption. Alpha amylase and glucosidase inhibitors are the potential targets in the development of lead compounds for the treatment of diabetes (Subramanian et al., 2008)

1.4.1 Alpha-amylase

The alpha-amylase enzyme catalyzes the endohydrolysis of alpha-1,4- glycosidic linkages in common polysaccharides (starches) from the diet, to regulate blood sugar levels (Nickavar et al., 2008). During the disease state of diabetes mellitus, this enzyme can be detrimental, due to the biochemical defect causing blood glucose levels to be elevated. Inhibition of the enzyme's activity would lower glucose absorption by the small intestine and would control the elevation of glucose levels. This would then allow more undigested starch to make it to the colon (Gautam, et al., 2013).

1.4.2 Alpha-glucosidase

Alpha-glucosidase inhibitors are drugs that inhibit the breakdown of carbohydrates in the gut and therefore the production and absorption of glucose may be employed in the treatment of patients with type 2 diabetes or impaired glucose tolerance (van der Laar, 2008). The membrane-bound intestinal alpha-glucosidase hydrolyzes oligosaccharides, trisaccharides, and

disaccharides to glucose and other monosaccharides in the small intestine. Inhibition of these enzyme systems reduces the rate of digestion of carbohydrates (Naquvi et al., 2011).

In recent years, many efforts have been made to identify effective alpha-glucosidase inhibitors from natural sources to develop a physiologic functional food or lead compounds for use against diabetes. Many alpha-glucosidase inhibitors, such as flavonoids, alkaloids, terpenoids, anthocyanins, glycosides, phenolic compounds, etc, have been isolated from plants (Mahajan et al., 2011).

Table 1. 2: **Most studied and commonly used antidiabetic medicinal plants**

Plant name	**Family**	**Part used**	**Reference**
Allium cepa (Onion)	Alliaceae	Onion bulbs	Sheela et al., 1995; Kumari et al., 1995; Eidi et al., 2006.
Allium sativum (Garlic)	Alliaceae	Garlic cloves	Sheela et al., 1992; Augusti and Shella 1996.
Aloe vera (*Aloe barbadensis*)	Aspholedeceae	Leaf pulp and gel	Rajasekaran., et al., 2004; Tanaka et al., 2006.
Catharanthus roseus	Apocynaceae	Fresh leaf juice	Benjamin et al., 1994; Marles and Farnsworth, 1995; Singh et al., 2001; Nammi et al., 2003.
Cinnamomum cassia	Lauraceae	Bark	Jarvill-Taylor et al., 2001; Chase and McQeen, 2007.
Coccinia indica	Cucurbitaceae	Leaves	Hossain et al., 1992; Kumar et al., 1993.
Fiscus benghalensis	Moraceae	Leaves and bark	Achrecker et al., 1991; Augusti et al., 1994
Gymnema sylvestre	Asclepiadaceae	Leaves	Shanmugasundaram, 1990; Chattopadhyay, 1999.
Ginseng	Araliaceae	Root and leaves	Yang et al., 1990; Petit et al., 1995.
Momordica charrant	Cucurbitaceae	Fruit pulp, seed, leaves	van de Venter et al., 2008.
Sclerocarya birrea	Anacardiaceae	Bark	Gondwe *et al*., 2008; Ojewole, 2004.
Securidaca longepedunculata	Polygalaceae	Stem bark and roots	Ojewole, 2004.
Sutherlandia frutescens	Fabaceae	Shoots	Chadwick et al., 2007.
Syzygium cordatum (Hochst.)	Myrtaceae	Leaves	Musabayane et al., 2005.
Psidium guajava	Myrtaceae	Leaves and roots	an de Venter et al., 2008.

Vinca major	Apocynaceae	Leaves and roots	van de Venter et al., 2008.

1.5 Tyrosinase

Tyrosinase is a multifunctional copper containing enzyme. Tyrosinase plays diverse physiological roles in different organisms. In fungi and vertebrates, tyrosinase catalyzes the initial step in the formation of the pigment melanin from tyrosine. In plants, the physiological substrates are a variety of phenolics. Tyrosinase oxidizes them in the browning pathway observed when tissues are injured. One possible role is protection of the wound from pathogens or insects. Tyrosinase is thought to be involved in wound healing and possibly sclerotization of the cuticle (Kim and Uyama, 2005). In addition, tyrosinase is known to be involved in the molting process of insects and adhesion of marine organisms (Bernard and Berthon, 2000; Haq et al. 2006; Kim et al. 2006; Lee et al. 2006).

1.6 Problem statement

In South Africa, different plant species are used to treat several diseases especially by the rural population where western medicine is not accessible or affordable. Nowadays, about 200,000 traditional healers exploit traditional medicine in South Africa and a high proportion of the population uses these traditional medicines as their primary source of health care (Lewis, 2009). The approach of using plant species in the discovery of therapeutics remains an ultimate strategy for the effective usage of plants (Hostettmann, 1998). For instance, *A. lignosus, C. odorata,* and *P. cynaroides* L. (King Protea) are common plants used traditionally as medicines for the treatment of several diseases, yet the phytochemical and biological activity of these plants has not been investigated in details. This thus necessitates more efficient research that will lead to innovative use of the above-mentioned plants. A comprehensive investigation for the presence of chemical constituents implicated in the various health benefits of these three selected medicinal plants will be pursued.

1.7 Rationale of the study

Medicinal plants have had a wide application in treating human ailments from prehistoric times because they produce many chemical compounds with biological functions, including defense against insects, fungi, and herbivorous mammals. (Shi, et al., 2010; Fabricant and Farnsworth, 2001).

Ethnobotanical studies are often significant in revealing locally important plant species especially for the discovery of crude drugs. Documentation of traditional knowledge, especially on the medicinal use of plants has provided many important drugs of modern days (Teklehaymanot and Giday, 2007). Over 50% of all modern clinical drugs are of natural origin and these natural products play an important role in drug development programs of the pharmaceutical industry (Farombi, 2003).

For a developing country such as South Africa, the world health organization (WHO) estimates that about 80% of the population relies on plant-based preparations used in their traditional medicine system and as basic needs for human primary health care (Kesaran et al., 2007). This is mainly because of the fact that African traditional medicine does not have as many side effects as their modern counterparts coupled with the fact that African traditional medicines are cost-effective (Benzie and Wachtel-Galor, 2011). South African traditional healers exploit a multitude of such plants to treat many health or medical conditions and these include *Aloe succotrina, Asparagus lignosus, Carpobrotus edulis, Cissampelos capensis, Chironia bassifera, Cliffortia odorata, Euclea undulata, Gethyllis multifolia, Kedrostris nana, Protea cynaroides, Psidium guajava,* and *Sutherlandia frutescens.* These plants were initially selected for investigation in this study as they are indigenous South African plants that have had applications in the treatment of arthritis, heart ailments, treatment of skin

disorders, stomach complaints, bladder and kidney ailments, cancer, treatment of back pain, colds and flu, cough, diabetes, feet ailments, hypertension, Tuberculosis (TB) and as a detoxification aid (Deutschländer et al., 2009a; Van Wyk and Gericke, 2000; Van Wyk, 2008). In spite of these uses, there is a lack of scientific study/validation concerning the safety evaluation and phytochemistry of the plants. And this necessitates to investigate the phytochemistry of these selected South African indigenous medicinal plants to validate their claimed medicinal use and to successfully characterize the metabolites that could be used to establish fingerprints or characteristic features of each species.

1.8 Justification

Natural products have provided biologically active compounds for many years and many of today's medicines are either obtained directly from a natural source or were developed from a lead compound originally obtained from a natural source (Koparde, Doijad, and Magdum., 2019). The use of medicinal plants as a fundamental component of the African traditional healthcare system is perhaps the oldest and the most assorted of all therapeutic systems. Since poorer societies rely mainly on traditional medicine for primary health care, there is an urgent need for phytochemical analysis of these medicinal plants to understand their mode of action, efficiency, and safety. This research should reveal the main constituents which may be used as part of commercial products for the cosmeceutical and pharmaceutical industries.

1.9 Aim of the study

To conduct a comprehensive phytochemical study on three indigenous South African medicinal plants (*Asparagus lignosus, Cliffortia odorata, Protea cynaroides*) and biologically evaluate their extracts and isolated compounds for enzymatic inhibition of alpha-glucosidase, alpha-amylase, and tyrosinase enzyme.

1.10 Objectives

1.10.1 General objectives

Preparation of aqueous and organic solvent extracts of *Asparagus lignosus*, *Clifortia odorata* and *Protea cynaroides*, isolation and structural elucidation of secondary metabolites, and biological evaluation of extracts and the isolates.

1.10.2 Specific objectives

- Collection and identification of the three plant species.
- Preparation of solvent extracts of *A. lignosus, C. odorata* and *P. cynaroides.*
- Isolation, identification, and structure elucidation of the chemical constituents from plant extracts using chromatographic and spectroscopic techniques.
- To determine anti-diabetic activity by studying enzyme inhibitory activity of extracts and isolated compounds on alpha-glucosidase and alpha-amylase enzymes.
- To determine anti-tyrosinase activity by studying the inhibitory activity of extracts and the isolated compounds on tyrosinase enzyme.

1.11 Structure of the thesis

Chapter 1: General introduction

Chapter 2: Literature review of medicinal plants studied

Chapter 3: Methodology

Chapter 4, 5 and 6: Phytochemical and biological investigation of the three studied plants

Chapter 7: Conclusions and recommendations

References

Achrekar, S., Kaklij, G.S., Pote, M.., Kelka, S.M., 1991. Hypoglycaemic activity of *Eugenia jambolana* and *Ficus bengalensis*: Mechanism of action. *In vivo* 5: 143-147.

Alberti, K.G.M.M. and Zimmet, P.Z., 1998. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus. Provisional report of a WHO consultation. *Diabetic medicine*, *15*(7), pp.539-553.

Atawodi, S.E., 2005. Antioxidant potential of African medicinal plants. *African Journal of Biotechnology*, *4*(2), pp.128-133.

Augusti, K.T., Daniel, R.S., Cherian, S., et al., 1994. Effect of leucopelargonin derivative from *Ficus bengalensis* Linn. on diabetic dogs. *Indian J Med Res* 99: 82-86.

Augusti, K.T., Sheela, C.G., 1996. Antiperoxide effect of S-allyl cysteine sulfoxide, an insulin secretagogue, in diabetic rats. *Experientia* 52: 115-120.

Benjamin, B.D., Kelkar, S.M., Pote, M.S., 1994. *Catharanthus roseus* cell cultures: Growth, alkaloid synthesis and antidiabetic activity. *Phytother Res.* 8: 185-186.

Benzie, I.F. and Wachtel-Galor, S. eds., 2011. Herbal medicine: *Biomolecular and Clinical Aspects.*

Bernard, P & Berthon, J.Y., 2000. Resveratrol: an original mechanism on tyrosinase inhibition. *International Journal of Cosmetic Science*, 22: 219-226.

Breyer-Brandwijk, M.G., 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa being an Account of their Medicinal and other Uses, Chemical Composition, *Pharmacological Effects and Toxicology in Man and Animal.*, (Edn 2).

Bruce, W.G.G., 1975. Medicinal properties in the *Aloe*. *Excelsa*, *5*, pp.57-68.

Cannell, R.J., 1998. How to approach the isolation of a natural product. In *Natural products isolation* (pp. 1-51). Humana Press.

Caturla, N., Pérez-Fons, L., Estepa, A. and Micol, V., 2005. Differential effects of oleuropein, a biophenol from *Olea europaea*, on anionic and zwiterionic phospholipid model membranes. *Chemistry and Physics of Lipids*, *137*(1), pp.2-17.

Chadwick, W.A., Roux, S., van de Venter, M., Louw, J. and Oelofsen, W., 2007. Anti-diabetic effects of *Sutherlandia frutescens* in Wistar rats fed a diabetogenic diet. *Journal of ethnopharmacology*, *109*(1), pp.121-127.

Chase, C.K., McQueen, C.E., 2007. Cinnamon in diabetes mellitus. *Am J Health Sst Pharm* 64: 1033-1035

Chattopadhyay, R.R., 1999. Possible mechanism of antihyperglycaemic effect of *Gymnema sylvestre* leaf extract, part I. *Gen Pharmacol* 31: 495-496.

Crouch, N., Symmonds, R., Spring, W. and Diederichs, N., 2006. Fact sheets for growing popular medicinal plant species. *Commercialising Medicinal Plants: A Southern African Guide. Sun Press, Stellenbosch, South Africa*, pp.100-102.

Daneman, D., 2006. Type 1 diabetes. *The Lancet*, *367*(9513), pp.847-858.

Deutschländer, M.S., Lall, N. and Van De Venter, M., 2009. Plant species used in the treatment of diabetes by South African traditional healers: An inventory. *Pharmaceutical Biology*, *47*(4), pp.348-365.

Deutschländer, M.S., Lall, N. and Van De Venter, M., 2009. Plant species used in the treatment of diabetes by South African traditional healers: An inventory. *Pharmaceutical Biology*, *47*(4), pp.348-365.

Drewes, S., Horn, M. and Khan, F., 2006. The chemistry and pharmacology of medicinal plants. *Commercialising medicinal plants. A Southern African guide. Sun Press, African Sun Media, Stellenbosch*, pp.89-95.

Eidi, A., Eidi, M. and Esmaeili, E., 2006. Antidiabetic effect of garlic (*Allium sativum* L.) in normal and streptozotocin-induced diabetic rats. *Phytomedicine*, *13*(9-10), pp.624-629.

Fabricant, D.S. and Farnsworth, N.R., 2001. The value of plants used in traditional medicine for drug discovery. *Environmental Health Perspectives*, 109(Suppl 1), p.69.

Farombi, E.O., 2003. African indigenous plants with chemotherapeutic potentials and biotechnological approach to the production of bioactive prophylactic agents. *African Journal of biotechnology*, *2*(12), pp.662-671.

Gondwe, M., Kamadyaapa, D.R., Tufts, M.A., Chuturgoon, A.A., and Musabayane, C.T., 2008. *Sclerocarya birrea* [(A. Rich.) Hochst.] [Anacardiaceae] stem-bark ethanolic extract (SBE) modulates blood glucose, glomerular filtration rate (GFR) and mean arterial blood pressure (MAP) of STZ-induced diabetic rat. *Phytomedicine* 15: 699-709.

Goswami, A., Barooah, P.K. and Sandhu, J.S., 2002. Prospect of herbal drugs in the age of globalization-Indian scenario. *Journal of Scientific Industrial Research*, *61*(6), pp.423-431.

Graham, J.H., 2001. What do root pathogens see in mycorrhizas?. *New Phytologist*, pp.357-359.

Gruenwald, J., 2009. Novel botanical ingredients for beverages. *Clinics in dermatology*, *27*(2), pp.210-216.

Haq, A., Malik, A., Khan, M.T.H., Haq, A., Khan, S.B., Ahmad, A & Choudhary, M.I., 2006. Tyrosinase inhibitory lignans from the methanol extract of the

roots of *Vitex negundo* Linn. and their structure-activity relationship.

Phytomedicine, 13: 255-260.

Hossain, M.Z, Shibib, B.A., Rahman, R., 1992. Hypoglycaemic effects of *Coccina indica*: inhibition of key gluconeogenic enzyme, glucose-6-phosphatase. *Indian Journal of Experimental Biology* 30: 418-420.

Hostettmann, K. 1998. Strategy for the biological and chemical evaluation of plant extracts. *Pure Applied Chemistry*, 70, 1- 9.

Hutchings, A., 1996. *Zulu medicinal plants: An inventory*. University of Natal press.

inhibition. *International Journal of Cosmetic Science*, 22: 219-226..

Jain, S. and Saraf, S., 2010. Type 2 diabetes mellitus—Its global prevalence and therapeutic strategies. *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*, *4*(1), pp.48-56.

Jaruvill-Taylor, K., 2001. A hydroxychalone polymer derived from cinnamon function as a mimentic for insulin in 3T3-L1 adipocytes. *J. Am Coll Nutr*. 204:327-336.

Jassim, S.A. and Naji, M.A., 2003. Novel antiviral agents: a medicinal plant perspective. *Journal of Applied Microbiology*, *95*(3), pp.412-427.

Khan, A., Zaman, G. and Anderson, R.A., 2009. Bay leaves improve glucose and lipid profile of people with type 2 diabetes. *Journal of clinical biochemistry and nutrition*, *44*(1), pp.52-56.

Kim, M., Meng, X & Kim, A., 2006. Developing a Long-Lasting Tyrosinase

Inhibitor from Morus alba L. *Cosmetic & Toiletries magazine*, 27(3): 91-102.

Kim, Y.J and Uyama H., 2005. Tyrosinase inhibitors from natural and synthetic

sources: structure, inhibition mechanism and perspective for the future. *CMLS*

Cellular and Molecular Life Sciences, 62: 1707-1723.

Kokwaro, J.O., 1993. Medicinal plants of east Africa. *Nairobi: Kenya Literature Bureau 401p. ISBN*, *9966*(44), p.190.

Koparde, A.A., Doijad, R.C. and Magdum, C.S., 2019. Natural products in drug discovery. In *Pharmacognosy-medicinal plants*. IntechOpen.

Kumar, G.P., Sudheesh, S., Vijayalakshmi, N.R., 1993. Hypoglycaemic effect of *Coccina indica*: Mechanism of action. *Planta Med*. 59: 330-332.

Kumari, K., Mathew, B.C. and Augusti, K.T., 1995. Antidiabetic and hypolipidemic effects of S-methyl cysteine sulfoxide isolated from *Allium cepa* Linn. *Indian journal of biochemistry & biophysics*, *32*(1), pp.49-54.

Laporta, O., Pérez-Fons, L., Mallavia, R., Caturla, N. and Micol, V., 2007. Isolation, characterization and antioxidant capacity assessment of the bioactive compounds derived from *Hypoxis rooperi* corm extract (African potato). *Food Chemistry*, *101*(4), pp.1425-1437.

Lee, H., Jang, H.C., Park, H.K., Metzger, B.E. and Cho, N.H., 2008. Prevalence of type 2 diabetes among women with a previous history of gestational diabetes mellitus. Diabetes Research and Clinical Practice. 81: 124-129.

Lee, M.H., Lin, Y.P., Hsu, F.L., Zhan, G.R & Yen, K.Y., 2006. Bioactive constituents of *Spatholobus suberctus* in regulating tyrosinase-related proteins and mRNA in HEMn cells. *Phytochemistry*, 67:1262-1270.

Long, H.S., Tilney, P.M. and Van Wyk, B.E., 2010. The ethnobotany and pharmacognosy of *Olea europaea* subsp. africana (Oleaceae). *South African Journal of Botany*, *76*(2), pp.324-331.

Louw, C.A.M., Regnier, T.J.C. and Korsten, L., 2002. Medicinal bulbous plants of South Africa and their traditional relevance in the control of infectious diseases. *Journal of Ethnopharmacology*, *82*(2-3), pp.147-154.

Malik, A., Khan, M.T.H., Khan, S.B., Ahmad, A. and Choudhary, M.I., 2006. Tyrosinase inhibitory lignans from the methanol extract of the roots of Vitex negundo Linn. and their structure–activity relationship. *Phytomedicine*, *13*(4), pp.255-260.

Mann, J., Davidson, R.S., Hobbs, J.B., Banthorpe, D.V. and Harborne, J.B., 1994. Natural products: Their Chemistry and Biological Significance. *Longman Scientific and Technical*.

Marles, R.J., Farnsworth, N.R., 1995. Antidiabetic plants and their active constituents. *Phytomedicine* 2:137–189.

Maruthur, N.M., Tseng, E., Hutfless, S., Wilson, L.M., Suarez-Cuervo, C., Berger, Z., Chu, Y., Iyoha, E., Segal, J.B. and Bolen, S., 2016. Diabetes medications as monotherapy or metformin-based combination therapy for type 2 diabetes: a systematic review and meta-analysis. *Annals of internal medicine*, *164*(11), pp.740-751.

Mayfield, J.A., 1998. Diagnosis and classification of diabetes mellitus: new criteria. *American family physician*, *58*(6), p.1355.

Mills, E., Cooper, C., Seely, D. and Kanfer, I., 2005. African herbal medicines in the treatment of HIV: Hypoxis and Sutherlandia. An overview of evidence and pharmacology. *Nutrition journal*, *4*(1), pp.1-6.

Musabayane, C. T., Mahlalela, N., Shode, F.O., and Ojewole, J.A.O., 2005. Effects of *Syzygium cordatum* (Hochst.) [Myrtaceae] leaf extract on plasma glucose and hepatic

glycogen in streptozotocin-induced diabetic rats. *Journal of Ethnopharmacology* 97(3):485-490.

Nammi, S., Boini, M.K., Lodagala, S.D., and Behara, R.B.S., 2003. The juice of fresh leaves of *Catharanthus roseus* Linn. Reduces blood glucose in normal and alloxan diabetic rabbits, *BMC Complementary and Alternative Medicine* 3: 4.-15.

Ojewole, J.A., 2006. Antinociceptive, anti-inflammatory and antidiabetic properties of Hypoxis hemerocallidea Fisch. & CA Mey.(Hypoxidaceae) corm [‘African Potato’] aqueous extract in mice and rats. *Journal of ethnopharmacology*, *103*(1), pp.126-134.

Ojewole, J.A.O., 2004. Analgesic, antiinflammatory and hypoglycemic effects of *Sutherlandia frutescens* R. BR. (variety Incana E. MEY.) [Fabaceae] shoot aqueous extract. Methods and findings in experimental and clinical pharmacology 26(6):409-16.

Petit, P.R., Sauvaire, Y.D., Hillaire-Buys, D.M., Leconte, O.M., Baissac, Y.G., Ponsin, G.R., Ribes, G.R., 1995. Steroid saponins from *Fenugreek* seeds extraction, purification, and pharmacological investigation on feeding behavior and plasma cholesterol. *Steroids* 60: 674–680.

Rahman, A.U. ed., 2012. Studies in natural products chemistry (Vol. 37). Elsevier.

Rajasekaran, S., Sivagnanam, K., Ravi, K. and Subramanian, S., 2004. Hypoglycemic effect of *Aloe vera* gel on streptozotocin-induced diabetes in experimental rats. *Journal of Medicinal food*, *7*(1), pp.61-66.

Rice-Evans, C.A., Miller, N.J. and Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free radical biology and medicine*, *20*(7), pp.933-956.

Shanmugasundaram, E.R., Rajeswari, G., Baskaran, K., Kumar, B.R., Shanmugasundaran, K., and Kizar, A.B., 1990. Use of *Gymnema sylvestre* leaf extract in the control of blood glucose in insulin-dependent diabetes mellitus. *Journal of Ethnopharmacol* 30: 281-294

Sheela, C.G. and Augusti, K.T., 1992. Antidiabetic effects of S-allyl cysteine sulphoxide isolated from garlic *Allium sativum* Linn. *Indian journal of experimental biology*, *30*(6), pp.523-526.

Sheela, C.G., Kumud, K. and Augusti, K.T., 1995. Anti-diabetic effects of onion and garlic sulfoxide amino acids in rats. *Planta Medica*, *61*(04), pp.356-357.

Shi, Q., Li, L., Huo, C., Zhang, M. and Wang, Y., 2010. Study on natural medicinal chemistry and new drug development. Zhongcaoyao= *Chinese Traditional and Herbal Drugs*, 41(10), pp.1583-1589.

Singh, S.N., Vats, P., Suri, S., Shyam, R., Kumria, M.M.L., Ranganathan, S., and Sridharan K., 2001. Effect of an antidiabetic extract of *Catharanthus roseus* on enzymic activities in streptozotocin induced diabetic rats. *Journal of Ethnopharmacology* 76: 269–277.

Smyth, S. and Heron, A., 2006. Diabetes and obesity: the twin epidemics. *Nature medicine*, *12*(1), pp.75-80.

Sparg, S.G., Van Staden, J. and Jäger, A.K., 2002. Pharmacological and phytochemical screening of two Hyacinthaceae species: *Scilla natalensis* and *Ledebouria ovatifolia*. *Journal of ethnopharmacology*, *80*(1), pp.95-101.

Steenkamp, V., Gouws, M.C., Gulumian, M., Elgorashi, E.E. and Van Staden, J., 2006. Studies on antibacterial, anti-inflammatory and antioxidant activity of herbal remedies used

in the treatment of benign prostatic hyperplasia and prostatitis. *Journal of Ethnopharmacology*, *103*(1), pp.71-75.

Street, R.A. and Prinsloo, G., 2013. Commercially important medicinal plants of South Africa: a review. *Journal of chemistry*, pp.1-16. doi.org/10.1155/2013/205048

Tanaka, M., Misawa, E., Ito, Y., Habara, N., Nomaguchi, K., Yamada, M., Toida, T., Hayasawa, H., Takase, M., Inagaki, M. and Higuchi, R., 2006. Identification of five phytosterols from *Aloe vera* gel as anti-diabetic compounds. *Biological and Pharmaceutical Bulletin*, *29*(7), pp.1418-1422.

Teklehaymanot, T. and Giday, M., 2007. Ethnobotanical study of medicinal plants used by people in Zegie Peninsula, Northwestern Ethiopia. *Journal of ethnobiology and Ethnomedicine*, *3*(1), pp.1-11.

van de Venter, M., Roux, S., Bungu, L.C., Louw, J., Crouch, N.R., Grace, O.M., Maharaj, V., Pillay, P., Sewnarian, P., Bhagwandin, N. and Folb, P., 2008. Antidiabetic screening and scoring of 11 plants traditionally used in South Africa. *Journal of ethnopharmacology*, *119*(1), pp.81-86.

Van Wyk, B.E. and Albrecht, C., 2008. A review of the taxonomy, ethnobotany, chemistry and pharmacology of *Sutherlandia frutescens* (Fabaceae). *Journal of ethnopharmacology*, *119*(3), pp.620-629.

Van Wyk, B.E. and Gericke, N., 2000. *People's plants: A guide to useful plants of Southern Africa*. Briza publications.

Van Wyk, B.E., 2008. A broad review of commercially important southern African medicinal plants. *Journal of ethnopharmacology*, *119*(3), pp.342-355.

Van Wyk, B.E., Oudtshoorn, B.V. and Gericke, N., 1997. *Medicinal Plants of South Africa*. Briza Publications, Pretoria, South Africa.

Whitelaw, B., Gayle, C., 2010. Gestational diabetes. Obstetrics, *Gynaecology and Reproductive Medicine*. 21:41-46.

WHO, 2016. World Health Organisation, Diabetes Fact sheet July 2016.

World Health Organization, 1999. *Definition, Diagnosis and Classification of Diabetes Mellitus and is complications. Part 1: Diagnosis and Classification of Diabetes Mellitus*. WHO/NCD/NCS/99.2 ed. Geneva, Warled Health Organization.

World Health Organization, 2004. WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems.

World Health Organization, 2006. *Prevention of blindness from diabetes mellitus: report of a WHO consultation in Geneva, Switzerland, 9-11 November 2005*. World Health Organization.

World Health Organization, 2013. *Diagnostic criteria and classification of hyperglycaemia first detected in pregnancy* (No. WHO/NMH/MND/13.2). World Health Organization.

Yang, M., Wang, B.X., and Jin, Y.L.,1990. Effects of ginseng polysaccharides on reducing blood glucose and liver glycogen. *Chung Kuo Yao Li Hsueh Pao* 11: 520-524.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Plants are a valuable source of new natural products. Natural products remain as one of the best reservoirs of new chemicals. A small proportion of the hundred thousand plant species around the world has been investigated both phytochemically and pharmacologically (Hostettmann, 1999).

Plants for the present study have been selected based on phytochemistry and ethnobotanical information. Plant leaves were investigated, however, based on literature review these plant parts have not been validated scientifically as yet. The aim of this study therefore, was to investigate phytochemical and biological properties of selected plants. A brief description, distribution, medicinal use and phytochemistry of each plant is discussed.

2.2 *Protea cynaroides*

2.2.1 Plant description

The Proteaceae or silk-oak family is one of the evergreen shrubs, trees, or sometimes sub herbaceous perennial plants. There are more than 1674 species from approximately 83 genera which are found largely in the dry, Mediterranean-type climatic regions of the Southern Hemisphere, especially in South Africa and Australia (Christenhusz and Byng, 2016; Moodley et al., 2013). The utmost diversity of genera and species are concentrated in Southwest Australia and the Western Cape Province of South Africa with 16 genera (682 species) and 14 genera (331 species), respectively (Lamont et al., 1985; Cowling and Lamont, 1998).

Proteas were the most prominent woody plants in the Cape Region of South Africa 300 years ago and were used by the early European settlers for a variety of their needs. The bark and

leaves of most *Protea* species may be regarded as astringent, and all have probably been used in the Cape Region for tanning. As early as 1720, "Syrupus Protea" was used as a cough syrup (Verotta *et al*., 1999).

Protea cynaroides (King Protea) is an upright and multi-stemmed shrub that grows between 0.3 and 2 m tall. It has thin branches, with hairless stems (Rebelo, 2000). The leaves are curved, oval, or narrowly elliptic, ranging from 50 to 120 mm in length and 50 to 75 mm in width. The flowerhead sizes range from 120 mm to 300 mm in diameter and the color of the bracts, which are either hairy or hairless, range from pink to creamy-white (Patterson-Jones, 2000).

Figure 2. 1: Photograph of *Protea cynaroides*

2.2.2. Distribution of *Protea cynaroides*

Protea cynaroides has one of the widest distribution ranges of all the Proteaceae and occurs from the Cedarberg in the northwest to Grahamstown in the east. It occurs on all mountain ranges in this area, except for the dry interior ranges, and at all elevations from sea level to 1500 meters high. The combination of the different climatic conditions with the large range of

localities has resulted in a large variety of leaf and flower sizes, as well as flower colors and flowering times. The different forms retain these characteristics even when grown under the same conditions on a commercial scale. This has made it possible to grow *Protea cynaroides* as a cut flower for a wide variety of export markets, where the flowers are needed at different times of the year. This South African protea is now grown in large quantities in New Zealand, Australia, and Hawaii. In the Mediterranean climates of Europe, America, and Australia, it can be grown successfully as a garden plant in the right type of soil.

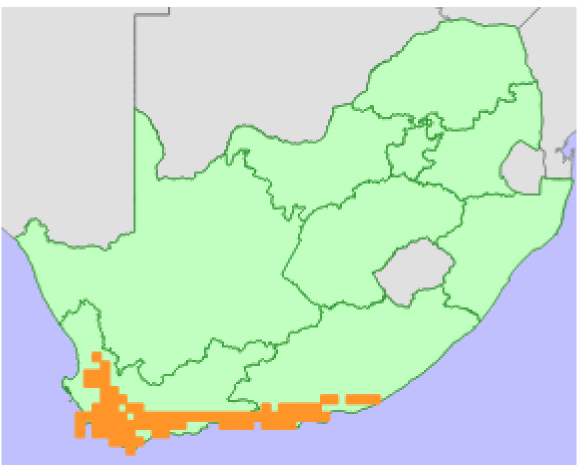


Figure 2. 2: Distribution map *Protea cynaroides* in South Africa

2.2.3 Ethnomedicinal use of *Protea cyanaroides*

A study conducted by Arendse (2013) revealed that the plant is used to treat bladder and kidney ailments as well as cancer and this is believed to be the main therapeutic benefit. As per this report, the tea is prepared by adding a few leaves to a cup of boiling water and allowed to cool and followed by straining. One cup is taken twice daily to treat bladder and kidney ailments and thrice daily for the treatment of cancer and diabetes.

2.2.4 Phytochemical studies

No conclusive studies have been carried out on the chemical substances present in this broad family. However, a few phenolic compounds (Perold and Carlton, 1989; Verotta et al., 1999; León et al., 2014) and polyol sugars (Bieleskiand Briggs,2005) have been identified as leaf constituents. Phenolic compounds in other Proteaceae members were also reported by Deans et al. (2018a,2018b) where they found for example quercetin glycosides and naphthoquinones in Lomatia species. Cyanogenic glycosides, derived from tyrosine, are often present as proanthocyanidins (delphinidin and cyanidin), flavonols (kaempferol, quercetin, and myricetin), and arbutin. Some members of the *Protea* genus have been shown to contain aromatic esters of aryl glycosides as typical leaf constituents (Perold et al., 1979).

Perold et al., (1973) reported that there are two classes of glycosides that are found in *Protea* sp., the *C*-glycosides proteacin or leucodrin and phenolic glycosides, which have 3,4-dihydroxybenzyl alcohol as a common aglycon. The known sugars involved in the β-glycosidic linkage are (+)-D-glucose and (+)-D-allose. Plant phenols have drawn an increasing attention due to their strong antioxidant properties and their marked effects in the prevention of various oxidative stress-associated diseases such as cancer (Dai and Mumper, 2010). Wu et al. (2007) reported the isolation of 3,4-dihydroxybenzoic acid from *P. cynaroides* along with other phenolic compounds such as caffeic, ferulic, gallic, and salicylic acids.

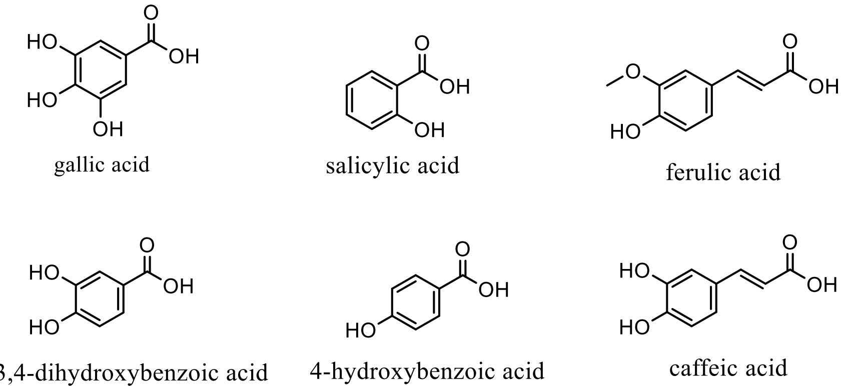


Figure 2. 3: **Some phenolic compounds isolated from *P. cynaroides***

Table 2. 1: **Some isolated compounds of *Protea* genera and their biological activities**.

Name of the plant	Isolated compound	Plant part	Activity	Ref
Protea rubropilosa	**Rubropilosin**	Leaves	Antioxidant	Perold et al., 1973
	Pilorubrosin	Leaves	Antioxidant	Perold et al., 1973
Protea hybrid *'Susara'*	**(6-ethoxy-3,5-dihydroxy-4-oxotetrahydro-2*H*-pyran-2-yl)methyl 4-hydroxybenzoate**	Leaves	Antioxidant	León et al., 2014
	2-ethoxy-4-(hydroxymethyl)phenol	Leaves	Antioxidant	León et al., 2014

Protea neriifolia	**2,4-Dihydroxyphenyl 6-*O*-benzoyl-β-D-glucopyranoside**	Leaves	Anticancer	Syarifah et al., 2011
Protea eximia	**Eximin (6-*O*-benzoylarbutin)**	Leaves	Antiviral	Huang et al., 2011
Protea caffra	**Phenol-2,4-bis (1,1-dimethylethyl)**	Twigs	Antioxidant Antifungal	Zhao et al., 2020 Ren et al., 2020
	Rimantadine	Twigs	Antiviral Antimicrobial	Vambe et al., 2020 Manchand et al., 1990
Protea lacticolor	**Lacticolorin**	Leaves		Perold et al., 1979

2.3 *Cliffortia odorata*

2.3.1 Plant description

Cliffortia L. (Rosaceae) comprises 135 species of woody evergreen plants species, 109 of which are endemic to the Cape Floristic Region (CFR) (Whitehouse 2002, 2004; Whitehouse and Fellingman 2007). Although the genus is found from sea level to the highest mountain summits in the CFR, further north it is restricted to high altitude areas.

Cliffortia odorata, commonly known as "Wildewingered" (wild vineyard) in Afrikaans, is a scrambling shrub growing 1-2 m in height (Adamson and Salter, 1950). The young stems are hairy and slightly pink, but later become hairless and rust-brown. The leaves are alternate with stipules at the base of the short petioles. The leaf shape is ovate with saw-toothed edges. The leaf is aromatic, dark to yellow-green, leathery, and hairy only on the lower surface whereas its surface is covered in parallel and straight lateral veins that are closely spaced and emerging at an angle from the midrib. These veins are conspicuously indented on the upper surface and run straight into the margins. It grows on mountain slopes of the Western Cape, the Eastern Cape, and KwaZulu-Natal in places where moisture is readily available (www.plantzafrica.com; Bond and Goldblatt, 1984). As per the literature search, there are no pharmacological and phytochemical investigations carried out on *Cliffortia* species.

Figure 2. 4: Photograph of *Cliffortia odorata.*

2.3.2. Distribution of *Cliffortia odorata*

The plant is widespread on damp mountain slopes of the Western and Eastern Cape Provinces, from Paarl to the Cape Peninsula and eastward to Port Elizabeth; also, north to KwaZulu-Natal.

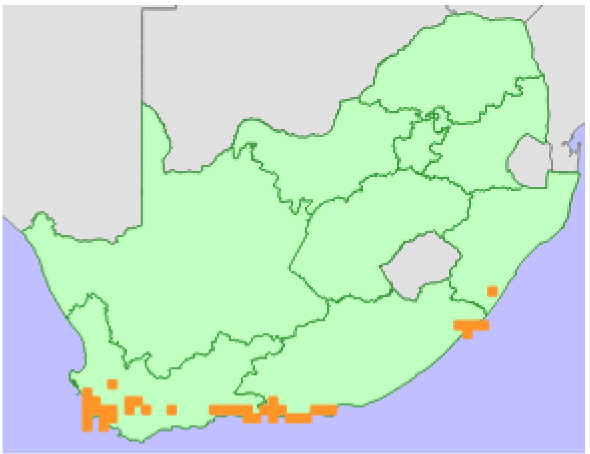


Figure 2.5: Distribution map of *Cliffortia odorata* in in South Africa

2.3.3 Ethnomedicinal use of *Cliffortia odorata*

The leaves of *C. odorata* have a variety of medicinal uses in traditional medicine with acknowledgment of some of its properties in modern pharmacology. This plant is used for the treatment of back pain, bladder and kidney ailments, cancer, colds and flu, cough, diabetes, feet ailments, fever, headache, hypertension, pain, stomach ailments, tuberculosis (TB), and as a detoxification aid (van Wyk, 2008). This treatment is achieved by adding a few leaves to 1 cup of boiling water and allowing it to cool down. Strain. Drink one cup daily to treat the ailments mentioned. For pain, add a handful of leaves to the bathwater and immerse body into the water. For the treatment of feet ailments, add a handful of leaves to a small foot bath and soak feet for 30 minutes.

2.3.4 Phytochemical studies

In the literature, no information could be found on the phytochemistry or bioactivity of *Cliffortia* species. However, preliminary microchemical tests indicated the presence of flavonoids, tannins, saponins, triterpenoid steroids, glycosides, and quinones. Alkaloids and essential oils were not detected (Arendse, 2013).

Although there is no phytochemical study reported on *Cliffortia* species, there are reports on the study of its immediate sister taxon namely, *Sanguisorba* and *Acaena*. The most investigated *Sanguisorba* species has been *Sanguisorba officinalis* (Cirovic et al., 2020) and among various active compounds isolated from the root and aerial parts of the *S. officinalis*, phenolic acids, triterpenoids, tannins and flavonoids have been reported as its major components (Nonaka et *al*., 1982a; Nonaka et *al*., 1982b; Tanaka et *al*., 1983; Tanaka et *al*., 1984; Tanaka et *al*., 1985and Chengand Yang, 1995; Han and Zhong, 2009).

Tanaka et *al*., 1984 have also reported various gallic acid derivatives such as methyl glucoside gallates and gallic acid glucoside gallate from *S. officinalis*. Tannins and related

compounds such as catechin, gallocatechin, procyanidin B-3, β-glucogallin, 1,2,3,6-tetra-*O*-galloyl-β-D-glucose, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose, 4,6-hexahydroxydiphenoyl-D-glucose, ped-unculagin, sanguiin H-6, and sanguiin H-11 have also been isolated from the roots of *S. officinalis* (Ishimaru et *al*., 1995). A representative flavonol glycoside rutin as another phenolic component from *S. officinalis* has also been reported (Kaneta et al., 1979).

Studies conducted by Ayoub (2003) and Tan et al. (2019), reported the isolation of phenolic carboxylic acids and ellagic acid from *S. minor* and *S. officinalis*, respectively.

In 2002, Backhouse et al. reported the isolation of four phenolic derivatives from *Acaena splendens* namely, tiliroside, epicatechin, 7-*O*-acetyl-3-*O*-β-D-glucosyl-kaempferol, and 7-β-D-glucosyloxy-5-hydroxy-chromone. Free radical scavengers such as quercetin, quercetin-3-O-β-D-glucoside, quercetin-3-O-β-D-galactoside, ellagic acid and catechin were isolated from *Acaena magellanica* by Feresin et al. (2002)

Table 2. 2: **Some isolated compounds and activities of *Cliffortia* genera**

Name of the plant	Isolated compound	Plant part	Activity	Ref
Sanguisorba minor	**Ellagic acid**	Whole plant	Antioxidant	Ayoub, 2003
Acaena magellanica		Whole plant	Anti-inflammatory	Feresin et al., *2002*
Acaena splendens	**Tiliroside**	Whole plant	Anti-inflammatory Antioxidant Anticarcinogenic Hepatoprotective	Backhouse et al., *2002*

Sanguisorba ternuifolia	R1 R2 R3 R4 R5 R6 1 = a-OH a-OH OH CH_3 CH_3 H 2 = H b-O-coumaroyl H CH_3 CH_3 H **1. Euscaphic acid** **2. *p*-coumaroylursolic acid**	*Roots*	Antihypoglycemic	Kuang et al., 2011
Sanguisorba hakusanensis	**Quercetin 3-*O*-(β-D-gl ucofuranoside)**	Leaves	Antioxidant	León et al., 2014
Sanguisorba obtusa	**Miquelianin**	Leaves	Antioxidant	Rice-Evans et al., 1996 Pietta, 2000
Acaena pinatifida	**Tormentic acid**	Roots	Anti-inflammatory antibacterial	Yang et al., 2016 Zang et al., 2007

Acaena magellanicas	**Catechin**	Roots	Cytotoxic	Yang et al., 2004
Sanguisorba alpina	**Prunasin**	Leaves	Antioxidant Anti-inflammatory Antibacterial Anticancer	Liu and Jia, 1993
Sanguisorba officinalis	**2α,3β-dihydroxy-28-norurs-12,17,19(20),21-tetraen-23-oic acid**	*Roots*	Antitumor	Liu et al., 2005
Sanguisorba officinalis	***Lyoniside***		Antioxidant Antifungal Allelopathic	Szakiel et al., 2011 Wang et al., 2020
Sanguisorba officinalis	***Syringin***		Anti-inflammatory Pain-suppressing Antioxidant	Li et al., 2013

2.4 *Asparagus lignosus*

2.4.1 Plant description

The genus *Asparagus* belongs to the Asparagaceae family and comprises about 200 species which are distributed around Africa, Asia, and Europe, of which nearly 88 are found in Southern Africa (http://pza.sanbi.org/asparagus-ramosissimus). It is a large genus of herbs or shrubs with stout, creeping, tuberous root-stocks, and erect or climbing stems. The leaves are reduced to spinescent structures and their function is carried on by slender, flattened, green, branched structures, called cladophylls (Stahl et al., 1978). African Asparagus species and the European Asparagus species are related, and they display a very interesting structure botanically. They do not have true leaves at all, but these are cladodes which may be the modified branches, while the spines are formed from modified branches or modified leaves (Mashele, 2019).

Asparagus lignosus is a rigid, upright shrub reaching 80 cm in height. The branches are zig-zagged, and pale grey-green to white. At each node along the branch, there is a single recurved 5 mm thorn. The stiff, linear (length 10-20mm), cylindrical, spine-tipped leaves are in tufts. The flowers are white with a green stripe on each petal. The anthers are orange and exist as single or in pairs.

Figure 2. 5: Photograph of *Asparagus lignosus*

2.4.2. Distribution of *Asparagus lignosus*

This species is indigenous to the Western Cape Province, South Africa. Its distribution is from Clanwilliam in the far north-west and Cape Town in the west, eastwards across the Little Karoo and Overberg regions, as far as Mossel Bay in the south-east. It occurs in rocky sandstone slopes, as well as rocky loamy soils in fynbos or renosterveld vegetation on lower slopes and flats.

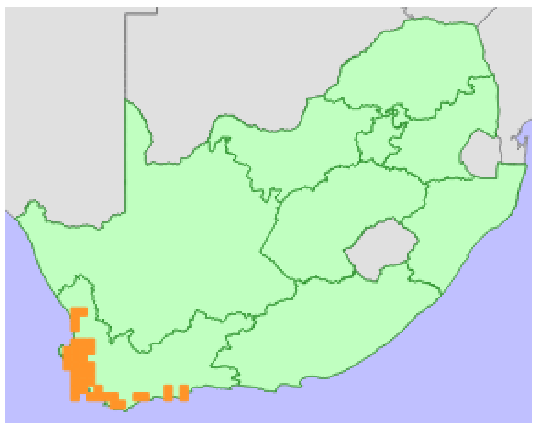


Figure 2. 6: Distribution map of *Asparagus lignosus* in South Africa

2.4.3 Ethnomedicinal use of *Asparagus lignosus*

Nothing has been reported on *A. lignosus* yet. However, a group of closely related African *Asparagus* species which includes *A. concinnus* and *A. microraphis* has been reported to have medicinal uses. According to Shale, (2013), *A. microraphis* is used by the southern Sotho and Xhosa people in treatment of venereal diseases. Extracts made from the whole plant can be taken orally or applied to patients with eruptions and gonorrhoea. Chauke et al., 2015 reported that the roots of *A. concinnus* are boiled to treat gonorrhoea and also in mixtures for treatment of all diseases.

2.4.4 Phytochemical studies

There are no pharmacological or phytochemical investigations on *Asparagus lignosus* in the literature. However, the study of this plant is motivated by the fact that the commercially available *Asparagus officinallis* commonly known as Asparagus for its rich bioactive compounds, now is widely consumed all over the world. Bioactive components, such as flavonoids, lignans and steroidal saponins were found in this plant (Fukushi et al., 2000; Hafizur et al., 2012).

The literature survey of the genus *Asparagus* showed that this genus is a rich source of saponins (Price et al., 1987, Schwarzbach et al., 2006). Saponin-rich plants have been found to improve growth, feed efficiency, and health in ruminants (Mader and Brumm, 1987). The roots of *Asparagus* are the main source of the drug shatavarin IV, the crude drug also used for increasing the secretion of milk and improving appetite in lactating women (Negi et al., 2010). The roots possess a variety of biological properties, such as antioxidant, immunostimulant, anti-inflammatory, antihepatotoxic, antibacterial, antioxytocic, and reproductive agents (Negi *et al*., 2010).

Table 2. 3: **Some isolated compounds and activities of *Asparagus* genera**

Name of the plant	Isolated compound	Plant part	Activity	Ref
Asparagus africanus Lam	**Nyasol**	Roots	Antimalaria	Oketch-Rabah et al., 1997
			Estrogenic	Yanget al., 2008
			Anti-inflammatory	Lim et al., 2009
Asparagus curillus	**Quercetin-3-glucuronide**	Leaves	Antioxidant	Rastogi and Mehrotra, 1990
Asparagus filicinus	**Tormentic acid**	Roots	Anti-inflammatory	Chang et al., 2011
			Anti-atherogenic	An et al, 2011, Fogo et al, 2009

	20-hydroxyecdysone	Roots	Antimicrobial Antioxidant	Wu et al., 2007 Nsimba et al., 2008
Asparagus gobicus	**Isoagatharesinol**	Roots	Cytotoxic	Yang et al., 2004
Asparagus maritimus	**Narcisin**	Aerial part	Antioxidant	Regalado et al., 2017
Asparagus officinalis	**Rutin**	Leaves	Antioxidant	Huang et al., 2008
Asparagus racemus	**Asparagamine A**	Root	Antitumor	Sekine et al., 1994

Conclusion

This chapter described the traditional uses, phytochemical constituents, and highlighted the biological importance of some notable chemical constituents isolated from each family/genus of the three studied plants

References

Adamson, R.S., Salter, T.M., 1950. Flora of the Cape Peninsula. Juta & Co., Ltd. Cape Town and Johannesburg.

An, H.J., Kim, I.T., Park, H.J., Kim, H.M., Choi, J.H. and Lee, K.T., 2011. Tormentic acid, a triterpenoid saponin, isolated from Rosa rugosa, inhibited LPS-induced iNOS, COX-2, and TNF-α expression through inactivation of the nuclear factor-κb pathway in RAW 264.7 macrophages. *International immunopharmacology*, *11*(4), pp.504-510.

Arendse, M.L., 2013. Medicinal plant use in the Dwarsrivier Valley, Stellenbosch (Doctoral dissertation, University of the Western Cape).

Ayoub, N.A., 2003. Unique phenolic carboxylic acids from *Sanguisorba minor*. *Phytochemistry*, *63*(4), pp.433-436.

Backhouse, N., Delporte, C., Negrete, R., Feliciano, S.S. and López-Pérez, J.L., 2002. Bioactive phenolic derivatives from *Acaena splendens* methanol extract. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, *16*(6), pp.562-566.

Bieleski, R.L. and Briggs, B.G., 2005. Taxonomic patterns in the distribution of polyols within the Proteaceae. *Australian Journal of Botany*, *53*(3), pp.205-217.

Bond, P., Goldblatt, P., 1984. Plants of the Cape flora. A descriptive catalogue. *Journal of South African Botany* (Suppl. 13). National Botanical Gardens of South Africa, Cape Town.

Chang, C.T., Huang, S.S., Lin, S.S., Amagaya, S., Ho, H.Y., Hou, W.C., Shie, P.H., Wu, J.B. and Huang, G.J., 2011. Anti-inflammatory activities of tormentic acid from suspension cells of *Eriobotrya Japonica ex* vivo and in vivo. *Food Chemistry*, *127*(3), pp.1131-1137.

Cheng, D. and Yang, P., 1995. Isolation and identification of the flavonoids from garden burnet (*Sanguisorba officinalis*). *Chinese Traditional And Herbal Drugs*, *26*, pp.570-571.

Christenhusz, M.J., M, Byng J.W. 2016. The number of known plants species in the world and its annual increase. *Phytotaxa*, *261*(201), pp.10-11646.

Cirovic, T., Barjaktarevic, A., Ninkovic, M., Bauer, R., Nikles, S., Brankovic, S., Markovic, M., Jovanovic, V.S., Ilic, M., Milovanovic, O. and Kojicic, K., 2020. Biological activities of *Sanguisorba minor* l. extracts ñ in vitro and in vivo evaluations. *Acta Poloniae Pharmaceutica*, *77*(5), pp.745-758.

Cowling, R.M. and Lamont, B.B., 1998. On the nature of Gondwanan species flocks: diversity of Proteaceae in Mediterranean south-western Australia and South Africa. *Australian Journal of Botany*, *46*(4), pp.335-355.

Dai, J. and Mumper, R.J., 2010. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules*, *15*(10), pp.7313-7352.

Deans, B.J., Kilah, N.L., Jordan, G.J., Bissember, A.C. and Smith, J.A., 2018. Arbutin derivatives isolated from ancient proteaceae: potential phytochemical markers present in *Bellendena, Cenarrhenes*, and *Persoonia Genera*. *Journal of natural products*, *81*(5), pp.1241-1251.

Deans, B.J., Tedone, L., Bissember, A.C. and Smith, J.A., 2018. Phytochemical profile of the rare, ancient clone *Lomatia tasmanica* and comparison to other endemic *Tasmanian* species *L. tinctoria* and *L. polymorpha*. *Phytochemistry*, *153*, pp.74-78.

Fan, R., Yuan, F., Wang, N., Gao, Y. and Huang, Y., 2015. Extraction and analysis of antioxidant compounds from the residues of *Asparagus officinalis* L. *Journal of food science and technology*, *52*(5), pp.2690-2700.

Feresin, G.E., Tapia, A., Angel, G.R., Delporte, C., Erazo, N.B. and Schmeda-Hirschmann, G., 2002. Free radical scavengers, anti-inflammatory and analgesic activity of *Acaena magellanica*. *Journal of pharmacy and pharmacology*, *54*(6), pp.835-844.

Fogo, A.S., Antonioli, E., Calixto, J.B. and Campos, A.H., 2009. Tormentic acid reduces vascular smooth muscle cell proliferation and survival. *European journal of pharmacology*, *615*(1-3), pp.50-54.

Fukushi, E., Onodera, S., Yamamori, A., Shiomi, N. and Kawabata, J., 2000. NMR analysis of tri-and tetrasaccharides from *Asparagus*. *Magnetic Resonance in Chemistry*, *38*(12), pp.1005-1011.

Han, W.A.N.G. and Zhong, Y.U.A.N., 2009. Triterpenes of *Sanguisorba officinalis* L.[J]. *Chinese Journal of Medicinal Chemistry*, *1*.

Hafizur, R.M., Kabir, N. and Chishti, S., 2012. *Asparagus officinalis* extract controls blood glucose by improving insulin secretion and β-cell function in streptozotocin-induced type 2 diabetic rats. *British Journal of Nutrition*, *108*(9), pp.1586-1595.

Hostettmann, K., 1999. Strategy for the biological and chemical evaluation of plant extracts. *Pure Appl. Chem*, *70*(11), pp.1-9.

Huang, X.F., Lin, Y.Y. and Kong, L.Y., 2008. Steroids from the roots of *Asparagus officinalis* and their cytotoxic activity. *Journal of Integrative Plant Biology*, *50*(6), pp.717-722.

Ishimaru, K., Hirose, M., Takahashi, K., Koyama, K. and Shimomura, K., 1995. *Sanguisorba officinalis* L. (Great Burnet): In Vitro Culture and Production of Sanguiin, Tannins, and Other Secondary Metabolites. In *Medicinal and Aromatic Plants VIII* (pp. 427-441). Springer, Berlin, Heidelberg.

Kaneta, M., Hikichi, H., Endo, S. and Sugiyama, N., 1979. Identification of flavones in nineteen Rosaceae species. *Agricultural and Biological Chemistry*, *43*(3), pp.657-658.

Kuang, H.X., Li, H.W., Wang, Q.H., Yang, B.Y., Wang, Z.B. and Xia, Y.G., 2011. Triterpenoids from the roots of *Sanguisorba tenuifolia* var. alba. *Molecules*, *16*(6), pp.4642-4651.

Lamont, B., 1985. The significance of flower colour change in eight co-occurring shrub species. *Botanical Journal of the Linnean Society*, *90*(2), pp.145-155.

León, F., Alfayate, C., Batista, C.V., López, A., Rico, M. and Brouard, I., 2014. Phenolic compounds, antioxidant activity and ultrastructural study from *Protea hybrid 'Susara'*. *Industrial Crops and Products*, *55*, pp.230-237.

Li, S., Li, S.K., Gan, R.Y., Song, F.L., Kuang, L. and Li, H.B., 2013. Antioxidant capacities and total phenolic contents of infusions from 223 medicinal plants. *Industrial Crops and Products*, *51*, pp.289-298.

Lim, H., Nam, J.W., Seo, E.K., Kim, Y.S. and Kim, H.P., 2009. (-)-Nyasol (cis-hinokiresinol), a norneolignan from the rhizomes of Anemarrhena asphodeloides, is a broad-spectrum inhibitor of eicosanoid and nitric oxide production. *Archives of pharmacal research*, *32*(11), pp.1509-1514.

Liu, X.Q. and Jia, Z.J., 1993. Chemical constituents of alpine burnet *Sanguisorba alpina*. *Chin. Tradit. Herb. Drugs*, *24*, pp.451-454.

Liu, X., Cui, Y., Yu, Q. and Yu, B., 2005. Triterpenoids from *Sanguisorba officinalis*. *Phytochemistry*, *66*(14), pp.1671-1679.

Mader, T.L. and Brumm, M.C., 1987. Effect of Feeding Sarsaponin in Cattle and Swine Diets 1. *Journal of Animal Science*, *65*(1), pp.9-15.).

Manchand, P.S., Cerruti, R.L., Martin, J.A., Hill, C.H., Merrett, J.H., Keech, E., Belshe, R.B., Connell, E.V. and Sim, I.S., 1990. Synthesis and antiviral activity of metabolites of rimantadine. *Journal of medicinal chemistry*, *33*(7), pp.1992-1995.

Mashele, S.S., 2019. Medicinal Properties of Selected Asparagus Species: A Review. *Phytochemicals in Human Health*.

Mei, N and Notten, A., *Asparagus ramosissimus* Baker. Kirstenbosch National Botanical Garden, May 2015, http://pza.sanbi.org/asparagus-ramosissimus. Accessed August 30, 2021

Moodley, D., Geerts, S., Richardson, D.M. and Wilson, J.R., 2013. Different traits determine introduction, naturalization and invasion success in woody plants: Proteaceae as a test case. *Plos one*, *8*(9), p.e75078.

Negi, J.S., Singh, P., Joshi, G.P., Rawat, M.S. and Bisht, V.K., 2010. Chemical constituents of Asparagus. *Pharmacognosy reviews*, 4(8), p.215.

Nonaka, G.I., Tanaka, T. and Nishioka, I., 1982. Tannins and related compounds. Part 3. A new phenolic acid, sanguisorbic acid dilactone, and three new ellagitannins, sanguiins H-1, H-2, and H-3, from *Sanguisorba officinalis*. *Journal of the Chemical Society, Perkin Transactions 1*, pp.1067-1073.

Nonaka, G., Tanaka, T., Nita, M. and Nishioka, I., 1982. A dimeric hydrolyzable tannin, sanguiin H-6 from *Sanguisorba officinalis* L. *Chemical and Pharmaceutical Bulletin*, *30*(6), pp.2255-2257.

Nsimba, R.Y., Kikuzaki, H. and Konishi, Y., 2008. Ecdysteroids act as inhibitors of calf skin collagenase and oxidative stress. *Journal of biochemical and molecular toxicology*, *22*(4), pp.240-250.

Oketch-Rabah, H.A., Dossaji, S.F., Christensen, S.B., Frydenvang, K., Lemmich, E., Cornett, C., Olsen, C.E., Chen, M., Kharazmi, A. and Theander, T., 1997. Antiprotozoal compounds from *Asparagus africanus*. *Journal of natural products*, *60*(10), pp.1017-1022.

Paterson-Jones, C., 2000. *Protea* family in southern Africa. Struik.

Perold, G.W., Beylis, P. and Howard, A.S., 1973. Metabolites of proteaceae. Part VIII. The occurrence of (+)-D-allose in nature: rubropilosin and pilorubrosin from *Protea rubropilosa* beard. *Journal of the Chemical Society, Perkin Transactions 1*, pp.643-649.

Perold, G.W., Rosenberg, M.E., Howard, A.S. and Huddle, P.A., 1979. Metabolites of proteaceae. Part 9. Eximin (6-O-benzoylarbutin) and the synthesis of aryl glycoside esters. *Journal of the Chemical Society, Perkin Transactions 1*, pp.239-243.

Perold, G.W. and Carlton, L., 1989. Neriifolin, an ester glucoside of benzene-1, 2, 4-triol. *Journal of the Chemical Society, Perkin Transactions 1*, (7), pp.1215-1217.

Pietta, P.G., 2000. Flavonoids as antioxidants. *Journal of natural products*, *63*(7), pp.1035-1042.

Price, K.R., Johnson, I.T., Fenwick, G.R. and Malinow, M.R., 1987. The chemistry and biological significance of saponins in foods and feedingstuffs. *Critical Reviews in Food Science and Nutrition*, 26(1), pp.27-135.

Rastogi, R.P. and Mehrotra, B.N., 1990. *Compendium of Indian medicinal plants*. *Central Drug Research Institute.*

Rebelo, A., 2000. Field guide to the proteas of the Cape Peninsula. Pretoria: Protea Atlas Project xiii, 130p.-illus. ISBN, 1919684220.

Regalado, J.J., Moreno, R., Castro, P., Carmona-Martin, E., Rodríguez, R., Pedrol, J., Larrañaga, N., Guillén, R., Gil, J. and Encina, C.L., 2017. *Asparagus macrorrhizus*

Pedrol, Regalado et López-Encina, an endemic species from Spain in extreme extinction risk, is a valuable genetic resource for asparagus breeding. *Genetic Resources and Crop Evolution*, *64*(7), pp.1581-1594.

Regalado, J.J., Moreno, R., Castro, P., Carmona-Martin, E., Rodríguez, R., Pedrol, J., Larrañaga, N., Guillén, R., Gil, J. and Encina, C.L., 2017. *Asparagus macrorrhizus* Pedrol, Regalado et López-Encina, an endemic species from Spain in extreme extinction risk, is a valuable genetic resource for asparagus breeding. *Genetic Resources and Crop Evolution*, *64*(7), pp.1581-1594.

Ren, J., Wang, J., Karthikeyan, S., Liu, H. and Cai, J., 2019. Natural anti-phytopathogenic fungi compound phenol, 2, 4-bis (1, 1-dimethylethyl) from Pseudomonas fluorescens TL-1. *Indian Journal of Biochemistry and Biophysics (IJBB)*, *56*(2), pp.162-168.

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

Schwarzbach, A., Schreiner, M. and Knorr, D., 2006. Effect of cultivars and deep freeze storage on saponin content of white asparagus spears (*Asparagus officinalis* L.). *European Food Research and Technology*, *222*(1-2), p.32.

Sekine, T., Fukasawa, N., Kashiwagi, Y., Ruangrungsi, N. and Murakoshi, I., 1994. Structure of asparagamine A, a novel polycyclic alkaloid from *Asparagus racemosus*. *Chemical and pharmaceutical bulletin*, *42*(6), pp.1360-1362.

Stahl, P.D., Rodman, J.S., Miller, M.J. and Schlesinger, P.H., 1978. Evidence for receptor-mediated binding of glycoproteins, glycoconjugates, and lysosomal glycosidases by alveolar macrophages. *Proceedings of the National Academy of Sciences*, 75(3), pp.1399-1403.

Syarifah, M.S., Nurhanan, M.Y., Haffiz, J.M., Ilham, A.M., Getha, K., Asiah, O., Norhayati, I., Sahira, H.L. and Suryani, S.A., 2011. Potential anticancer compound from *Cerbera odollam*. *Journal of Tropical Forest Science*, pp.89-96.

Szakiel, A., Voutquenne-Nazabadioko, L. and Henry, M., 2011. Isolation and biological activities of lyoniside from rhizomes and stems of *Vaccinium myrtillus*. *Phytochemistry Letters*, *4*(2), pp.138-143.

Tan, Y.H., Shudo, T., Yoshida, T., Sugiyama, Y., Si, J.Y., Tsukano, C., Takemoto, Y. and Kakizuka, A., 2019. Ellagic acid, extracted from *Sanguisorba officinalis*, induces G1 arrest by modulating PTEN activity in B16F10 melanoma cells. *Genes to Cells*, *24*(11), pp.688-704.

Tanaka, G., Tanaka, T., Nita, M. and Nishioka, I., 1982. A dimeric hydrolyzable tannin, sanguiin H-6 from Sanguisorba officinalis L. *Chemical and Pharmaceutical Bulletin*, *30*(6), pp.2255-2257.

Tanaka, T., Nonaka, G.I. and Nishioka, I., 1983. 7-O-galloyl-(+)-catechin and 3-O-galloylprocyanidin B-3 from *Sanguisorba officinalis*. *Phytochemistry*, *22*(11), pp.2575-2578.

Tanaka, T., Nonaka, G. and Nishioka, I., 1984. Tannins and related compounds. XVI. Isolation and characterization of six methyl glucoside gallates and a gallic acid glucoside gallate from *Sanguisorba officinalis* L. *Chemical and pharmaceutical bulletin*, *32*(1), pp.117-121.

Tanaka, T., Nonaka, G.I. and Nishioka, I., 1985. Tannins and related compounds. XXVIII: Revision of the structures of sanguiins H-6, H-2, and H-3, and isolation and characterization of sanguiin H-11, a novel tetrameric hydrolysable tannin, and seven related tannins, from *Sanguisorba officinalis*. *Journal of chemical research. Synopses (Print)*, (6), pp.176-177.

The Plant List (2021). Sanguisorba. Available at:
http://www.theplantlist.org./tpl1.1/search?q=Sanguisorba (Accessed August 30, 2021).

Vambe, M., Aremu, A.O., Chukwujekwu, J.C., Gruz, J., Luterová, A., Finnie, J.F. and Van Staden, J., 2020. Antibacterial, Mutagenic Properties and Chemical Characterisation of Sugar Bush (Protea caffra Meisn.): A South African Native Shrub Species. *Plants*, *9*(10), p.1331.

Van Wyk, B.-E., 2008. A broad review of commercially important southern African medicinal plants. *Review. Journal of Ethnopharmacology* 119, 342-355.

Verotta, L., Orsini, F., Pelizzoni, F., Torri, G. and Rogers, C.B., 1999. Polyphenolic glycosides from African proteaceae. *Journal of Natural Products*, 62(11), pp.1526-1531.

Wang, R., Sun, J., Ye, C., Wang, J., Zong, T., Zhou, W. and Li, G., 2020. Chemical constituents isolated from the roots of *Sanguisorba officinalis* L. and their chemotaxonomic significance. *Biochemical Systematics and Ecology*, *89*, p.103999.

Watt, J.M., Breyer-Brandwijk, B.N., 1962. Medicinal and poisonous plants of Southern and Eastern Africa, second ed. Edinburgh, Churchill Livingstone.

Whitehouse CM, Fellingham AC. 2007. New species and notes on the genus *Cliffortia* (Rosaceae). Bothalia 37: 9–22.

Whitehouse CM. 2002. Systematics of the genus *Cliffortia* L. (Rosaceae). PhD thesis. University of Cape Town.

Whitehouse CM. 2004. The genus *Cliffortia* (Rosaceae) in KwaZulu-Natal. Bothalia 34:1–10.

Wu, H.C., Du Toit, E.S., Reinhardt, C.F., Rimando, A.M., Van der Kooy, F. and Meyer, J.J.M., 2007. The phenolic, 3, 4-dihydroxybenzoic acid, is an endogenous regulator of rooting in *Protea cynaroides*. *Plant Growth Regulation*, *52*(3), pp.207-215.

Yang, D., Liang, J., Xie, H. and Wei, X., 2016. Norsesquiterpenoids and triterpenoids from strawberry cv. Falandi. *Food chemistry*, *203*, pp.67-72.

Yang, C.X., Huang, S.S., Yang, X.P. and Jia, Z.J., 2004. Nor-lignans and steroidal saponins from *Asparagus gobicus*. *Planta medica*, *70*(05), pp.446-451.

Yang, H., Baggett, S., Staub, R., Chow, S., Bjeldanes, L.F., Leitman, D. and Cohen, I., 2008. Norlignans from Anemarrhenae asphoeloides Displaying Selective Estrogen Beta (ER) Activity. *Planta Medica*, *74*(03), pp.P-78.

Zhang, Y., Bao, F., Hu, J., Liang, S., Zhang, Y., Du, G., Zhang, C. and Cheng, Y., 2007. Antibacterial lignans and triterpenoids from *Rostellularia procumbens*. *Planta medica*, *73*(15), pp.1596-1599.

Zhao, F., Wang, P., Lucardi, R.D., Su, Z. and Li, S., 2020. Natural sources and bioactivities of 2,4-di-tert-butylphenol and its analogs. *Toxins*, *12*(1), p.35.

CHAPTER THREE

EXPERIMENTAL PROCEDURE

3.1 Materials and instrumentation

Plant materials: *Asparagus lignosus*
Cliffortia odorata
Protea cynaroides

3.2. Reagents and solvents

Hexane, Dichloromethane, Ethyl acetate, Methanol and Butanol, vanillin, and deuterated chloroform, DMSO, methanol and pyridine were supplied by Merck (Cape Town, South Africa). Acetonitrile, Dimethyl sulfoxide (DMSO), formic Acid, and methanol were of HPLC grade were supplied by Fisher (Cape Town, South Africa).

3.3 Chromatography

3.3.1 Thin Layer Chromatography (TLC)

Thin layer chromatography was carried out on pre-coated silica gel 60 F_{254} plates (Merck) with a 0.2 mm layer thickness. Visualisation of the TLC spots was carried out under UV light at 254nm and/or 366nm, and further detection of compounds was achieved by spraying with vanillin spray reagent (prepared by dissolving 15g of vanillin in 250 mL ethanol followed by the addition of 2.5 mL concentrated sulphuric acid). After spraying, the TLC plates were heated on a hot plate until spots became visible.

3.3.2 Column chromatography (CC)

Glass columns (20-25 mm diameter) were packed with silica gel 60 (0.040-0.063mm) (230-400 Mesh ASTM, Merck) were used for column chromatography.

3.3.3.3 Electrospray ionization mass spectroscopy (ESI-MS)

High resolution mass spectroscopy (HRMS) analysis was performed on waters Quattro micro API with electrospray ionization (ESI) interface working in the positive mode.

3.4 Spectroscopy

3.4.1 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded at 25^0 C, using deuterated methanol (CD_3OD), DMSO-d_6 (CD_3SOCD_3), Pyridine (C_5D_5N) and chloroform ($CDCl_3$) as solvents, on a Bruker Avance 400 MHz NMR spectrometer that uses a 5mm BBO probe, based at the Department of Chemistry, University of the Western Cape, South Africa. Chemical shifts of ^{13}C and ^{1}H were measured relative to tetramethyl silane as internal reference. The chemical shifts were expressed in δ (ppm), and coupling constants (*J*) in Hz.

3.4.2 Liquid chromatography mass spectrometry (LC-MS)

Liquid chromatography mass spectrometry (LC-MS) Analysis. A Waters Synapt G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS) connected to a Waters Acquity ultra-performance liquid chromatograph (UPLC) (Waters, Milford, MA, USA) was used for high-resolution UPLC-MS analysis. Electrospray ionization was used in negative mode with a cone voltage of15V, desolvation temperature of 275 °C, desolvation gas at 650 L/h, and the rest of the MS settings optimized for best resolution and sensitivity.

3.4.3 Infrared (IR) spectroscopy

Attenuated total internal reflectance FTIR measurements were performed using Spectrum 100 (Perkin Elmer Corporation). Spectra recording were achieved using the interface "Spectrum". Samples were dissolved using Dichloromethane (DCM).

3.4.4 Ultra violet (UV) spectroscopy

UV Nicolet Evolution (EV-100 ver. 4.60) spectrophotometer (Therma Electron Corporation, Madison, USA) was used for measurement of absorbance maxima between the wavelengths 200-400 nm.

3.5 Plant material

3.5.1 Collection and identification of plant material

Fresh leaves of *A. lignosus* were collected in November 2020 from the Cape Nature Reserve at UWC in Cape Town, South Africa.

The aerial parts of *Cliffortia odorata* and *Protea cynaroides* were collected in July 2018 in Kirstenbosch Botanical Garden in Cape Town, South Africa. The plant samples from the three plants were identified with the help of a taxonomist in the field. They were washed thoroughly using running tap water and then air-dried at room temperature (20-25 °C) over the benches in chemistry laboratory. The dry plant materials were then ground into fine powders using a milling machine.

3.5.2 Plant preparation

All the plants collected were separately washed thoroughly using running tap water and then air-dried at room temperature (20-25 °C) over the benches in chemistry laboratory. The dried plant materials were then ground into fine powders using a milling machine. All the powdered plant materials were stored in well labeled air-tight containers prior to analysis.

A. lignosus leaves = 155g

C. odorata leaves = 298g

P. cynaroides leaves = 445g

3.6 Extraction and fraction of plants

General sequential extraction

The leaves of the plant material was blended and extracted with 80% methanol (1.5-2. L) at room temperature (25 ^{o} C) stirring for 24 h. The methanol extract was filtered and evaporated to dryness under reduced pressure at 40 ^{o} C. The total extract was concentrated under vacuum to remove the methanol for freeze drying. The freeze-dried material was suspended in water and partitioned successively with n-hexane, DCM, EtOAc and BuOH. Each extract was concentrated to dryness under reduced pressure. Purification and isolation of natural products was achieved through one or a combination of chromatographic techniques. The process was the same for all three plant materials in the study.

3.6.1. Extraction and isolation of compounds from EtOAc extract of *Protea cynaroides*

The EtOAc extract (3.27 g) was pre-adsorbed on silica gel and fractionated on a column by gravity elution using the mixture of DCM elution as follows: 1L of 100%, then 1L volumes of mixtures with EtOAc in the following ratios (80:20), (60:40), (40:60), (20:80), (10:90) and finally 100% EtOAc. Finally, MeOH was introduced in the mixture with EtOAc up to 15% MeOH, (95:5), (90:10), (85:15). A total of thirty-one fractions were analysed by TLC using $CHCl_3$: MeOH: H_2O (200:52:6). Fractions with similar Rf values were pooled together. The combined fraction from vials **F_9-F_{12}** (55 mg) and **F_{19}-F_{21}** (1.5 g) were each preadsorbed on silica gel and loaded on separate columns for further separation. **F_9-F_{12}** was subjected to a successive silica gel column and eluted with DCM: MeOH (90:10) isocratically and yielded a

total of nineteen subfractions and this led to the isolation of compound **1** as white crystals (15.0 mg). **F_{19}-F_{21}** was also subjected to a successive silica gel column and eluted with DCM: MeOH (90:10) isocratically and yielded a total of twenty-four subfractions and this led to the isolation of compound **2** as light-brown crystals (980.0 mg).

3.6.2. Extraction and isolation of compounds from BuOH extract of *Protea cynaroides*

BuOH extract (6.82 g) was pre-adsorbed on silica gel and fractionated on a column by gravity elution using the mixture of DCM elution as follows: 1L of 100%, then 1L volumes of mixtures with EtOAc in the following ratios (50:50), (40:60), (30:70), (20:80), (10:90) and finally 100% EtOAc. Finally, MeOH was introduced in the mixture with EtOAc up to 15% MeOH, (95:5), (90:10), (85:15). A total of thirty-five fractions were analysed by TLC using DCM: MeOH: H_2O (90:10). Fractions with similar Rf values were pooled together. The combined fraction from vials **F_6-F_9** (95 mg), **F_{12}-F_{14}** (210 mg), **F_{16}** (305.0 mg), **F_{18}-F_{20}** (5.10 mg), **F_{22}** (150.0 mg) and **F_{24}** (75.0 mg) were each separately preadsorbed on silica gel and loaded onto a column for further separation. **F_6-F_9** was subjected to a successive silica gel column and eluted with EtOAc: MeOH (95:5) isocratically and this led to the isolation of compound **3** as white crystals (36.0 mg).

Fraction **F_{12}-F_{14}** was further separated by column chromatography using 100 %EtOAc. This resulted into eleven subfractions (I-XI). Further separation of fraction V-VII on a column using DCM-MeOH (95:5) yielded compound **4** (45.8 mg). Fractions **F_{16}**, **F_{18}-F_{20}** and **F_{22}** were all prepared for sephadex LH-20 using 95% ethanol as the eluent isocratically. **F_{16}** yielded compound **5** (60.5 mg), **F_{18}-F_{20}** yielded compound **6** (202.6 mg) and similarly **F_{22}** yielded compound **7** (20.5 mg).

F_{24} was further separated by column chromatography using DCM: MeOH (90:10, 85:15) and a total of eight fractions were obtained and (1-9) and 4-6 were combined and rechromatographed and led to the isolation of compound **8** (12.2 mg).

3.6.3. Extraction and isolation of compounds from EtOAc extract of *Cliffortia odorata*

EtOAc extract (2.47 g) was pre-adsorbed on silica gel and fractionated on a column by gravity elution using the mixture of DCM elution as follows: 1L of 100%, then 1L volumes of mixtures with EtOAc in the following ratios (80:20), (60:40), (40:60), (20:80), (10:90) and finally 100% EtOAc. Small portions of MeOH were introduced in the mixture with EtOAc up to 15% MeOH, (95:5), (90:10), (85:15). A total of fifty-one fractions were analysed by TLC using DCM: MeOH: H_2O (90:10). Fractions with similar R_f values were pooled together. The combined fraction from vials **F_3-F_6** (55.0 mg), **F_{10}** (75.0 mg), **F_{13}-F_{15}** (90.0 mg), **F_{22}-F_{25}** (25.0 mg) and **F_{31}-F_{33}** (35.0 mg) were preadsorbed on silica gel and loaded onto a column for further separation. **F_3-F_6** was subjected to a successive silica gel column and eluted with DCM: EtOAc in the following ratios (80:20), (70:30), (60:40), (50:50) and finally 100% EtOAc and this led to the isolation of compound **9** as white powder (10.05 mg).

Fraction **F_{10}** was further separated by column chromatography using 100 %EtOAc and compound **10** (20.1 mg) was obtained precipitating on the tip of the column as a white crystalline solid.

Fractions **F_{13}-F_{15}** was loaded on a sephadex LH-20 column using 100% ethanol as the eluent isocratically and this yielded compound **11** (60.5 mg) as a yellow powder.

F_{22}-F_{25} and **F_{31}-F_{33}** were further separated by column chromatography individually using the same solvent system DCM: MeOH (90:10, 85:15) and a total of eight and ten subfractions respectively. Those that were similar were combined and rechromatographed and led to the isolation of compound **12** (6.50 mg) and **13** (8.0 mg).

3.6.4. Extraction and isolation of compounds from BuOH extract of *Cliffortia odorata*

The BuOH extract (8.12 g) was chromatographed using the solvent mixture of DCM: EtOAc (50:50), (30:70), (10:90), (0:100) and small portions of MeOH were introduced in the mixture with EtOAc up to 15% MeOH, (95:5), (90:10), (85:15). A total of forty-two fractions were obtained.

Fractions **F_3-F_4**, **F_8-F_{10}**, **F_{19}** were loaded on a sephadex LH-20 column individually using 90% aqueous ethanol. Fractions of 2 mL each were collected and evaporated using rotary evaporator. Fractions obtained were developed on TLC using solvent system C and the fractions that displayed the same profiles on the TLC plate were combined and from this compound **14** (20.5 mg), **15** (6,8 mg) and **16** (30.8 mg).

Fractions **F_{24}-F_{26}**, and **F_{33}-F_{36}** were further separated by column chromatography individually using the same solvent system EtOAc: MeOH: H_2O (75:20:5) and both compound **17** 80.6 mg), and **18** (120.5 mg) were obtained individually.

3.6.5. Extraction and isolation of compounds from BuOH extract of *Asparagus lignosus*

The BuOH extract (2.25g) was chromatographed using the solvent mixture of DCM: EtOAc (50:50), (30:70), (10:90), (0:100) and small portions of MeOH were introduced in the mixture with EtOAc up to 15% MeOH, (95:5), (90:10), (85:15). A total of forty-two fractions were obtained.

Fraction **F_6-F_{10}** was subjected to a successive silica gel column and eluted with DCM: MeOH in the following ratios (95:5) and (90:10) and this led to the isolation of compound **19** as white powder (120.0 mg mg).

Fractions **F_{15}-F_{18}** and **F_{22}-F_{24}** were loaded on a sephadex LH-20 column individually using 90% aqueous ethanol. Fractions of 2 mL each were collected and evaporated using rotary

evaporator. Fractions obtained were developed on TLC using solvent system DCM: MeOH (90:10) and the fractions that displayed the same profiles on the TLC plate were combined and from this compound **20** (15.0 mg) and **21** (25.0 mg) were obtained.

3.7 General experimental procedure for biological assays

3.7.1. Reagents

alpha-glucosidase (Saccharomyces cerevisiae), alpha-amylase (procaine pancreas) and 3, 5, di-nitro salicylic acid (DNS), 4-nitro-phenyl-α-D-glucopyranoside (pNPG), sodium carbonate (Na_2CO_3), sodium dihydrogen phosphate, di-sodium hydrogen phosphate secured purchased from Sigma-Aldrich, South Africa.

3.7.2. alpha-glucosidase inhibition activity

Alpha-glucosidase inhibitory activity of the isolated compounds was carried out according to the standard method employed by (Telagari & Hullatti, 2015), with a slight modification. In a 96-well plate, the reaction mixture containing 50 µL of phosphate buffer (100 mM, = 6.8), 10 µL of alpha-glucosidase (1 U/mL), and 20 µL of isolated compounds was pre-incubated at 37°C for 15 minutes. Next, 20 µL of p-NPG (5 mM) was added as a substrate and incubated further at 37°C for 20 minutes. The reaction was then stopped by adding 50 µL of sodium carbonate (Na_2CO_3, 0.1 M). The absorbance of the released *p*-nitrophenol was measured at 405 nm using a Multiplate reader (Multiskan thermo scientific, version 1.00.40, Vantaa, Finland). Acarbose at various concentrations was used as a standard. Each experiment was performed in triplicates.

The results were expressed as a percentage inhibition, which was calculated using formula for (1)

Inhibitory activity (%) = (1-A/B) x 100, where A is the absorbance in the presence of the test substance and B is the absorbance of the control.

3.7.3. alpha-amylase inhibition activity

Alpha-amylase inhibitory activity of extracts was carried out according to the standard method described by Kim et al., 2005 with some modification. In a 96-well plate, reaction mixture containing 50 µL phosphate buffer (100 mM, pH = 6.8), 10 µL alpha–amylase (2 U/mL), and 20 µL of plant extracts at 2.0 mg/mL and was preincubated at 37°C for 20 min. Then, the 20 µl of 1% soluble starch (100 mM phosphate buffer pH 6.8) was added as a substrate and incubated further at 37°C for 30 min; 100 µL of the DNS colour reagent was then added and boiled for 10 min. The absorbance of the resulting mixture was measured at 540 nm using Multiplate Reader (Multiska thermo scientific, version 1.00.40). Acarbose was used as a control. The extracts or isolated compounds were measured in triplicates. The results for both α-amylase and α-glucosidase were expressed as percentage inhibition, which was calculated using the formula,

Inhibitory activity (%) = (1 − As/Ac) ×100

Where:

As is the absorbance in the presence of test substance and Ac is the absorbance of control.

3.7.4. Tyrosinase inhibition assay

Tyrosinase inhibition assay was executed in the course of the study following the approach used by Curto et al. (1999) and Nerya et al. (2003). Extracts were dissolved in DMSO (dimethyl sulphoxide) to a final concentration of 20 mg/mL. This extract stock solution was then diluted to 100 µg/mL and 200 µg/mL in 50 mM potassium phosphate buffer (pH 6.5). 'Kojic acid' was used as control drugs (Lee et al. 1997; Kim et al, 2006). In the wells of a

96well plate, 70 µL of each extract dilution was combined with 30 µL of tyrosinase (500 Units/mL in phosphate buffer) in triplicate. After incubation at room temperature for 5 minutes, 110 µL of substrate (2 mM L-tyrosine) was added to each well. Final concentrations of the extract samples and positive controls ranged from 0.2 to 1000 µg/mL. Incubation commenced for 30 minutes at room temperature by measuring the absorbance at 490 nm with the AccuReader M965 Metertech (V1.11). Equation 1 was employed in determining the percentage of tyrosinase inhibition.

Equation 1: Percentage of tyrosinase inhibition;

$$\% \text{ Of tyrosinase inhibition} = \frac{(A_{control} - A_{blank\ 1}) - (A_{sample} - A_{blank}}{(A_{control} - A_{blank})} \text{ X } 100\%$$

Where $\boldsymbol{A_{control}}$ is the absorbance of the control with the enzyme, $\boldsymbol{A_{blank\ 1}}$ is the absorbance of the control without the enzyme, $\boldsymbol{A_{sample}}$ is the absorbance of the test sample with the enzyme and $\boldsymbol{A_{blank\ 2}}$ is the absorbance of the test sample without the enzyme.

3.8 Statistical Analysis

All the measurements were repeated three times and IC_{50} values were calculated using GraphPad Prism 9 version 9.0.0 (121) (Graph pad software, Inc., La Jolla, CA, USA.) statistical software. The data presented are means ± SD obtained from 96 well plate readers for all in vitro experiments.

References

Curto, E.V., Kwong, C., Hermersdörfer, H., Glatt, H., Santis, C., Virador, V., Hearing, V.J & Dooley, T.P., 1999. Inhibitors of mammalian melanocyte tyrosinase: In vitro comparisons of alkyl esters of gentisic acid with other putative inhibitors. *Biochemical Pharmacology*, 57: 663-672.

Kim, M., Meng, X & Kim, A., 2006. Developing a Long-Lasting Tyrosinase Inhibitor from *Morus alba* L. *Cosmetic and Toiletries magazine*, 27(3): 91-102.

Lee, K.T., Kim, B.J & Kim, J.H., 1997. Biological screening of 100 plant extracts for cosmetic use (I): inhibitory activities of tyrosinase and DOPA autooxidation. *International Journal of Cosmetic Science*, 19: 291-298.

Nerya, O., Vaya, J., Musa, R., Izrael, S., Ben-Arie, R & Tamir, S., 2003. Glabrene and Isoliquiritigenin as Tyrosinase inhibitors from Licorice roots. *Journal of Agriculture and Food Chemistry*, 51: 1201-1207.

Telagari, M and Hullatti,K. 2015. In-vitro alpha-amylase and alpha-glucosidase inhibitory activity of *Adiantum caudatum* Linn. and *Celosia argentea* Linn. extracts and fractions. *Indiam Journal of Pharmacology*, 47, 425-429.

CHAPTER 4

CHEMICAL CHARACTERIZATION AND BIOLOGICAL INVESTIGATION OF *PROTEA CYNAROIDES*

In the current study, a sequential extraction involving solvents of decreasing polarity (n-hexane, DCM, EtOAc and BuOH) was used to extract and isolate bioactive compounds from the leaves of *Protea cynaroides* and this afforded eight compounds, four of which are new (compounds **2**, **5** , **7**, and **8**) and identified as 4-hydroxybenzoic acid **(1)**, 3,4-*bis*(4-hydroxybenzoyl)-1,5-anhydro-D-glucitol **(2)**, 1,5-anhydro-D-glucitol **(3)** ,3,4-dihydroxybenzoic acid **(4)**, 4-hydroxybenzoyl-1,5-anhydro-D-glucitol **(5)** ,3-hydroxykojic acid **(6)** ,2-(hydroxymethyl)-4-oxo-4*H*-pyran-3-yl-6-*O*-benzoate-β-D-glucopyranoside **(7)** and, 3-hydroxy-7,8-dihydro-β-ionone 3-*O*-β-D-glucopyranoside **(8)**. The chemical structures of the isolated compounds were determined on the basis of 1D and 2D NMR, FTIR, and LC-MS spectroscopy and in comparison, with literature data. Five of the compounds were isolated for the first time from *Protea cynaroides*. Evaluation of tyrosine inhibitory activity showed strong inhibitory activities for compound **4** and **6** with IC_{50} values of 0.8776 ± 0.2 and 0.7771 ± 0.09 µg/mL respectively while **1** and **3** demonstrated weak activity and only compound **1** showed activity towards alpha-glucosidase with an IC_{50} value of 162.52 ± 1.21. This is the first scientific report on the isolation and of compounds from *P. cynaroides* combined with evaluation of their inhibitory activity towards alpha glucosidase, alpha amylase and tyrosinase.

4.1 *Protea cynaroides*

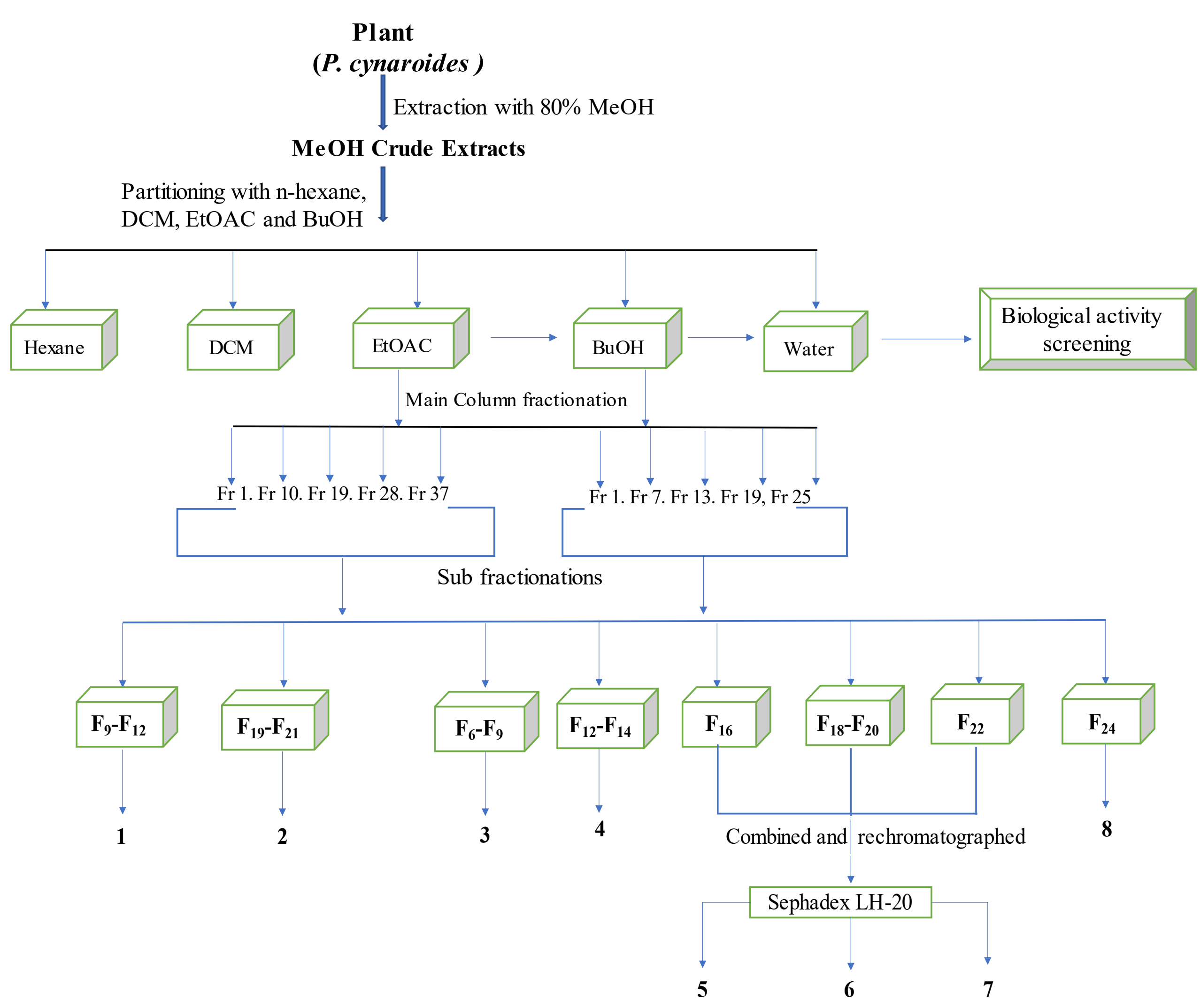
The dried powdered leave material (445 g) was sequentially extracted with hexane, ethyl acetate, and finally with butanol. The hexane (1.09g), DCM (0.55g), ethyl acetate
(3.27g) and butanol (6.82g) extracts were phytochemically studied for their bio-active constituents.

4.1.1 Isolation of constituent of the ethyl acetate extract fraction

Column chromatography of the ethyl acetate extract (3.27g) afforded thirty-one main fractions. $\mathbf{F_9}$ to $\mathbf{F_{12}}$ and $\mathbf{F_{19}}$-$\mathbf{F_{21}}$ were combined and rechromatographed. This afforded compound **1** (15.0 mg) and compound **2** (980 mg) respectively.

4.1.2 Isolation of constituent of the butanol extract

On running the column chromatography of the butanolic extract (6.82 g), thirty-five fractions were obtained. Fractions $\mathbf{F_6}$-$\mathbf{F_9}$, $\mathbf{F_{12}}$-$\mathbf{F_{14}}$, $\mathbf{F_{16}}$, $\mathbf{F_{18}}$-$\mathbf{F_{20}}$, $\mathbf{F_{22}}$ and $\mathbf{F_{24}}$ were rechromatographed due to their high percentage yield. This afforded compound **3** (36.0mg), compound **4** (45,8 mg), compound **5** (60.5 mg), compound **6** (202.6 mg), compound **7** (20.5 mg) and compound **8** (12.2 mg) as shown in scheme **4.1**.



Scheme 4. 1: **A scheme of experimental procedure for the isolation of compounds from *P. cynaroide***

4:2 Structural elucidation and characterization of compounds

4.2.1: Structural elucidation of compound 1

Figure 4. 1: Chemical structure of compound 1

Compound **1** was obtained as white crystalline solid (15 mg). The structure of the compound was established on the basis of 1D (^{1}H, ^{13}C NMR) (Table **4.1**), FTIR and MS data (Appendix **1A**).

^{1}H-NMR spectrum of compound **1** (Apendix **1A**) showed that all proton signals appeared in the aromatic region confirming the presence of a benzene ring.

Two intense doublet signals were observed at δ_H 7.79 (2H, *d*, *J* = 8.8 Hz, H-2/H-6) and 6.82 (2H, *d*, *J* = 8.8 Hz, H-3/ H-5) suggesting the presence of 1,4-*para*-substituted benzene ring.

In the ^{13}C-NMR spectrum of compound **1**, the presence of five carbon signals between 100 – 170 ppm indicated the presence of aromatic ring carbons (Apendix **1A**). Two intense aromatic methine carbon were observed at δ_C 115.6 (C-3/5) and 131.9 (C-2/6), two aromatic fully substituted carbons were seen at δ_C 121.8 (C-1), 162.5 (C-4) while a very downfield signal at δ_C 167.4 was assigned to the carbonyl function of a carboxylate function at C-7. Based on its 1D data as well as comparison with literature data (Silva et al., 2007), compound **1** was determined to be **4-hydroxybenzoic acid**. This compound was first reported from the same plant by Wu (2007). 4-Hydrobenzoic acid has been identified as potential allelopathic agent and is also used as preservatives in cosmetics and pharmaceuticals (Einhellig, 2004). Cho et al (1998) reported that 4-hydroxybenzoic acid has antibacterial activity against most of Gram +

ve and some of Gram - ve bacteria at 50% inhibitory concentration of 160 and 100-170 µg/mL respectively.

Table 4. 1: **^{1}H and ^{13}C NMR spectral data of compounds 1**

Position	**δ_H (*J* in *Hz*)**	**δ_C**	**4-hydroxybenzoic acid** (Silva et al., 2007)
1	-	121.8	121.5
2	7.79 (*d*, 8.8)	131.9	131.4
3	6.82 (*d*, 8.8)	115.6	115.1
4	-	162.1	161.5
5	6.82 (*d*, 8.8)	115.6	115.1
6	7.79 (*d*, 8.8)	131.9	131.4
7	-	167.6	167.2

4.2.2: Structural elucidation of compound 2

Figure 4. 2: Chemical structure of compound **2**

Compound **2** was obtained as light-brown crystals (980 mg). The molecular formula was established as $C_{20}H_{20}O_9$, by the positive-ion ESI-MS, which showed molecular ion $[M+H]^+$ at *m/z* 405.1195 (calculated value: 404.3742). The structure of the compound was established on the basis of 1D (^1H, ^{13}C and DEPT 135) and 2D-NMR experiments (HSQC, and HMBC), FTIR and MS data.

The IR spectrum of **1** showed absorption bands for hydroxyl groups (3335 cm^{-1}), conjugated ester (1693 cm^{-1}), α, β-unsaturated carbonyl (1633 cm^{-1}) and aromatic C=C (1600 cm^{-1}) functionalities.

The ^1H NMR data (Table **4.2**) of compound **2** indicated the presence of eight protons resonating as doublets in the aromatic region at δ_H 7.71 (2H, *d*, *J* = 8.8 Hz, H-2/H-6), 7.68 (2H, *d*, *J* = 8.8 Hz, H-2″/H-6″) and 6.78 (4H, *d*, *J* = 8.8 Hz, H-3/3', H-5/5') indicating the presence of two *p*-hydroxybenzoate moieties. The other protons resonated δ_H 5.27 (1H, *t*, *J* = 9.3, H-3), 5.05 (1H, *t*, *J* = 9.7, H-4), 3.94 (1H, *dd*, *J* = 11.1, 5.5, H-1_a), 3.76 (1H, *m*, H-2), 3.59 (1H, *ddd*, *J* = 4.2, 2.3, 2.2, H-5), 3.45 (1H, *dd*, *J* = overlapped, H-6_b), 3.38 (1H, *dd*, *J* = overlapped, H-6_a) and

3.33 (1H, *t*, $J = 11.3$, H-1_b) showed the presence of a sugar moiety. The absence of the anomeric proton suggested that the sugar moiety was a 1,5-anhydro-D-glucitol.

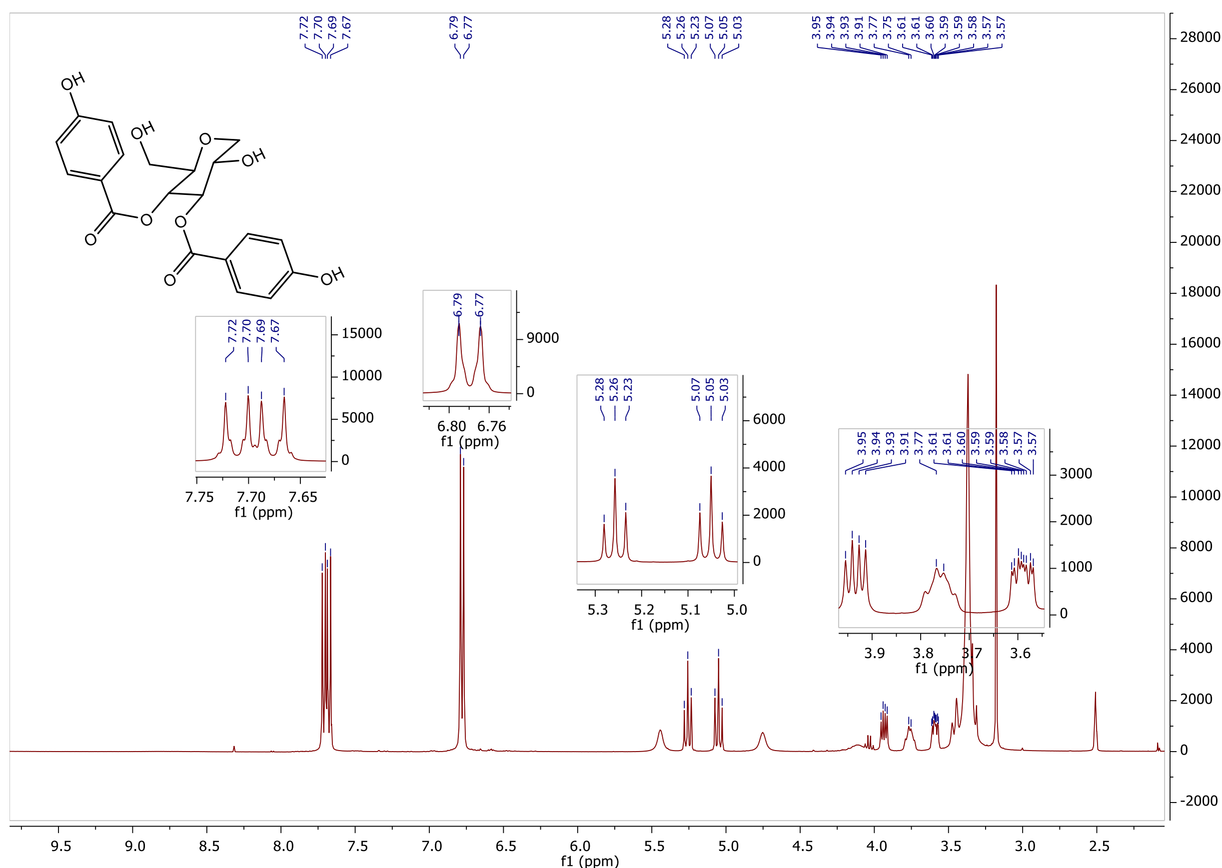


Figure 4. 3: ^{1}H NMR spectrum proton of compound **2** in DMSO-d_6

The ^{13}C NMR spectrum of compound **2** showed sixteen carbon signals.

Apart from the *p*-hydroxybenzoate carbon signals, six oxygenated carbon signals at δ_C 79.5, 77.5, 69.9, 69.7, 68.3 and 61.2 were observed in the ^{13}C NMR spectrum, which also supported the presence of a sugar moiety (Table **4.2**). The other carbon signals were observed at δ_C 115.6, 115.7, 120.1, 120.8, 131.9,132.0, 162.3, 162.6, 165.0, 165.6, which were all in duplicates indicated that there were two *p*-hydroxybenzoate moieties.

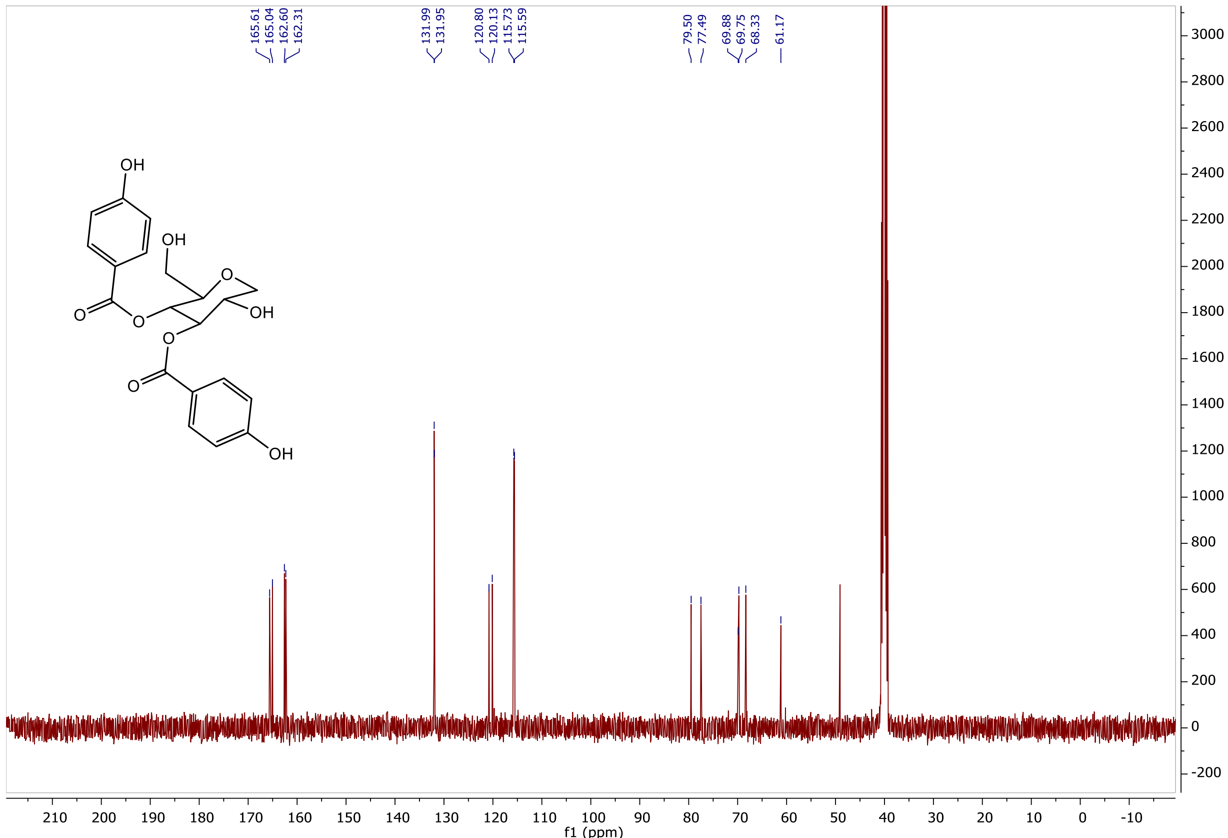


Figure 4. 4: ^{13}C NMR spectrum of compound **2** in DMSO-d_6

Further combined analysis of the ^{1}H–^{1}H COSY, HSQC and HMBC spectra allowed the establishment of the structure of compound **2**.

The HSQC spectra allowed the assignment of all the protons attached to their corresponding carbons.

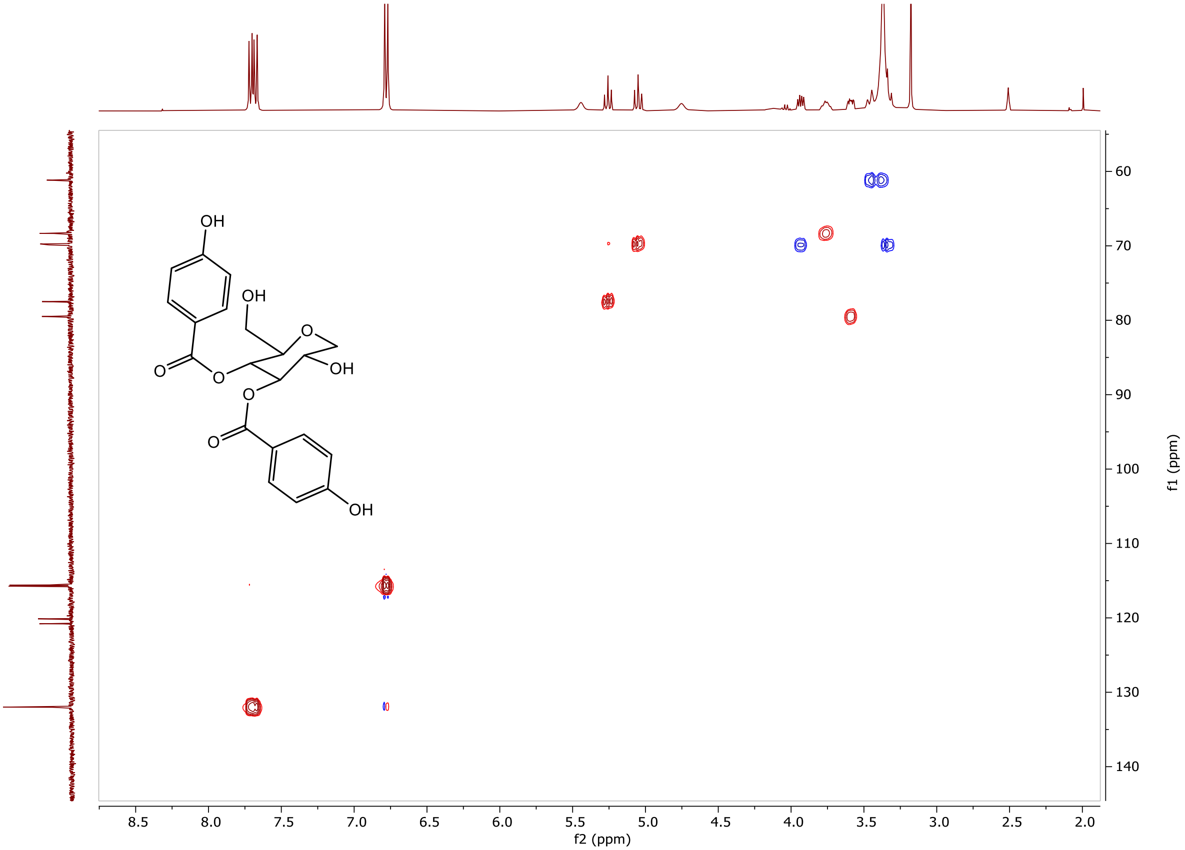


Figure 4. 5: HSQC spectra of compound **2**

In addition, the attachment of the acyl groups to the sugar moiety was confirmed by HMBC cross peaks between δ_H 5.27 (H-3) to δ_C 165.6 (C-7') and 5.05 (H-4) to δ_C 165.0 (H-7'') thus the structure of compound **2** was established as **3,4-bis(4-hydroxybenzoyl)-aceritol**. A Sci-Finder database search provided no evidence for compound **2** as having been previously reported, therefore it is a new compound. Similar structures have been isolated before but with a pyrogallol moiety instead of a *p*-hydroxybenzoate. These kinds of compounds are known to show different bioactive such as alpha-glucosidase, alpha-amylase inhibition and anti-oxidant activities (Machida et *al*., 2019).

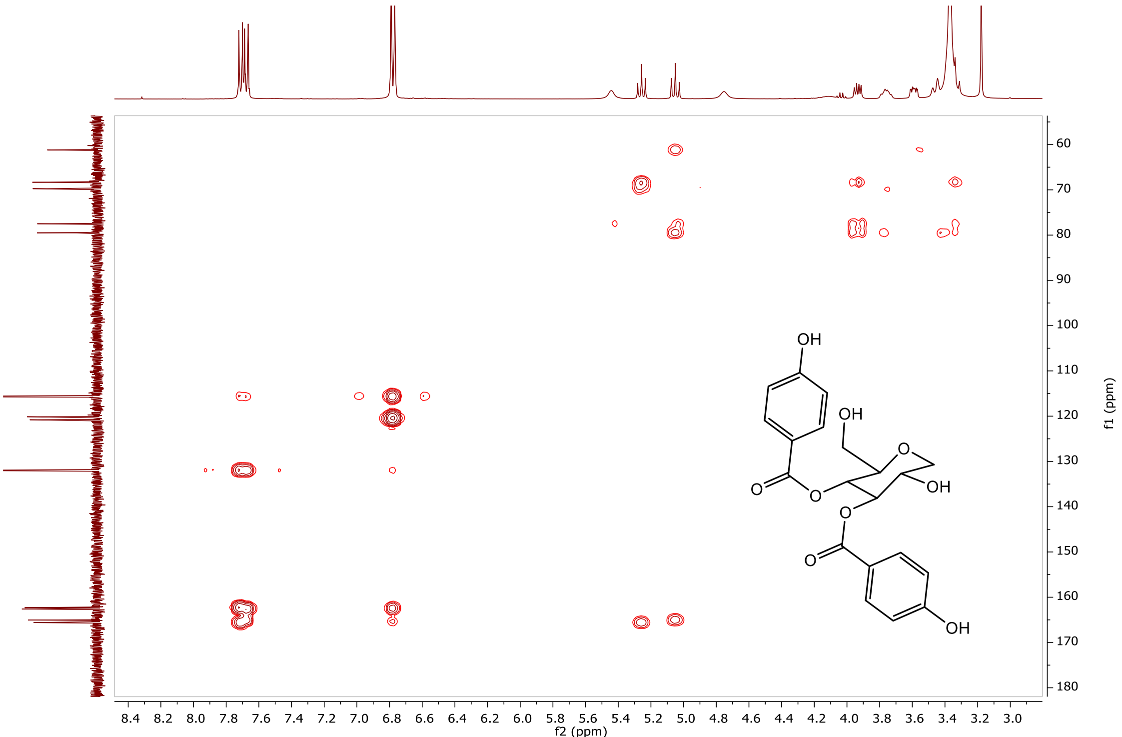


Figure 4. 6: HMBC spectrum of compound **2**

Figure 4. 7: Key HMBC (⟶) and COSY (▬) correlations of compound **2**

Table 4. 2. ^{1}**H and** ^{13}**C NMR spectral data of compound 2 in DMSO-d$_6$**

compound **2**		δ_C	COSY	HMBC
Position	**δ_H (*multi*, *J* in Hz)**			
1a	3.94 (*dd*, 11.1, 5.5)	69.9	H-1b, H-2	C-2, C-3, C-5
1b	3.33 (*t*, 11.3)		H-1a, H-2	C-2, C-3, C-5
2	3.76 (*m*)	68.3	H-1, H-3	C-1, C-3, C-5
3	5.27 (*t*, 9.3)	77.5	H-2, H-4	C-1, C-2, C-4, C-7’
4	5.05 (*t*, 9.7)	69.7	H-3, H-5	C-3, C-5, C-6, C-7”
5	3.59 (*ddd*, 4.2, 2.3, 2.2)	79.5	H-4, H-6	C-6
6a	3.38 (*dd*, *)	61.2	H-5, H-6b	C-5
6b	3.45 (*dd*, *)		H-5, H-6a	C-5
1’	-	120.8	-	-
2’	7.71 (*d*, 8.8)	132.0	H-3’	C-1’, C-3’/5’, C-4’, C-7’
3’	6.78 (*d*, 8.5)	115.6	H-2’	C-1’, C-2’/6’, C-4’, C-7’
4’	-	162.3	-	-
5’	6.78 (*d*, 8.5)	115.6	H-6’	
6’	7.71 (*d*, 8.8)	132.0	H-5’	
7’	-	165.6	-	-
1”	-	120.1	-	-
2”	7.68 (*d*, 8.8)	131.9	H-3’’	C-1”, C-3”/5”, C-4”, C-7”
3”	6.78 (*d*, 8.5)	115.7	H-2’’	C-1”, C-2”/6”, C-4”, C-7”
4”	-	162.6	-	-
5”	6.78 (*d*, 8.5)	115.7	H-6’’	
6”	7.68 (*d*, 8.8)	131.9	H-5’’	
7”	-	165.0	-	-

* = overlapped signals, could determine the *J* values

4.2.3: Structural elucidation of compound 3

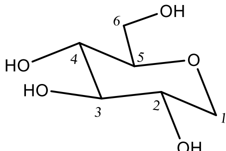


Figure 4. 8: Chemical structure of compound **3**

Compound **3** was obtained as white crystals (36.0 mg). Its chemical structure was elucidated by 1D NMR (^{1}H, ^{13}C and DEPT) and it was compared with those from an authentic sample of aceritol. Its chemical formula was determined to be $C_6H_{12}O_5$.

^{1}H-NMR showed proton signals between 3.10 – 4.00 ppm (Table **4.3**). Protons of two methylenes were observed at 3.84 (1H, *dd*, *J* =11.2 Hz, 5.4 Hz, H-1b), 3.13 (1H, *t*, *J* =10.9, H-1a), 3.74 (1H, *dd*, *J* = 12.2 Hz, 1.4 Hz, H-6a) and 3.54 (1H, *dd*, 1H, *J* = 12.3, 1.6, H-6b) and protons of four methines at 3.43 (1H, *m*, H-2), 3.28 (1H, *m*, H-3), 3.21 (2H, *m*, H-4, H-5) and this information suggested that compound **3** was a sugar and the absence of an anomeric proton led into identifying the compound as a 1-deoxyglycoside (Appendix **1A**).

The ^{13}C NMR and DEPT 135 exhibited the presence of six carbon resonances, two of those were the two methylenes at δ 68.8 and δ 60.9 attributed to C-1 and C-6 respectively and the remaining four carbon signals were the methines at δ_C 80.3(C-5), 77.5(C-3), 69.7(C-4), and 69.4(C-2). The structural identity of compound **3**, was tentatively established as 1,5-anhydro-D-glucitol (aceritol) based on its 1D NMR spectra as well as by comparison with literature data (Kamori *et al*., 2016). This compound was first identified by Plouvier (1964) from *Protea cynaroides*, and from seven other *Protea* species such as *P. compacta, P. eximia, P. lepidocarpodendron, P. neriifolia, P. obtusifolia, P. pityphylla* and *P. repens*. Boeyens et al.,

(1983) reported the occurrence of 1,5-anhydro-D-glucitol as a major leaf compound in three South African *Protea* species namely *P. arborea, P. barbigera, and P. roupelliaea*.

Table 4. 3: **^{1}H and ^{13}C NMR (400MHz) spectral data of compounds 3 in D_2O**.

Position	*δ* ^{1}H	*δ* ^{13}C	**Aceritol** (Kamori *et al*., 2016)
1a	3.84 (*dd*, 11.2, 5.4)	68.8	68.0
1b	3.13 (*t*, 10.9)		
2	3.43 (*m*)	69.4	68.5
3	3.28 (*m*)	77.5	76.6
4	3.21 (*m*)	69.7	68.8
5	3.21 (*m*)	80.3	79.4
6a	3.74 (*dd*, 12.2, 1.4)	60.9	60.1
6a	3.84 (*dd*, 12.2, 1.4)		

4.2.4: Structural elucidation of compound 4

Figure 4. 9: Chemical structure of compound **4**

Compound **4** was obtained as reddish-brown crystals (20 mg) and gave molecular ion at *m/z* 154.0267 which was consistent with molecular formula $C_7H_6O_4$. The compounds gave dark blue colour when treated with ferric chloride indicated the presence of phenolic hydroxyl function. The structure of the compound was established on the basis of 1D (^1H, ^{13}C NMR) (Table **4.4**), FTIR and MS data (Appendix **1A**).

The ^1H NMR spectrum exhibited three proton signals in the aromatic region confirming the presence of a benzene ring. Three doublets were observed at δ_H 7.14 (1H, *d*, J = 2.1 Hz), 7.11 (1H, *dd*, J = 8.2, 2.1 Hz) and 6.58 (1H, *d*, J = 2.1 Hz) which were assigned to H-5, H-6, and H-2, respectively, based on their coupling constants and mutual couplings and this suggested a 1,3,4 trisubstituted benzene ring.

The ^{13}C NMR and DEPT 135 spectra of compound **4** (Table **4.4**) exhibited the presence of seven carbon resonances, three substituted aromatic carbons at δ 147.0 (C-4), 143.1 (C-3) and 128.4 (C-1), three protonated aromatic carbons at δ 122.4 (C-6), 116.8 (C-5, and 115.1 (C-2), a very downfield signal at δ 174.5 was assigned to a carboxylic group at C-7. Based on these spectroscopic data and the literature information, the structure of compound **4** was concluded to be **3,4-dihydroxybenzoic acid** (protocatechuic acid), previously reported from the stem of *Protea cynaroides* (Wu *et al*, 2007).

It is reported to exhibit antifungal activity, anti-inflammatory, antihepatotoxic (Sroka and Cisowski, 2003), anti-oxidant (Hur *et al*., 2003), free radical scavenger (Yip *et al*., 2006), cytotoxic (Vari *et al*., 2010), chemopreventive, apoptotic (Yin *et al*., 2009), antiplatelet aggregation, neuroprotective and LDL oxidation inhibitor activity (Khadem and Marles, 2010).

Table 4. 4: **^{1}H and ^{13}C NMR (400 MHz) spectral data of compound 4 in pyridine d_5**

Position	**δ_H (J in Hz)**	**δ_C**	**Protocatechuic acid** (Wu *et al*, 2007)
1	-	128.4	123.9
2	6.58 (*d*, 2.1)	131.9	131.4
3	-	143.1	146.2
4	-	147.0	151.5
5	7.14 (*d*, 2.1)	116.5	115.8
6	7.11 (*dd*, 8.2, 2.1)	122.4	123.8
7	-	174.5	171.0

4.2.5: Structural elucidation of compound 5

Figure 4. 10: Chemical structure of compound **5**

Compound **5** was obtained as white clear crystals. The molecular formula was established as $C_{13}H_{16}O_7$, by the positive-ion HR-ESI-MS, which showed molecular ion $[M+H]^+$ at *m/z* 285.0978 (calc. for 284.3659). The structure of the compound was established on the basis of 1D (^1H, ^{13}C and DEPT 135) and 2D-NMR experiments (HSQC, COSY and HMBC), FTIR and MS data.

Compound **5** was somewhat similar to that of compound **5** indicating that the structures of both compounds were closely related, and the only difference was likely the absence of an additional *p*-benzoate moiety in compound **5**.

The ^1H NMR data indicated the presence of two intense protons resonating as doublets in the aromatic region at δ_H 7.83 (2H, *d*, *J* = 8.8 Hz, H-2'/H-6'), and 6.75 (2H, *d*, *J* = 8.8 Hz, H-3'/H-5') indicating the presence of *p*-hydroxybenzoate moiety. The other protons resonated δ_H 4.81 (1H, *t*, *J* = 9.3 Hz, H-4), 3.89 (1H, *dd*, *J* = 11.2, 4.6, H-1_a), 3.51 (1H, *m*, H-2), 3.51 (1H, *m*, H-3), 3.49 (1H, *m*, H-6_b), 3.40 (1H, *m*, H-6_a), 3.38 (1H, *m*, H-5) and 3.16 (1H, *t*, *J* = 10.6 Hz, H-1_b) suggested the presence of a sugar moiety. The absence of the anomeric proton suggested that the sugar moiety was a 1,5-anhydro-D-glucitol.

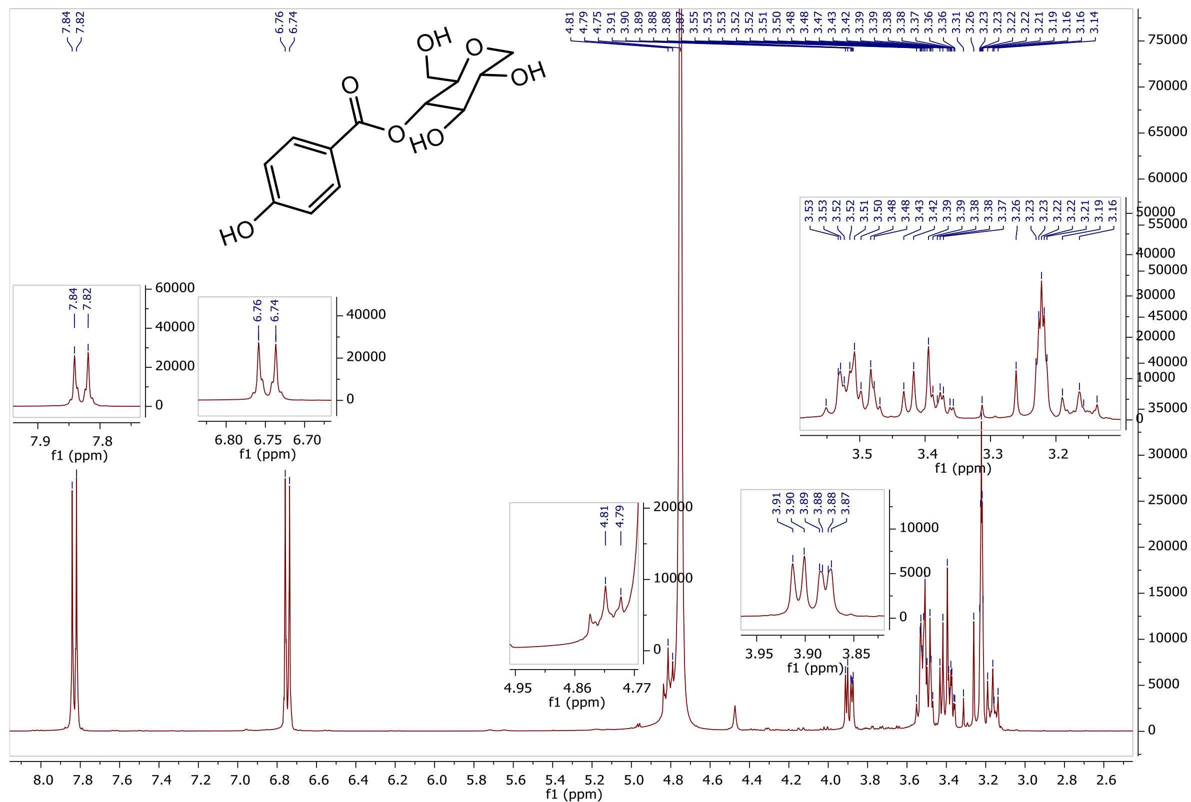


Figure 4. 11: ^{1}H NMR spectrum proton of compound **5** in CD_3OD

Inspection of the ^{13}C NMR data showed signals indicative of 4-hydroxybenzoyl moiety resonating at δ 167.7 (C-7'), 163.8 (C-4'), 133.2 (C-3'/5'), 122.1 (C-1') and 116.3 (C-2'/6'). Apart from the 4-hydroxybenzoyl carbon signals, six oxygenated carbon signals at δ_C 81.0, 77.9, 73.0, 71.7, 71.1 and 62.9 were observed in the ^{13}C NMR spectrum, which also supported the presence of a sugar moiety.

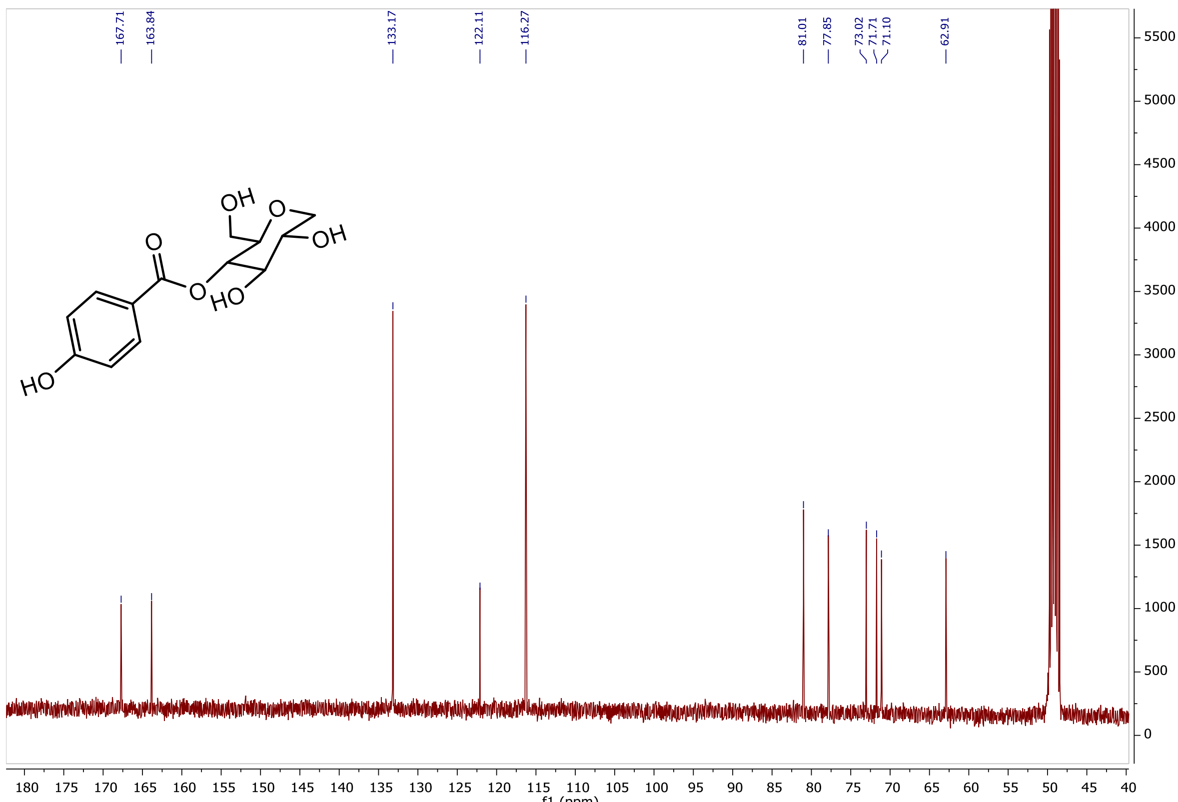


Figure 4. 12: ^{13}C NMR spectrum of compound **5**

Further combined analysis of the ^{1}H–^{1}H COSY, HSQC and HMBC spectra allowed the establishment of the structure of compound **5**. The HSQC spectrum allowed the assignment of all the protons attached to their corresponding carbons.

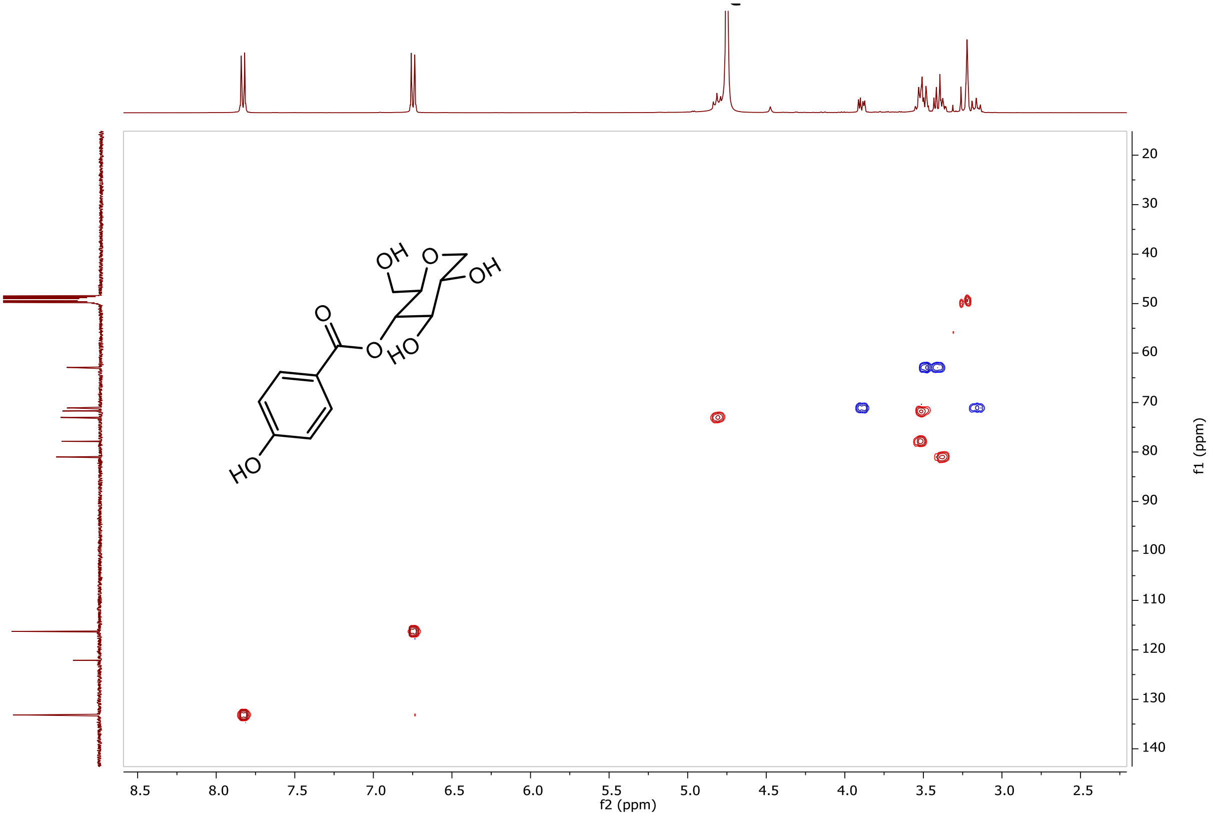


Figure 4. 13: HSQC spectra of compound **5**

The HMBC correlations between H-4 and the ester carbonyl (C-7′) indicated that the 4-*para*-hydroxybenzoate group was linked at C-4 of the 1,5-anhydro-glucitol moiety thus the structure of compound **5** was established as 4,5-dihydroxy-2-(hydroxymethyl)tetrahydro-2*H*-pyran-3-yl 4-hydroxybenzoate. A database search provided no evidence for compound **5** as having been previously reported, therefore it is a new compound.

The nearest analogue of compound **5** found in literature is 4-*O*-galloyl-1,5-anhydro-D-glucitol (maplexin B), published by Wan et al., 2012. Only the aromatic ring of this molecule differs by having three hydroxyl groups on the 3,4,5-carbons, whereas compound **5** has only one hydroxyl groups on C-4 position of.

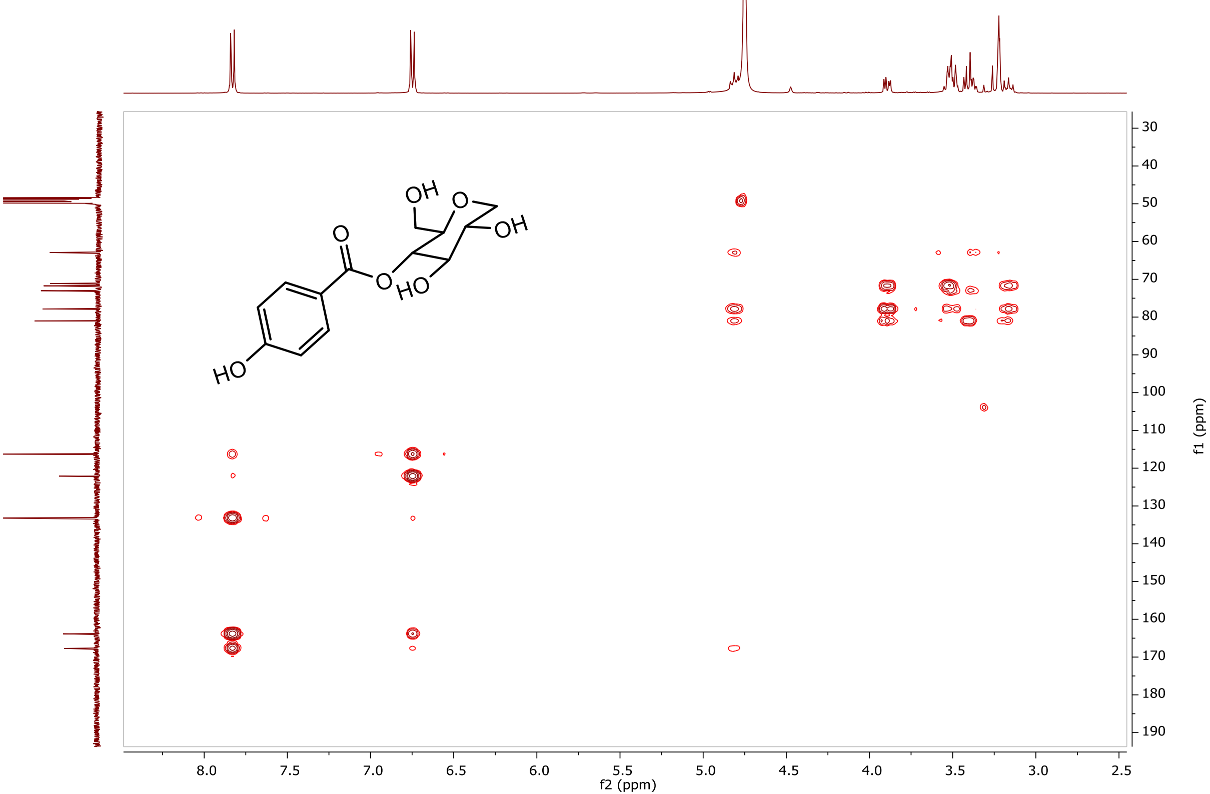


Figure 4. 14: HMBC spectra of compound **5**

Figure 4. 15: **Key HMBC (→) and COSY (▬) correlations of compounds 5**

Table 4. 5: **^{1}H and ^{13}C NMR spectral data of compounds 5 in CD_3OD**.

Position	Compound **5** δ_H (*J* in Hz)	δ_C	COSY	HMBC
1a	3.89 (*dd*, 11.1, 5.5)	71.1	H-1b, H-2	C-2, C-3, C-5
1b	3.16 (*t*, 10.6)		H-1a, H-2	C-2, C-3, C-5
2	3.51 (*m*)	71.7	H-1, H-3	C-1, C-3, C-5
3	3.51 (*m*,)	77.8	H-2, H-4	C-1, C-2, C-4,
4	4.81 (*t*, 9.3)	73.0	H-3, H-5	C-3, C-5, C-6, C-7’
5	3.38 (*m*)	81.0	H-4, H-6	C-6
6a	3.38 (*m*)	62.9	H-5, H-6b	C-5
6b	3.45 (*m*)		H-5, H-6a	C-5
1’	-	122.1	-	-
2’	7.83 (*d*, 8.8)	133.2	H-3’	C-1’, C-3’/5’, C-4’, C-7’
3’	6.75 (*d*, 8.5)	116.3	H-2’	C-1’, C-2’/6’, C-4’, C-7’
4’	-	163.8	-	-
5’	6.75 (*d*, 8.5)	116.3	H-6’	-
6’	7.83 (*d*, 8.8)	133.2	H-5’	-
7’	-	167.7	-	-

4.2.6: Structural elucidation of compound 6

Figure 4. 16: Chemical structure of compound **6**

Compound **6** was obtained as a light brown solid (200 mg). The molecular formula was established as $C_6H_6O_5$, by the positive-ion HRMS, which showed molecular ion $[M+H]^+$ at *m/z* 159.5810 (calc. for 158.3628). The IR spectrum of **6** showed absorption bands for hydroxyl groups (3270.8 cm^{-1}), cyclic-C=O (1663 cm^{-1}), aromatic C=C (1611 cm^{-1}), cyclic C-O-C (1074) functionalities.

The ^{1}H NMR spectrum of compound **6** displayed the following signals: two singlets, each integrating for one proton each appearing as a doublet at δ_H 9.05 (*s*) and 9.03 (*s*) which were assigned to the two -OH groups at C-3 and C-5, one olefinic methine at δ_H 8.04 (1H, *s*, H-6), a singlet appeared at δ_H 4.40 (2H, *s*) was attributed to H-7 along with a small singlet signal at δ_H 5.40 (*s*) which was from the OH group attached to the methylene at position 7 which were characteristics of a 2,3,5-trisubstituted 4-pyrone.

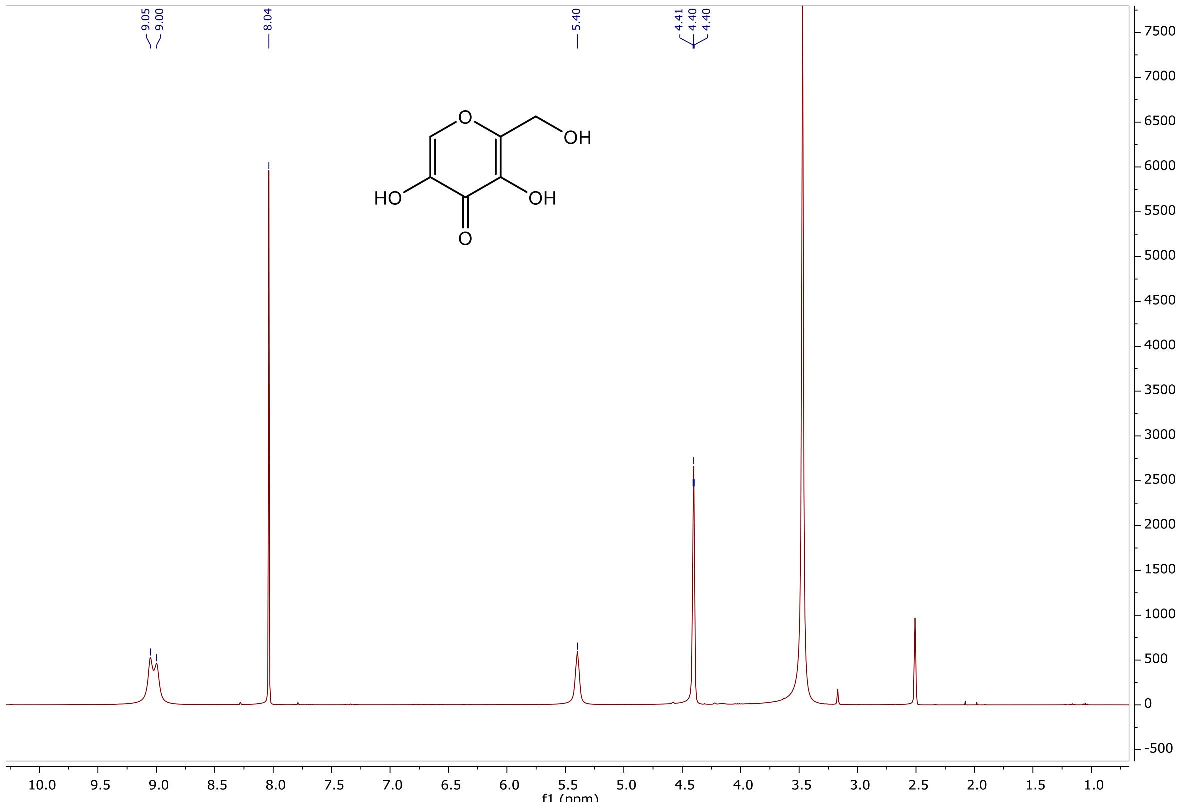


Figure 4. 17: ^{1}H NMR spectrum proton of compound **6**

The ^{13}C NMR and DEPT-135 spectra revealed signals for six carbons: one carbonyl carbon resonating at 169.6 (C-4), three olefinic fully substituted enol carbons at 150.7, 144.8 and 141.7 which were assigned to C-2, C-5 and C-3 respectively, one olefinic methine at 139.9 (C-6) and an oxymethylene at 55.8 attributed to C-7. All these ^{13}C NMR signals together with the ^{1}H NMR signals established the structure of **3-oxykojic acid**. Further confirmation was obtained through HSQC and HMBC correlations

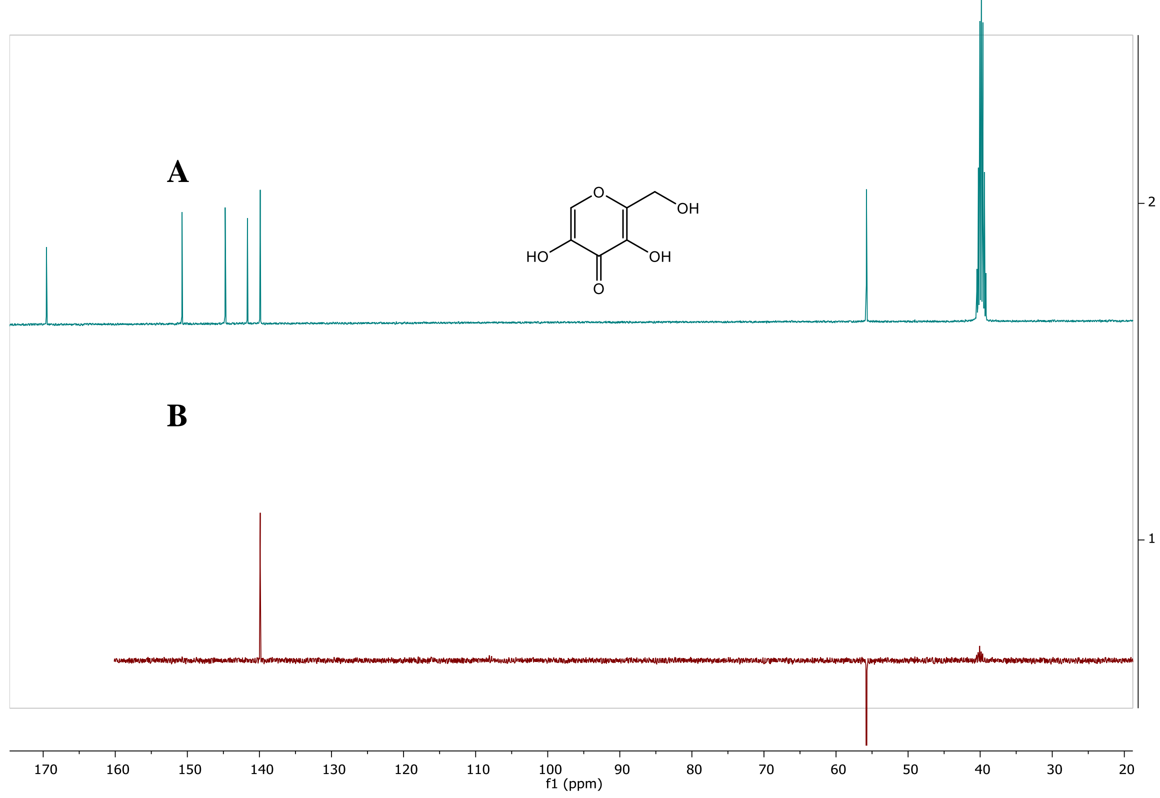


Figure 4. 18: Stacked ^{13}C and DEPT 135 spectrum of **6**

A – ^{13}C NMR spectrum of **6**
B – DEPT-135 of **6**

Table 4. 6: **^{1}H and ^{13}C NMR spectral data of compounds 6 in DMSO-d_6**

Compound **6**			
Position	**δ_H (*J* in Hz)**	δ_C	**HMBC**
2	-	150.7	-
3	-	141.7	-
4	-	169.6	-
5	-	144.8	-
6	8.04 (*s*)	139.9	C-3, C-4, C-5
7	4.40 (*s*)	55.8	C-2, C-3

This compound was first isolated from the culture broth of *Gluconobacter cerinus var. ammoniac* (Terada *et al*., 1961) and from *Gaultheria leucocarpa*, and is used for the treatment of rheumatoid arthritis, swelling pain, trauma, chronic tracheitis, colds and vertigo (Liu *et al*., 2013). Yao et al., 2005, reported that the asymmetric unit in the crystal structure

of this compound consists of two nearly parallel molecules with different conformations linked by a strong intermolecular O—H· · · ·O hydrogen bond.

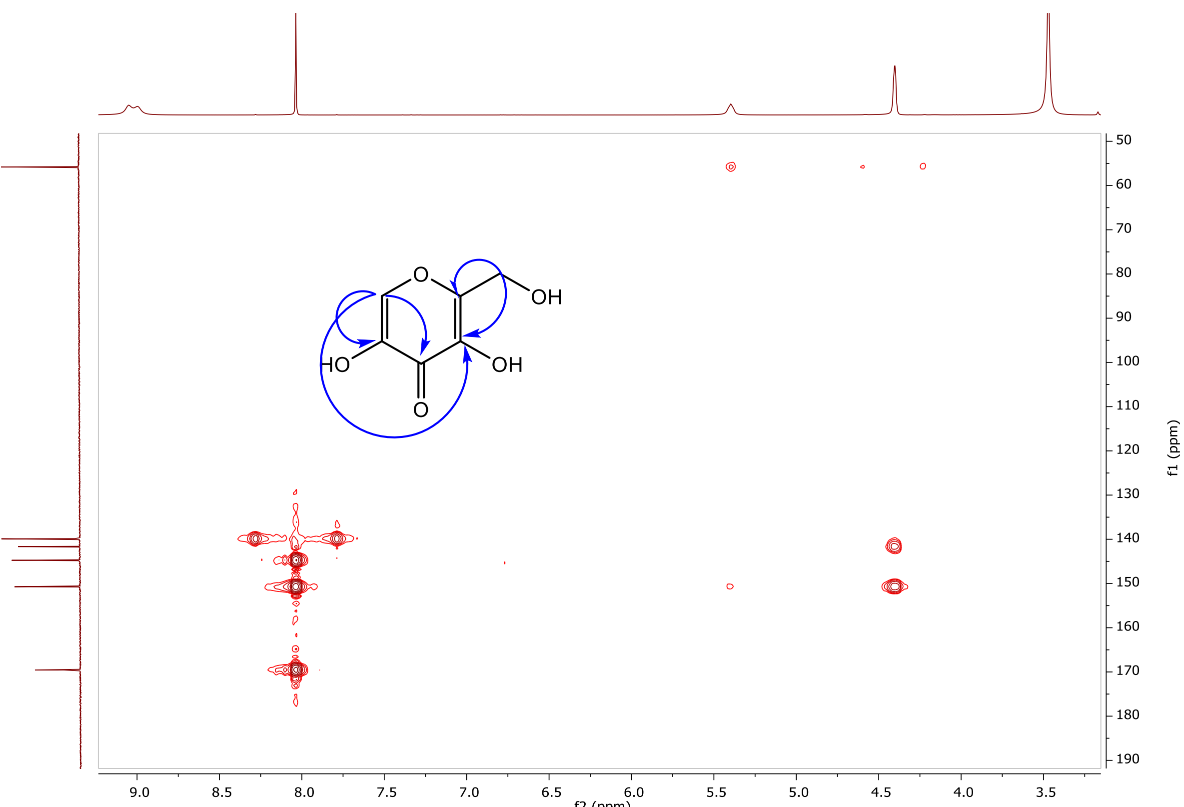


Figure 4. 19: HMBC spectra of compound **6**

4.2.7: Structural elucidation of compound 7

Figure 4. 20: Chemical structure of compound **7**

Compound **7** was obtained as light brown solid. The molecular formula was established as $C_{19}H_{20}O_{11}$, by the positive-ion HRMS, which showed molecular ion $[M+H]^+$ at *m/z* 425.1094 (calc. for 425.3689). The IR spectrum of **7** showed absorption bands for hydroxyl groups (3225 cm^{-1}), conjugated ester (1688 cm^{-1}), alpha, beta-unsaturated carbonyl (1644 cm^{-1}) and aromatic C=C (1612 cm^{-1}) functionalities.

The ^{1}H NMR spectrum showed the presence of one set of *ortho*-coupled aromatic protons at δ_H 7.97 (1H, *d*, *J* = 5.6 Hz, H-6) and δ_H 6.31 (1H, *d*, *J* = 5.6 Hz, H-5), also an oxymethylene protons at δ_H 4.69 (1H, *d*, *J* = 14.1 Hz, H-7*a*) and 4.47 (1H, *d*, *J* = 14.1 Hz, H-7*b*) which were characteristics of a 1,2-disubstituted 4-pyrone unit and another set of *ortho*-coupled aromatic protons at δ_H 7.84 (2H, *d*, *J* = 8.8 Hz, H-2″/H-6″) and δ_H 6.83 (2H, *d*, *J* = 8.8 Hz, H-3″/H-5″), which indicated the presence of a 1,4-disubstituted benzoate unit. In addition, one sugar moiety was deduced by the presence of an anomeric proton resonating at δ_H 4.94 (1H, *d*, *J* = 7.7 Hz) and other signals in the range, δ_H 3.12–3.55 (m).

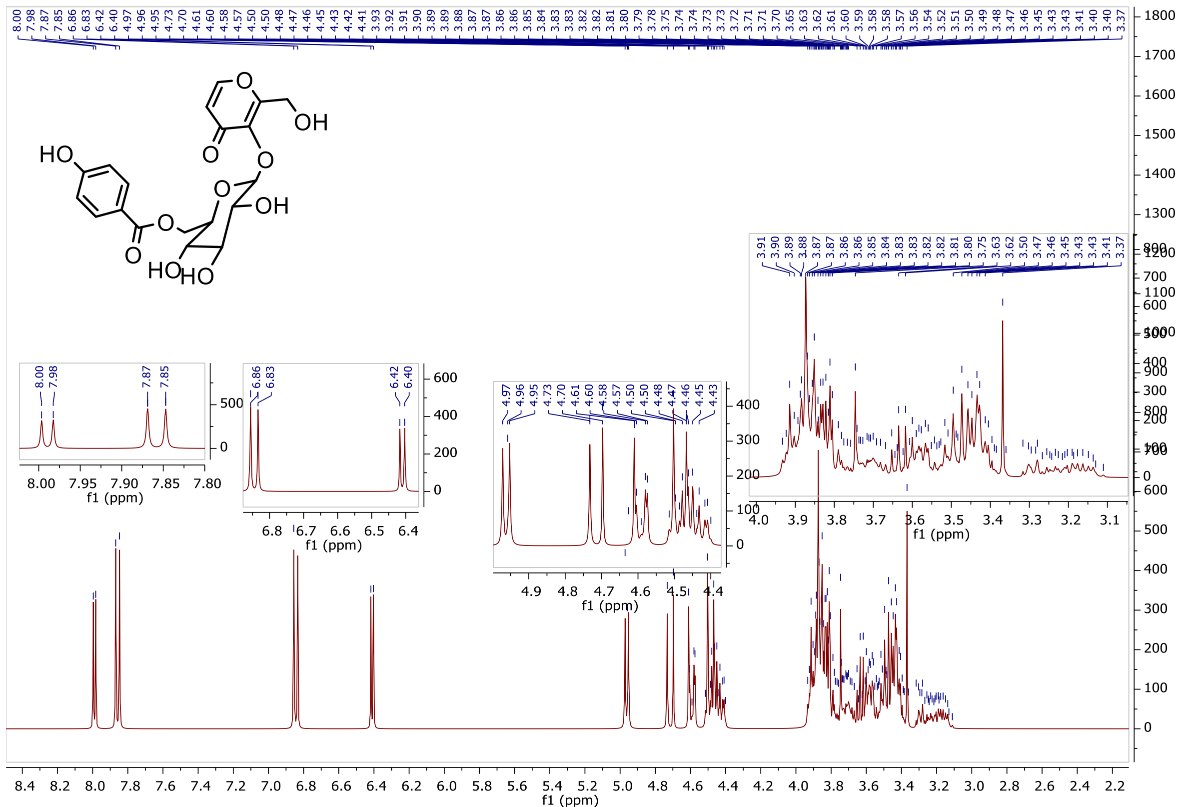


Figure 4. 21: ^{1}H NMR spectrum of compound **7** in CD_3OD

The ^{13}C NMR and DEPT-135 spectra showed that compound **7** possessed 16 signals counted for 18 carbons, including characteristic signals of 4-hydroxybenzoyl moiety at δ 167.9, 163.7, 133.0 x 2, 122.1 and 116.4 x 2, a pyranone moiety at δ_C 177.4, 163.9, 157.5, 142.3 and 117.7; a glucopyranosyl unit at δ_C 104.0, 77.8, 76.2, 75.4, 71.7 and 64.4 and an oxymethylene at δ_C 57.7.

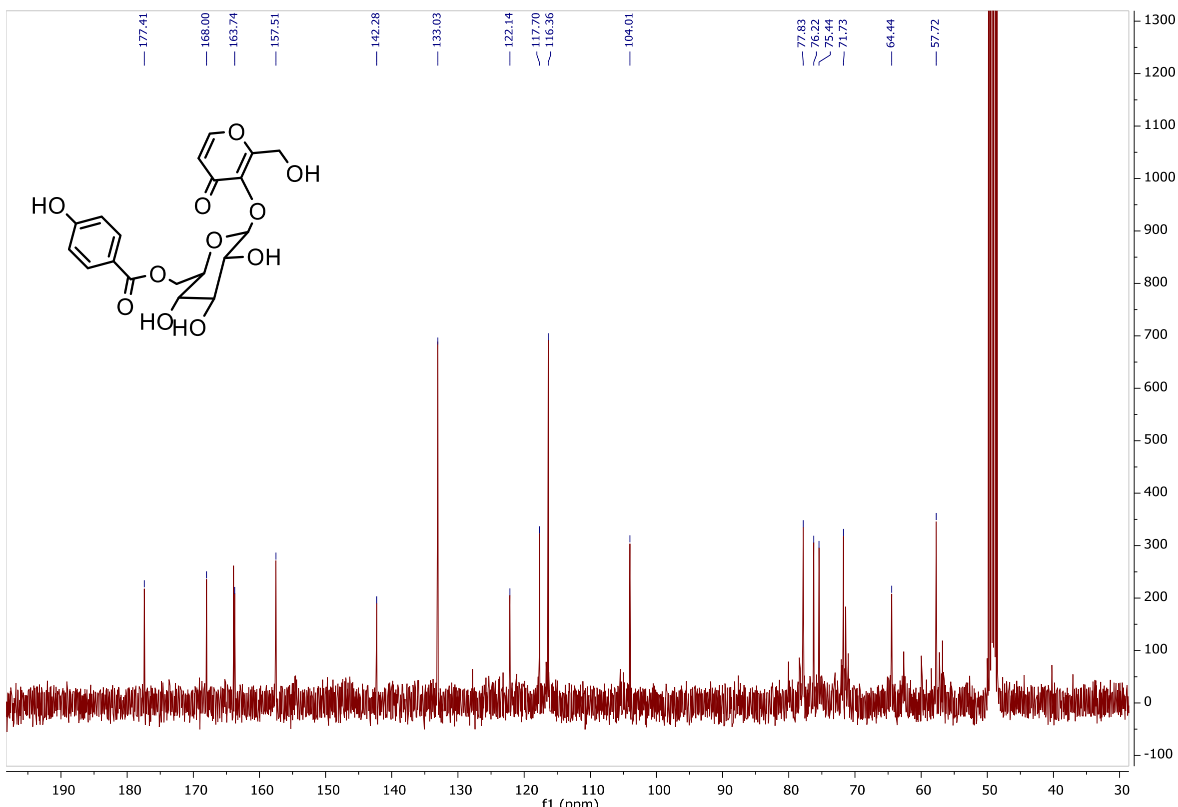


Figure 4. 22: ^{13}C NMR spectrum of compound **7** in CD_3OD

Further combined analysis of the ^{1}H–^{1}H COSY, HSQC and HMBC spectra allowed the establishment of the structure of compound **7**. The HSQC spectrum allowed the assignment of all the protons attached to their corresponding carbons.

The observed correlation of H-6’ from the sugar unit with C-7″ (δ 167.9) in the HMBC spectrum indicated the 4-hydroxybenzoyl moiety was attached at C-6’ of the glucosyl. Furthermore, HMBC experiment indicated a correlation of the anomeric proton H-1’ of the glycosyl (δ_H 4.94) unit with δ 142.3 which is attributed to C-3 of the pyrone unit. Also, the oxymethylene was located at position C-2 of the pyrone unit which was confirmed by the HMBC correlations between H-7 (δ_H 4.69a, 4.47b) and C-2 (δ_C 142.3) and (δ_C 163.9) (Figure **4.7.4**). Therefore, **7** was determined as 2-(hydroxymethyl)-4-*oxo*-4*H*-pyran-3yl-6-*O*-

benzoate-β-D-glucopyranoside. A database search provided no evidence for compound **7** as having been previously reported, therefore it was a new compound.

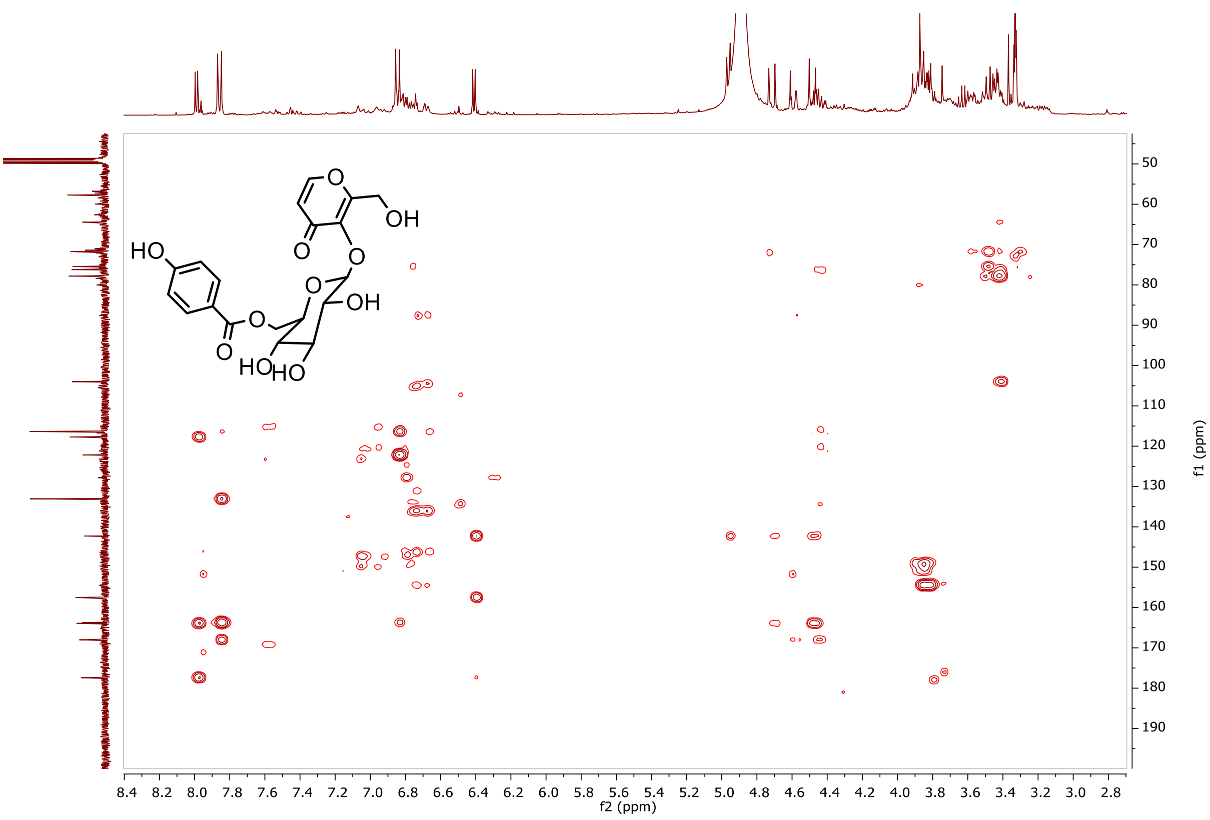


Figure 4. 23: HMBC spectra of compound **7**

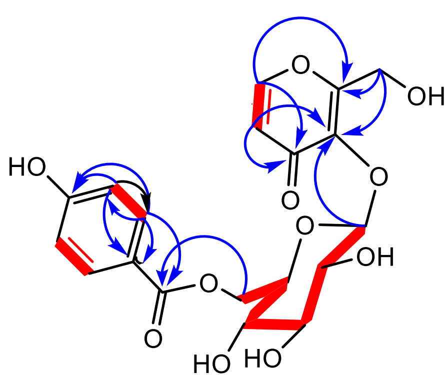


Figure 4. 24: Key HMBC (⟶) and COSY (▬) correlations of compounds **7**

Table 4. 7: **^{1}H and ^{13}C NMR spectral data of compounds 7 in CD_3OD.**

	Compound **7**			
	δ_H	**δ_C**	**COSY**	**HMBC**
2	-	163.9	-	-
3	-	142.3	-	-
4	-	177.4	-	-
5	6.39 (*d*, 5.6)	117.7	H-6	C-3, C-4, C-6
6	7.97 (*d*, 5.6)	157.5	H-5	C-2, C-4, C-5
7a	4.69 (*d*, 14.1)	57.7	H-7b	C-2, C-3
7b	4.47 (*d*, 14.1)		H-7a	
1'	4.94 (*d*, 7.7)	104.0	H-2'	C-3
2'	3.41 (*m*)	75.4	H-1', H-3'	C-1', C-3'
3'	3.45 (*m*)	77.8	H-2', H-4'	C-2', C-4'
4'	3.43 (*m*)	71.7	H-3', H-5'	C-3', C-5', C-6'
5'	3.56 (*m*)	76.2	H-4', H-6'	C-4', C-6'
6a'	4.58 (*dd*, 11.8, 2.2)	64.4	H-6b', H-5'	C-4', C-7"
6b'	4.45 (*dd*, 11.8, 2.2.)		H-6a', H-5'	C-5', C-7"
1"	-	122.1	-	-
2"	7.84 (*d*, 8.7)	133.0	H-3''/H-6''	C-4", C-5", C-7"
3"	6.83 (*d*, 8.7)	116.4	H-2''/H-5''	C-1" C-4", C-6"
4"	-	163.7		-
5"	7.84 (*d*, 8.7)	133.0	H-3''/H-6''	C-3", C-4", C-7"
6"	6.83 (*d*, 8.7)	116.4	H-2''/H-5''	C-1" C-3", C-4"
7"	-	167.9	-	-

4.2.8: Structural elucidation of compound 8

Figure 4. 25: Chemical structure of compound **8**

Compound **8** was obtained as white solid. The molecular formula was established as $C_{19}H_{32}O_8$, by the positive-ion HRMS, which showed molecular ion $[M+H]^+$ at *m/z* 389.2177 (calc. for 388.4615). The IR spectrum of **8** showed absorption bands for hydroxyl groups (3179 cm^{-1}), conjugated ester (1693 cm^{-1}), α, β-unsaturated carbonyl (1633 cm^{-1}) and aromatic C=C (1600 cm^{-1}) functionalities.

The ^1H NMR spectrum showed signals of two methyl protons at δ_H 0.86 (3H, *s*, H-12), 1.01 (3H, *s*, H-11), a vinyl methyl at 1.56 (3H, *s*, H-13) and an acetyl methyl at δ_H 2.07 (3H, *s*, H-10). Three methylene protons were observed resonating at δ_H 2.00 (1H, *dd*, J = 17.4, 6.4 Hz, H-4a), 2.31 (1H, *dd*, J = 17.4, 6.4 Hz, H-4b), 2.09 (1H, *m*, H-7a), 2.16 (1H, *m*, H-7b) and 2.45 (2H, *t*, J = 8.1 Hz, H-8). Also, two oxymethine protons were seen at δ_H 3.16 (1H, *d*, J = 2.9 Hz) and 3.70 (1H,) and were attributed to H-2 and H-3 respectively. A resonance at δ_H 4.28 (1H, *d*, J = 7.8 Hz) was an indication of an anomeric proton and the J coupling constant suggested that it's a beta anomeric proton. This then implied that the structure has sugar moiety. The other sugar peaks were clustered between 3.0 and 3.70.

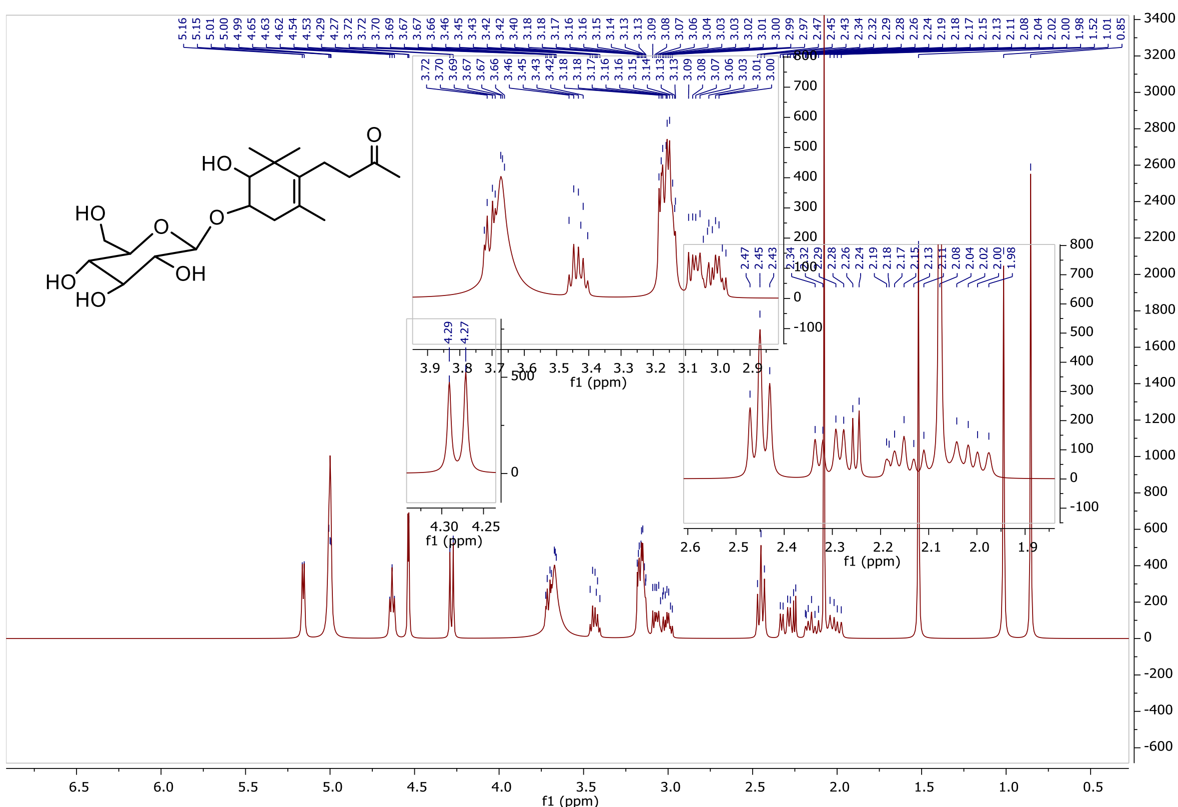


Figure 4. 26: ^{1}H NMR spectrum of compound **8** in DMSO-d_6

The ^{13}C NMR and DEPT-135 spectra showed that compound **8** displayed nineteen carbon resonances, including, four methyls at δ_C 19.5 (C-13), 22.0 (C-12), 25.6 (C-11) and 30.1 (C-10), three methylene at δ_C 22.2 (C-7), 37.9 (C-4) and 43.9 (C-8), two oxymethines at δ_C 75.7 (C-2) and 76.9 (C-3) and four fully substituted carbon resonances at δ_C 42.2 (C-1), 123.9 (C-5), 136.0 (C-6) and including one carbonyl at δ_C 208.9 (C-9) for the aglycone group. The ^{13}C NMR displayed a characteristic anomeric carbon resonance at δ_C 101.4 (C-1'),

which corresponded to a typical anomeric proton resonance at δ_H 4.28 (*d*, $J = 7.8$ *Hz*) in the HSQC spectrum and this suggested that compound **8** possessed one sugar group. The other sugar moiety signals appeared at δ_C 61.4 (C-6'), 70.4 (C-4'), 73.7 (C-2'), 77.2 (C-3') and 76.8 (C-5').

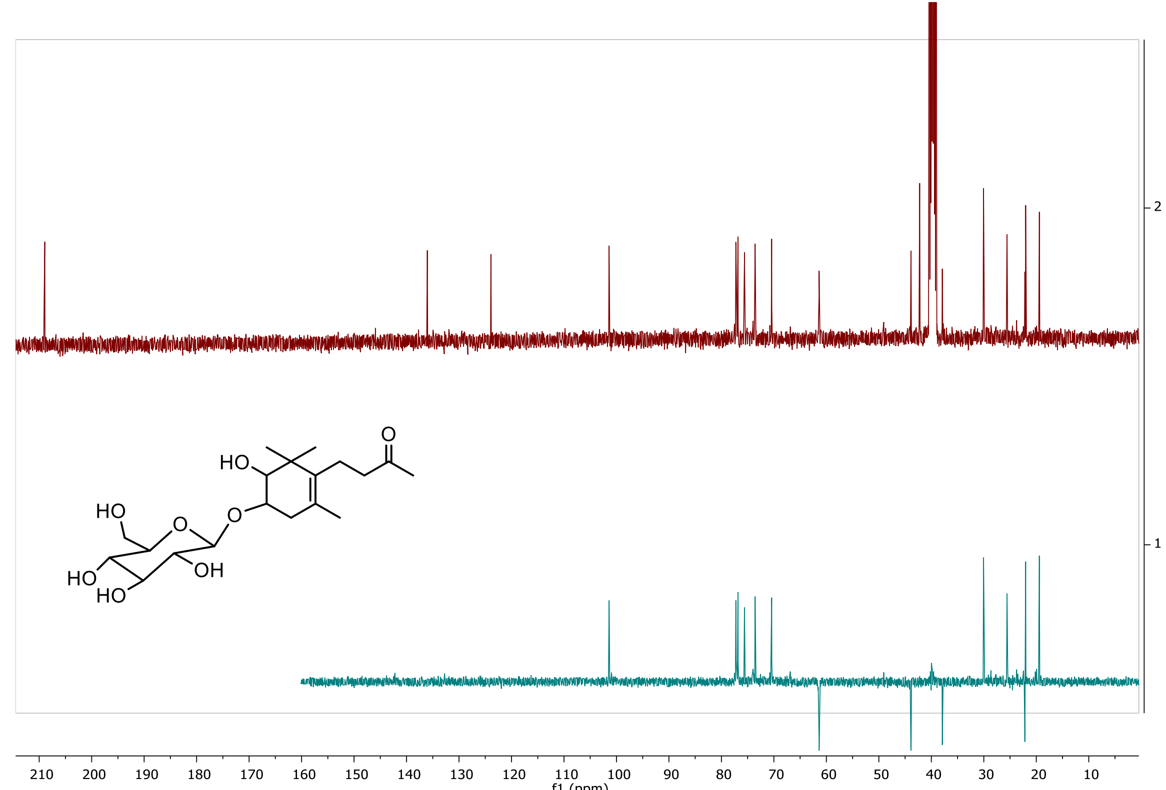


Figure 4. 27: ^{13}C and DEPT-135 NMR spectra of compound **8**

The coupling of H-3 (δ_H 3.70) with H-2 (δ_H 3.16) and H-4 (δ_H 2.00 and 2.31) was observed in the COSY ^1H-^1H. H-7 (δ_H 2.09 and 2.16) with H-8 (δ_H 2.45).

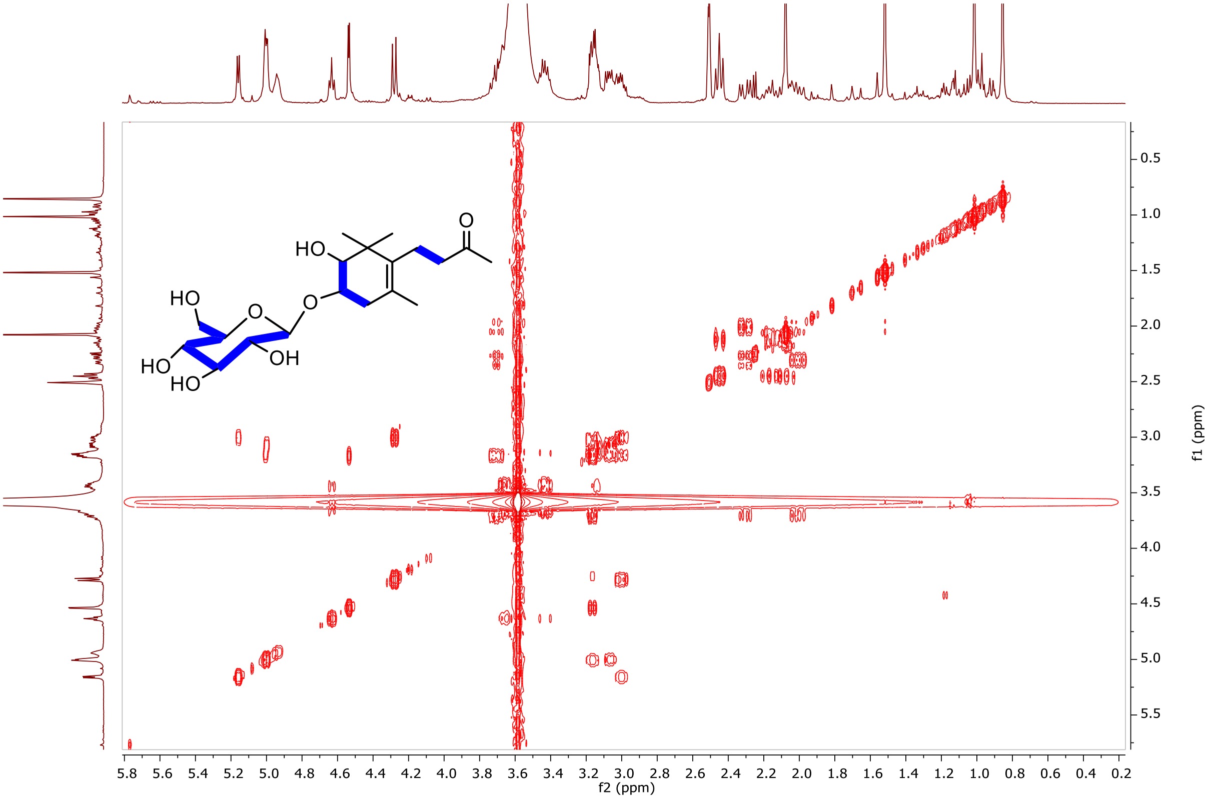


Figure 4. 28: ^{1}H-^{1}H COSY NMR spectra of compound **8**

The HSQC spectra allowed the assignment of all the protons attached to their corresponding carbons.

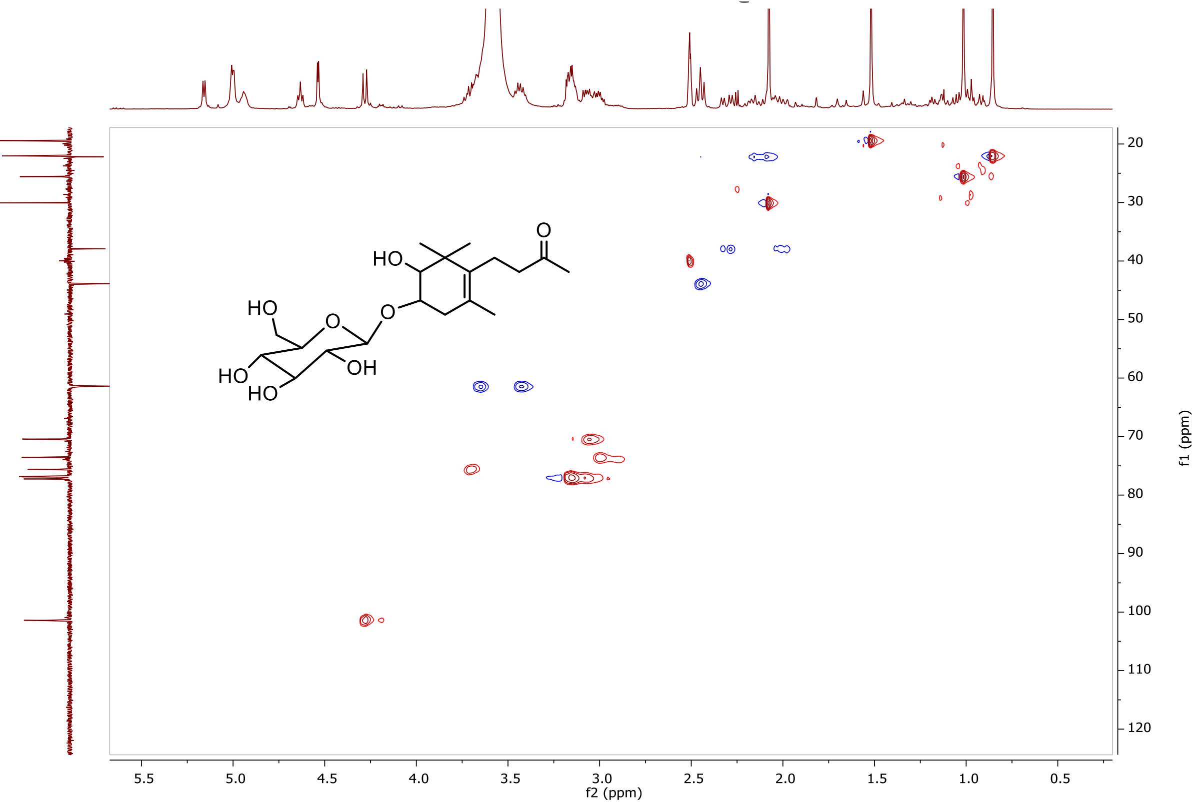


Figure 4. 29: HSQC NMR spectra of compound **8**

In the HMBC spectra, the observed correlation of the anomeric proton H-1' from the sugar unit with (δ 75.7) which was attributed to C-3 of the aglycone unit, confirming the position of the sugar moiety attachment.

Furthermore, HMBC experiment indicated a correlation of the oxymethine proton at H-2 with C-11 (25.6), C-12 (22.0) and C-3 (75.7). Also, a correlation between H-13 and C-4 (37.9), C-5 (123.9) and C-6 (136.1) confirmed the position of the methylene at position 4. (**Figure: 4.30**)

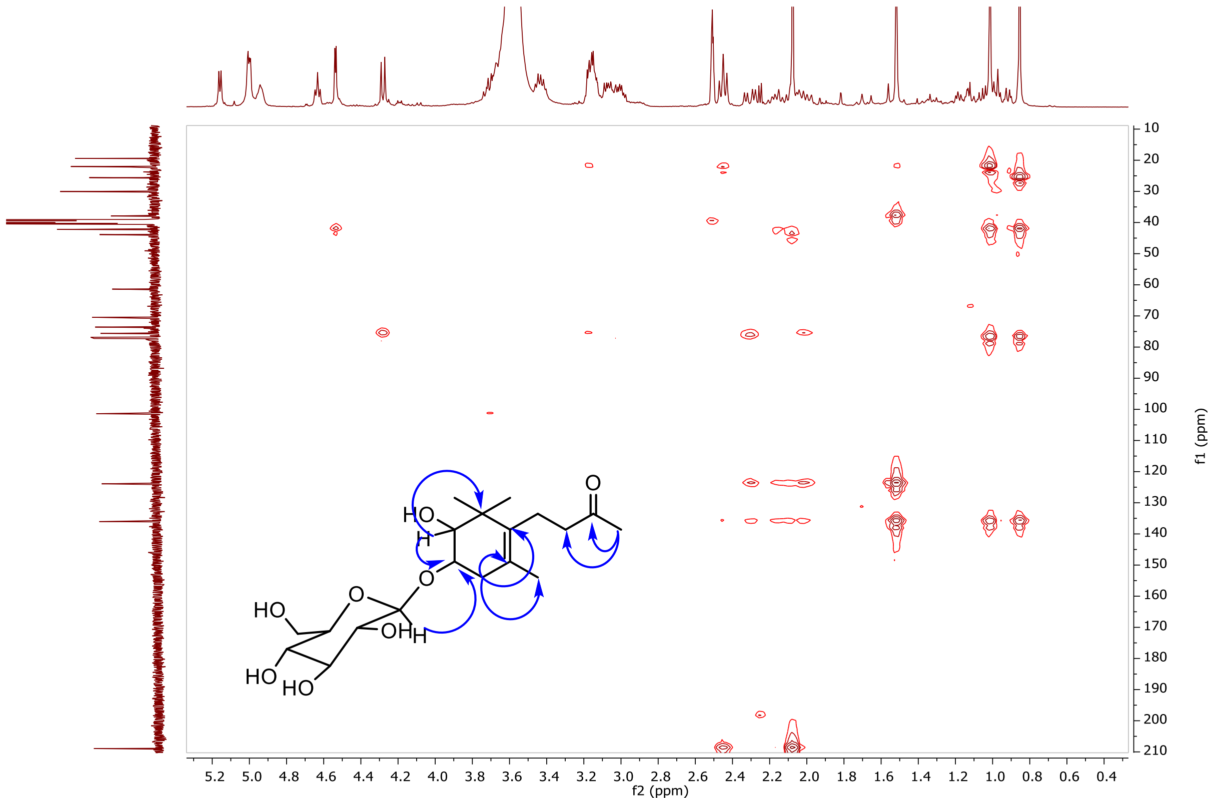


Figure 4. 30: HMBC NMR spectra of compound **8**

According to the above information, compound **8** was determined as **2-hydroxy-7,8-dihydro-ionone-3-*O*-β-D-glucopyranoside**. A database search provided no evidence for compound **8** as having been previously reported; therefore, it was a new compound. According to our findings, compound **8** is an isomer of a compound called 3,4-dihydroxy-7,8-dihydro-ionone-3-*O*-β-D-glucopyranoside (icariside B_8). This compound was first isolated from *Epimedium diphyllum* (Miyase et *al*., 1989).

Table 4. 8: **^{1}H and ^{13}C NMR spectral data of compounds 8 in DMSO-*d_6***

Position	^{1}H (*J* in Hz)	^{13}C	DEPT-135	COSY	HMBC
1	–	42.2	C	–	–
2	3.16 (*m*)	76.9	CH	H-3	C-1, C-3, C-11, C-12
3	3.70 (*d*, 6.7)	75.7	CH	H-2, H-4	C-2, C-1’
4a	1.99 (*dd*, 9.5, 7.7)	37.9	CH_2	H-3	C-2, C-3, C-5, C-6
4b	2.31 (*dd*, 10.9., 6.8)			H-3	C-2, C-3, C-5, C-6, C-13
5	–	123.9	–	–	–
6	–	136.1	–	–	–
7a	2.09 (*m*)	22.2	CH_2	H-8	C-1, C-5, C-6, C-8
7b	2.16 (*m*)			H-8	C-1, C-5, C-6, C-8
8	2.45 (*t*, 8.1)	43.9	CH_2	H-9	C-6, C-7, C-9
9	–	208.9	C	–	–
10	2.08 (*s*)	30.1	CH_3	–	C-8, C-9
11	1.01 (*s*)	25.6	CH_3	–	C-1, C-2, C-6, C-12
12	0.86 (*s*)	22.0	CH_3	–	C-1, C-2, C-6, C-11
13	1.52 (*s*)	19.5	CH_3	–	C-4, C-5, C-6
1’	4.28 (*d*, 7.8)	101.4	CH	H-2	C-3, C-2’
2’	3.00 (*m*)	73.6	CH	H-1, H-3	C-1’, C-3’
3’	3.15 (*m*)	77.3	CH	H-4	C-2’, C-4’, C-5’
4’	3.05 (*m*)	70.5	CH	H-3, H-5	C-2’, C-5, C-6’
5’	3.16 (*m*)	76.8	CH	H-6	C-6’, C-4’
6a’	3.44 (*m*)	61.5	CH_2	H-5	–
6b’	3.65 (*m*)			H-5	–

Figure 4. 31: Chemical structures of the isolated compounds from *P. cynaroides*

4.3 Physico-chemical data of isolated compounds from *P. cynaroides*

4.3.1: 4-hydroxybenzoic acid (1)

Molecular formula $C_7H_6O_3$ MW 138.0800 g/mol

Description: White amorphous powder

Mass spectrum: ESI- MS m/z (ES+) 139.2158 $[M+H]^+$

NMR spectrum: ^1H and ^{13}C see Table **4.1**

4.3.2: 3,4-bis(4-hydroxybenzoyl)-1,5-Anhydro-D-glucitol (2) New

Molecular formula $C_{20}H_{20}O_9$ MW 404.6812 g/mol

Description: Light brown powder

Mass spectrum: ESI- MS *m/z* (ES+) 405.1192 $[M+H]^+$

NMR spectrum: ^1H and ^{13}C see Table **4.2**

4.3.3: 1,5-Anhydro-D-glucitol (3)

Molecular formula $C_6H_{12}O_5$ MW 164.1888 g/mol

Description: White crystals

Mass spectrum: ESI- MS *m/z* (ES+) 165.4571 $[M+H]^+$

NMR spectrum: ^1H and ^{13}C see Table **4.3**

4.3.4: 3,4-dihydroxybenzoic acid (4)

Molecular formula $C_7H_6O_4$ MW 154.0227 g/mol

Description: Reddish brown amorphous powder

Mass spectrum: ESI- MS *m/z* (ES+) 155.5756 $[M+H]^+$

NMR spectrum: ^1H and ^{13}C see Table **4.4**

4.3.5: 4-hydroxybenzoyl-1,5-Anhydro-D-glucitol (5) New

Molecular formula $C_{13}H_{16}O_7$ MW 284.1861 g/mol

Description: White clear amorphous powder

Mass spectrum: ESI- MS *m/z* (ES+) 285.0978 $[M-H]^{+-}$

NMR spectrum: ^{1}H and ^{13}C see Table **4.5**

4.3.6: 3-hydroxykojic acid (6)

Molecular formula $C_6H_6O_5$ MW 158.0287 g/mol

Description: Light brown rough solid

Mass spectrum: ESI- MS *m/z* (ES+) 159.8521 $[M+H]^{+}$

NMR spectrum: ^{1}H and ^{13}C see Table **4.6**

4.3.7: 2-(hydroxymethyl)-4-*oxo*-4*H*-pyran-3yl-6-*O*-benzoate-β-D-glucopyranoside (7) New

Molecular formula $C_{19}H_{20}O_{11}$ MW 424.3281 g/mol

Description: Light brown solid

Mass spectrum: ESI- MS *m/z* (ES+) 425.1094 $[M+H]^{+}$

NMR spectrum: ^{1}H and ^{13}C see Table **4.7**

4.3.8: 3-Hydroxy-7,8-Dihydro-β-Ionone 3-*O*-β-D-glucopyranoside (8) New

Molecular formula $C_{19}H_{32}O_8$ MW 388.2561 g/mol

Description: White needles

Mass spectrum: ESI- MS *m/z* (ES+) 389.2177 $[M+H]^{+}$

NMR spectrum: ^{1}H and ^{13}C see Table **4.8**

4.4 Biological evaluation

To validate the ethnopharmacological applications and to explore the therapeutic potential of the plant *P. cynaroides*, its extracts and the respective isolated compounds were evaluated for their activity to inhibit alpha-glucosidase, alpha-amylase, and tyrosinase enzyme.

4.4.1 Alpha-glucosidase and alpha amylase inhibition

The extracts (EtOAc, BuOH and aqueous) of *P. cynadoides* were screened for their alpha-glucosidase and alpha-amylase inhibitory activity against alpha-glucosidase and alpha-amylase enzyme. This preliminary screening was done at a working concentration of 0.2 mg/mL of the extracts. As shown in Figure **4.8.9**, EtOAc and BuOH extracts of the leaves of *P. cynaroides* demonstrated the highest percentage inhibition against alpha-glucosidase activity (43% and 58% respectively). The aqueous extracts yielded the lowest inhibitory activity (15%) against α-glucosidase. The extracts didn't show any inhibitory activity for alpha-amylase and they showed negative inhibitory activity and this could be that the inhibitor is not acting as an inhibitor, but is instead stimulating the enzyme.

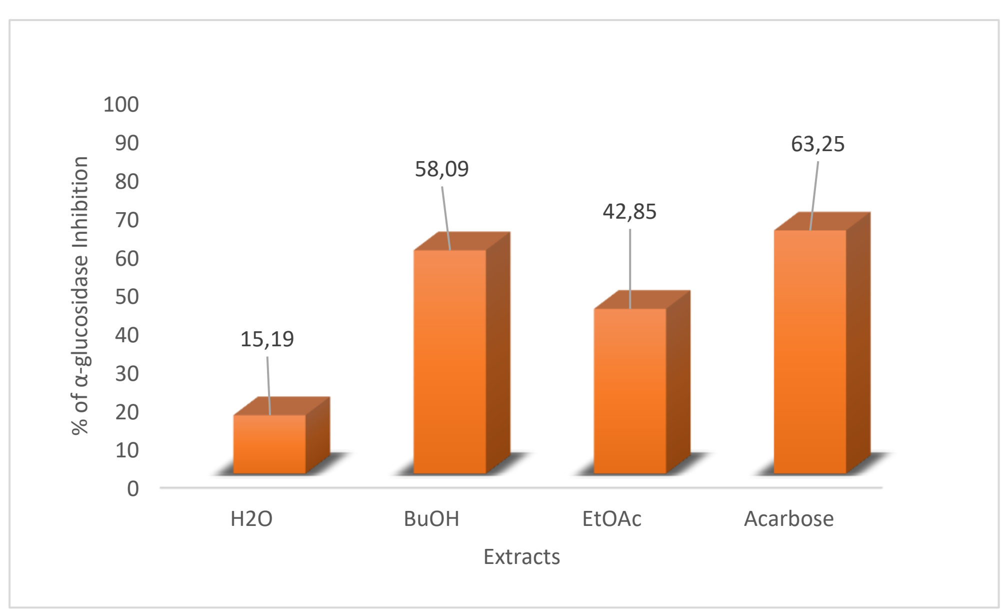


Figure 4. 32: Average % inhibition of alpha-glucosidase of the plant extracts

The IC_{50} values of isolated compounds from *P. cynaroides* (which showed strong alpha glucosidase inhibitory activities during screening) were investigated and the results showed that only compound **1** demonstrated weak alpha-glucosidase inhibitory activity with IC_{50} value of 162.56 ± 1.21 µg/mL as well as the IC_{50} of acarbose. The other tested compounds showed very low activity against alpha-glucosidase and no activity for alpha-amylase inhibitory activity as summarised in **Table 4.9**

Table 4. 9: **IC_{50} of isolated compounds and acarbose on alpha-glucosidase and alpha-amylase**

Tested Samples	**% Inhibition (0.2 mg mL^{-1})**	**alpha-glucosidase IC_{50} (µg/mL)**	**% Inhibition (0.2 mg mL^{-1})**	**alpha-amylase IC_{50} (µg/mL)**
1	60.7	162.56 ± 1.21	NA	NA
2	7.1	-	NA	NA
3	2.7	-	NA	NA
4	6.8	-	NA	NA
5	4.8	-	NA	NA
6	7.6	-	NA	NA
7	16.5	-	NA	NA
8	3.6	-	NA	NA
Acarbose	59.9	101 ± 1.05	97.5	7.65 ± 0.34

NA: not active at the tested concentrations. The results are expressed as mean ±SEM for n =3

4.4.2: Mushroom L-Tyrosinase assay

4.4.2.1: Anti-tyrosinase activity of various extracts from *P. cynaroides*

Inhibition of tyrosinase activity of EtOAc, BuOH and water extracts of *P. cynaroides* was measured using L-tyrosine. The BuOH extract exhibited 45% tyrosinase inhibitory activity at 0.2 mg/mL. It showed the highest anti-tyrosinase activity as compared to the EtOAc and water extracts which showed the lowest values (**Figure 4.33**).

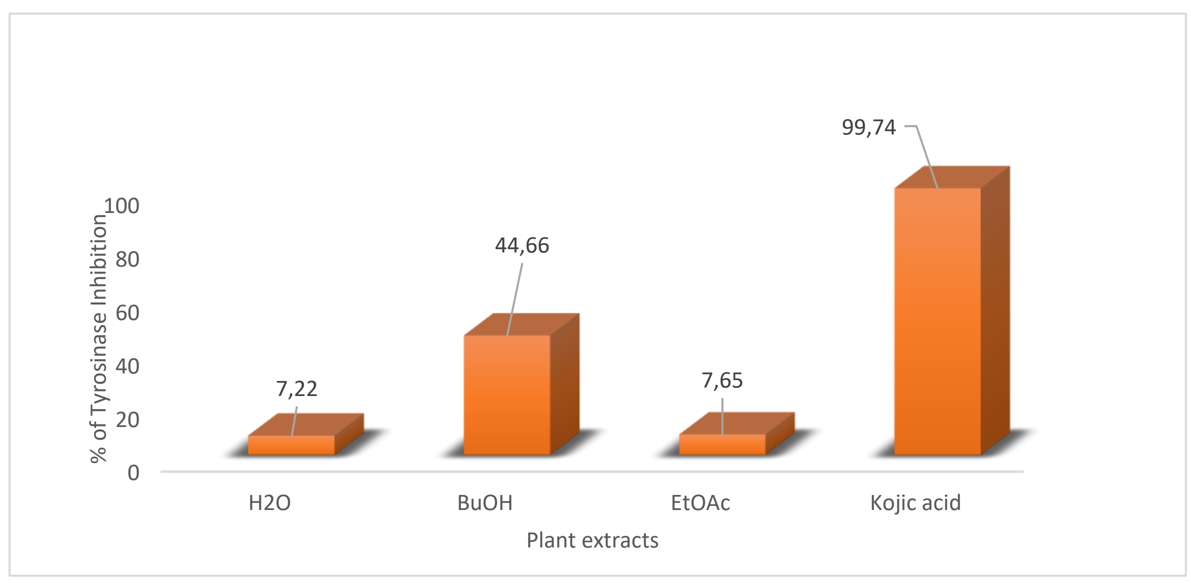


Figure 4. 33: Average % inhibition of tyrosinase by the extracts from *P. cynaroides*

4.4.2.2: Anti-tyrosinase activity of isolated compounds from *P. cynaroides*

The average tyrosinase inhibition percentages of the isolated compounds screened at 0.2 mg/mL revealed that compound **4** and **6** had an inhibition of 100%, while **1** showed a moderate inhibition of 55% and **3** showed weak inhibition of 25%. The rest of compounds were shown to be ineffective inhibitors of mushroom tyrosinase with their inhibition percentage less than 10%. The compounds with the high inhibition percentage were then investigated further for their IC_{50} values. Compounds **4** and **6** showed an IC_{50} values of 0,8776 ± 0.12 and 0.7771 ± 0.09 µg/mL respectively and the two compounds showed similar inhibition activity compared to of kojic acid (KA), a well-known tyrosinase inhibitor (**Figure 4.34**).

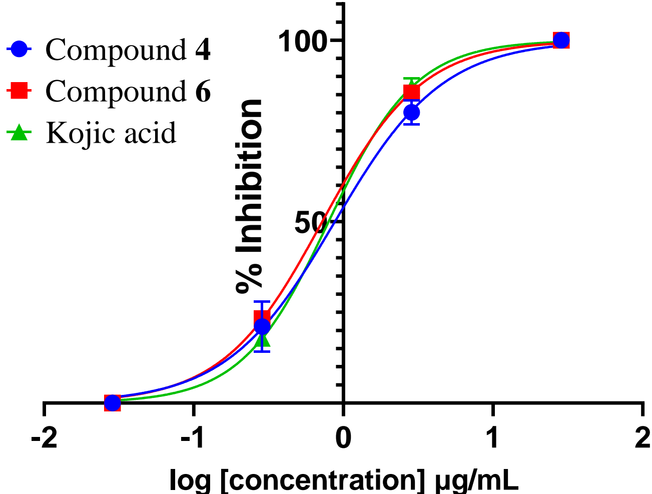


Figure 4. 34: IC_{50} plot of compound 4 and 6 from *P. cynaroides* against kojic acid

Table 4. 10: **% inhibition of tyrosinase and IC_{50} of compound** 4, 6 **and** Koic acid

	% inhibition (µg/mL)				
Tested Samples	**200**	**20**	**2**	**0.2**	**IC_{50}**
4	99.7	82.3	30.9	3.9	0,8776
6	99.9	86.2	27.7	5.8	0,7215
KOJIC ACID	99.8	88.9	31.5	8.2	0,7062

The other two compounds showed weak inhibition and their IC_{50} values were determined. Compound **1** showed an IC_{50} value of 149.2 ± 1.06 µg/mL and compound **3** exhibited an IC_{50} value of 274.5 ± 2.12 µg/mL among the tested isolated compounds.

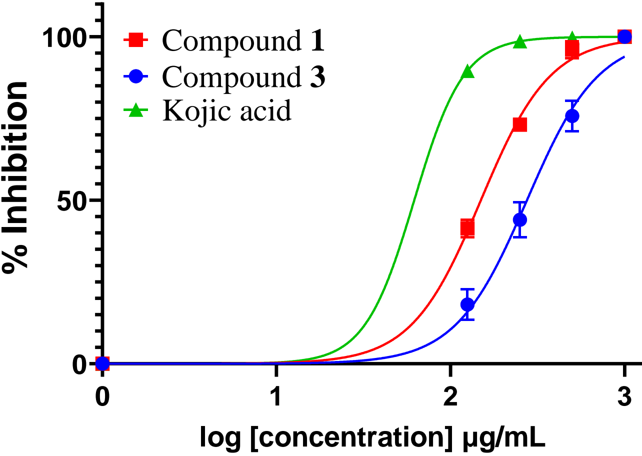


Figure 4. 35: IC_{50} plot of compound 1 and 3 from *P. cynaroides*

Table 4. 11: **IC_{50} values of isolated compounds of *P. cynaroides***

	% inhibition(µg/mL)				
Tested Samples	**1000**	**500**	**250**	**125**	**IC_{50}**
1	78.2	75.3	56.6	32.7	149.2
2	ND	ND	ND	ND	-
3	62.3	29.8	15.6	6.5	274.5
5	ND	ND	ND	ND	-
7	ND	ND	ND	ND	-
8	ND	ND	ND	ND	-
KOJIC ACID	99.8	98.9	98.5	98.21	3.8

ND: not determined at the tested concentrations. The results are expressed as mean ±SEM for n =3

4.5: Conclusion

Phytochemical and biological investigation of the EtOAc and BuOH soluble fractions of *Protea cynaroides* leaf extract using column chromatography isolation techniques afforded eight compounds, with four of them being (compounds **2, 5**, **7** and **8**). The compounds were identified as 4-hydroxybenzoic acid **(1)**, 3,4-*bis*(4-hydroxybenzoyl)-1,5-anhydro-D-glucitol **(2)**, 1,5-Anhydro-D-glucitol **(3)** ,3,4-dihydroxybenzoic acid **(4)**, 4-hydroxybenzoyl-1,5-anhydro-D-glucitol **(5)** ,3-hydroxykojic acid **(6)** , 2-(hydroxymethyl)-4-*oxo*-4*H*-pyran-3-yl-6-*O*-benzoate-β-D-glucopyranoside **(7)** and, 3-hydroxy-7,8-dihydro-β-ionone 3-*O*-β-D-glucopyranoside **(8)** as its bioactive constituents with significant anti-tyrosinase inhibitory activities as well as weak alpha glucosidase inhibitory activities. Five of the isolated compounds are also reported for the first time from the extracts of *Protea cynaroides* with four of them being novel compounds. Good anti-tyrosinase activity exhibited by three of these compounds warrants further investigation of the use of *P. cynaroide* given the activity that has now been shown by the plant. This study highlights the importance of *Protea cynaroides*, the national plant of South Africa, as a medicinal plant with therapeutic potentia

References

Bieleski, R.L. and Briggs, B.G., 2005. Taxonomic patterns in the distribution of polyols within the Proteaceae. *Australian Journal of Botany*, *53*(3), pp.205-217.

Boeyens, J.C.A., Marais, J.L.C. and Perold, G.W., 1983. The occurrence, conformation and crystal structure of 1, 5-anhydro-d-glucitol in *Protea* spp. *Phytochemistry*, *22*(9), pp.1959-1960.

Cho, J.Y., Moon, J.H., Seong, K.Y. and Park, K.H., 1998. Antimicrobial activity of 4-hydroxybenzoic acid and trans 4-hydroxycinnamic acid isolated and identified from rice hull. *Bioscience, biotechnology, and biochemistry*, *62*(11), pp.2273-2276.

Einhellig, F.A., Galindo, J.C.G., Molinillo, J.M.G. and Cutler, H.G., 2004. Mode of allelochemical action of phenolic compounds. *Allelopathy: Chemistry and mode of action of allelochemicals*, pp.217-238.

Hur, J.M., Park, J.G., Yang, K.H., Park, J.C., Park, J.R., Chun, S.S., Choi, J.S. and Choi, J.W., 2003. Effect of methanol extract of *Zanthoxylum piperitum* leaves and of its compound, protocatechuic acid, on hepatic drug metabolizing enzymes and lipid peroxidation in rats. *Bioscience, biotechnology, and biochemistry*, *67*(5), pp.945-950.

Kamori, A., Kato, A., Miyawaki, S., Koyama, J., Nash, R.J., Fleet, G.W., Miura, D., Ishikawa, F. and Adachi, I., 2016. Dual action of acertannins as potential regulators of intracellular ceramide levels. *Tetrahedron: Asymmetry*, *27*(22-23), pp.1177-1185.

Khadem, S. and Marles, R.J., 2010. Monocyclic phenolic acids; hydroxy-and polyhydroxybenzoic acids: occurrence and recent bioactivity studies. *Molecules*, *15*(11), pp.7985-8005.

Liu, W.R., Qiao, W.L., Liu, Z.Z., Wang, X.H., Jiang, R., Li, S.Y., Shi, R.B. and She, G.M., 2013. *Gaultheria*: Phytochemical and pharmacological characteristics. *Molecules*, *18*(10), pp.12071-12108.

Machida, S., Mukai, S., Kono, R., Funato, M., Saito, H. and Uchiyama, T., 2019. Synthesis and comparative structure–activity study of carbohydrate-based phenolic compounds as α-glucosidase inhibitors and antioxidants. *Molecules*, *24*(23), p.4340.

Miyase, T., Ueno, A., Takizawa, N., Kobayashi, H. and Oguchi, H., 1989. Ionone and lignan glycosides from *Epimedium diphyllum*. *Phytochemistry*, *28*(12), pp.3483-3485.

Plouvier, V., 1964. Sur la recherche des polyalcools et des heterosides cyanogenetiques chez quelques Proteacees. *Comptes Rendus Hebdomadaires Des Seances De Lacademie Des Sciences*, *259*(3), p.665.

Silva, V.C.D., Carvalho, M.G.D. and Silva, S.L.D.A.C., 2007. Chemical constituents from roots of *Andira anthelmia* (Leguminosae). *Rev Latinoamer de Quim*, *35*(1-2), pp.13-19.

Sroka, Z. and Cisowski, W., 2003. Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. *Food and Chemical Toxicology*, *41*(6), pp.753-758.

Terada, O., Suzuki, S. and Kinoshita, S., 1961. 3-Oxykojic Acid, a New γ-Pyrone formed by *Gluconobacter*. *Agricultural and Biological Chemistry*, *25*(10), pp.802-803.

Varì, R., D'Archivio, M., Filesi, C., Carotenuto, S., Scazzocchio, B., Santangelo, C., Giovannini, C. and Masella, R., 2011. Protocatechuic acid induces antioxidant/detoxifying enzyme expression through JNK-mediated Nrf2 activation in murine macrophages. *The Journal of Nutritional Biochemistry*, *22*(5), pp.409-417.

Wagner, H. and Bladt, S., 1996. *Plant drug analysis: a thin layer chromatography atlas*. Springer Science & Business Media.

Wan, C., Yuan, T., Li, L., Kandhi, V., Cech, N.B., Xie, M. and Seeram, N.P., 2012. Maplexins, new α-glucosidase inhibitors from red maple (*Acer rubrum*) stems. *Bioorganic & medicinal chemistry letters*, *22*(1), pp.597-600.

Wu, H.C., Du Toit, E.S., Reinhardt, C.F., Rimando, A.M., Van der Kooy, F. and Meyer, J.J.M., 2007. The phenolic, 3, 4-dihydroxybenzoic acid, is an endogenous regulator of rooting in *Protea cynaroides*. *Plant Growth Regulation*, *52*(3), pp.207-215.

Yao, G.M., Wang, Y.B., Wang, L.Q. and Qin, G.W., 2005. 3, 5-Dihydroxy-2-hydroxymethyl-4H-pyran-4-one. *Acta Crystallographica Section E: Structure Reports Online*, *61*(5), pp.o1403-o1405.

Yin, M.C., Lin, C.C., Wu, H.C., Tsao, S.M. and Hsu, C.K., 2009. Apoptotic effects of protocatechuic acid in human breast, lung, liver, cervix, and prostate cancer cells: potential mechanisms of action. *Journal of agricultural and food chemistry*, *57*(14), pp.6468-6473.

Yip, E.C.H., Chan, A.S.L., Pang, H., Tam, Y.K. and Wong, Y.H., 2006. Protocatechuic acid induces cell death in HepG2 hepatocellular carcinoma cells through a c-Jun N-terminal kinase-dependent mechanism. *Cell biology and toxicology*, *22*(4), pp.293-302.

CHAPTER 5

CHEMICAL CHARACTERIZATION AND BIOLOGICAL INVESTIGATION OF *CLIFFOTIA ODORATA*

The phytochemical investigation of *Cliffortia odorata* afforded ten compounds and identified as 6-methoxychroman-4-one (**9**), 2,4-dihydroxy-6-methoxyacetophenone (**10**), catechin (**11**), butyl-α-D-fructofuranoside (**12**), butyl-β-D-fructofuranoside (**13**), isolariciresinol (**14**), kaempferol (**15**), kaempferol 3,7-*O*-disulphate (**16**), sucrose (**17**), mixture of β-Dglucopyranose, α-D-glucopyranose, β-D-fructopyranose and β-D-fructofuranose **(18)** as its bioactive constituents with significant alpha glucosidase inhibitory activities as well as weak antityrosinase inhibitory activities. To the best of our knowledge, all of the isolated compounds are reported for the first time from the extracts of *C. odorata*. Compound **15** and **16** demonstrated moderate to remarkable alpha glucosidase inhibitory activities with the IC_{50} values of 16.6 ± 1.02 µg/mL and 3.8 ± 0.32 µg/mL respectively and on the other hand compounds **11** and **12** demonstrated weak antityrosinase inhibitory activities with the IC_{50} values of 131.9 ±1.05 µg/mL and 231.8 ±1.26 µg/mL and this could mean there is a need to validate the use of *C. odorata* in cosmetic and pharmaceutical industries upon further biological and clinical investigation.

5.1 *Cliffortia odorata*

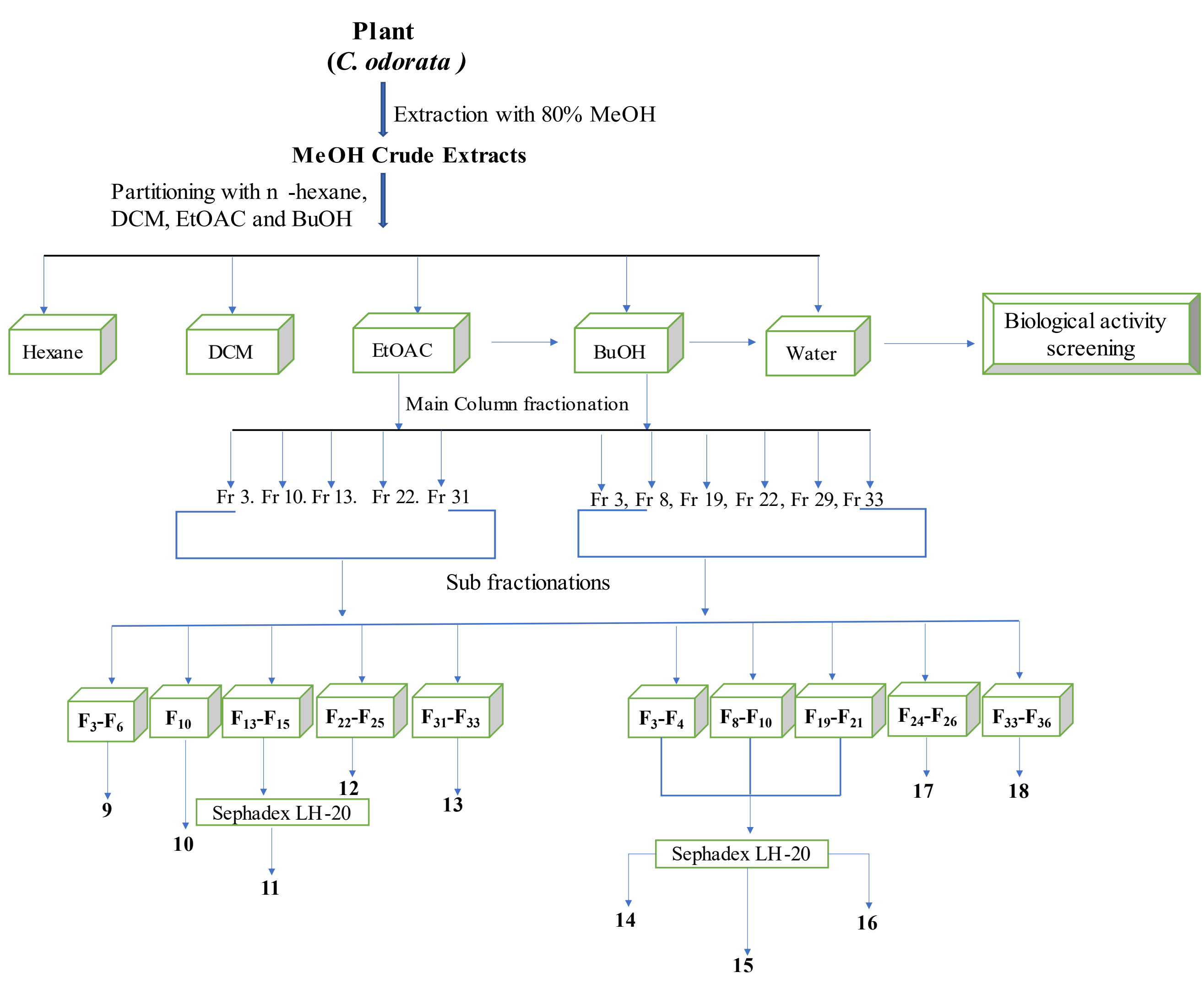
The dried powdered leave material (298 g) was macerated in 80% methanol and sequentially extracted with hexane, DCM, ethyl acetate and finally butanol. The hexane (1.05 g), DCM (852 mg), ethyl acetate (2.47 g), butanol (8.12 g) and water (68.21 g) extracts were phytochemically studied for their bioactive constituents

5.1.1 Isolation and identification of the chemical constituents of the ethyl acetate fraction

Column chromatography of the ethyl acetate extract (2.47 g) afforded fifty-one main fractions (Table 6). **F_3-F_6**, **F_{10}, F_{13}-F_{15}**, **F_{22}-F_{25}** and **F_{31}-F_{33}** were combined based on similar profile on TLC and rechromatographed. This afforded compounds **9** (10.05 mg), **10** (20.1 mg), **11** (30.5 mg), **12** (6.5 mg) and **13** (8.0 mg)

5.1.2 Isolation and identification of the chemical constituents of the butanol fraction

On running the column of the butanoic extract (8.12 g), forty-two fractions were obtained. Fractions **F_3-F_4**, **F_8-F_{10}**, **F_{19}, F_{24}-F_{26},** and **F_{33}-F_{36}** were rechromatographed. This afforded compound **14** (20.5 mg), **15** (6,8 mg), **16** (30.8 mg), **17** (80.6 mg), and **18** (120.5 mg) as shown in section **scheme 5.1**.



Scheme 5. 1: **A scheme of experimental procedure for the isolation of compounds from *C. odorata***

5:2 Structural elucidation and characterization of compounds

5.2.1: Structural elucidation of compound 9

Figure 5. 1: Chemical structure of compound **9**

Compound **9** was obtained as a white powder (3.6 mg). The negative ESI (-)-HRMS revealed a molecular ion $[M-H]^-$ peak at m/z 178.0800, corresponding to the molecular formula $C_{10}H_{10}O_3$. Compound **9** was identified as a chroman-4-one derivative on the basis of 1D and 2D NMR experiments (see Table **5.1**, Appendix **1B**).

The ^1H NMR spectrum revealed the presence of aromatic protons, a doublet of doublets at δ_H 7.54 (1H, *dd*, *J* = 8.2, 1.8, Hz, H-7) and it is overlapping with a singlet at δ_H 7.54 (1H, *s*, H-5) and another doublet resonating at δ_H 6.95 (1H, *d*, *J* = 8.2 Hz) representing 1,2,4-trisubstituted benzene ring. A sharp singlet δ_H 3.96 (3H, *s*, OCH_3) integrating for three protons indicate the aromatic methoxyl group being present and two triplets resonating at δ_H 4.00 (2H, *t*, *J* = 5.3 Hz, H-2) and δ_H 3.18 (2H, *t*, *J* = 5.3 Hz, H-3) were attributed to two sets of methylene protons, one being adjacent to the carbonyl group (H-3) and the other one being connected to an oxygen (H-2).

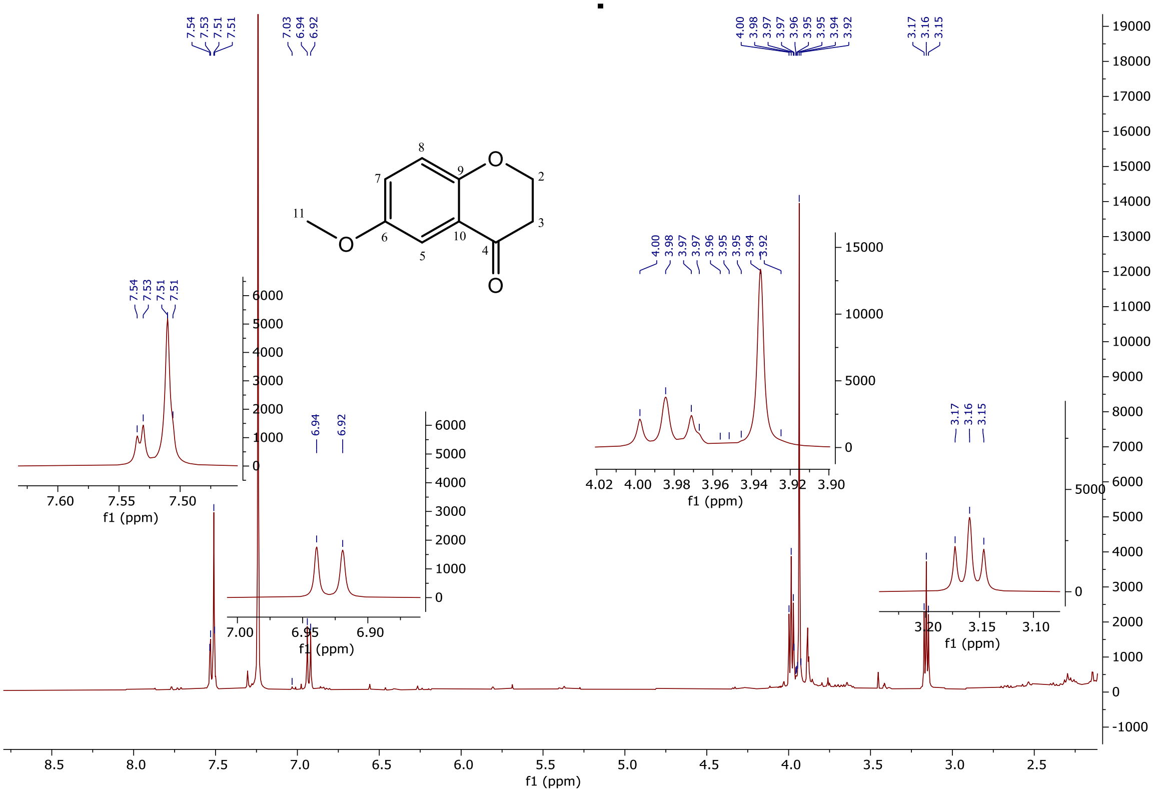


Figure 5. 2: ^{1}H NMR spectrum of compound **9** in $CDCl_3$

Analyses of the ^{13}C NMR and DEPT 135 data revealed the presence of ten carbons, seven of them were aromatic carbons at δ_C 199.1, 150.8, 146.8, 129.6, 123.7, 113.9 and 109.6, two methylenes at δ_C 56.1 and 39.7 and one oxygenated methyl group at δ_C 56.1. These results suggested that compound **9** has a monosubstituted chromanone structure. The locations of the substituent was established primarily on the basis of HMBC. The HMBC correlation of methoxy protons to C-7 established the position of the aryl methoxy group. Correlations of the protons of the -OCH_2CH_2-unit with the carbonyl at δ_C 199.1 and two adjacent aryl carbons (C-9 and C-10) as shown in Figure **5.1** required formation of a pyranone ring fused to the benzenoid moiety and this led to the complete assignment of the structure.

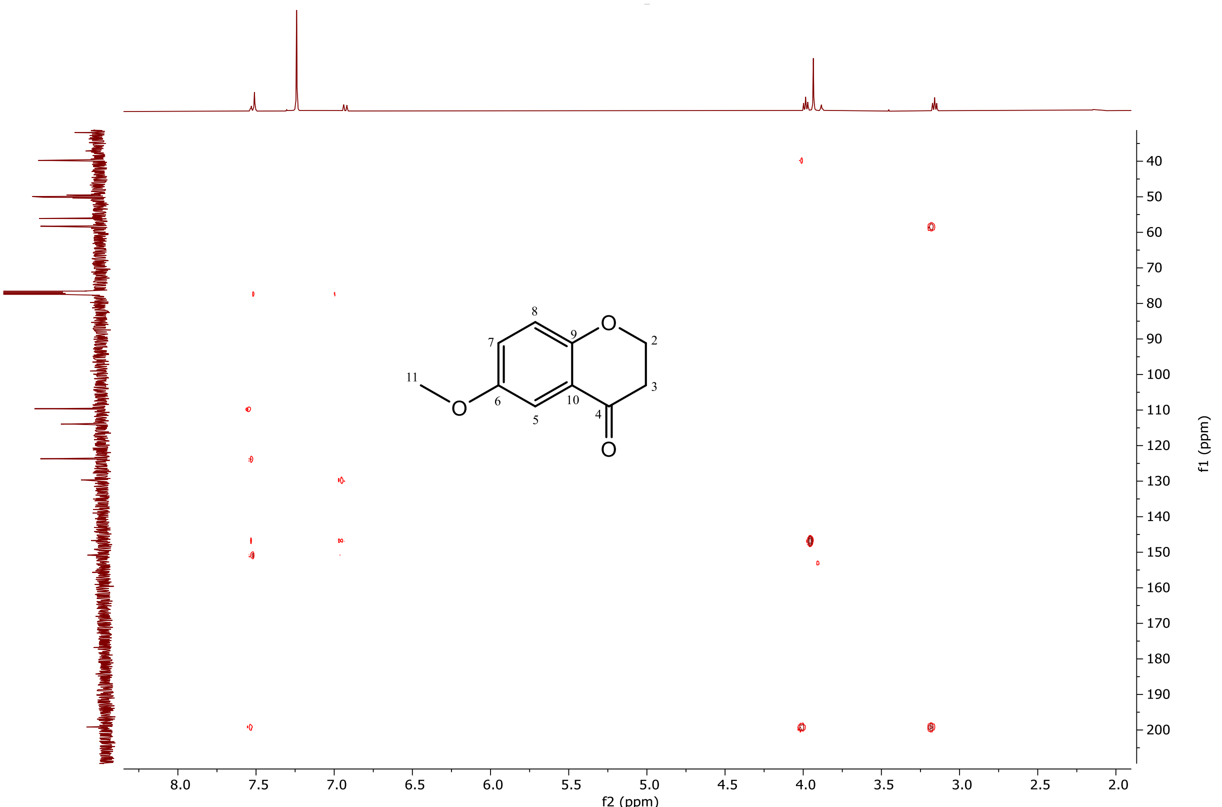


Figure 5. 3: HMBC spectra of compound **9**

Based on this data the structure of compound **9** was identified as 6-methoxychroman-4-one. Chromanone derivatives have been isolated from a variety of fungi and plants (Lee, *et. Al.*, 2007). As far as the literature is concerned, there hasn't been any report on the isolation of compound **9** from natural products, although it has been synthesized.

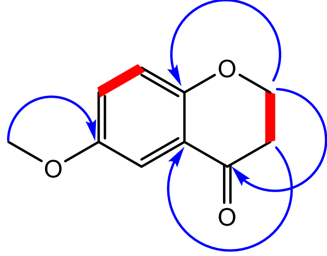


Figure 5. 4 : HMBC and COSY correlations of compound **9**

Table 5. 1: **^{1}H and ^{13}C NMR spectral data of compounds** 9 **in $CDCl_3$**

Position	δ_H (*J* in Hz)	δ_C	HMBC
2	4.00 (*t*, 5.3)	58.3	C-3, C-4, C-9
3	3.18 (*t*, 5.3)	39.8	C-2, C-4, C-10
4	-	199.2	-
5	7.54 (*br s*)	109.6	C-4, C-6, C-7, C-9, C-10
6	-	146.8	-
7	7.54 (*dd*, 8.2, 1.8)	123.7	C-5, C-6, C-8, C-9
8	6.95 (*d*, 8.2)	113.9	C-6, C-7, C-9, C-10
9	-	150.8	-
10	-	129.6	-
OCH_3	3.95 (*s*)	56.1	C-6

5.2.2: Structural elucidation of compound 10

Figure 5. 5: Chemical structure of compound **10**

Compound **10** was obtained as a white crystalline solid (20.1 mg). The negative ESIMS revealed a molecular ion $[M-H]^-$ peak at *m/z* 182.6800, corresponding to the molecular formula $C_9H_{10}O_4$. Compound **10** was identified as an acetophenone derivative on the basis of 1D and 2D NMR experiments (see Table **5.2**, Appendix **1B**).

The ^{1}H NMR spectrum of compound **10** indicated the presence of four protons. The spectrum showed two aromatic proton signals at δ 5.92 (1H, *d*, *J* = 2.1 Hz, H-5) and 5.88 (1H, *d*, *J* = 2.1 Hz, H-3) in the *meta* position and this suggested that the compound is 1, 2, 4, 6-tetrasubstituted benzene. The spectrum also showed two 3H signals at δ 3.85 (*s*) and δ 2.55 (*s*) assigned to the methoxy and acetyl methyl protons respectively. The ^{1}H-NMR chemical shift assignments are presented in the Table **5.2**

The ^{13}C-NMR and DEPT-135 spectra of the compound **10** showed the presence of nine carbon atoms in the molecule. Four substituted aromatic carbons at δ_C 166.0 (C-6), 164.5 (C-4), 163.1 (C-2) and 104.2 (C-1). Two protonated aromatic carbons at δ_C 89.9 (C-3) and 94.8 (C-5), one carbonyl carbon at δ_C 202.2 (C-7), one methoxyl carbon at δ_C 54.1 and one methyl carbon at δ_C 31.1 (C-8). On the basis of above spectral data the compound **10** was identified as **2,4-dihydroxy-6-methoxyacetophenone**. This compound has been reported to occur in the *Artemisia annua* (Singh et al., 1997) and *Inula viscosa* (Grande et al., 1985)

Table 5. 2: **^{1}H and ^{13}C NMR spectral data of compounds** 10 **in CD_3OD**

Position	**δ ^{1}H (*J* in Hz)**	**δ ^{13}C**
1	-	104.2
2	-	163.1
3	5.93 (*d*, 2.1)	89.9
4	-	164.5
5	5.89 (*d*, 2.1)	94.8
6	-	166.0
7	-	202.2
8	2.55 (*br s*)	31.1
OCH_3	3.85 (*br s*)	54.1

5.2.3: Structural elucidation of compound 11

Figure 5. 6: Chemical structure of compound **11**

Compound **11** was isolated as a yellow powder (40 mg). The negative ESIMS revealed a molecular ion $[M-H]^-$ peak at m/z 289.0800, corresponding to the molecular formula $C_{15}H_{14}O_6$. Compound **11** was identified as a flavanol on the basis of 1D and 2D NMR experiments (see Table **5.3**, Appendix **1B**).

^{1}H NMR spectrum has shown 1,3,4-trisubstituted aromatic ring with protons appears at δ_H 6.72 (1H, *d*, J = 1.8 Hz), 6.68 (1H, *d*, J = 8.0 Hz) and 6.59 (1H, *dd*, J = 8.0, 1.8 Hz). These protons were related to H-2', H-5', and H-6' respectively. Signals of two *meta* coupled protons were shown resonating at δ_H 5.88 (1H, *d*, J = 2.1 Hz) and 5.68 (1H, *d*, J = 2.1 Hz) and were related to H-6 and H-8. Two methine protons were identified at δ_H 4.47 (1H, *d*, J = 2.1 Hz) and 3.81 (1H, *m*) for H-2 and H-3 respectively. Finally, a methylene group was also identified at δ_H 2.65 (1H, *dd*, J = 16.0, 5.2 Hz, H-4eq) and 2.35 (1H, *dd*, J = 16.0, 8.1 Hz, H-4ax). A strong J coupling constant between δ_H 4.47 (H-2α) and 3.81 (H-3α) was observed, supporting a *trans*-configuration between these two protons (Foo, 1987).

The ^{13}C and DEPT-135 NMR experiment detected 15 carbon signals including twelve aromatic carbons, one oxygenated aliphatic carbon, one aliphatic methine and one aliphatic methylene carbon. The aromatic methine signals were observed at δ_C 118.4, 115.1, 114.5, 95.1 and 93.8 ppm for C-6', C-5', C-2', C-6 and C-8 respectively and the remaining seven

signals were the aromatic substituted carbons resonating at δ_C 156.5 (C-7), 156.2 (C-5), 155.4 (C-9), 144.8 (C-3'/4'), 130.6 (C-1') and 99.1 (C-10). The oxymethine signal was seen at 81.0 (C-3) and the aliphatic methine was observed at δ_C 66.3 (C-2). The methylene signal resonated at δ_C 27.9 and was allocated to C-4. The above data showed that compound **11** consists of a flavan-3-ol backbone. On the basis of a thorough literature review; it was identified as catechin, a common procyanidin monomer typically isolated from *Wedelia prostrata* and *Scurrula liquidambaricolus* (Shen *et al.*, 1993).

Table 5. 3: **^{1}H and ^{13}C NMR spectral data of compound 11 in DMSO-*d*$_6$**

Position	**^{1}H (*J* in Hz)**	**^{13}C**	**DEPT-135**	**Lit** (Shen *et al.*, 1993)
2	3.81 (*m*)	66.3	CH	66.4
3	4.47 (*d*, 2.1)	81.0	CH	81.0
4a	2.35 (*dd*, 16.0, 8.1)	27.9	CH_2	27.7
4b	2.65 (*dd*, 16.0, 5.2)			
5	–	156.2	C	156.1
6	5.88 (*d*, 2.1)	95.1	CH	95.3
7	–	156.5	C	156.4
8	5.68 (*d*, 2.1)	93.8	CH	94.0
9	–	155.4	C	155.3
10	–	99.1	C	99.2
1'	–	130.6	C	130.7
2'	6.72 (*d*, 1.8)	114.5	CH	114.9
3'	–	144.8	C	144.8
4'	–	144.8	C	144.8
5'	6.68 (*d*, 8.0)	115.1	CH	115.1
6'	6.59 (*dd*, 8.0, 1.8)	118.4	CH	118.4

5.2.4 Structural elucidation of compound 12

Figure 5. 7: Chemical structure of compound **12**

Compound **12** was obtained as amorphous powder (6 mg). The $[M+Na]^+$ at *m/z*: 259.6648 in the HR-ESI-MS spectrum corresponds to the molecular formula $C_{10}H_{20}O_6$ (calc. 236.2202), indicating one degrees of unsaturation. Compound **12** was identified on the basis of 1D and 2D NMR experiments (see Table **5.4**, Appendix **1B**).

The ^{1}H-NMR data showed a triplet at δ 0.93 (3H, *t*, *J* =7.3 Hz,) for terminal methyl protons attributed to H-4', three multiplets at δ 1.40 (2H, *m*), 1.55 (2H, *m*) and 3.53 (2H, *m*) for H-3', H-2' and H-1' respectively integrating for two protons each, consistent with three methylene groups which suggested the presence of an *n*-butoxyl moiety. In these spectra, it is still observed that the presence of signals in the region between δ 3.40–4.2 ppm referring to the presence of a sugar unit.

The ^{13}C and DEPT-135 NMR experiment indicated the ten carbons signals, one methyl, three methines, five methylenes and one quaternary. The presence of an anomeric carbon at δ_C 107.4 and, also the presence of two hydroxymethyls at δ_C 62.8, 61.9 ppm and three hydroxymethine groups at δ_C 84.2, 83.5 and 78.8 ppm suggested that the structure contained a sugar probably in the furanose form. The other four remaining signals resonating at δ_C 62.2, 33.7, 20.7 and 14.5 ppm indicated the presence of a butyl moiety as the aglycone part. Consequently, the structure of compound **12** was confirmed by comparison with the previously reported data (Zhang *et al.*, 1996). Compound **12** was identified as *n*-butyl-α-D-fructofuranoside. This

compound was first reported by Zhang et al (1996), he isolated it from *Cynomorium songaricum* grows in the northwest of China and is used in traditional Chinese medicine for the treatment of kidney disorders.

5.2.5: Structural elucidation of compound 13

Figure 5. 8: Chemical structure of compound **13**

Compound **13** was obtained as amorphous powder (8 mg). The $[M+Na]^+$ at *m/z*: 259.2593 in the HR-ESI-MS spectrum corresponds to the molecular formula $C_{10}H_{20}O_6$ (calc. 236.2202), indicating one degrees of unsaturation.

The ^{1}H-NMR data of compound **13** showed signals in the region between 0.9-3.5 ppm the same pattern of multiplicity of signals found in compound **12**, which together with the number of hydrogens estimated by the integral also confirmed the presence of a butyl group. In the spectrum, the presence of signals in the region between δ 3.40-4.2 was also observed, referring to the presence of a sugar unit.

^{13}C NMR and DEPT-135 spectra were in agreement with the suggested molecular formula, as it showed the presence of ten well resolved carbon signals. The signals at δ_C 62.3 and 62.0 belong to the C-6 and C-1 carbons of sugar, confirming fructofuranose by the presence of two methylenic groups. compound **13** also showed the presence of an anomeric carbon at δ_C 105.5. Based on the above information and comparison with the previously reported

literature, compound **13** was established as *n*-butyl-β-D-fructofuranoside (Dudíková *et al.*, 2007).

Table 5. 4: **^{1}H and ^{13}C NMR spectral data of compound** 12 **and** 13 **in CD_3OD**

	n-Butyl-α-D-fructofuranoside		n-Butyl-β-D-fructofuranoside	
Position	**^{13}C**	**^{1}H (*J* in Hz)**	**^{13}C**	**^{1}H (*J* in Hz)**
1a	61.9	3.71 (*d,* 11.7)	62.5	3.66 (*d,* 11.8)
1b		3.62 (*d*, 12.0)		3.52 (*d,* 11.7)
2	109.1	–	105.5	–
3	83.5	4.05 (*d*, 4.9)	78.7	4.11 (*d*, 8.1)
4	78.8	3.89 (*dd*,7.0, 4.9)	77.6	3.92 (*t*,7.8)
5	84.2	3.84 (*m*)	83.6	3.85 (*m*)
6	62.8	3.70 (*m*)	65.3	3.68 (*m*)
1’	62.2	3.53 (*m*)	62.2	3.50 (*m*)
2’	33.7	1.55 (*m*)	33.7	1.52 (*m*)
3’	20.7	1.40 (*m*)	20.7	1.39 (*m*)
4’	14.5	0.93 (*t*, 7.3)	14.6	0.95 (*t*, 7.3)

5.2.6: Structural elucidation of compound 14

Figure 5. 9: Chemical structure of compound **14**

Repeated column chromatography of the BuOH extract yielded compound **14** (15.0 mg) as a pale yellow amorphous solid. On TLC it showed an active spot under UV and a purple prominent spot after spraying with vanillin/H_2SO_4 reagnet. The molecular formula $C_{20}H_{24}O_6$ was established by the ion at *m/z* 397.1705 g/mol $[M+Na]^+$ in the ESI-MS spectrum and. Theoretical mass of this compound is 374.1158 g/mol. The structure of the compound was established on the basis of 1D (^1H, ^{13}C and DEPT 135) and 2D-NMR experiments (HSQC, COSY and HMBC), (Table **5.6**), FTIR and MS data.

The ^1H NMR data showed that compound **14** has an ABX coupling system assignable to one 1,3,4-trisubstituted benzene ring δ_H 6.79 (1H, *d*, *J* = 1.7 Hz, H-5’), 6.75 (1H, *d*, *J* = 8.1 Hz, H-2’) and 6.68 (1H, *br s*, H-2), two aromatic protons δ_H 6.62 (1H, *dd*, *J* = 8.1, 1.7 Hz, H-6’), and 6.22 (1H, *br s*, H-5) which suggested the presence of a 1,3,4,6-tetrasubstituted benzene ring in the structure; two methoxy groups at δ_H 3.83 and 3.82; two sets of oxygen connected methylenes each with two non-equivalent protons at δ_H 4.12 (1H, *dd*, *J* = 9.8, 2.9 Hz, H-9’a), 3.89 (1H, *dd*, *J* = 9.8, 2.9 Hz, H-9’b) and δ_H 3.79 (1H, *dd*, *J* = 10.1, 3.4 Hz, H-9a), 3.74 (1H, *d*, *J* = 11.7, 6.2 Hz, H-9b) respectively; one non-oxygenated methylene protons at δ_H 2.86 (2H, *brd*, *J* = 10.5 Hz, H-7) and three methine protons resonating at δ_H 3.95 (1H, *d*, *J*= 10.5 Hz, H-7’), 2.08 (1H, *m*, H-8’) and 1.93 (1H, *m*, H-8).

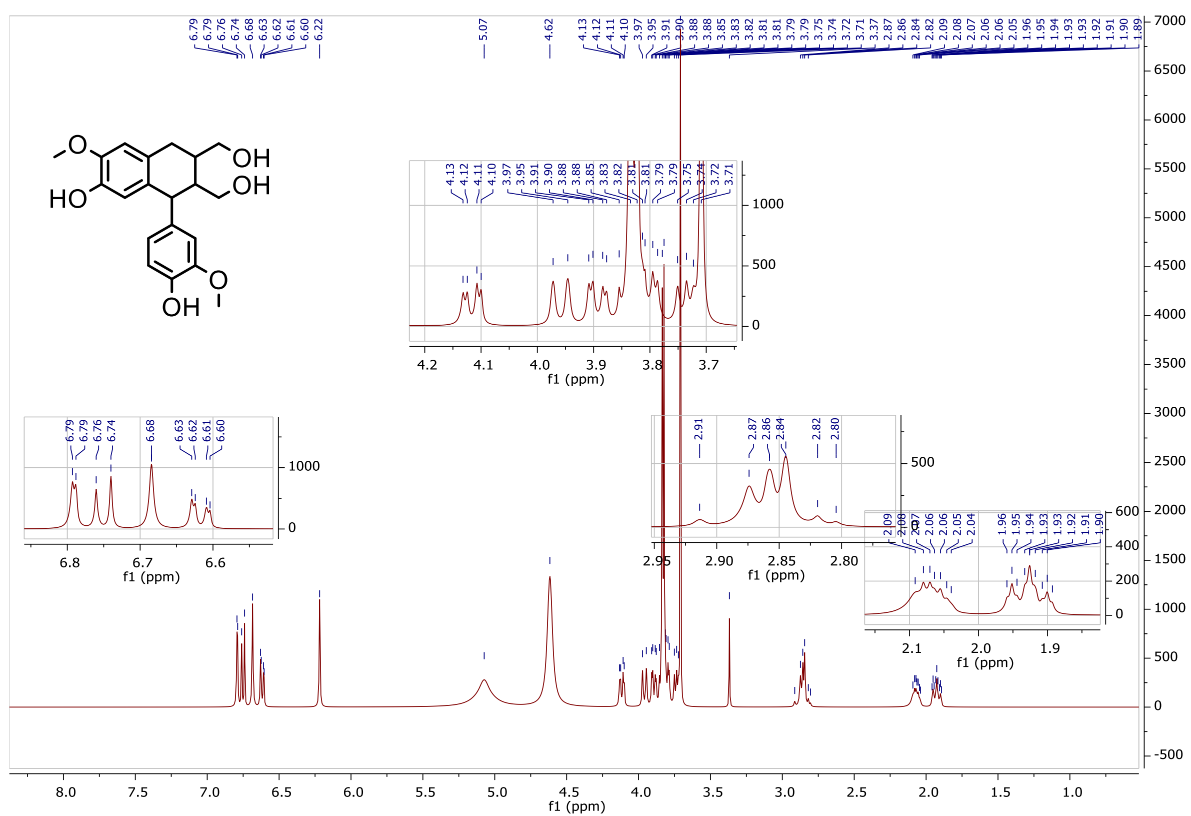


Figure 5. 10: ^{1}H NMR spectrum of compound **14** in CD_3OD

Analyses of the ^{13}C NMR and DEPT 135 data revealed the presence of twenty carbons, twelve of them were aromatic carbons (δ_C 147.4, 145.8, 144.4, 143.8, 137.1, 132.5, 127.8, 121.7, 115.9, 114.7, 112.9, 110.9); three methylenes (δ_C 66.1, 63.4 and 32.2); two oxygenated methyls overlapped at δ_C 54.9 and three methines at δ_C 46.5, 43.8 and 37.7.

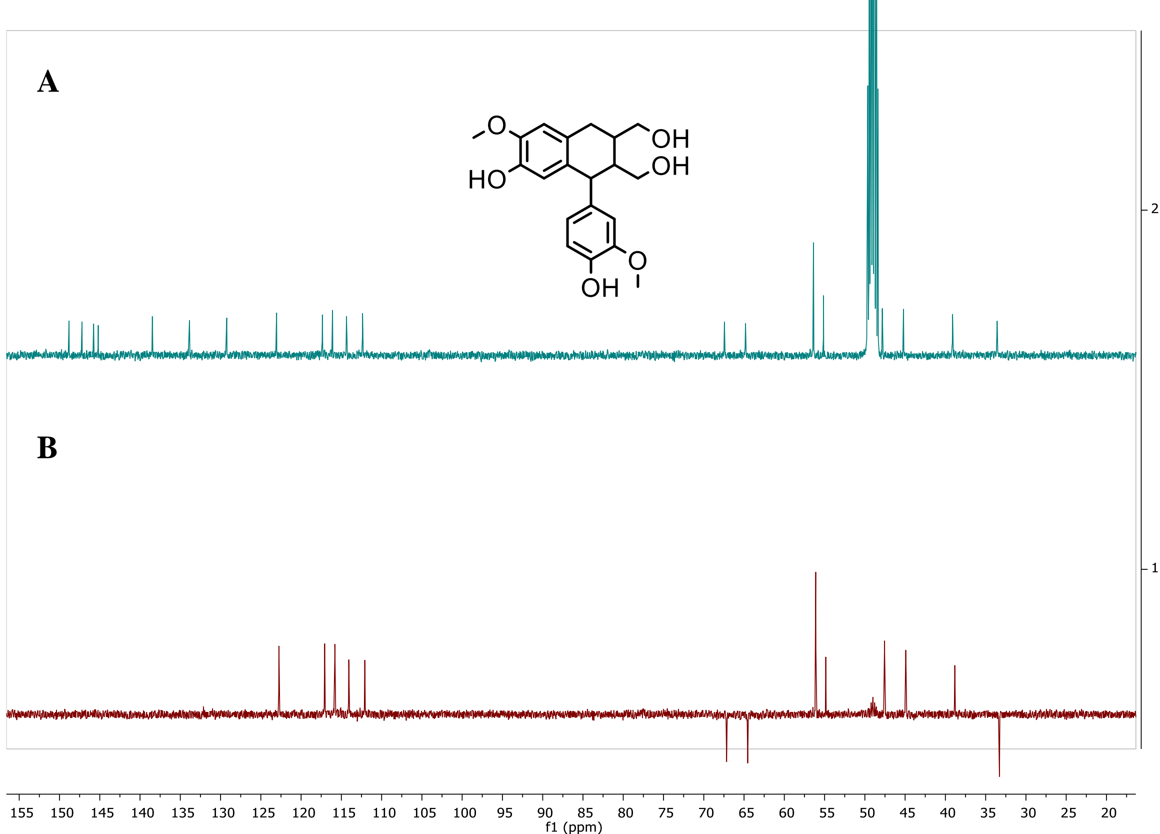


Figure 5. 11: Stacked ^{13}C and DEPT 135 spectrum of **14** in CD_3OD

The COSY ^1H-^1H cross peaks observed, were between H-7' (δ_H 3.95)/ H-8' (δ_H 1.93); H-8 (δ_H 2.08)/ H-7 (δ_H 2.85), H-8/H-9 (δ_H 3.79a and 3.74b), and H-8′/ H-9′ (δ_H 4.12a and 3.89b) (see Figure **5.11** and Table **5.6**).

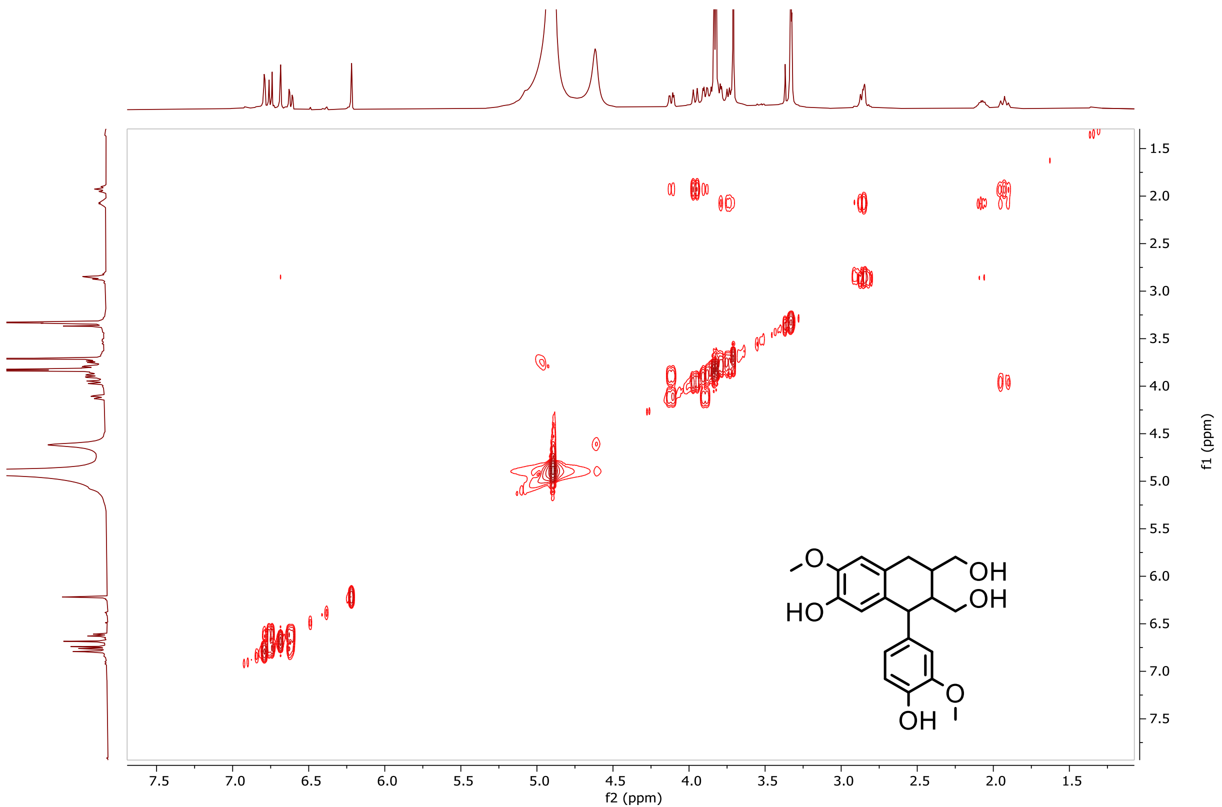


Figure 5. 12: COSY spectrum of compound **14** in CD_3OD

HSQC experiment was used to assign proton signals to the corresponding carbon signals; the five HSQC correlations are well resolved and are shown in Figure **5.13**.

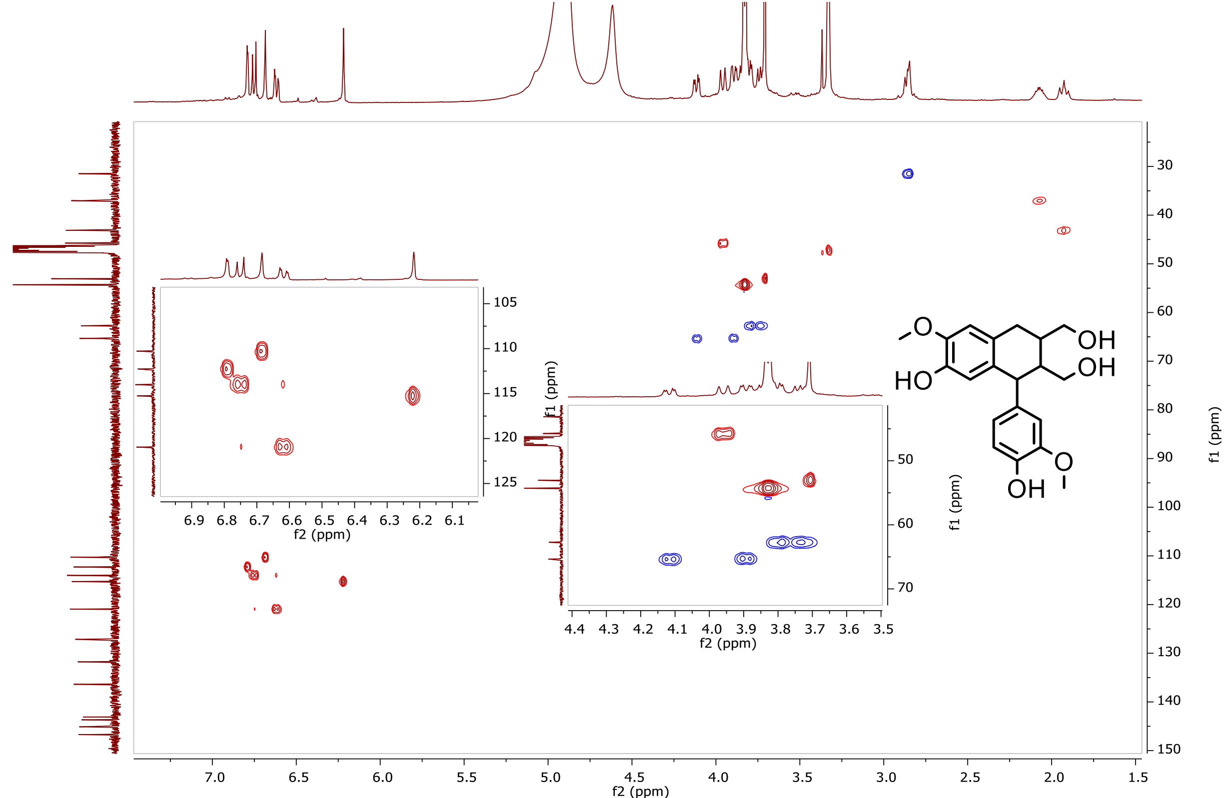


Figure 5. 13: HSQC spectrum of compound **14** in CD_3OD

The HMBC correlations (Table **5.5**) long range cross-peaks of methylene protons at H-7 with C-1, C-2, C-6, C-8, and C-8' revealed that C-7 was attached at a disubstituted benzene ring. HMBC cross-peaks of H-2 to C-1, C-3, C-4, C-6, and C-7 indicated that a one methoxyl group was attached at disubstituted aromatic ring. The correlations of H-7' to C-1, C-6, C-1', C-2' C-5' and C-6' deduced that H-7' attached to 1,3,4-trisubstituted benzene ring. The connectivity between C-8 and C-9 was supported by the HMBC correlation between H-8 / C-9 and H-9 / C-8

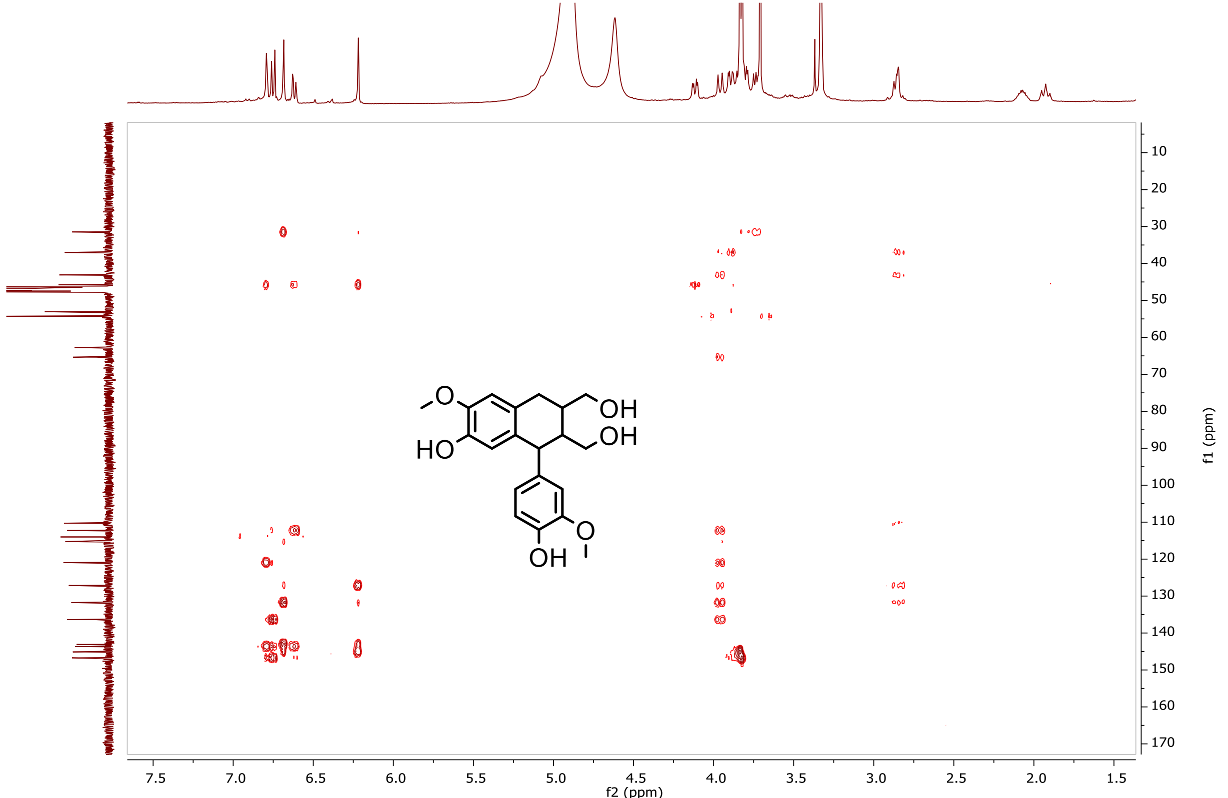


Figure 5. 14: HMBC spectrum of compound **14** in CD_3OD

Figure 5. 15: Key HMBC and COSY correlations of **14**

The above information and comparison with literature (Fonseca et al., 1978; Okuyama et al., 1987; Thomford et al., 2018) suggested compound **14** to be isolariciresinol and to the best of

our knowledge this compound is reported for the first time from *C. odorata*. Isolariciresinol is a lignan and according to MacRae and Towers, 1984, and Arroo et al., 2002, lignans are known to possess a wide range of biological activities, including anti-cancer, antibacterial, antifungal, antiviral, antioxidant and anti-inflammatory effects.

Table 5. 5: **^{1}H and ^{13}C NMR spectral data of compound** 14 **in CD_3OD**

Position	**^{1}H (*J* in Hz)**	**^{13}C**	**COSY (^{1}H-^{1}H)**	**HMBC**
1	–	127.8	–	–
2	6.68 (s)	110.9	–	C-3, C-4, C-6, C-7
3	–	145.8	–	–
4	–	143.8	–	–
5	6.22 (s)	115.9	–	C-1, C-3, C-4, C-7’
6	–	132.5	–	–
7	2.86 (*br d,* 10.5)	32.2	H-8	C-1, C-2, C-6, C-8, C-8’
7’	3.95 (*d*, 10.5)	46.5	H-8’	C-1, C-6, C-1’, C-2’ C-5’, C-6’
8	2.08 (*m*)	37.7	H-7, 8’, 9a, 9b	–
8’	1.93 (*m*)	43.8	H-7’, 8, 9’a, 9’b	C-1’, C-7’
9a	3.79 (*dd*, 3.4, 6.5)	63.4	H-8, 9b	C-7, C-8
9b	3.74 (*dd*, 3.4, 6.5)		H-8, 9a	C-7
9’a	4.12 (*dd*, 2.7, 9.8)	66.1	H-8’, 9’b	C-7’
9’b	3.89 (*dd*, 2.7, 9.8)		H-8’, 9a’	C-7’, C-8
1’	–	137.1	–	–
2’	6.79 (*d*, 8.1)	112.9	H-6’	C-3’, C-4’, C-6’, C-7'
3’	–	144.4	–	–
4’	–	147.4	–	–
5’	6.75 (*d*, 1.7)	114,7	H-6’	C-1’, C-2’, C-3’, C-4’
6’	6.62 (*dd*, 1.7, 8.1)	121.7	H-2’, 5’	C-2’, C-3’, C-4’, C-7’
OCH_3	3.82 (*s*)	54.9	–	147.4
OCH_3	3.83 (*s*)	54.9	–	145.8

5.2.7: Structural elucidation of compound 15

Figure 5. 16: Chemical structure of compound **15**

Compound **15** was obtained as a yellow powder (7 mg). The negative ESIMS revealed a molecular ion $[M-H]^-$ peak at m/z 272.0800, corresponding to the molecular formula $C_{15}H_{12}O_5$. The structure of the compound was established on the basis of 1D (^1H, ^{13}C and DEPT-135) (see Table **5.7**, Appendix **1B**).

The ^1H NMR spectrum analysis revealed four diagnostic signals in the aromatic region. The presence of two pairs of intense doublets at δ 7.24 (2H, *d*, *J* = 8.8 Hz, H-2'/H-6') and 6.73 (2H, *d*, *J* = 8.8 Hz, H-3'/H-5') suggested an existence of a 1,4-disubstituted benzene ring. Also, a set of two meta coupled aromatic protons at δ 6.29 (1H, *d*, *J* = 2.1 Hz, H-8) and δ 6.08 (1H, *d*, *J* = 2.1 Hz, H-6) confirming a tetra-substituted benzene ring.

The ^{13}C NMR and DEPT 135 spectra of compound **15** exhibited the presence of thirteen signals counted for fifteen carbons at δ_C 179.1 (C-4), 163.0 (C-5), 165.6 (C-7), 161.2 (C-4'), 158.5 (C-2), 158.3 (C-9), 132.1 (C-2'/6'), 123.0 (C-1'), 116.1 (C-3'), 105.9 (C-10), 99.7 (C-6), 94.6 (C-8), Based on the obtained results and comparison with literature data, the compound **15** was identified as kaempferol (Telange et al., 2016; Wang et al., 2010).

5.2.8: Structural elucidation of compound 16

Figure 5. 17: Chemical structure of compound **16**

Compound **15** was obtained as a brown solid (16 mg). The positive ESIMS revealed a molecular ion $[M+H]^+$ peak at m/z 446.9704, corresponding to the molecular formula $C_{15}H_{10}O_{12}S_2$ and another fragment at m/z 367.0129 corresponding to $[M-HO_3S]^+$, and indicated the presence of two sulphate groups. The structure of the compound was established on the basis of 1D (^{1}H, ^{13}C and DEPT-135), FTIR and MS data (see Table **5.7**, Appendix **1B**).

The ^{1}H NMR spectrum analysis showed only aromatic signals, indicating the absence of aliphatic substituents on the flavonoid nucleus. The spectrum exhibited the characteristic 5-OH proton signal at δ 12.55. *Ortho* coupled doublets were observed at δ 8.15 (2H, *d*, *J* = 8.9 Hz, H-2'/H-6') and 6.88 (2H, *d*, *J* = 8.9 Hz, H-3'/H-5') suggesting the existence of a 1,4-disubstituted benzene ring. Also, a set of two *meta* coupled aromatic protons at δ 6.97 (1H, *d*, *J* = 2.0 Hz, H-8) and δ 6.58 (1H, *d*, *J* = 2.0 Hz, H-6) confirming a 5, 7-disubstituted ring.

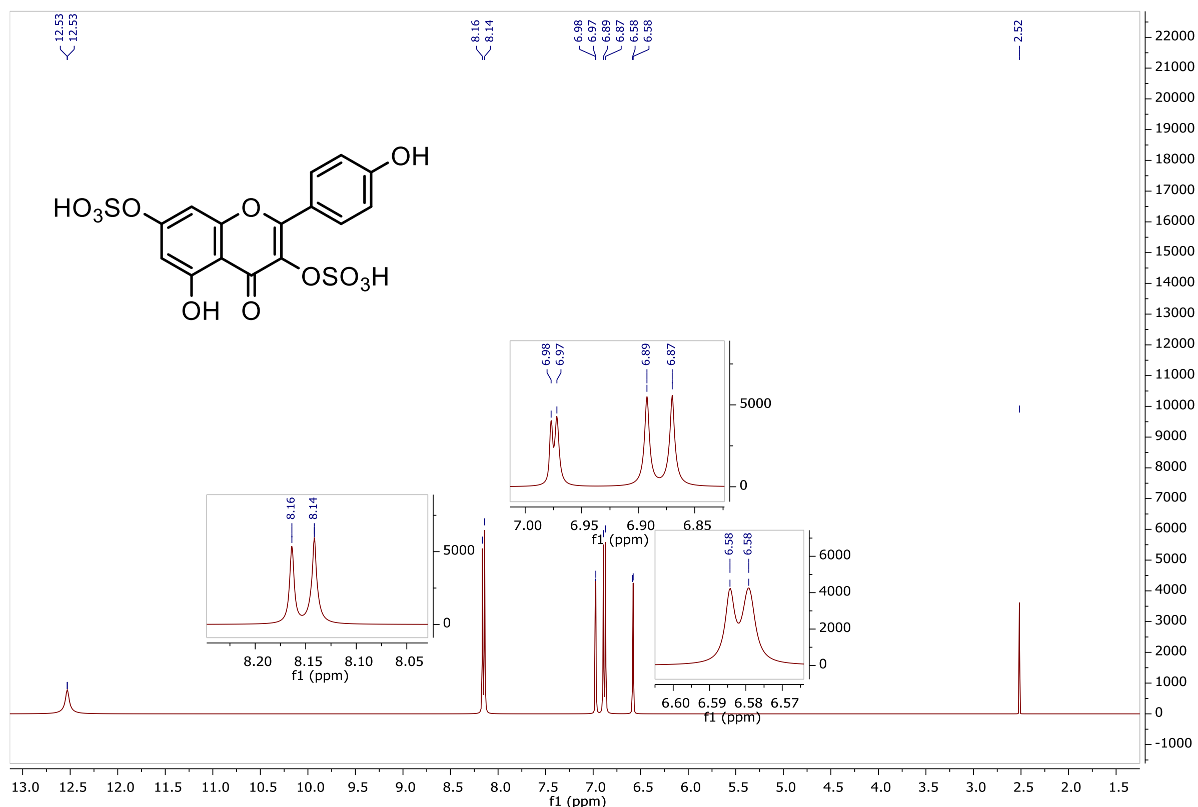


Figure 5. 18: ^{1}H NMR spectrum of compound **16** in DMSO-d_6

^{13}C NMR spectrum of compound **16** also exhibited only thirteen aromatic signals at δ_C 178.3 (C-4), 160.7 (C-5), 160.6 (C-4'), 159.9 (C-7), 157.5 (C-2), 155.7 (C-9), 133.0 (C-3), 131.3 (C-2'/6'), 121.4 (C-1'), 115.7 (C-3'/5'), 106.5 (C-10), 102.3 (C-6) and 97.9 (C-8) which were all typical kaempferol signals besides the change of C-3 and C-2 chemical shifts, which suggested that the sulphate was attached at position C-3.

The other sulphate group was placed in position C-7 based on the fact that both C-6 and C-8 signals were shifted to the low field when compared to kaempferol (Table **5.7**). The structure of compound **16** was identified as **kaempferol 3,7-di-*O*-sulphate**.

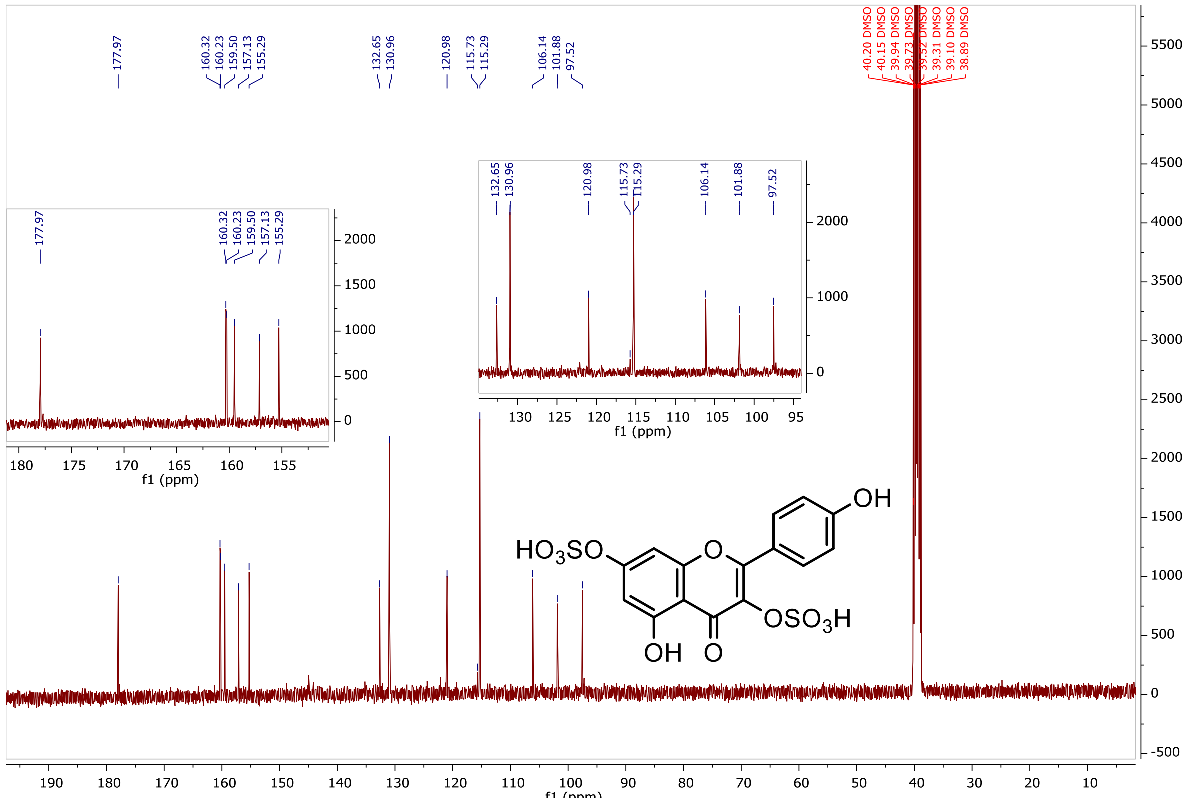


Figure 5. 19: ^{13}C NMR spectrum of compound 16 in DMSO-d_6

The first flavonoid sulphate was reported in 1937, named isorhamnetin 3-sulphate (persicarin), isolated from *Polygonum hydropiper L.* (Polygonaceae). Later, other related flavonoids were isolated, usually from plants found in swampy areas (Teles *et al*., 2018).

Flavonoids are known for their great variety of biological activities and several studies have demonstrated pharmacological properties for sulphated flavonoids, highlighting their anticoagulant, anti-inflammatory, and antitumor activities (Barron et al., 1988; Guglielmone et al., 2005; Gledhill et al., 2007; Calzia et al., 2015; Teles et al., 2015).

Table 5. 6: ^{1}H **and** ^{13}C **NMR spectral data of compound 14 and 16 in DMSO-*d₆***

	Compound **15**		Compound **16**	
Position	**^{1}H (*J* in Hz)**	**^{13}C**	**^{1}H (*J* in Hz)**	**^{13}C**
2	–	147.2	–	157.5
3	–	135.8	–	133.0
4	–	177.6	–	178.3
5	–	156.5	–	160.7
6	6.15 (*d*, 2.1)	98.6	6.58 (*d*, 2.0)	102.3
7	–	164.2	–	159.9
8	6.39 (*d*, 2.1)	93.8	6.97 (*d*, 2.0)	97.9
9	–	161.1	–	155.7
10	–	103.4	–	106.5
1’	–	121.9	–	121.4
2’	8.00 (*d*, 8.8)	129.9	8.15 (*d*, 8.9)	131.3
3’	6.88 (*d*, 8.8)	115.8	6.88 (*d*, 8.9)	115.7
4’	–	159.5	–	160.6
5’	6.88 (*d*, 8.8)	115.8	6.88 (*d*, 8.9)	115.7
6’	8.00 (*d*, 8.8)	129.9	8.15 (*d*, 8.9)	131.3

5.2.9: Structural elucidation of compound 17

Figure 5. 20: Chemical structure of compound **17**

Compound **17** was obtained as a white crystal (100 mg). The structure of the compound was established on the basis of 1D (^{1}H, ^{13}C and DEPT-135) (see Table **5.8**, Appendix **1B**).

The ^{1}H-NMR spectrum showed a downfield doublet at δ_H 5.32 (1H, *d*, *J*= 3.8 Hz, H-1*α*) which is characteristic of the anomeric proton of a glucosyl moiety and another doublet resonating at δ_H 4.12 (1H, *d, J*= 8.8 Hz) which was also a characteristic of the H-3' of the furanoside moiety. Three triplets appeared at 3.96 (1H, *t*, *J* = 8.5 Hz), 3.67 (1H, *t*, *J* = 9.8 Hz) and 3.38 (1H, *t*, *J* = 9.5 Hz) and were attributed to H-4', H-3 and H-4 respectively. A singlet appeared at 3.59 (1H, *s*) was assigned to H-1' and a doublet of doublets at 3.47 (1H, *dd*, J= 9.9, 3.9, Hz) was assigned to H-2.

^{13}C-NMR and DEPT-135 spectra showed the presence of twelve signals, one quaternary at δ_C 106.2 (C-2'), eight methines at δ_C 94.7 (C-1), 83.9 (C-5'), 78.9 (C-3'), 76.5 (C-4'), 75.0 (C-3), 74.9 (C-5), 73.6 (C-2), 71.7 (C-4) and three methylenes at δ_C 64.9 (C-6') 63.8 (C-1') and 62.6 (C-6), which were characteristic of a disaccharide. Based on the above information and comparison with the previously reported literature, compound **17** was established as α-D-glucopyranosyl (1→2) β-Dfructofuranose (sucrose) ((De Bruyn and Van Loo, 1991; Zhang *et al*., 2015).

Table 5. 7: ^{1}H **and** ^{13}C **NMR spectral data of compound 17 in D_2O**

Position	^{1}H (***J* in Hz**)	^{13}C	**DEPT 135**	**Lit** (Zhang *et al*., 2015)
1	5.32 (*d*, 3.8)	94.7	CH	92.2
2	3.47 (*dd*, 9.9, 3.9)	73.6	CH	71.1
3	3.68 (*t*, 9.8)	75.1	CH	72.6
4	3.38 (*t*, 9.5)	71.7	CH	69.2
5	3.76 (*m*)	74.9	CH	72.4
6	3.72 (*d*, 2.8)	62.6	CH_2	60.1
1’	3.59 (*s*)	63.8	CH_2	61.4
2’	–	106.2	C	103.7
3’	4.12 (*d*, 8.8)	78.9	CH	76.5
4’	3.96 (*t*, 8.5)	76.5	CH	74.0
5’	3.80 (*m*)	83.9	CH	81.4
6’	3.73 (*d*, 2.9)	64.8	CH_2	62.4

5.2.10: Structural elucidation of compound 18

Figure 5. 21: Chemical structures of compound **18**

Compound **18** was obtained as an off-white powder (41 mg). The structure of the compound was established on the basis of 1D (^{1}H, ^{13}C and DEPT-135) (Appendix **1B**).

The ^{1}H-NMR spectrum clearly indicated the presence of two doublets at δ_H 5.12 (1H, *d*, *J* = 3.7 Hz) and 4.56 (1H, *d*, *J* = 7.9 Hz) which were characteristics of the alpha and beta anomeric protons of a glucose respectively and also one doublet appeared at 4.03 (1H, *d*, *J* = 3.7 *Hz*) which was a clear indication of the presence of an alpha fructofuranose H-3 proton. An up-field doublet of doublets resonation at 3.16 (1H, *dd*, *J* = 8.1, 1.1 Hz) which were also characteristics of H-2 protons of the glucose was observed. The rest of the peaks were just a cluster between 3.78 and 3.29 ppm (see Figure**: 5.21**)

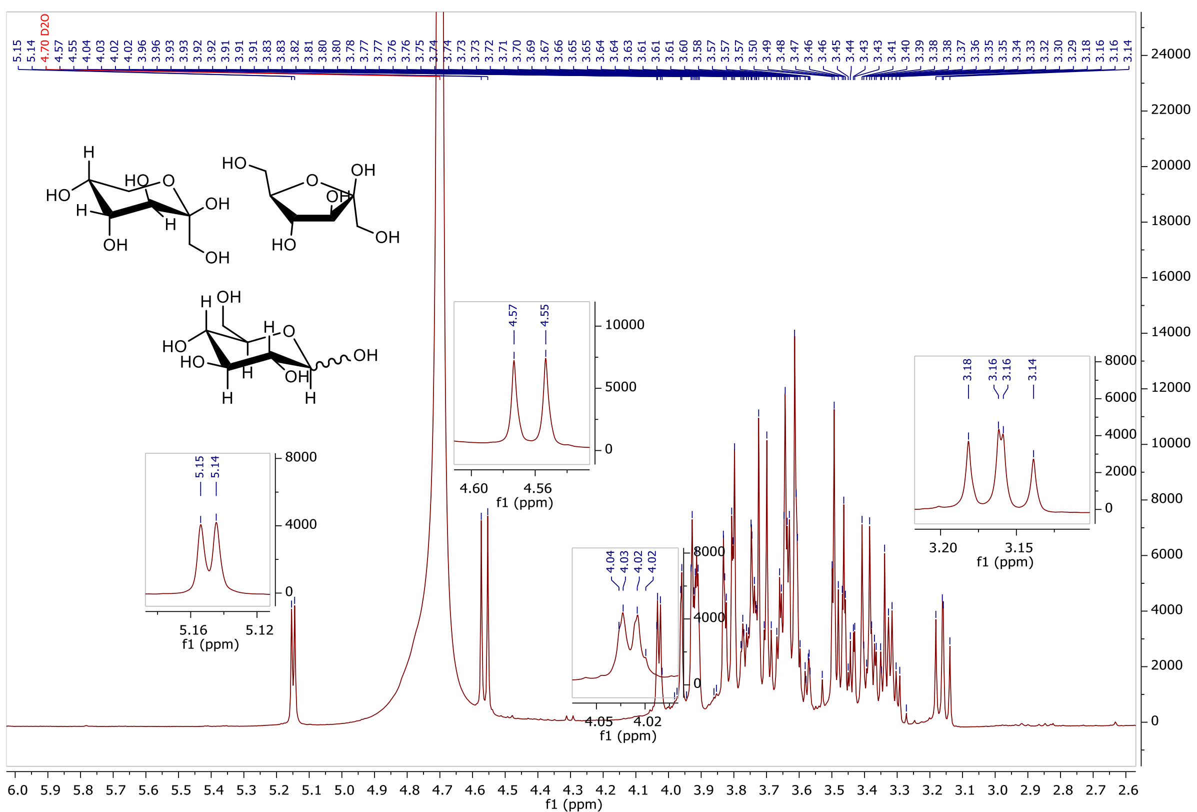


Figure 5. 22: ^{1}H NMR spectrum of compound **18** in D_2O

In addition, the ^{1}H NMR of compound **18** was run against commercially available sugars (glucose, fructose and sucrose) (Figure **5.22**), from which we were able to rule out that compound **18** could be a mixture of β-D-glucopyranose, α-D-glucopyranose, β-D-fructopyranose and β-D-fructofuranose.

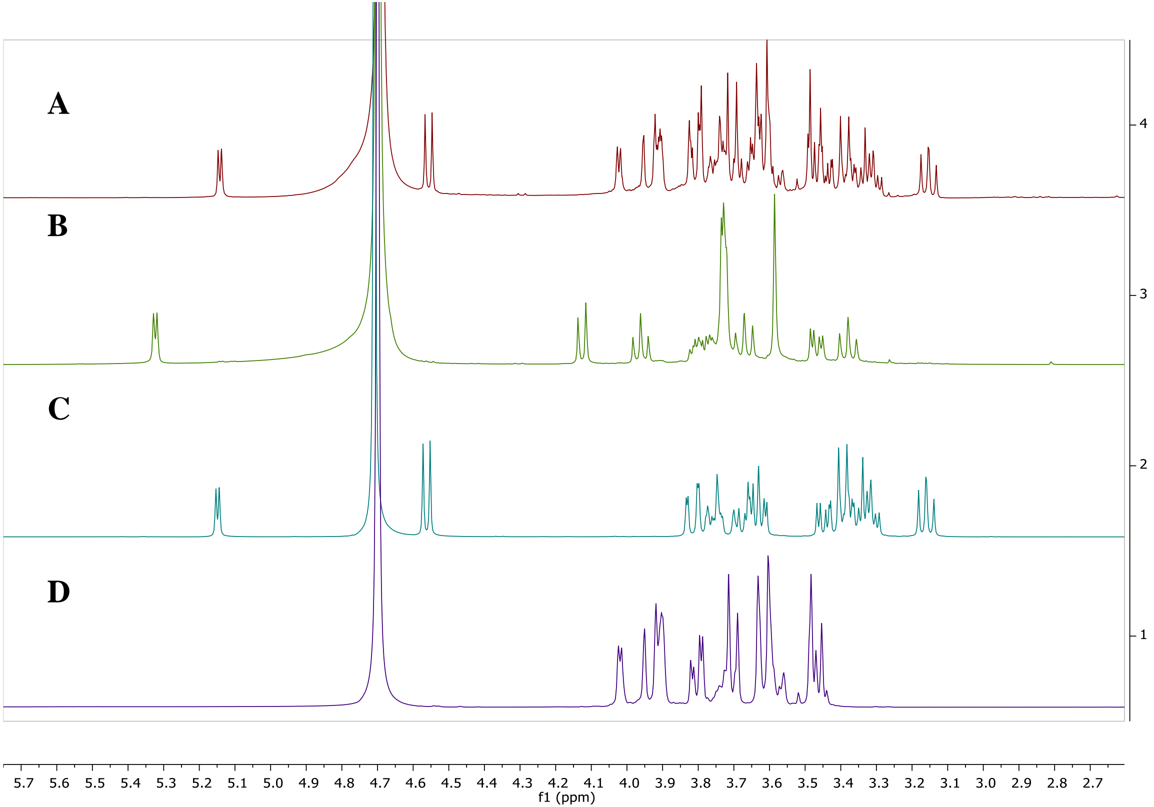


Figure 5. 23: Stacked ^{1}H NMR spectra of compound **18**, sucrose, glucose, and fructose in D_2O. Where **A** – compound **18**, **B** – sucrose, **C** – glucose, **D** – fructose.

The presence of glucose in leaves could be there awaiting the synthesis of glucosides or some role in attracting insects or birds, which might ultimately be related to propagation of the species and glucose is one of those metabolites that every biological fluid is likely to have as a primary metabolite and primary source of energy.

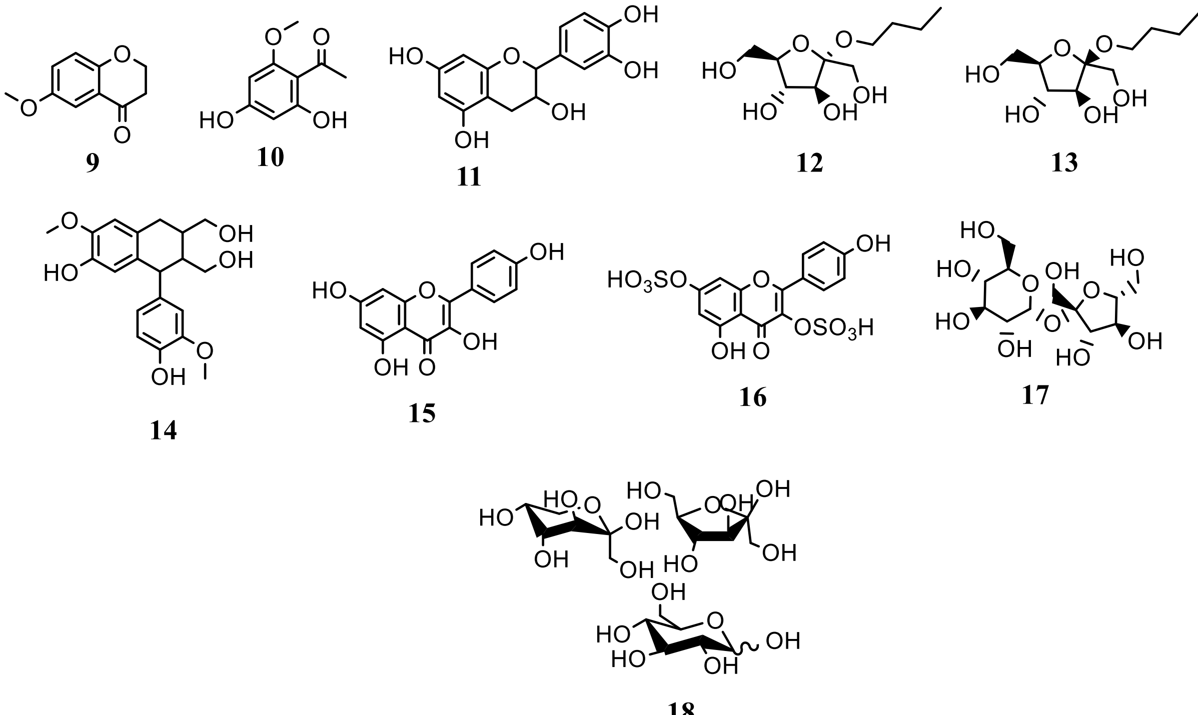


Figure 5. 24: Chemical structures of the isolated compounds from *C. odorata*

5.3 Physico-chemical data of isolated compounds from *C. odorata*

5.3.1: 6-methoxychroman-4-one (9)

Molecular formula $C_{10}H_{10}O_3$ MW 178.0806 g/mol

Description: White powder

Mass spectrum: ESI- MS m/z (ES+) 179.2111 $[M+H]^+$

NMR spectrum: ^1H and ^{13}C see Table **5.1**

5.3.2: 2,4-dihydroxy-6-methoxyacetophenone (10)

Molecular formula $C_9H_{10}O_4$ MW 182.6808 g/mol

Description: White powder

Mass spectrum: ESI- MS *m/z* (ES+) 183.6776 $[M+H]^+$

NMR spectrum: ^1H and ^{13}C see Table **5.2**

5.3.3: Catechin (11)

Molecular formula $C_{15}H_{14}O_6$ MW 290.8012 g/mol

Description: Yellow powder

Mass spectrum: ESI- MS *m/z* (ES+) 291.4558 $[M+H]^+$

NMR spectrum: ^1H and ^{13}C see Table **5.3**

5.3.4: *n*-butyl-alpha-D-fructofuranoside (12)

Molecular formula $C_{10}H_{20}O_6$ MW 236.452 g/mol

Description: Yellow gummy oil

Mass spectrum: ESI- MS *m/z* (ES+) 237.6156 $[M+H]^+$

NMR spectrum: ^1H and ^{13}C see Table **5.4**

5.3.5: *n*-butyl -beta-D-fructofuranoside (13)

Molecular formula $C_{10}H_{20}O_6$ MW 236.655 g/mol

Description: Yellow gummy oil

Mass spectrum: ESI- MS *m/z* (ES+) 237.2888 $[M+H]^+$

NMR spectrum: ^1H and ^{13}C see Table **5.4**

5.3.6: Isolariciresinol (14)

Molecular formula $C_{20}H_{24}O_6$ MW 360.1170 g/mol

Description: Pale yellow amorphous solid

Mass spectrum: ESI- MS *m/z* (ES+) 378.1252 $[M+NH_4]^+$

NMR spectrum: ^1H and ^{13}C see Table **5.5**

5.3.7: Kaempferol (15)

Molecular formula $C_{15}H_{10}O_6$ MW 286.1980 g/mol

Description: Yellow powder

Mass spectrum: ESI- MS *m/z* (ES-) 285.3808 $[M+H]^-$

NMR spectrum: ^1H and ^{13}C see Table **5.6**

5.3.8: Kaempferol 3,7-*O*-disulphate (16)

Molecular formula $C_{15}H_{10}O_{12}S_2$ MW 445.9123 g/mol

Description: Brown pwoder

Mass spectrum: ESI- MS *m/z* (ES+) 446.9704 $[M+H]^+$

NMR spectrum: ^1H and ^{13}C see Figure **5.6**

5.3.9: Sucrose (17)

Molecular formula $C_{21}H_{26}O_6$ MW 342.3571 g/mol

Description: White crystals

Mass spectrum: ESI- MS *m/z* (ES+) 343.3005 $[M+H]^+$

NMR spectrum: ^1H and ^{13}C see Table **5.7**

5.3.10: β-D-glucopyranose, α-D-glucopyranose, β-D-fructopyranose and β-D-fructofuranose (Honey). (18)

Molecular formula mixture

Description: Brown amorphous solid

NMR spectrum: ^1H and ^{13}C see Figure **5.21 and 5.22**

5.4 Biological evaluation

To validate the ethnopharmacological applications and to explore the therapeutic potential of the plant *C. odorata*, its extracts and the respective isolated compounds were evaluated for their activity to inhibit alpha-glucosidase, alpha-amylase, and tyrosinase enzyme.

5.4.1 Alpha-glucosidase inhibition

EtOAc, BuOH, and water extracts of the leaves of *C. odorata* were screened for their alpha-glucosidase inhibitory activity at 0.2 mg/mL. As shown in Figure **5.24**, EtOAc and BuOH extracts demonstrated the highest percentage inhibition against alpha- glucosidase activity (100% and 100% respectively). The water extract yielded the lowest inhibitory activity (4%).

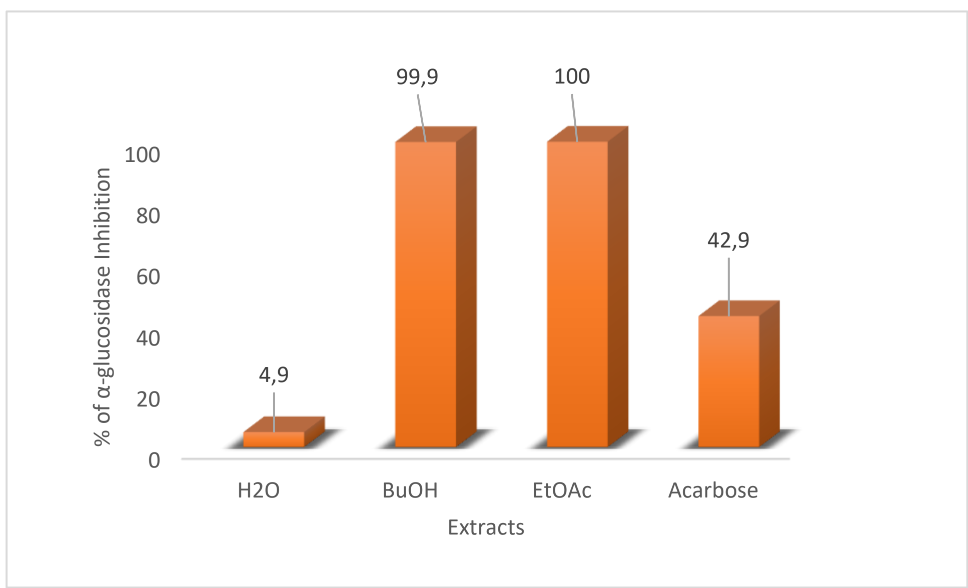


Figure 5. 25: Average % inhibition of alpha-glucosidase of the plant extracts in triplicates

Table 5. 8: **Inhibitory activity (%) of *C. odorata* extracts against alpha-glucosidase enzyme**

Inhibitor/extract	Percentage %	Concentration (µg/mL)
EtOAc	100	200
BuOH	100	200
Water	4.00	200
Acarbose	45.0	200

The ten isolated compounds from *C. odorata* were also screened for their alpha-glucosidase inhibitory activity. Compound **15** and **16** demonstrated the highest percentage inhibition against alpha- glucosidase activity (99% and 60% respectively), and the rest of the compounds yielded the lowest percentage inhibition (Table 5.10).

Table 5. 9: **Inhibitory activity (%) of *C. odorata* compounds against alpha-glucosidase enzyme**

Inhibitor/compound	Percentage %	Concentration (µg/mL)
9	NA	200
10	7.50	200
11	9.50	200
12	1.80	200
13	3.00	200
14	NA	200
15	60.0	200
16	99.0	200
17	NA	200
18	NA	200
Acarbose	38.50	200

NA not active at the tested concentrations

IC_{50} values of compound **15** and **16** (which showed strong alpha glucosidase % inhibitory activities during screening) as well as the IC_{50} of acarbose determined from corresponding dose-response curves of percentage inhibition versus inhibitor concentration. Compound **16** demonstrated the highest alpha-glucosidase inhibitory activity IC_{50} value of 3.8 ± 0.32 µg/mL followed by compound **15** with IC_{50} value of 16.6 ± 1.02 µg/mL. The IC_{50} value of compound **15** was in agreement with the reported value of 16.5 ± 0.81 µg/mL (Dewi and Maryani, 2015).

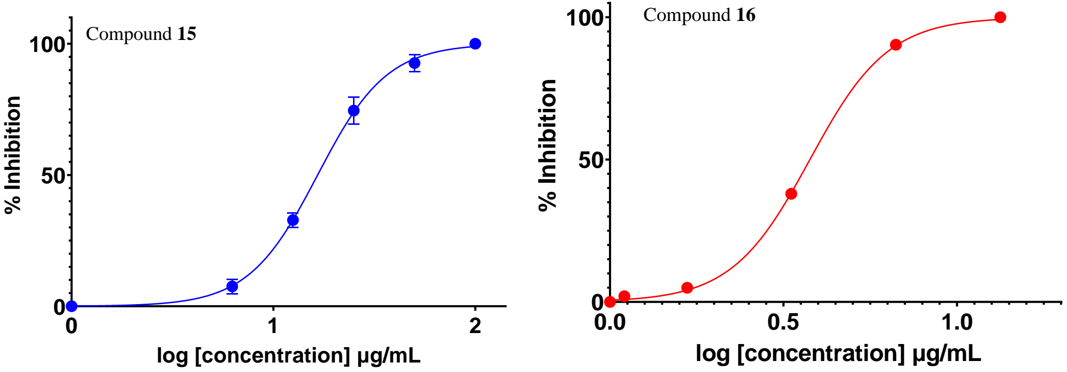


Figure 5. 26: alpha-glucosidase inhibitory activity estimation of IC_{50} values isolated compounds **15** and **16**

Table 5. 10: **IC_{50} values of tested compounds on alpha-glucosidase**

Inhibitor/extract	**alpha-glucosidase IC_{50}** (µg/mL)
15	16.6 ±1.02
16	3.8 ± 0.32
Acarbose	215 ± 0.55

The IC_{50} values of both compounds **15** and **16** were significantly lower than that of acarbose, a positive reference (Table **5.10**). These results implies that these compounds may be competitive inhibitors of alpha glucosidase with the ability to bind to the enzyme or substrate (López, et al., 2015).

5.4.2 Alpha-amylase inhibition

The extracts of *C. odorata* (EtOAc, BuOH, and water) and isolated compounds were screened to determine their inhibition activity against alpha-amylase enzyme. The extracts and the compounds were tested at 0.2 mg/mL. The results for enzyme of both the extracts and the isolated compounds showed no significant inhibition on the alpha-amylase inhibitory. Most of the results were negative and this could be that the inhibitor is not acting as an inhibitor, but is instead stimulating the enzyme.

5.4.3: Mushroom L-tyrosinase assay

5.4.3.1: Anti-tyrosinase activity of various fractions and isolated compounds from *C. odorata*

Inhibition of tyrosinase activity of EtOAc, BuOH and water extracts of *C. odorata* was measured using L-tyrosine. EtOAc extract exhibited 16.5% tyrosinase inhibitory activity at 0.2 mg/ml. It showed the highest anti-tyrosinase activity as compared to BuOH and water extracts which showed the lowest values (**Figure 5.26**). The average inhibition percentage of the isolated compounds at 0.2 mg/mL was determined and it revealed that compound **11** and **12** had an inhibition of 50% and 30% respectively. The rest of the other compounds showed not to be effective inhibitors of mushroom Tyrosinase with their inhibition percentage less than 10%. The two compounds with high inhibition percentage were then investigated for their IC_{50} values.

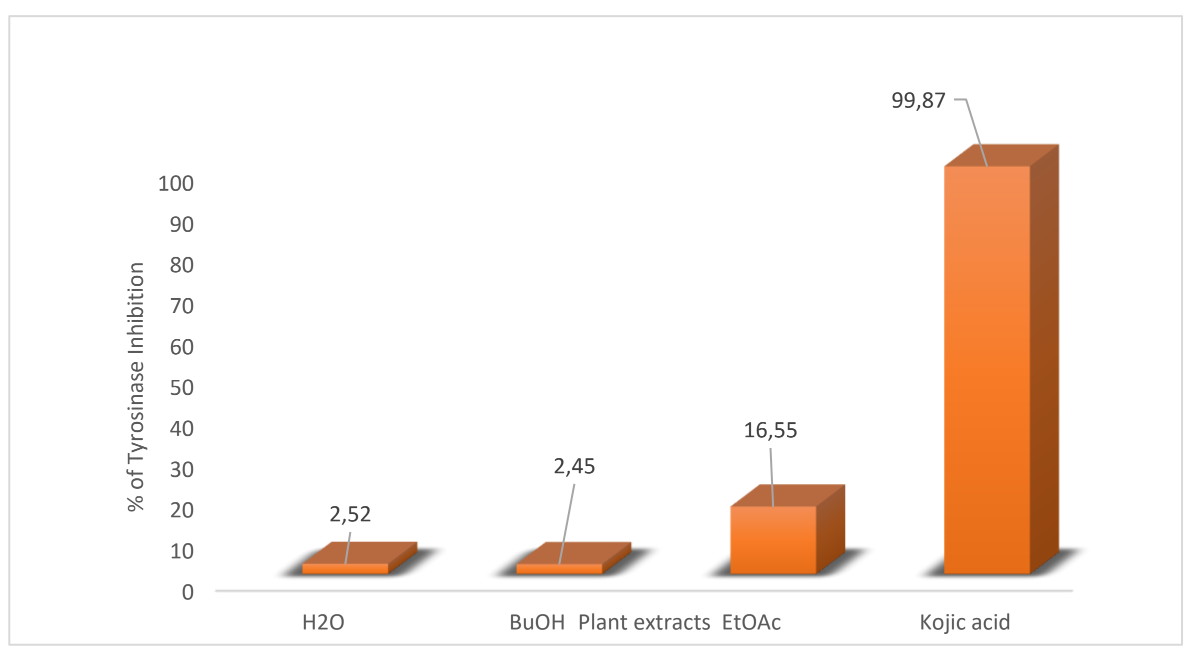


Figure 5. 27: Average % inhibition of Tyrosinase of the plant extracts

Compound **11** and **12** showed IC_{50} values of 131.9 ± 1.05 and 274.5 ± 1.26 µg/mL respectively.

Table 5. 11: **IC_{50} values of isolated compounds from EtOAc extract of *C. odorata***

	% inhibition(µg/mL)				
Tested Samples	**1000**	**500**	**250**	**125**	**IC_{50}**
9	NA	NA	NA	NA	-
10	NA	NA	NA	NA	-
11	76.5	71.9	63.2	35.7	131.9
12	61.9	47.9	30.3	18.04	231.8
13	NA	NA	NA	NA	-
KOJIC ACID	99.8	98.9	98.5	98.21	3.1

NA: not active at the tested concentrations. The results are expressed as mean ±SEM for n =3

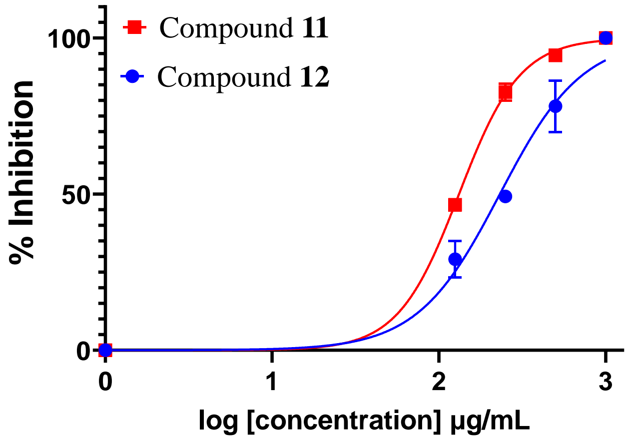


Figure 5. 28: IC_{50} plot of compound **11** and **12**

5.5: Conclusion

Phytochemical and biological investigation of the EtOAc and BuOH soluble fractions of *Cliffortia odorata* leaf extract using column chromatography isolation techniques afforded ten bioactive compounds with significant alpha glucosidase inhibitory activities as well as weak antityrosinase inhibitory activities. To the best of our knowledge, all of the isolated compounds are reported for the first time from the extracts of *C. odorata*. Compound **15** and **16** demonstrated remarkable alpha glucosidase inhibitory activities and on the other hand compounds **11** and **12** demonstrated weak antityrosinase inhibitory activities and this could mean that *C. odorata* could be a good candidate in cosmetic and pharmaceutical industries upon further biological and clinical investigation.

References

Arroo, R.R.J., Alfermann, A.W., Medarde, M., Petersen, M., Pras, N. and Woolley, J.G., 2002. Plant cell factories as a source for anti-cancer lignans. *Phytochemistry Reviews*, 1(1), pp.27-35.

Barron, D., Varin, L., Ibrahim, R.K., Harborne, J.B. and Williams, C.A., 1988. Sulphated flavonoids—an update. *Phytochemistry*, *27*(8), pp.2375-2395.

Bohm, B.A. and Stuessy, T.F., 2001. *Flavonoids of the sunflower family (Asteraceae). Springer Science and Business Media*.

Calzia, D., Oneto, M., Caicci, F., Bianchini, P., Ravera, S., Bartolucci, M., Diaspro, A., Degan, P., Manni, L., Traverso, C.E. and Panfoli, I., 2015. Effect of polyphenolic phytochemicals on ectopic oxidative phosphorylation in rod outer segments of bovine retina. *British journal of pharmacology*, *172*(15), pp.3890-3903.

Dewi, R.T. and Maryani, F., 2015. Antioxidant and α-glucosidase inhibitory compounds of Centella asiatica. *Procedia Chemistry*, *17*, pp.147-152.

Dudíková, J., Mastihubová, M., Mastihuba, V. and Kolarova, N., 2007. Exploration of transfructosylation activity in cell walls from *Cryptococcus laurentii* for production of functionalised *β*-D-fructofuranosides. *Journal of Molecular Catalysis B: Enzymatic*, *45*(1-2), pp.27-33.

Fang, X.K., Gao, J. and Zhu, D.N., 2008. Kaempferol and quercetin isolated from Euonymus alatus improve glucose uptake of 3T3-L1 cells without adipogenesis activity. *Life Sciences*, *82*(11-12), pp.615-622.

Fonseca, S.F., de Paiva Campello, J., Barata, L.E. and Rúveda, E.A., 1978. 13C NMR spectral analysis of lignans from *Araucaria angustifolia*. *Phytochemistry*, *17*(3), pp.499-502.

Foo, L.Y., 1987. Phenylpropanoid derivatives of catechin, epicatechin and phylloflavan from *Phyllocladus trichomanoides*. *Phytochemistry*, *26*(10), pp.2825-2830.

Gledhill, J.R., Montgomery, M.G., Leslie, A.G. and Walker, J.E., 2007. Mechanism of inhibition of bovine F1-ATPase by resveratrol and related polyphenols. *Proceedings of the National Academy of Sciences*, *104*(34), pp.13632-13637.

Grande, M., Piera, F., Cuenca, A., Torres, P. and Bellido, I.S., 1985. Flavonoids from *Inula viscosa*. *Planta medica*, *51*(05), pp.414-419.

Guglielmone, H.A., Agnese, A.M., Montoya, S.C.N. and Cabrera, J.L., 2005. Inhibitory effects of sulphated flavonoids isolated from *Flaveria bidentis* on platelet aggregation. *Thrombosis research*, *115*(6), pp.495-502.

Harborne, J.B., 1975. Flavonoid sulphates: a new class of sulphur compounds in higher plants. *Phytochemistry*, *14*(5-6), pp.1147-1155.

Ho, L.K., Lin, C.C. and Chang, Y.S., 1993. Investigation of Chemical Components from *Scurrula Liquidambaricolus* (Hayata) Danser. *4*(2), pp.151-155.

Lee, N.H., Gloer, J.B. and Wicklow, D.T., 2007. Isolation of chromanone and isobenzofuran derivatives from a fungicolous isolate of *Epicoccum purpurascens*. *Bulletin of the Korean Chemical Society*, *28*(5), pp.877-879.

López, D., Cherigo, L., Spadafora, L., Loza-Mejía, M.A., Martínez-Luis, S. 2015. Phytochemical composition, antiparasitic and alpha-glucosidase inhibition activities from *Pelliciera rhizophorae. Chemistry Central Journal*, 9: 53.

MacRae, W.D. and Towers, G.N., 1984. Biological activities of lignans. *Phytochemistry*, 23(6), pp.1207-1220.

Okuyama, E., Suzumura, K. and Yamazaki, M., 1995. Pharmacologically active components of *Todopon Puok* (Fagraea racemosa), a medicinal plant from Borneo. *Chemical and pharmaceutical bulletin*, *43*(12), pp.2200-2204.

Pelter, A., Ward, R.S. and Gray, T.I., 1976. The carbon-13 nuclear magnetic resonance spectra of flavonoids and related compounds. *Journal of the Chemical Society, Perkin Transactions 1*, (23), pp.2475-2483.

Singh, A.K., Pathak, V. and Agrawal, P.K., 1997. Annphenone, a phenolic acetophenone from *Artemisia annua*. *Phytochemistry*, *44*(3), pp.555-557.

Telange, D.R., Patil, A.T., Pethe, A.M., Tatode, A.A., Anand, S. and Dave, V.S., 2016. Kaempferol-phospholipid complex: formulation, and evaluation of improved solubility, in vivo bioavailability, and antioxidant potential of kaempferol. *Journal of excipients and food chemicals*, *7*(4), p.1174.

Teles, Y.C., Horta, C.C.R., Agra, M.D.F., Siheri, W., Boyd, M., Igoli, J.O., Gray, A.I. and De Souza, M.D.F.V., 2015. New sulphated flavonoids from *Wissadula periplocifolia* (L.) C. Presl (Malvaceae). *Molecules*, *20*(11), pp.20161-20172.

Thomford, A.K., Abdelhameed, R.F.A. and Yamada, K., 2018. Chemical studies on the parasitic plant *Thonningia sanguinea* Vahl. *RSC advances*, *8*(37), pp.21002-21011.

Wagner, H. and Bladt, S., 1996. *Plant drug analysis: a thin layer chromatography atlas*. Springer Science & Business Media.

Wang, H., Du, Y.J. and Song, H.C., 2010. α-Glucosidase and α-amylase inhibitory activities of guava leaves. *Food chemistry*, *123*(1), pp.6-13.

Zhang, C.Z., Xu, X.Z. and Li, C., 1996. Fructosides from *Cynomorium songaricum*. *Phytochemistry*, *41*(3), pp.975-976.

CHAPTER 6

CHEMICAL CHARACTERIZATION AND BIOLOGICAL INVESTIGATION OF *ASPARAGUS LIGNOSUS*

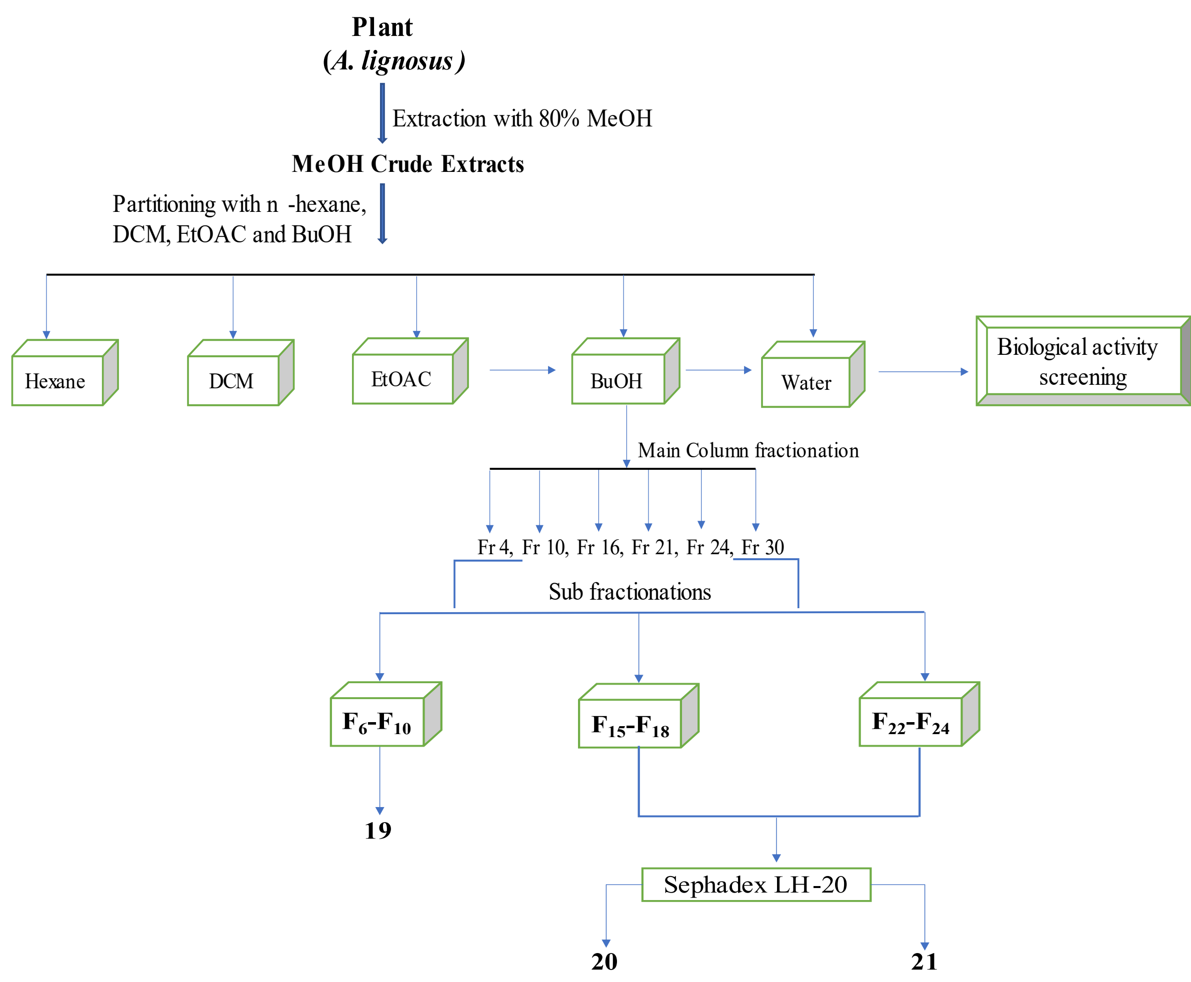
In this study, phytochemical and biological investigation of the BuOH fraction of *Asparagus lignosus* afforded three known compounds, 20-hydroxyecdysone **(19)**, quercetin **(20)**, and isorhamnetin-3-*O*-rutinoside (narcissin) **(21)**. All isolated compounds are reported for the first time from *A. lignosus*; however, these compounds are common in the Asparagaceae family. The isolated compounds were assayed for their alpha-glucosidase, alpha-amylase and tyrosinase activity and only compound **20** showed weak to moderate activity on both alpha-glucosidase and tyrosinase, with the IC_{50} values of 110.8 ± 2.52 µg/mL and 128.9 ± 2.12 µg/mL respectively.

6.1 *Asparagus lignosus*

The dried powdered leave material (155g) was extracted with 80% MeOH and sequentially extracted with hexane, ethyl acetate, methanol and finally with water. The hexane (0.12g), DCM (0.08g), EtOAc (0.25g), BuOH (2.25g) and water (12.21g) extract and due to the limited yield of the hexane and DCM extracts, only BuOH was phytochemically studied for its bioactive constituents

6.1.1 Isolation of constituent of the BuOH extract fraction

Column chromatography of the BuOH extract (2.25g) afforded thirty-eight main fractions. **F_6-F_{10}**, **F_{15}-F_{18}** and **F_{22}-F_{24}** were combined and rechromatographed, see Scheme 6.1. This afforded compound **19** (150mg), **20** (15mg), and **21** (25mg).



Scheme 6. 1: **A scheme of experimental procedure for the isolation of compounds from *A. lignosus***

6.2: Structural elucidation and characterization of compounds

6.2.1: Structural elucidation of compound 19

Figure 6. 1: Chemical structure of compound **19**

Compound **19** was obtained as a white powder (120 mg). The molecular formula was established as $C_{27}H_{44}O_7$, by the positive-ion HR-ESI-MS, which showed molecular ion $[M+H]^+$ at *m/z* 504.2185 (calc. for 503.36). The structure of the compound was established on the basis of 1D and 2D (NMR) (Table **6.1**), (Appendix **1C**).

The ^{1}H NMR spectrum of compound **19** in displayed five methyl signals, two of them appears as two singlet signals at δ 0.89 (3H, s, H-18) and 0.97 (3H, s, H-19), the other three observed as singlet integrated for nine protons at 1.21 (9H, s, H-21, H-26 and H-27); three signals at δ 3.33 (1H, *m*, H-22), 3.84 (1H, *m*, H-3) and 3.96 (1H, *m*, 1H, H-2) each integrating for one proton is due to oxymethine protons indicating that three oxymethine groups are present in the compound. An olefinic proton was observed as a singlet adjacent to carbonyl at 5.82 (1H, s, H-7).

The ^{13}C NMR and DEPT 135 experiments of compound **19** showed the presence of twenty-seven carbon signals which indicated that the compound might possess a steroidal skeleton with a side chain. DEPT revealed the presence of twenty protonated carbon signals. Thus, the other remaining seven signals could be attributed to fully substituted carbons. One

carbonyl carbon resonated at δ_C 206.6 (C-6), five quaternary carbons at δ_C 85.4, 78.5, 71.4, 49.9 and 40.4 were attributed to C-14, C-20, C-25, C-13 and C-10 respectively and one fully substituted olefinic carbon at δ_C 168.1 (C-8). Seven methine groups were observed at δ_C 122.3 (C-7) 78.0 (C-22), 68.8 (C-3), 68.6 (C-2), 51.9 (C-5), 50.6 (C-17), and 35.3 (C-9). The methylene (seven) carbon peaks appeared at δ_C 43.6 (C-24), 38.5 (C-1), 34.0 (C-12), 33.6 (C-12), 32.9 (C-4), 28.6 (C-23) and 22.7 (C-11) while the five methyl carbons absorbed at δ_C 30.9 (C-26), 30.1 (C-27), 25.6 (C-19), 22.2 (C-21) and 19.2 (C-18). Based on the data obtained for compound **19** and upon comparison with literature data (Girault and Lafont, 1988; Vokáč et al., 1998) confirmed the structure as **20-hydroxyecdysone.**

20-Hydroxyecdysone is a naturally occurring ecdysteroid hormone which regulates the ecdysis moulting in insects. These types of compounds are found abundantly in plant species and have anabolic effects in vertebrates (Isenmann et al., 2019).

Ahmed et al (1996) and Khaliq-uz-Zaman et al (2000) reported the isolation of 20-hydroxyecdysone from *A. dumosus* and it being responsible for anti-microbial activity of the extracts of the species.

Table 6. 1: **^1H and ^{13}C NMR spectral data of compound 19 in CD_3OD**

Position	^1H (mult. J_{Hz})	^{13}C	**Lit** (Vokáč *et al.*, 1998)
1a	1.82 (*m*)	38.5	37.4
1b	1.44 (*m*)		
2	3.96 (*m*)	68.6	68.7
3	3.84 (*m*)	68.8	68.5
4a	1.99*	32.9	32.9
4b	1.61*		
5	2.388*	51.9	51.9
6	–	206.6	206.5
7	5.82 (*br s*)	122.3	122.1
8	–	168.1	167.9
9	3.16 (*m*)	35.3	35.1
10	–	40.4	39.3
11a	1.99*	22.6	21.5
11b	1.81*		

12	2.14*	32.6	32.5
13	–	49.9**	49.6*
14	–	85.4	85.2
15a	1.99*	33.9	31.8
15b	1.61*		
16a	1.96*	22.7	21.6
16b	1.72*		
17	2.39 (*m*)	50.6	50.5
18	0.89 (*s*)	18.2	18.1
19	0.97 (*s*)	24.5	24.4
20	–	78.5	77.9
21	1.20 (*s*)	21.2	21.1
22	3.33 (*br d*, 14)	78.0	78.4
23a	1.69 (*m*)	28.6	27.3
23b	1.29 (*m*)		
24a	1.81 (*m*)	42.5	42.4
24b	1.43 (*m*)		
25	–	71.4	71.3
26	1.20 (*s*)	30.1[+]	29.7
27	1.21 (*s*)	30.9[+]	28.9

+ Interchangeable

*The proton chemical shift overlapped, determined from 2D spectra (overlapped signals)

**The chemical shift overlapped with the solvent peak

6.2.2: Structural elucidation of compound 20

Figure 6. 2: Chemical structure of compound **20**

Compound **20** was obtained as a yellow powder (17 mg). The HRES-MS showed a pseudo molecular peak at m/z 303.3851 $[M+H]^+$ corresponding to the molecular formula $C_{15}H_{10}O_7$. The structure of the compound was established on the basis of 1D (^1H, ^{13}C and DEPT-135) (see Table **6.2**, Appendix **1C**).

^1H NMR spectrum showed two *meta* coupled aromatic protons at δ_H 6.29 (1H, *d*, *J* = 2.1 Hz) and δ_H 6.09 (1H, *d*, *J* = 2.1 Hz), assigned for H-6 and H-8 respectively, confirming a 5, 7 dihydroxy substituted ring A. Other three aromatic protons appeared at δ_H 7.64 (1H, *d*, *J* = 2.1), 7.54 (1H, *dd*, *J* = 8.5, 2.1 Hz) and 6.79 (1H, *d*, *J* = 8.5) were assigned as H-2', H-6', and H-5' of the B-ring respectively, suggesting a 3', 4'substitued B-ring of a flavonoid.

The ^{13}C NMR spectrum of compound **20** (Table **6.2**) exhibited the presence of fifteen carbon resonances at 177.6 (C-4), , 165.9 (C-7), 162.8 C-5, 158.5 C-9, 149.0 (C4'), 148.2 (C-2), 146.5 (C-3'), 137.5 (C-2), 124.4 (C-1'), 121.9 (C-6'), 116.5 (C-5)', 116.3 (C-2'), 104.8 (C-10), 99.5 (C-6) and, 94.7 (C-8), Based on the obtained results and comparison to the literature, the compound **20** was identified as quercetin (Dhasan et al., 2008; Abdel-Sattar et al., 2009; Mohammed, 2015).

This compound has been isolated from other *Asparagus* species such *A. officinalis* (Grubben, and Denton., 2004) and *A. racemosus* (Anthony et al., 2012). Quercetin has various biological properties, including antioxidant, anti-inflammatory, antibacterial, antiviral,

radical-scavenging, gastroprotective, and immune-modulatory activities (David et al., 2016; Massi et al., 2017). It is reported to inhibit the uptake of glucose from the blood, blocks the fat cell production, and enhances fat cell necrosis (Strobel et al., 2005, Yang et al., 2008) and it also has potential anticancer properties which include antiproliferative, growth factor suppression, and antioxidant (Lamson and Brignall, 2000)

Table 6. 2: **^{1}H and ^{13}C NMR spectral data of compound 20 in CD_3OD**

Position	**^{1}H (*J* in Hz)**	**^{13}C**
2	–	148.2
3	–	137.5
4	–	177.6
5	–	162.8
6	6.29 (*d*, 2.1)	99.5
7	–	165.9
8	6.09 (*d*, 2.1)	94.7
9	–	158.5
10	–	104.8
1’	–	124.4
2’	7.64 (*d*, 2.1)	116.3
3’	–	146.5
4’	–	149.0
5’	6.79 (*d*, 8.5)	116.5
6’	7.54 (*dd*, 8.5, 2.1)	121.9

6.2.3: Structural elucidation of compound 21

Figure 6. 3: Chemical structure of compound **21**

Compound **21** was obtained as a yellow powder (25 mg). The molecular formula was established as $C_{28}H_{32}O_{16}$, by the positive-ion HR-ESI-MS, which showed molecular ion $[M+H]^+$ at *m/z* 625.7538 (calc. for 624.5441). The structure of the compound was established on the basis of 1D and 2D (NMR) (Table **6.3**), FTIR and MS data (Appendix **C**).

The ^{1}H NMR spectrum of compound **21** exhibited a doublet at δ_H 7.75 (1H, *d*, *J* = 1.8 Hz), a doublet of doublets at δ_H 7.44 (1H, *dd*, *J* = 8.5, 1.8 Hz) and a doublet at δ_H 6.69 (1H, *d*, *J* = 8.5 Hz) and they were assigned to H-2', 6' and 5' protons of 1,3,4-trisubstituted ring B from a flavonoid framework respectively. Additionally, the ^{1}H NMR spectrum exhibited one singlet at δ_H 3.83 (3H, *s*), indicating the presence of a methoxy group. There were also two anomeric proton signals at δ_H 4.89 (1H, *d*, *J* = 7.4 Hz) and δ_H 4.34 (1H, *s*), which together with overlapped proton signals at δ_H 3.76–3.18, demonstrating the glycosylated nature of compound a rutinoside unit. Observing a doublet at δ_H 0.95 (3H, *d*, *J* = 6.2 Hz), typical of rhamnose methyl group.

In the ^{13}C-NMR spectrum of compound **21** established fifteen carbon signals for the flavonoid nucleus at δ_C 94.9 (C-8), 97.3 (C-6), 104.1 (C-10), 115.1 (C-2'), 116.9 (C-5'), 124.6 (C-6'), 123.3 (C-1'), 135.8 (C-3), 149.2 (C-3'/C-4'), 152.4 (C-2), 158.6 (C-9), 159.7 (C-5), 163.1 (C-7) and 178.9 (C-2). Two anomeric carbons at δ_C 105.9 (C-1'') and δ_C 103.2 (C-1''') were observed suggesting the presence of two sugar moieties, one of which is L-rhamnose confirmed

by a doublet at δ_H 0.95 ppm. Signals attributed to the glucose moiety were observed to resonate at δ_C 69.3 (C-6''), 72.8 (C-4''), 76.6 (C-2''), 77.9 (C-5''), 78.9 (C-3''). While those which indicated L-rhamnose moiety were observed at 18.6 (C-6'''), 70.5 (C-5'''), 72.2 (C-2'''), 72.9 (C-3'''), 74.6 (C-4''').

The HMBC spectral data (Appendix C) showed correlation of methoxy protons resonating at δ_H 3.83 to C-3''/C-4'' established the position of the aryl methoxy group. The linkage between the flavonoid and the sugar was observe through a correlation between H-1'' at δ_H 4.98 correlated with C-3 at δ_C 135.9, also a correlation between the anomeric proton of rhamnose H-1''' with the with C-6'' of glucose at δ_C 69.3 was observed which confirmed the link between the two sugar units.

The chemical structure of compound **21** was proposed to be isorhamnetin-3-*O*-rutinoside (narcissin) by comparison with the previously reported data (Yeskaliyeva et al., 2006; Olennikov et al., 2012; Alhozaimy et al., 2017). This compound has been previously isolated from many genera including *Atractylis* (Chabani et al., 2013), *Coyza* (Calzada et al., 2001), *Daniellia* (Afifi et al., 2006), *Ferula*, (Znati et al., 2014), and *Strumpfia* (Hsu et al., 1981). Barros et al. also reported that the flavonoid profile of the young stems of *A. acutifolius* comprised of rutin, narcissin and nicotiflorin.

Figure 6. 4: Key HMBC (→) correlations of compounds **21**

Table 6. 3: **^{1}H and ^{13}C NMR spectral data of compound 21 in CD_3OD**

Position	**^{1}H (*J* in Hz)**	**^{13}C**	**Narcissin** (Yeskaliyeva et al., 2006)
2	–	158.4	158.9
3	–	135.8	135.5
4	–	178.9	179.4
5	–	163.1	163.1
6	6.11 (*br s*)	97.4	99.7
7	–	165.7	166.1
8	5.89 (*br s*)	93.8	94.9
9	–	158.7	158.5
10	–	104.1	105.7
1’	–	123.3	123.0
2’	7.75 (*d*, 1.8)	115.1	114.6
3’	–	149.2	148.3
4’	–	149.2	150.8
5’	6.68 (*d*, 8.0)	116.9	116.1
6’	7.43 (*dd*, 8.0,	124.6	123.9
OCH_3	3.83 (*s*)	57.3	56.8
		3-*O*-C(6)-Glucosyl	
1’’	4.97 5.68 (*d*,	105.9	104.4
2’’	3.37 (*m*)	76.6	75.9
3’’	3.35 (*m*)	79.1	78.2
4’’	3.52 (*m*)	72.8	71.6
5’’	3.25 (*m*)	78.3	77.4
6’’a	3.28 (*m*)	69.3	68.5
6’’b	3.71 (*m*)		
		6’’-*O*-Rhamnosyl	
1’’’	4.42 (*d*, 0.9)	103.2	102.5
2’’’	3.15 (*m*)	72.2	72.1
3’’’	3.40 (*m*)	72.9	72.3
4’’’	3.17 (*m*)	74.7	73.8
5’’’	3.31 (*m*)	70.5	69.8
6’’’	0.95 (*d*, 6.2)	18.6	17.9

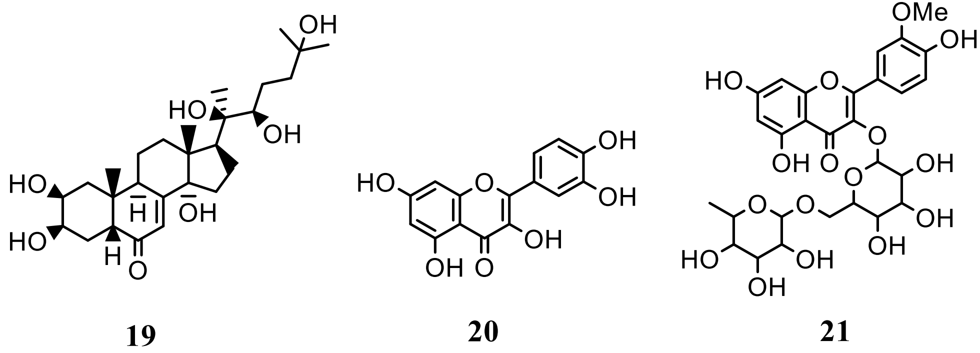


Figure 6. 5: Chemical structures of the isolated compounds from *A. lignosus*

6.3. Physico-chemical data of isolated compounds from *A. lignosus*

6.3.1: 20-Hydroxyecdysone (19)

Molecular formula $C_{27}H_{44}O_7$ MW 503.7751 g/mol

Description: White solid

Mass spectrum: ESI- MS *m/z* (ES+) 504.2185 $[M+H]^+$

NMR spectrum: ^1H and ^{13}C see Table **6.1**

6.3.2: Quercetin (20)

Molecular formula $C_{15}H_{10}O_7$ MW 302.1775 g/mol

Description: Yellow powder

Mass spectrum: ESI- MS *m/z* (ES+) 303.3851 $[M+H]^+$

NMR spectrum: ^1H and ^{13}C see Table **6.2**

6.3.3: Isorhamnetin-3-*O*-rutinoside (narcissin) (21)

Molecular formula $C_{28}H_{32}O_{16}$ MW 624.5441 g/mol

Description: Yellow needles

Mass spectrum: ESI- MS *m/z* (ES+) 625.7538 $[M+H]^+$

NMR spectrum: ^1H and ^{13}C see Table **6.3**

6.4 Biological evaluation

Two extracts of the *A. lignosus* plant material namely BuOH, and water were screened for their inhibitory activities against alpha-amylase, alpha-glucosidase and Tyrosinase.

6.4.1 Alpha-glucosidase inhibition

BuOH, and water extracts of the leaves of *A. lignosus* were screened for their alpha-glucosidase inhibitory activity at 0.2 mg/mL. As shown in Figure **6.8**, BuOH extracts demonstrated a moderate but not high inhibitory activity on alpha-glucosidase and the water extract had no inhibitory activity (Table **6.4** and Figure **6.8**).

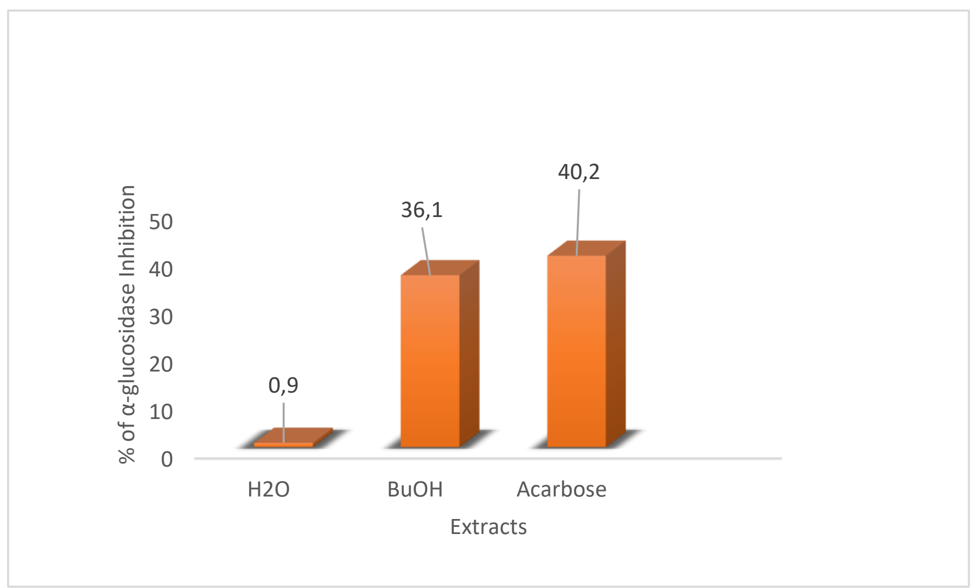


Figure 6. 6: Average % inhibition of alpha-glucosidase of the plant extracts

Table 6. 4: **Inhibitory activity (%) of *C. odorata* extracts against alpha-glucosidase enzyme**

Inhibitor/extract	Percentage %	Concentration (µg/mL)
BuOH	34.7	200
Water	1.00	200
Acarbose	39.4	200

The isolated compounds from *A. lignosus* were also screened for their alpha-glucosidase inhibitory activity. Only compound **20** showed activity against alpha- glucosidase (42%), and the rest of the compounds yielded the lowest percentage inhibition (Table **6.5**).

Table 6. 5: **Inhibitory activity (%) of *A. lignosus* compounds against alpha-glucosidase enzyme**

Inhibitor/compound	Percentage %	Concentration µg/mL
19	NA	200
20	42.0	200
21	5.55	200
Acarbose	**38.7**	**200**

IC_{50} values of compound **20** as well as the IC_{50} of acarbose determined from corresponding dose-response curves of percentage inhibition versus inhibitor concentration. Compound **20** demonstrated alpha-glucosidase inhibitory activity IC_{50} value of 110.8 ± 2.52 µg/mL.

6.4.2 Alpha-amylase inhibition

The fractions of *A. lignosus* BuOH, and water, and isolated compounds were screened to determine their inhibition activity against alpha-amylase enzyme. The extracts and the compounds were tested at 0.2 mg/mL. The results for enzyme of both the extracts and the isolated compounds showed no inhibitory activity on the alpha-amylase. The results were negative and this could be either because the solvent enhances the activity of the enzyme or because the extinction coefficient of the product is higher when the solvent is present.

6.4.3 Mushroom L-tyrosinase assay

6.4.3.1 Anti-tyrosinase activity of various extracts and isolated compounds from *A. lignosus*

Inhibition of tyrosinase activity of BuOH and water extracts of *A. lignosus* was measured using L-tyrosine. BuOH extract exhibited moderate tyrosinase inhibitory activity at 0.2 mg/ml. It showed the highest anti-tyrosinase activity as compared to the water extract which showed the lowest values (**Figure 6.7**). The average inhibition percentage of the isolated compounds at 0.2 mg/mL was determined and it revealed that compound **20** had an inhibition of 55%. The rest of the other compounds showed not to be effective inhibitors of mushroom Tyrosinase with their inhibition percentage less than 5%. Compound **20** was then investigated for its IC_{50} value and showed IC_{50} values of 128.7 ± 2.12 µg/mL (**Figure:6.8**) and this value was in agreement with the reported value 130 µg/mL (Chen and Kubo, 2002).

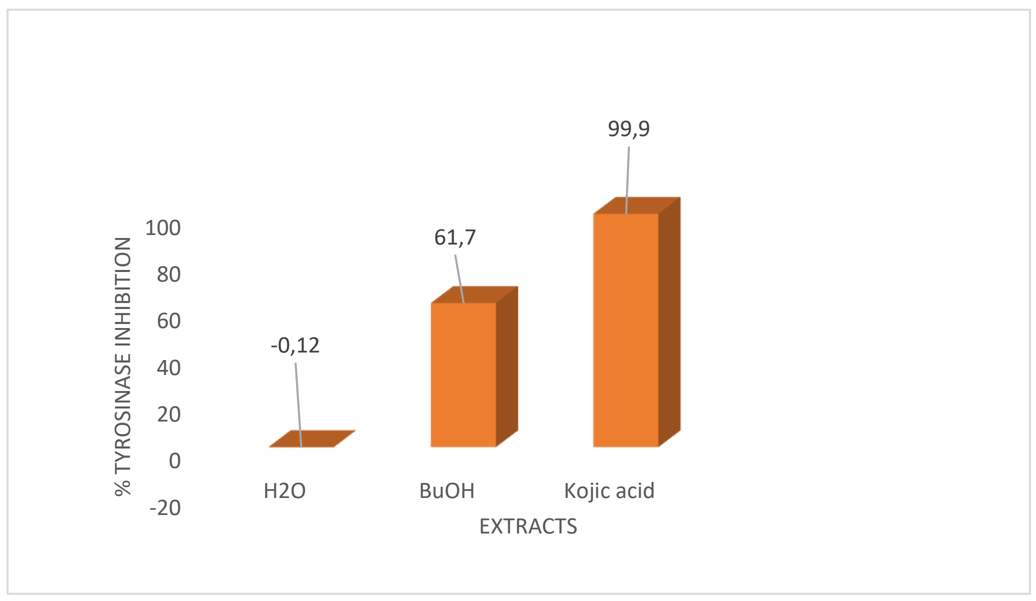


Figure 6. 7: % of Tyrosinase inhibition by A. lignosus extracts

Table 6. 6: **IC_{50} values of isolated compounds from BuOH extract of *A. lignosus***

	% inhibition(µg/mL)				
Tested Samples	**1000**	**500**	**250**	**125**	**IC_{50}**
19	ND	ND	ND	ND	-
20	74.1	68.2	51.2	25.3	128.9
21	ND	ND	ND	ND	-
Kojic Acid	99.8	98.9	98.5	98.21	3.1

ND: not active at the tested concentrations. The results are expressed as mean ±SEM for n =3
ND-: Not determined

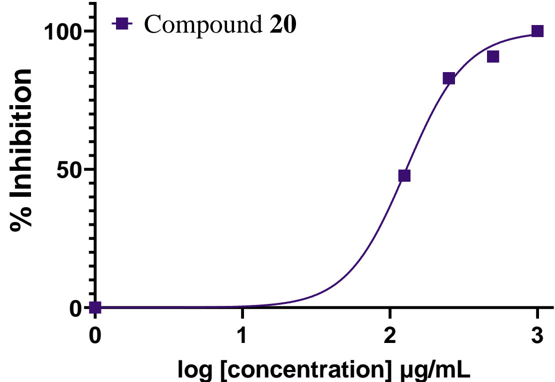


Figure 6. 8: IC_{50} plot of compound **20**

6.5 Conclusion

Investigation of the BuOH fraction of *Asparagus lignosus* afforded two known compounds, 20-hydroxyecdysone **(19)**, quercetin **(20)**, and isorhamnetin-3-*O*-rutinoside **(21)**. All isolated compounds are reported for the first time from *A. lignosus*; however, these compounds are common in the Asparagaceae family. The isolated compounds were assayed for their apha-glucosidase, alpha-amylase and tyrosinase activity and only compound **20** showed weak to moderate activity on both alpha-glucosidase and tyrosinase.

.

6.6 References

Abdel-Sattar, E., Abd El-Monem, A.R. and Sleem, A.A., 2009. Biological and chemical study of *Cleome paradoxa* B. Br. *Pharmacognosy Research*, *1*(4).

Afifi, M.S., Salama, O.M., Gohar, A.A. and Marzouk, A.M., 2006. Iridoids with antimicrobial activity from *Plumeria alba* L. *Bulletin of Pharmaceutical Sciences. Assiut*, *29*(1), pp.215-223.

Alhozaimy, G.A., Al-Sheddi, E.S. and Ibrahim, T.A., 2017. Biological activity and isolation of compounds from stem bark of *Plumeria acutifolia*. *Pharmacognosy magazine*, *13*(Suppl 3), p.S505.

Anthony, M.P., Burrows, J.N., Duparc, S., JMoehrle, J. and Wells, T.N., 2012. The global pipeline of new medicines for the control and elimination of malaria. *Malaria journal*, *11*(1), pp.1–25.

Barros, L., Dueñas, M., Ferreira, I.C., Carvalho, A.M. and Santos-Buelga, C., 2011. Use of HPLC–DAD–ESI/MS to profile phenolic compounds in edible wild greens from Portugal. *Food Chemistry*, *127*(1), pp.169-173.

Bathori, M., Mathe, I., Girault, J.P., Kalasz, H. and Lafont, R., 1998. Isolation and structural elucidation of two plant ecdysteroids, gerardiasterone and 22-*epi*-20-hydroxyecdysone. *Journal of Natural Products*, *61*(3), pp.415-417.

Calzada, F., Cedillo-Rivera, R. and Mata, R., 2001. Antiprotozoal Activity of the Constituents of Conyza f ilaginoides. *Journal of Natural Products*, *64*(5), pp.671-673.

Chabani, S., Haba, H., Lavaud, C., Benkhaled, M. and Harakat, D., 2013. Flavonoid glycosides and triterpenoids from *Atractylis flava*. *Phytochemistry Letters*, *6*(1), pp.9-13.

Chen, Q.X. and Kubo, I., 2002. Kinetics of mushroom tyrosinase inhibition by quercetin. *Journal of Agricultural and Food Chemistry*, *50*(14), pp.4108-4112.

David, A.V.A., Arulmoli, R. and Parasuraman, S., 2016. Overviews of biological importance of quercetin: A bioactive flavonoid. *Pharmacognosy reviews*, *10*(20), p.84.

Dhasan, P., Jegadeesan, M. and Kavimani, S., 2008. Cucurbitacins isolated from the fruits of *Momordica cymbalaria* Hook f. *Pharmacognosy Magazine*, *4*(14), p.96.

Girault, J.P. and Lafont, R.D., 1988. The complete 1H-NMR assignment of ecdysone and 20-hydroxyecdysone. *Journal of insect physiology*, *34*(7), pp.701-706.

Grubben, G.J.H. and Denton, O.A., 2004. Plant resources of tropical Africa 2. Vegetables. *Plant resources of tropical Africa 2. Vegetables.*

Hsu, C.C., Dobberstein, R.H., Bingel, A.S., Fong, H.H.S., Farnsworth, N.R. and Morton, J.F., 1981. Biological and phytochemical investigation of plants XVI: *Strumpfia maritima* (Rubiaceae). *Journal of Pharmaceutical Sciences*, *70*(6), pp.682-683.

Isenmann, E., Ambrosio, G., Joseph, J.F., Mazzarino, M., de la Torre, X., Zimmer, P., Kazlauskas, R., Goebel, C., Botrè, F., Diel, P. and Parr, M.K., 2019. Ecdysteroids as non-conventional anabolic agent: performance enhancement by ecdysterone supplementation in humans. *Archives of Toxicology*, *93*(7), pp.1807-1816.

Khaliq-uz-Zaman, S.M., Simin, K. and Ahmad, V.U., 2000. Chemical constituents from *Asparagus dumosus*. *Fitoterapia*, *71*(3), pp.331-333.

Lamson DW, Brignall MS. 2000. Antioxidants and cancer, part 3: Quercetin. alternative *Med Rev*. 5: pp 196–208.

Mamadalieva, N.Z., El-Readi, M.Z., Janibekov, A.A., Tahrani, A. and Wink, M., 2011. Phytoecdysteroids of *Silene guntensis* and their in vitro cytotoxic and antioxidant activity. *Zeitschrift für Naturforschung C*, *66*(5-6), pp.215-224.

Massi, A., Bortolini, O., Ragno, D., Bernardi, T., Sacchetti, G., Tacchini, M. and De Risi, C., 2017. Research progress in the modification of quercetin leading to anticancer agents. *Molecules*, *22*(8), p.1270.

Olennikov, D.N., Tankhaeva, L.M., Partilkhaev, V.V. and Rokhin, A.V., 2012. Chemical constituents of *Caragana bungei* shoots. *Revista Brasileira de Farmacognosia*, *22*, pp.490-496.

Strobel P, Allard C, Perez-Acle T, Calderon R, Aldunate R, Leighton F. 2005. Myricetin, Myricetin, quercetin and catechin-gallate inhibit glucose uptake in isolated rat adipocytes. *Biochemistry Journal*. 386(Pt 3): pp 471–478.

Thuy, T.T., Tam, N.T., Anh, N.T.H., Hau, D.V., Phong, D.T., Adorisio, S., Van Sung, T. and Delfino, D.V., 2017. 20-Hydroxyecdysone from *Dacrycarpus imbricatus* bark inhibits the proliferation of acute myeloid leukemia cells. *Asian Pacific journal of tropical medicine*, *10*(2), pp.157-159.

Vokáč, K., Buděšnský, M., Harmatha, J. and Kohoutová, J., 1998. Ecdysteroid constituents of the mushroom *Tapinella panuoides*. *Phytochemistry*, *49*(7), pp.2109-2114.

Yang JY, Della-Fera MA, Rayalam S, Ambati S, Hartzell DL, Park HJ, et al. 2008. Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin. Life Science. 82: pp 1032–1039.

Yeskaliyeva, B., Mesaik, M.A., Abbaskhan, A., Kulsoom, A., Burasheva, G.S., Abilov, Z.A. and Choudhary, M.I., 2006. Bioactive flavonoids and saponins from *Climacoptera obtusifolia*. *Phytochemistry*, *67*(21), pp.2392-2397.

Znati, M., Ben Jannet, H., Cazaux, S., Souchard, J.P., Harzallah Skhiri, F. and Bouajila, J., 2014. Antioxidant, 5-lipoxygenase inhibitory and cytotoxic activities of compounds isolated from the *Ferula lutea* flowers. *Molecules*, *19*(10), pp.16959-16975.

CHAPTER 7

CONCLUSION AND RECOMMENDATIONS

Plants have been used since time immemorial to provide humans with food and medicine. The various ways that humans utilise plants are complex and dynamic (Heinrich et al., 2006). Phytochemical and pharmacological evaluation of plants has led to the elucidation of novel structures to manufacture new drugs (Freund and Hegeman, 2020). Ethnobotanical surveys provide a platform to identify potential plants for further investigation as well as plants which could possibly be at risk of becoming extinct. As a result, documentation of medicinal plants isimperative.

In screening several plant species from an inventory of common medicinal plants from South Africa for diverse medicinal purposes, 3 plants were selected based on their interesting and useful ethnomedicinal values. These plants are *Asparagus lignosus*, *Cliffortia odorata* and *Protea cynaroides*. The extractions were carried out using hexane, dichloromethane, ethyl acetate and, butanol. Fractionation was carried out using standard chromatographic techniques. A total of twenty-one compounds were isolated from the three plants namely *Asparagus lignosus*, *Cliffortia odorata* and *Protea cynaroides*. The isolates were characterised and their structures were unambiguously established by spectroscopic methods including 1D and 2D NMR, FTIR, HRMS and UV spectroscopy.

The phytochemical investigation of *Protea cynaroides* afforded eight compounds, from which **2**, **5**, **7** and **8** were reported for the first time from a natural source, while **6** was isolated for the first time from *Protea cynaroides*. Bio-evaluation against tyrosinase showed strong inhibitory activities for compound **4** and **6** with IC_{50} values of 0.8776 ± 0.2 and 0.7771 ± 0.09 µg/mL respectively while **1** and **3** demonstrated weak activity and only compound **1** showed activity towards alpha-glucosidase with an IC_{50} value of 162.52 ± 1.21. All

compounds showed no activity on alpha-amylase. This is the first scientific report on the phytochemical isolation and bio-evaluation of alpha-glucosidase, alpha-amylase and tyrosinase inhibitory activities of *Protea cynaroides*.

The phytochemical investigation of *Cliffortia odorata* afforded ten compounds, from which **9-18** were reported for the first time from *Protea cynaroides*. Bio-evaluation against alpha-glucosidase showed strong inhibitory activities for compound **16** with an IC_{50} value of and **6** with IC_{50} values of 3.8 ± 0.32 µg/mL and **15** with an IC_{50} value of 16.6 ± 1.02 µg/mL. No activity was detected on alpha-amylase for all the compounds while on the other hand **11** and **12** demonstrated weak antityrosinase inhibitory activities with the IC_{50} values of 131.9 ±1.05 µg/mL and 231.8 ±1.26 µg/mL. This is the first scientific report on the phytochemical isolation and bio-evaluation of alpha glucosidase, alpha amylase and tyrosinase inhibitory activities of *Cliffortia odorata*.

The phytochemical investigation of *Asparagus lignosus* led to the isolation of three known compounds, **19-21** which were reported for the first time from *Asparagus lignosus*. The isolated compounds were assayed for their alpha-glucosidase, alpha-amylase and tyrosinase activity and only compound **20** showed weak to moderate activity on both alpha-glucosidase and tyrosinase, with the IC_{50} values of 110.8 ± 2.52 µg/mL and 128.9 ± 2.12 µg/mL respectively. This is the first scientific report on the phytochemical isolation and bio-evaluation of alpha glucosidase, alpha amylase and tyrosinase inhibitory activities of *Asparagus lignosus*.

The enzyme inhibitory effects indicated by the plants extracts and compounds isolated afford these plants an opportunity to be used as agents for antidiabetic management and in cosmetic and pharmaceutical industries further biological and clinical investigation. To a certain degree, the study validated some of the ethnomedicinal uses of the plants.

This study has therefore contributed to the body of knowledge relating to the phytochemistry of some South African medicinal plants in particular and the chemistry and biological activities of natural products in general.

Recommendations

A reasonable amount of plant material of the examined plant species must be used in future, so to be able to obtain the available constituents in the plant of interest in adequate amounts; which in return will allow further relative biological studies on such isolated constituents.

Since some of the isolated compounds are novel, and their biological activities have not been completely explored, there is a need to further bio-evaluation against different biological targets.

Since some of the isolated compounds are active on the biological activity of interest, a rapid search to verify their structural integrity and binding effect to targets of the alpha-glucosidase and tyrosinase activity through molecular docking may be of importance.

Reference

Eljounaidi, K. and Lichman, B.R., 2020. Nature's chemists: the discovery and engineering of phytochemical biosynthesis. *Frontiers in Chemistry*, *8*, p.1041.

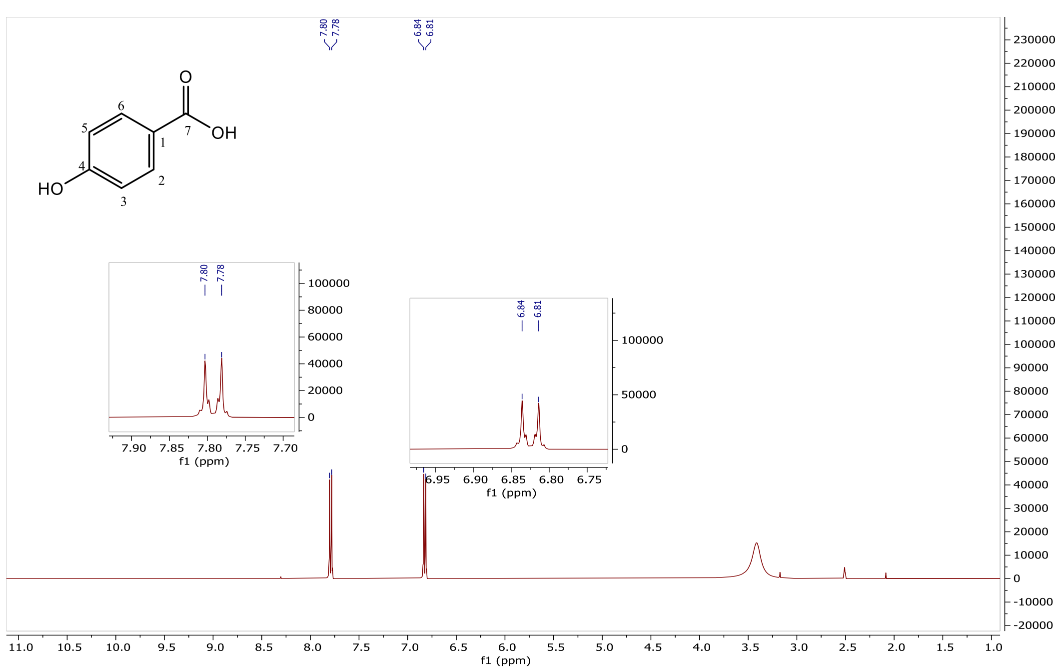
Geyid, A., Abebe, D., Debella, A., Makonnen, Z., Aberra, F., Teka, F., Kebede, T., Urga, K., Yersaw, K., Biza, T. and Mariam, B.H., 2005. Screening of some medicinal plants of Ethiopia for their anti-microbial properties and chemical profiles. *Journal of ethnopharmacology*, *97*(3), pp.421-427.

Heinrich, M., Kufer, J., Leonti, M. and Pardo-de-Santayana, M., 2006. Ethnobotany and ethnopharmacology—Interdisciplinary links with the historical sciences. *Journal of ethnopharmacology*, *107*(2), pp.157-160

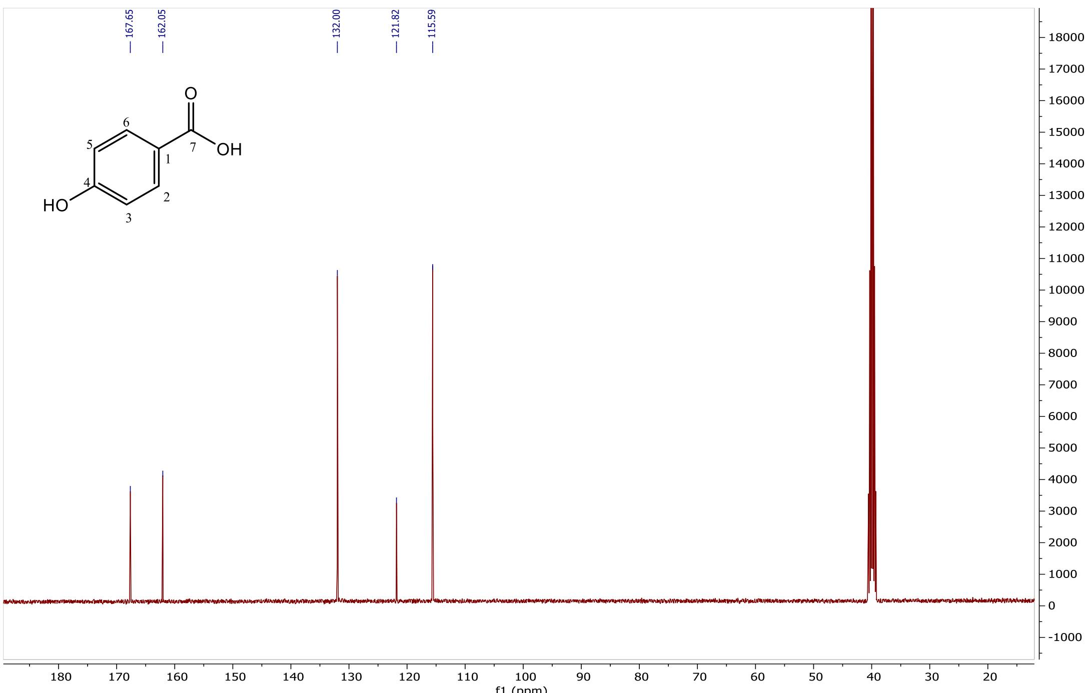
Appendix 1A

NMR spectra of isolated compounds from *Protea cynaroides*

Compound **1**

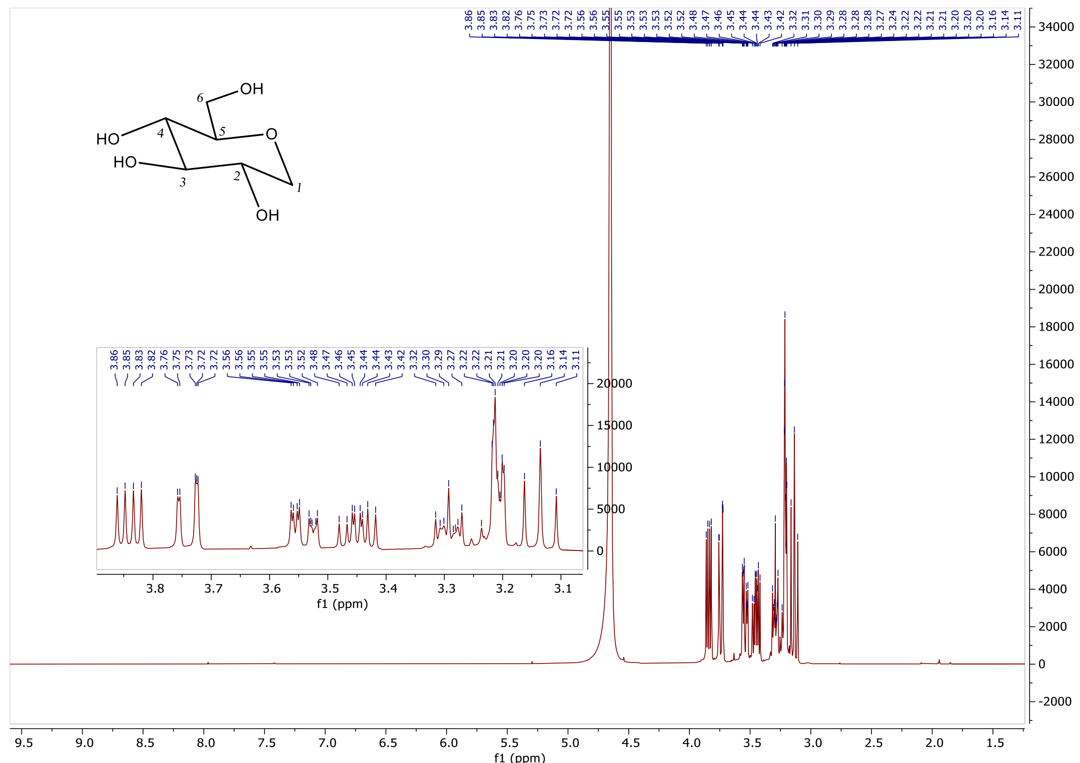


^{1}H NMR spectrum of compound **1**

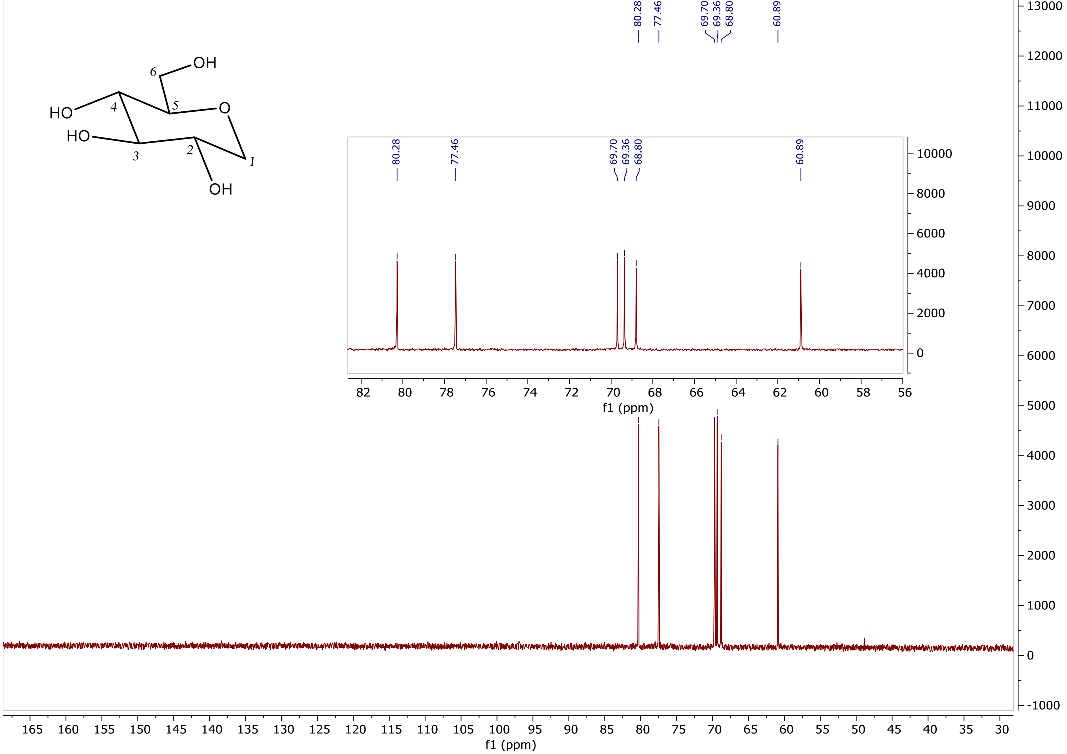


^{13}C NMR spectrum of compound **1**

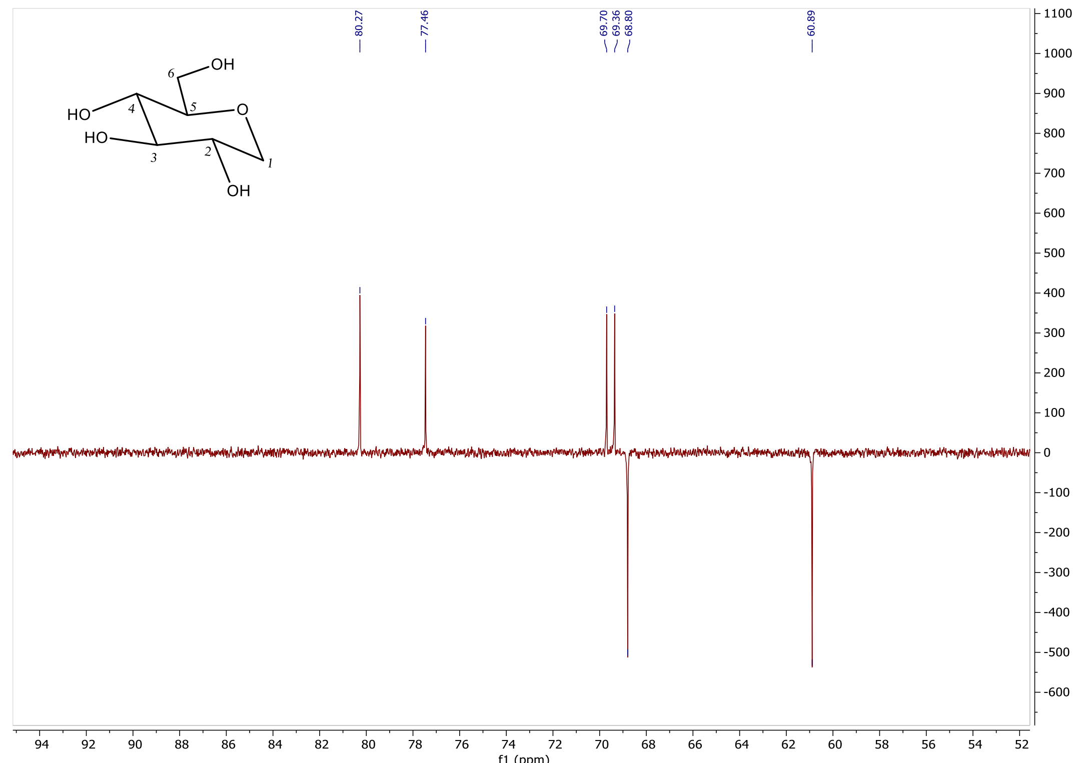
Compound **3**



^{1}H NMR spectrum of compound **3**

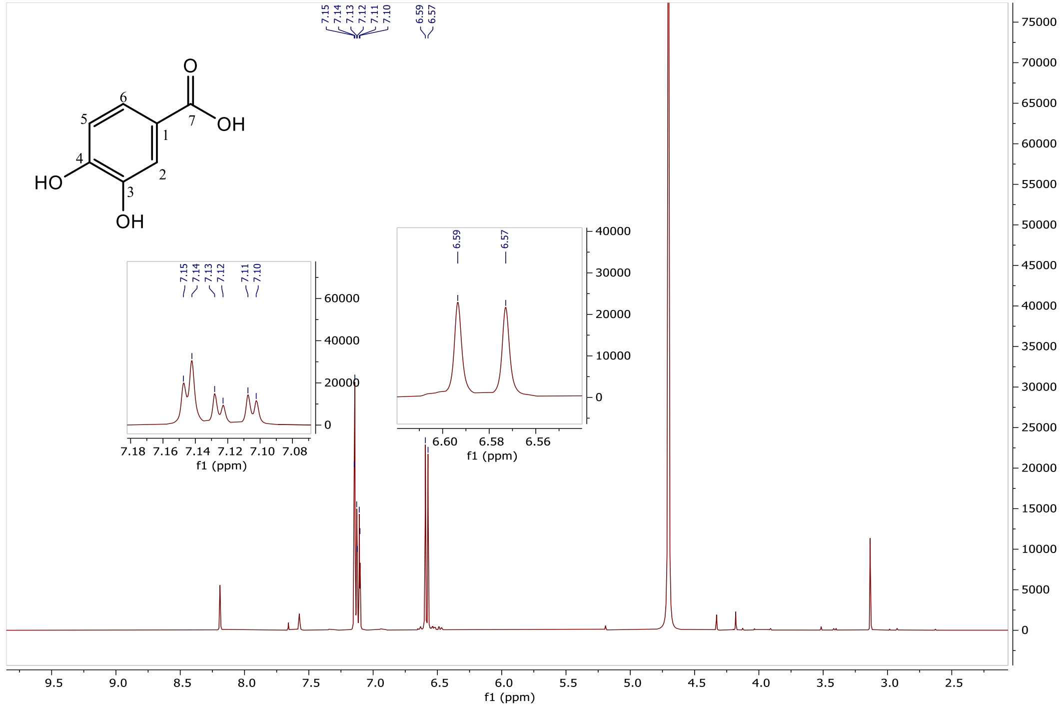


^{13}C NMR spectrum of compound **3**

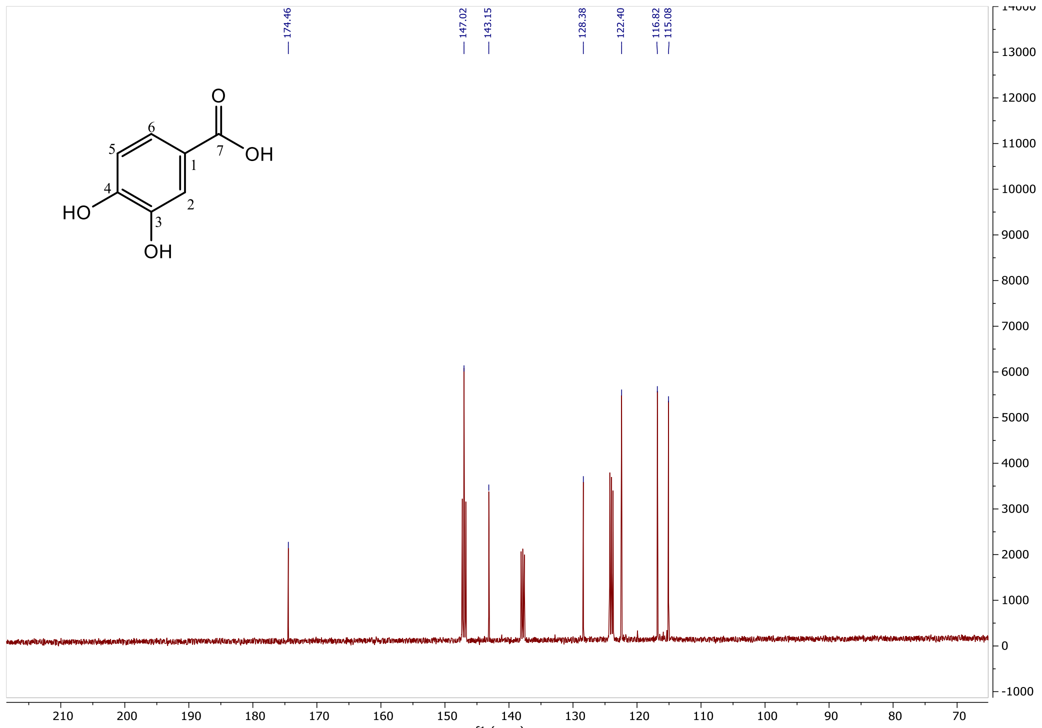


DEPT-135 NMR spectrum of compound **3**

Compound **4**



^{1}H NMR spectrum of compound **4**

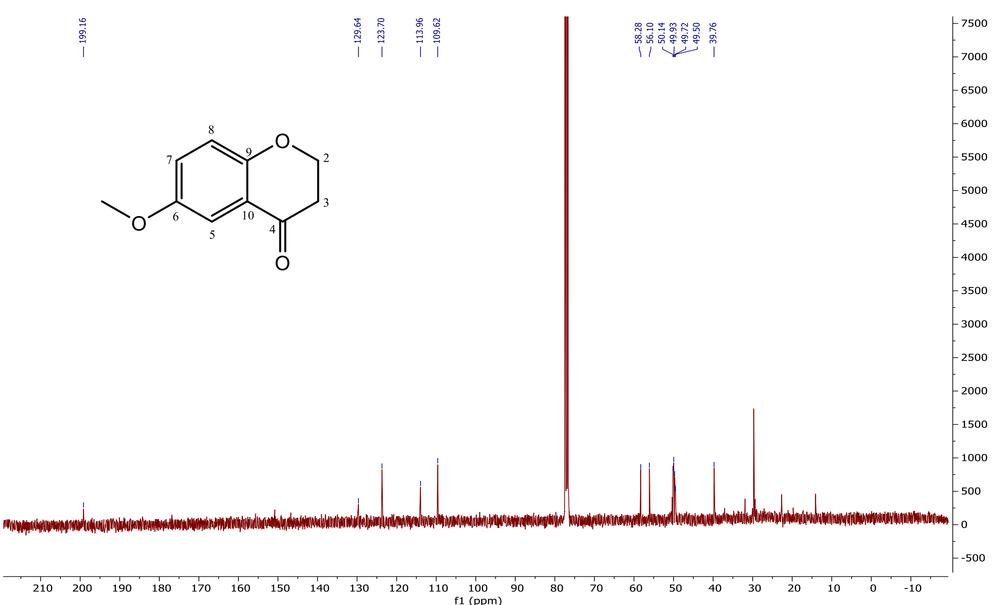


^{13}C NMR spectrum of compound **4**

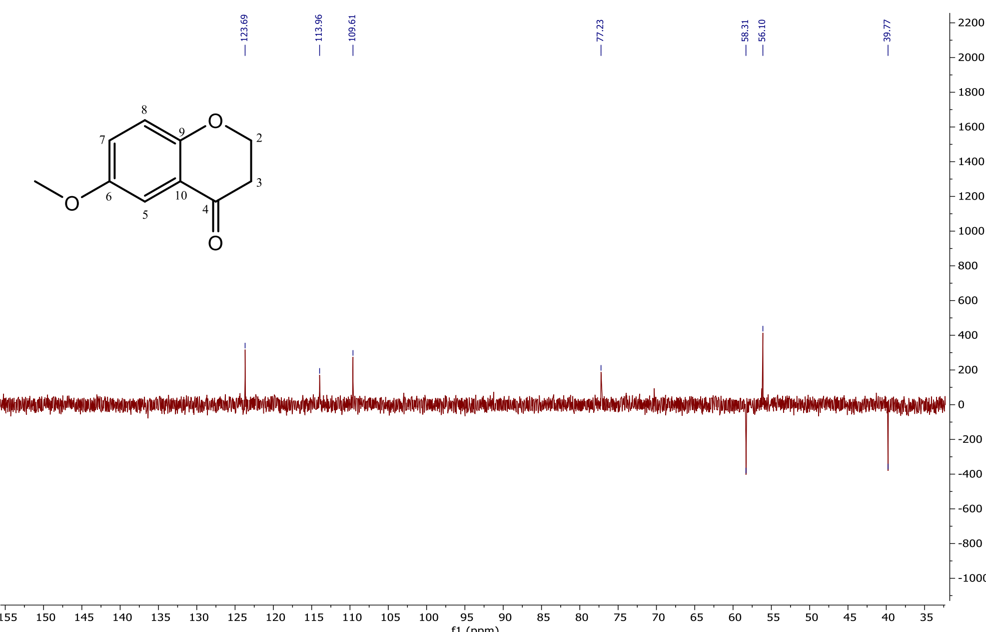
Appendix 1B

NMR spectra of isolated compounds from *Cliffortia odorata*

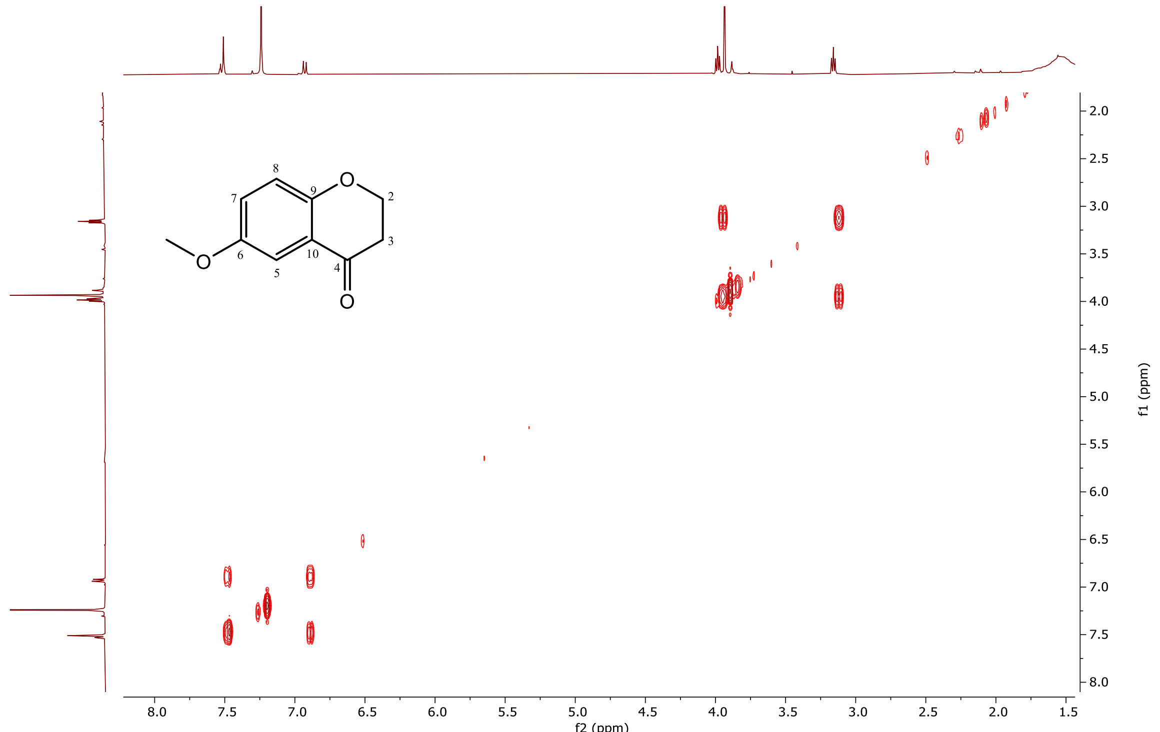
Compound **9**



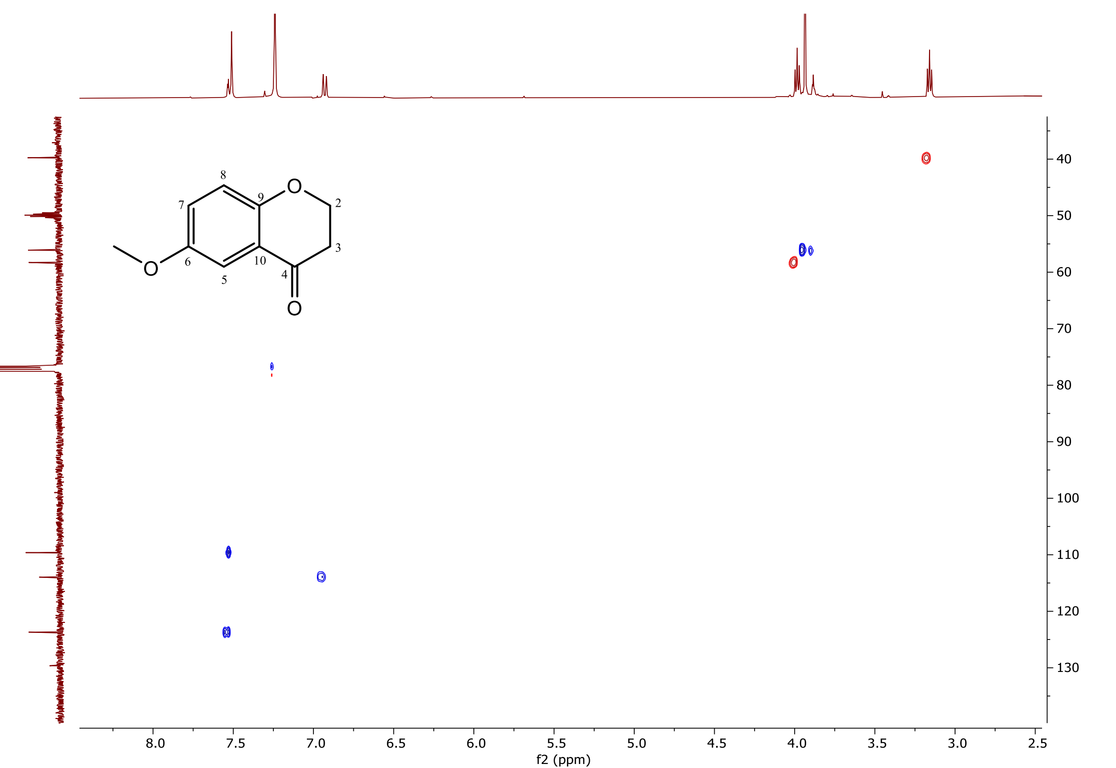
^{13}C NMR spectrum of compound **9**



DEPT-135 NMR spectrum of compound **9**

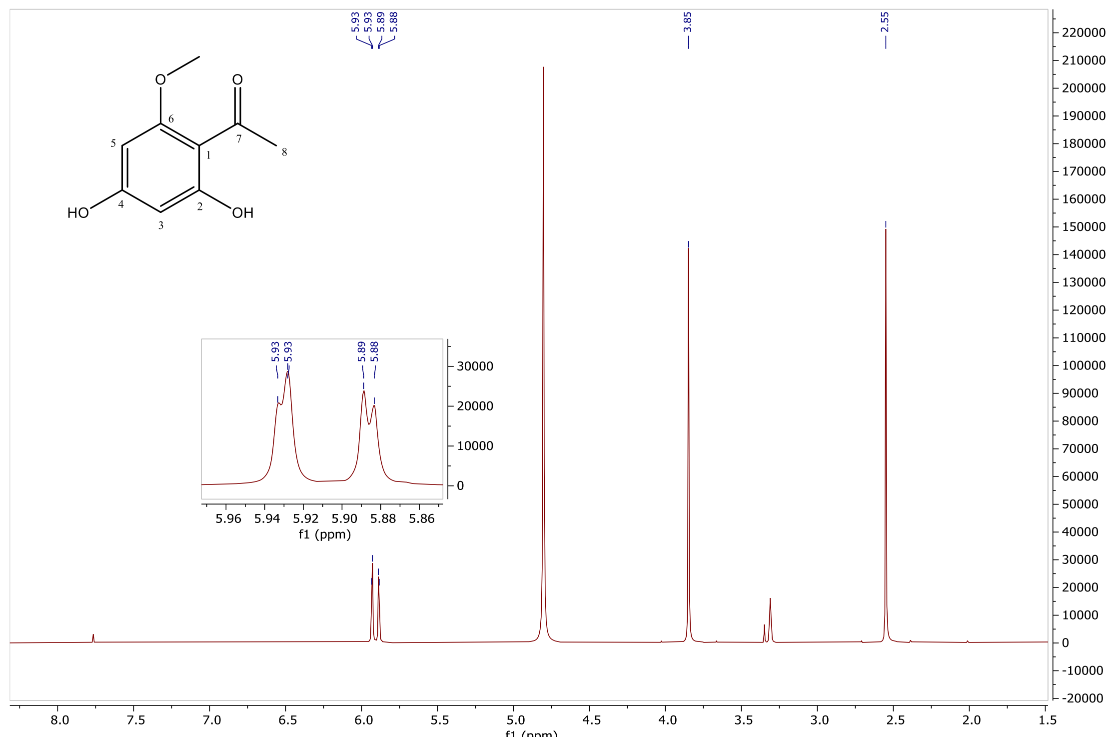


^{1}H-^{1}H COSY NMR spectra of compound **9**

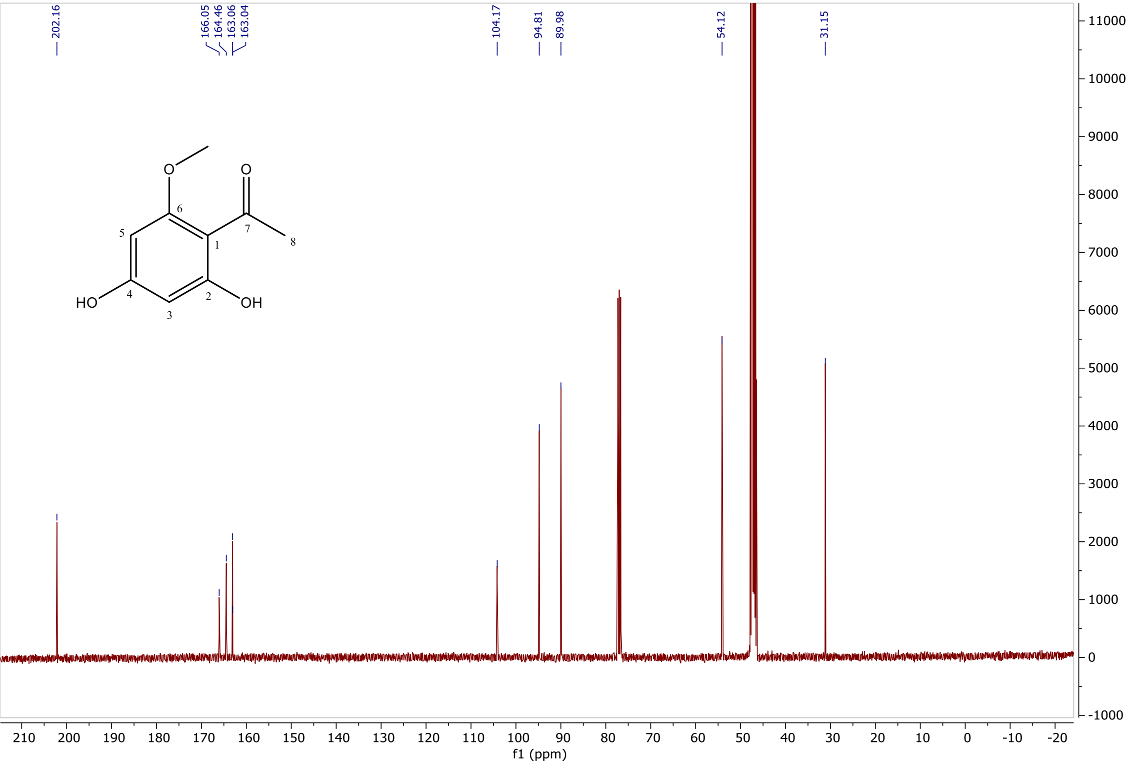


HSQC NMR spectra of compound **9**

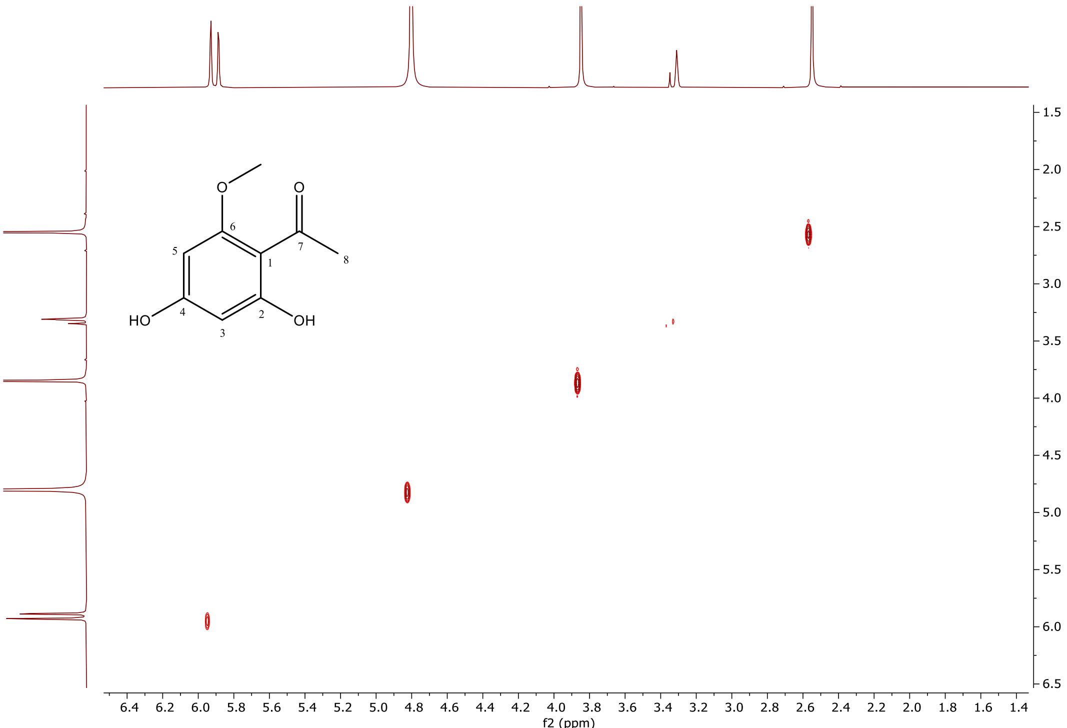
Compound **10**



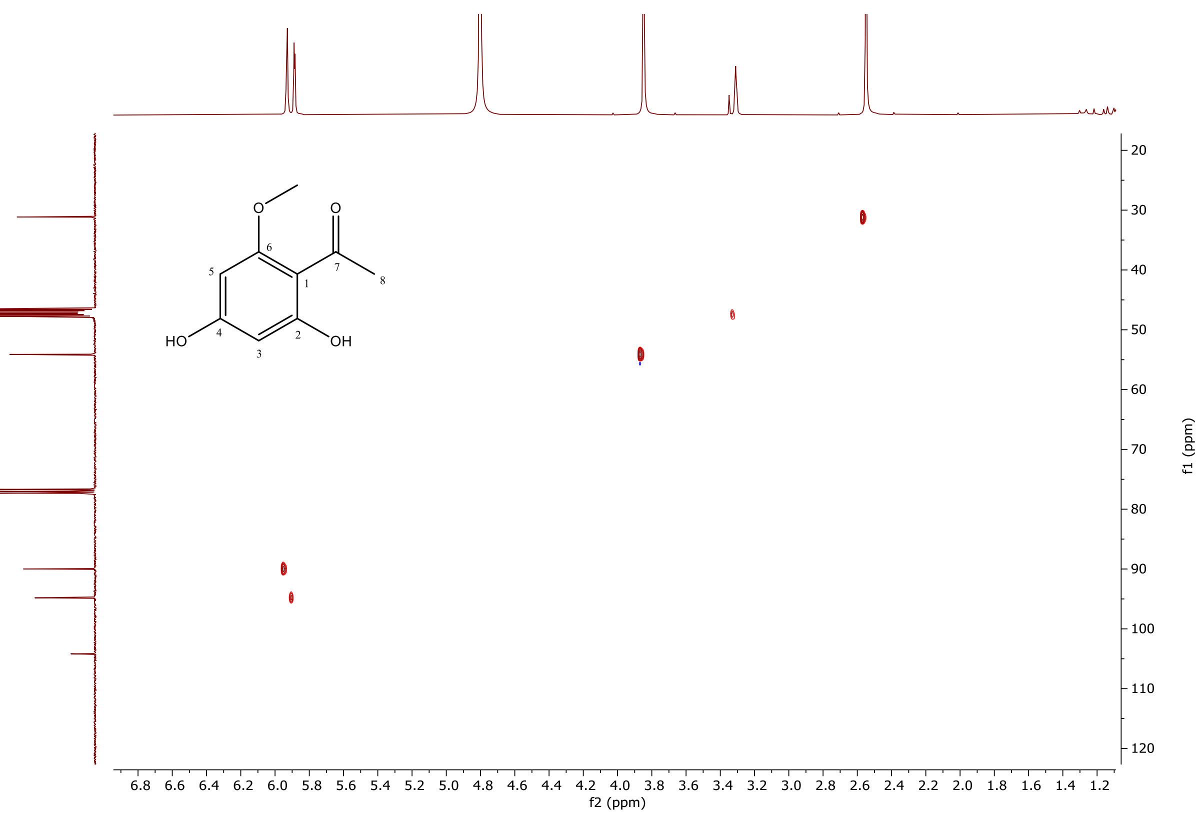
^{1}H NMR spectrum of compound **10**



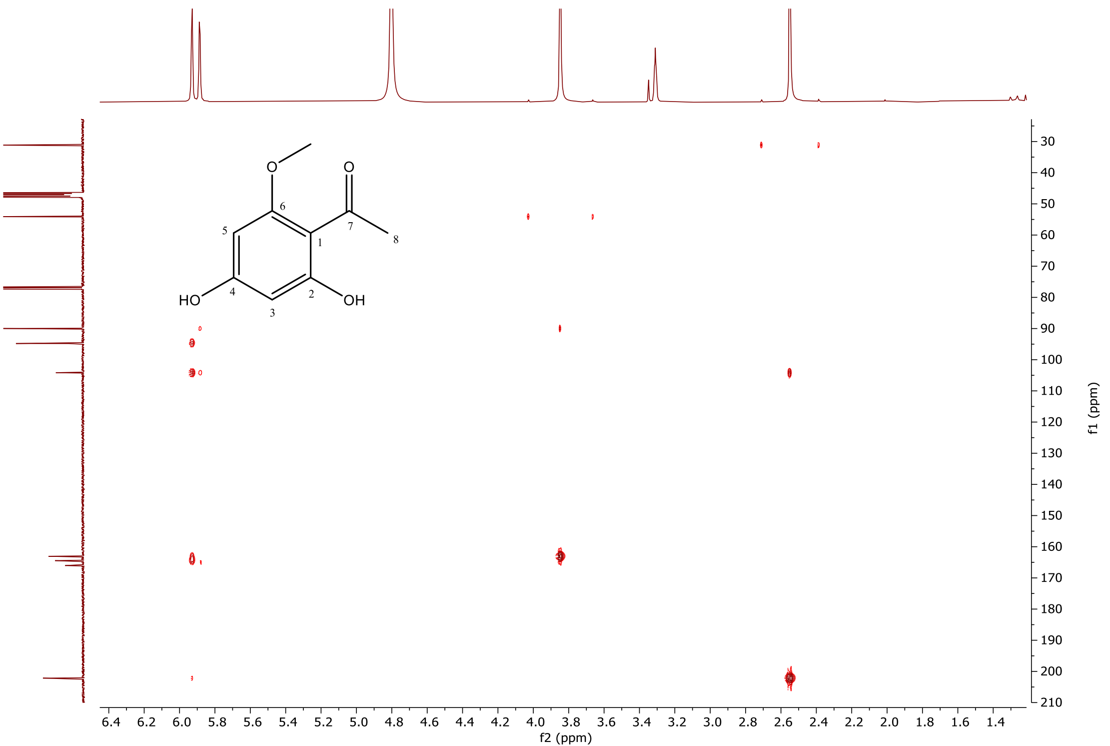
^{13}C NMR spectrum of compound **10**



^{1}H-^{1}H COSY NMR spectra of compound **10**

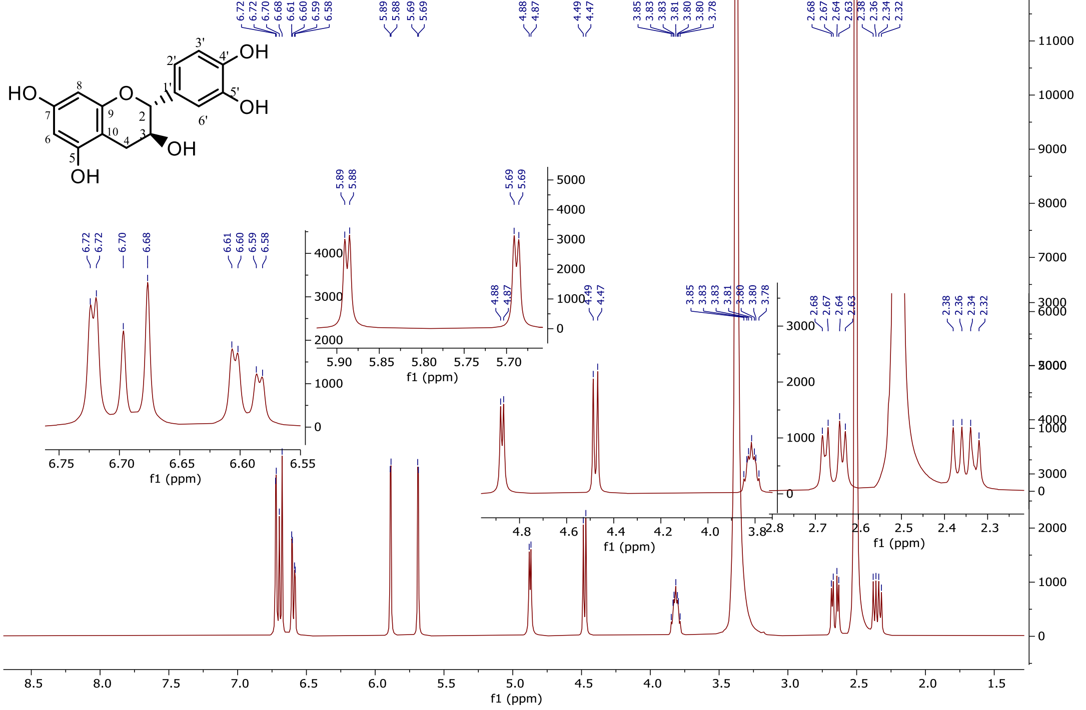


HSQC NMR spectra of compound **10**

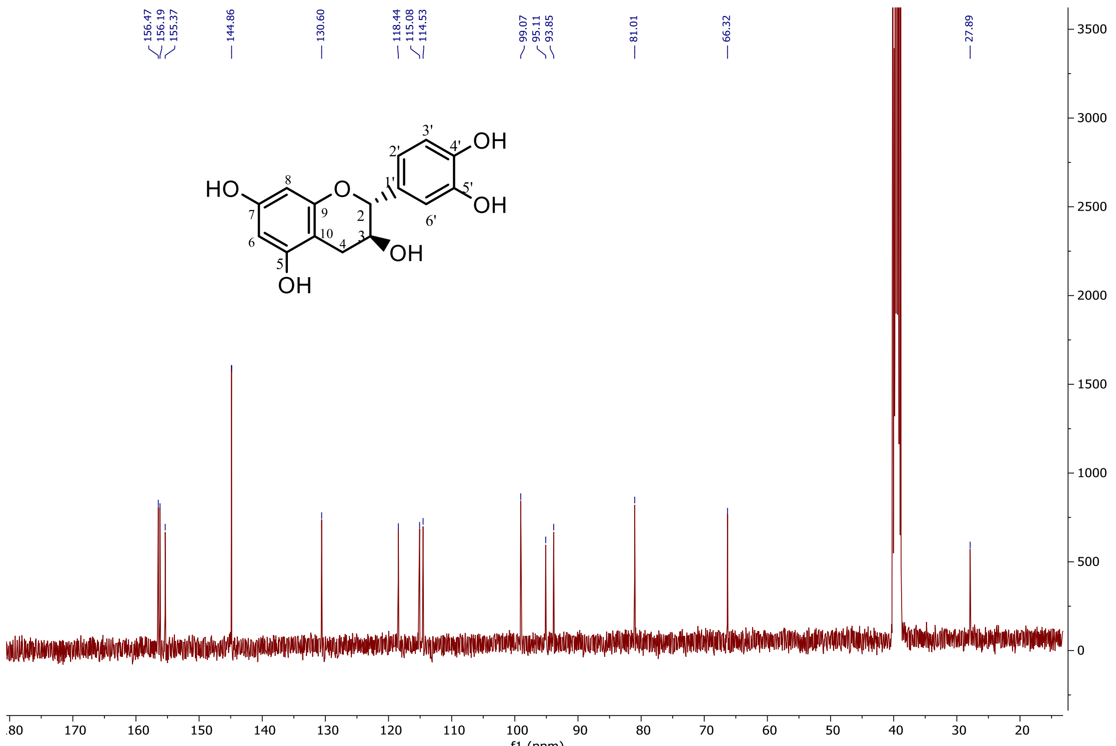


HMBC NMR spectra of compound **10**

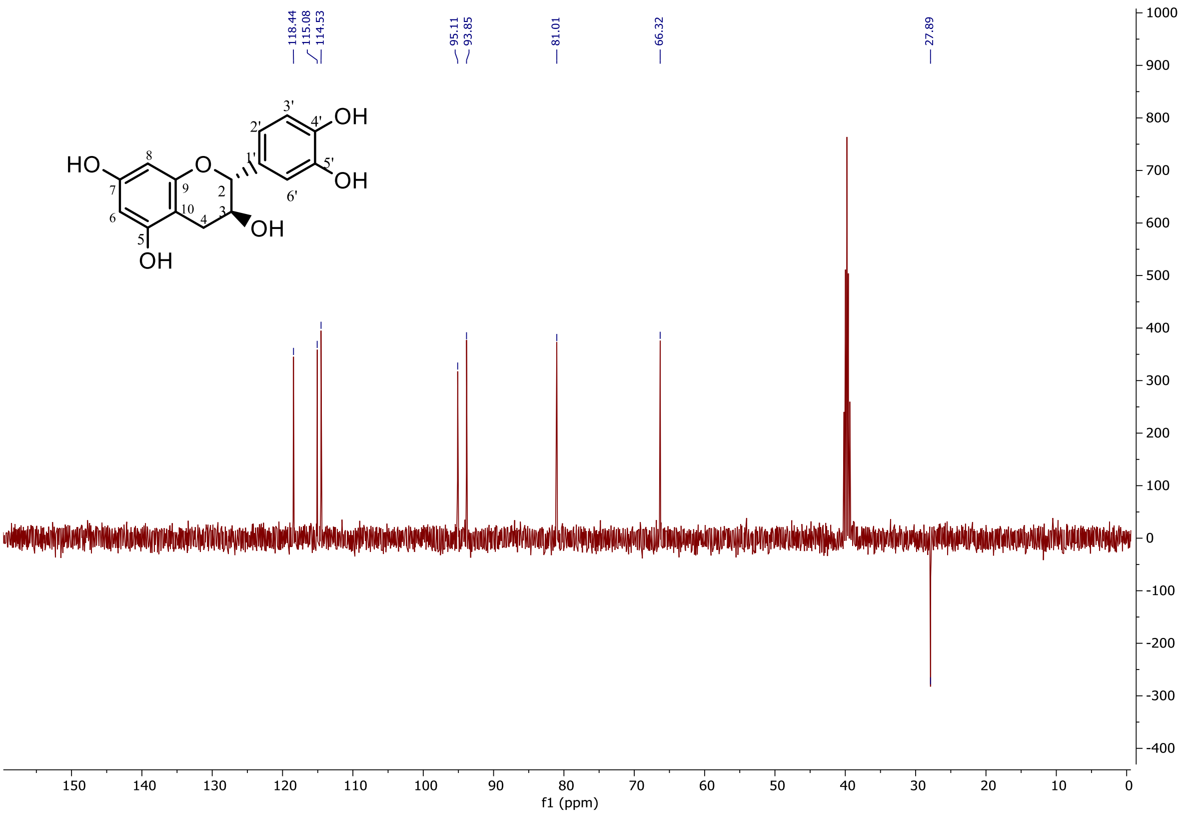
Compound **11**



^{1}H NMR spectrum of compound **11**

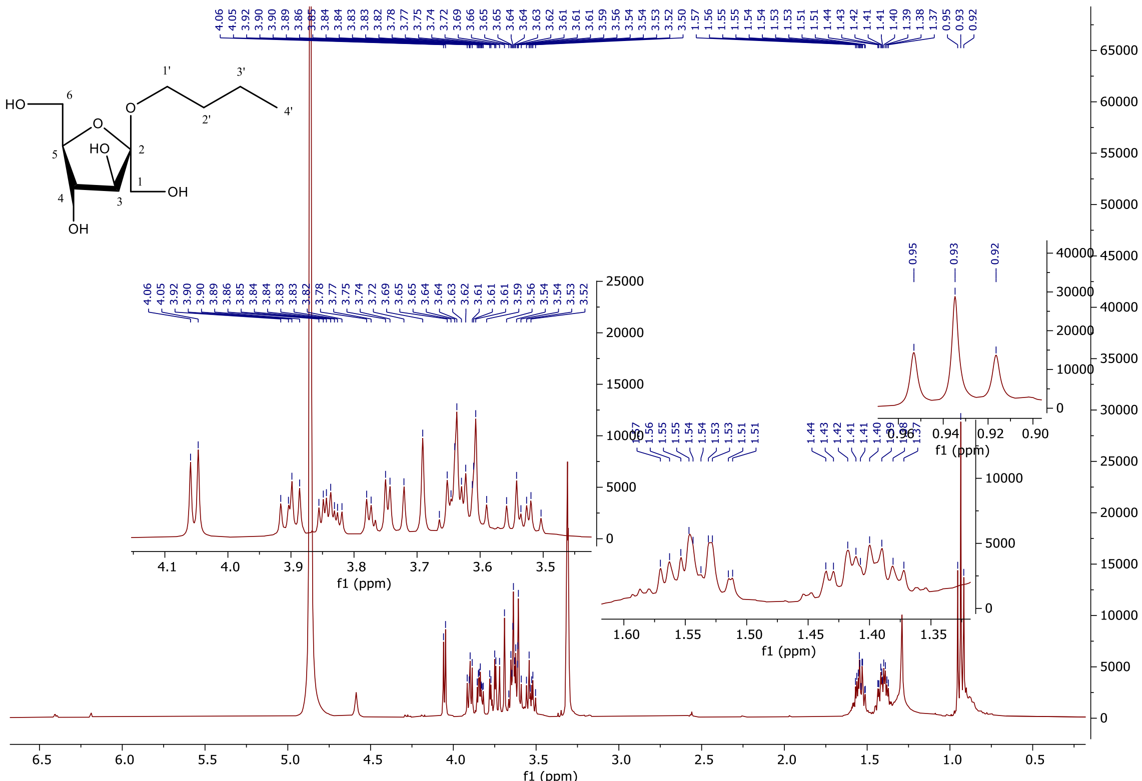


^{13}C NMR spectrum of compound **11**

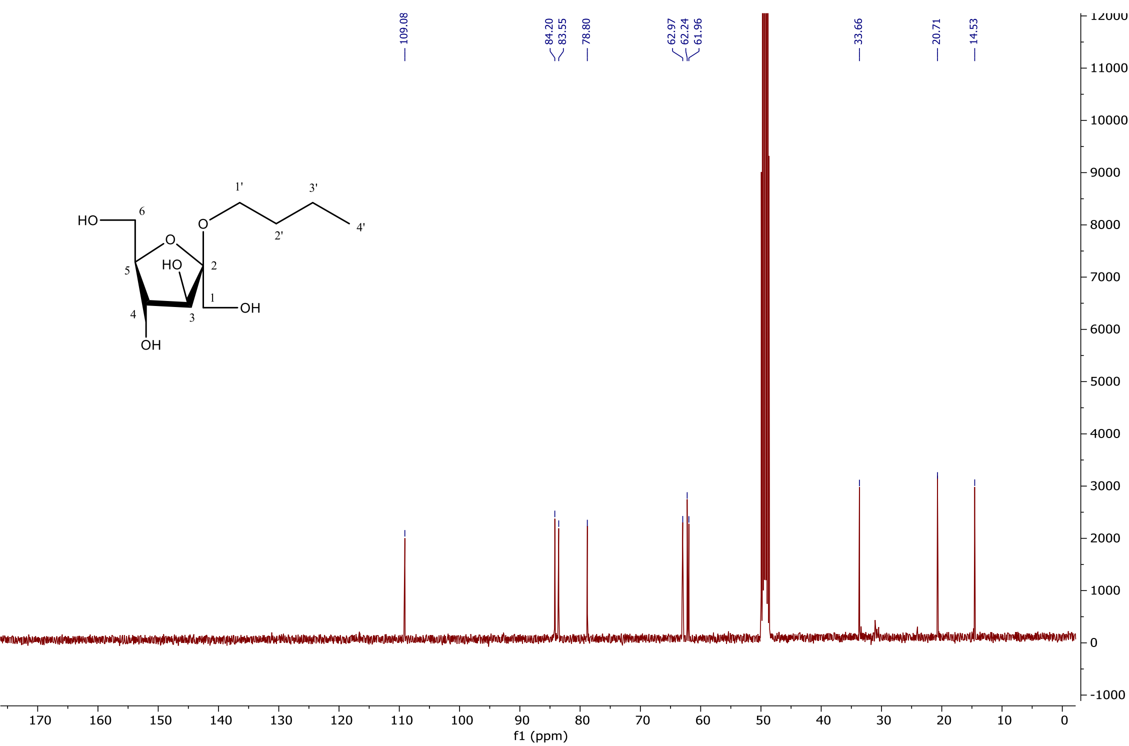


DEPT-135 NMR spectrum of compound **11**

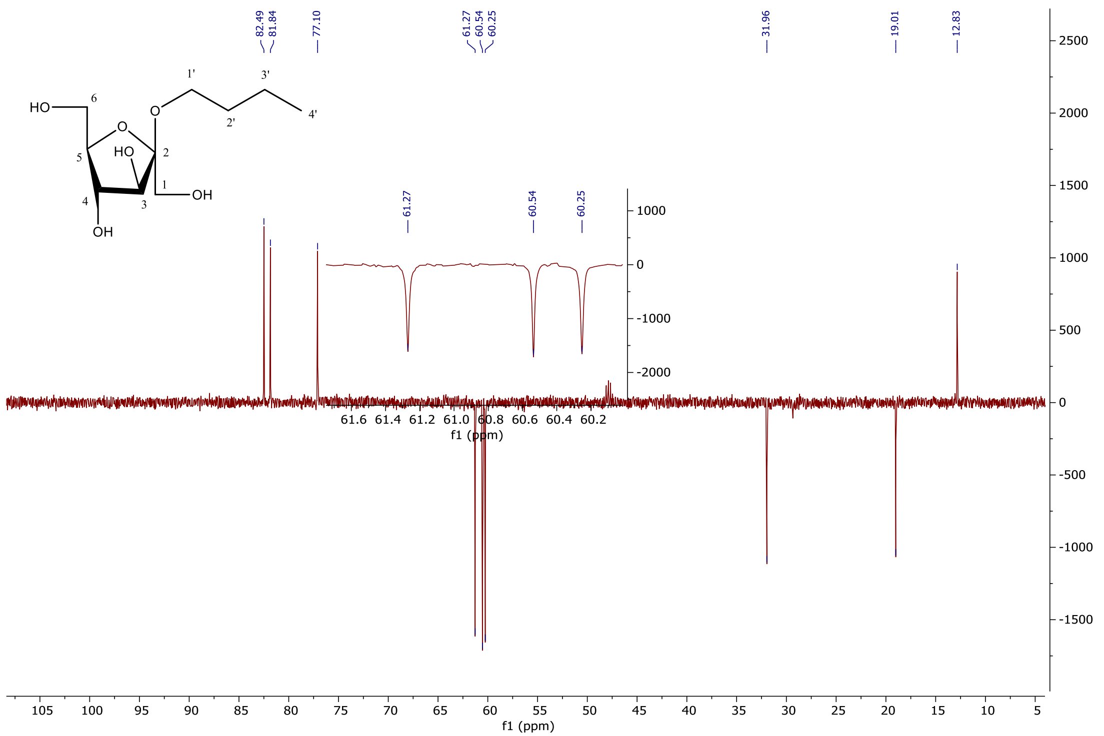
Compound **12**



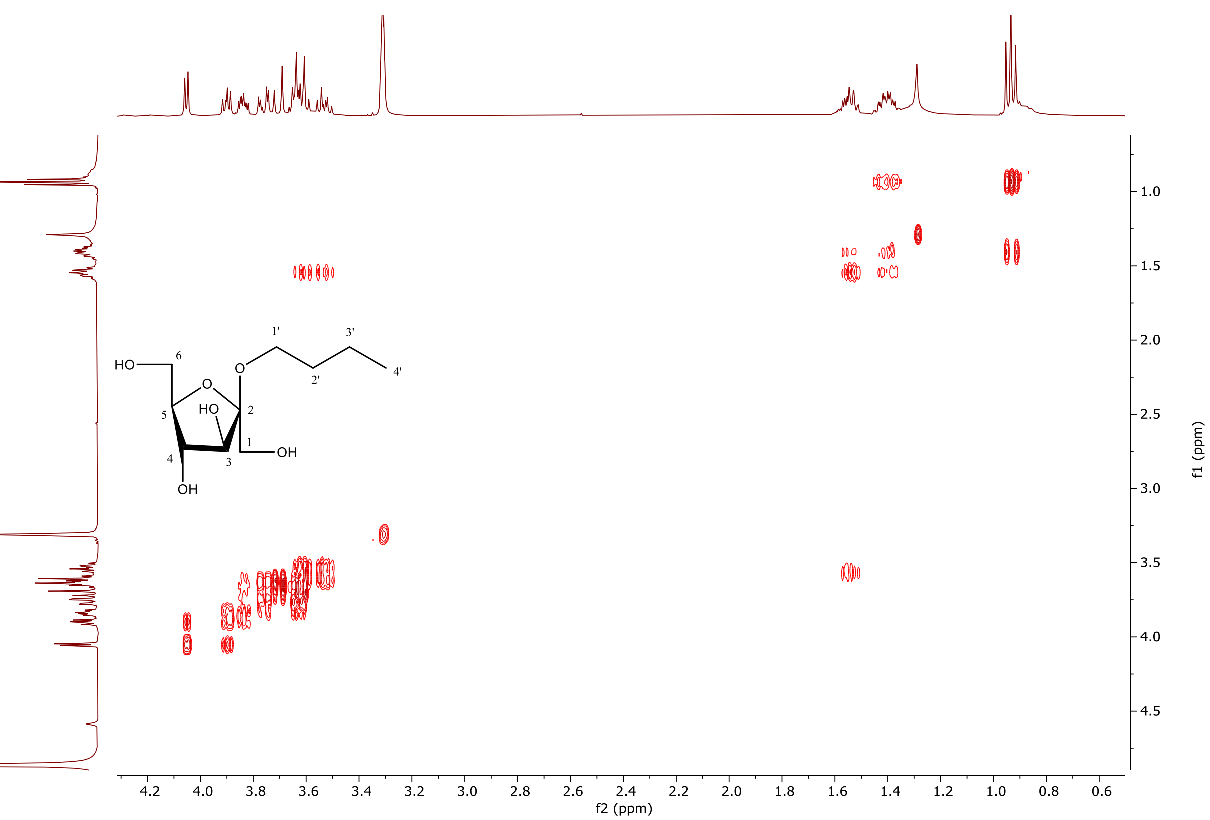
^{1}H NMR spectrum of compound **12**



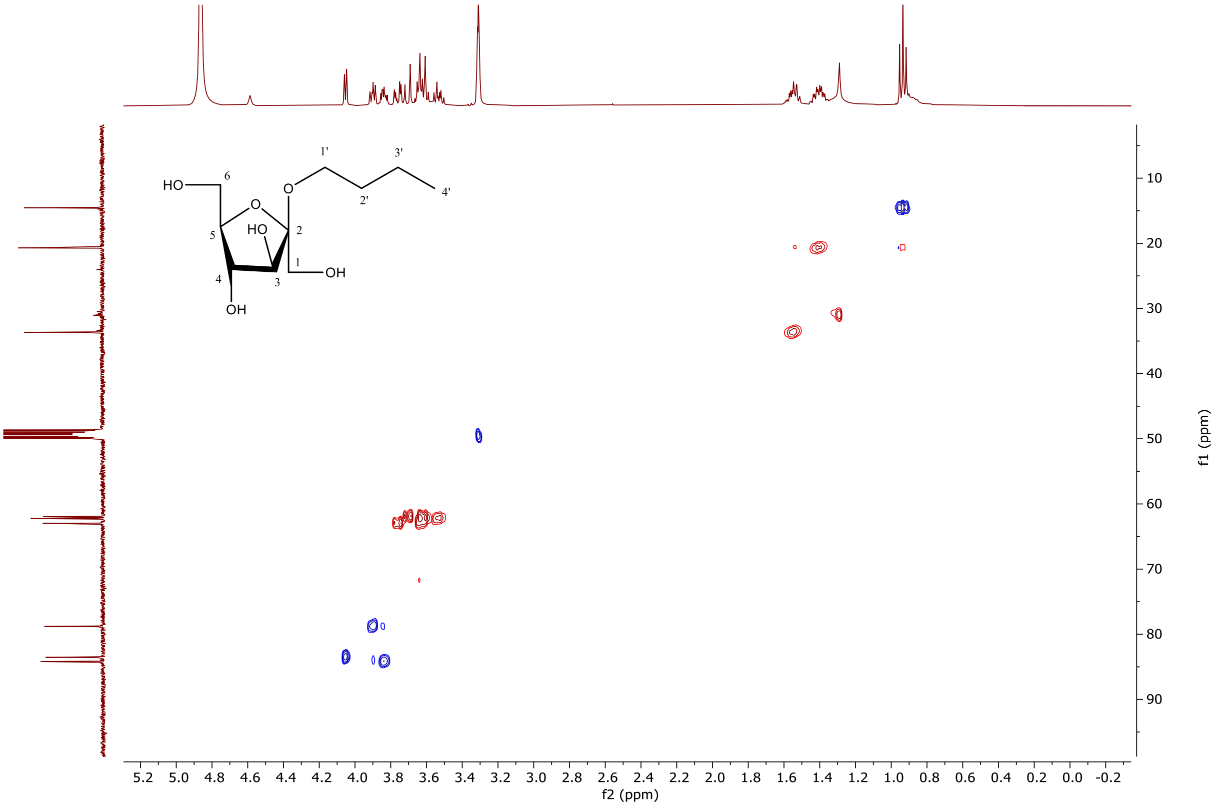
^{13}C NMR spectrum of compound **12**



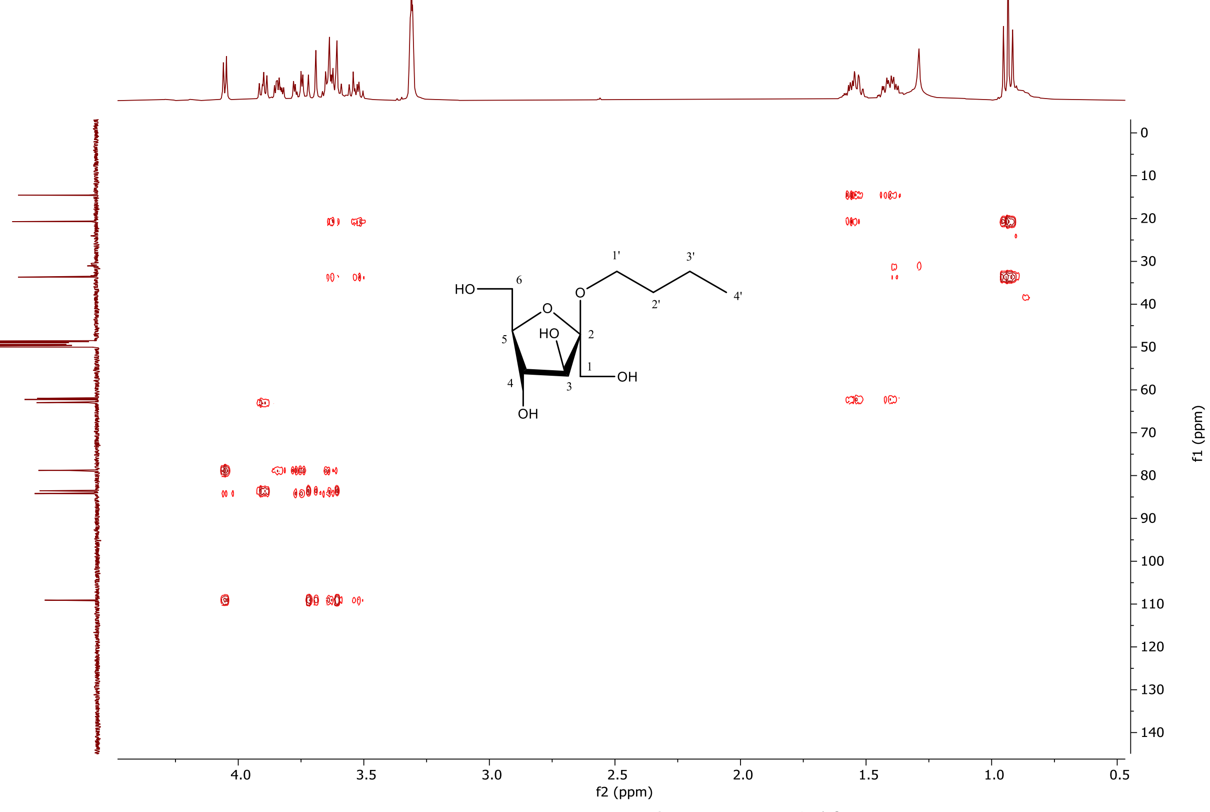
DEPT-135 NMR spectrum of compound **12**



^{1}H-^{1}H COSY NMR spectra of compound **12**

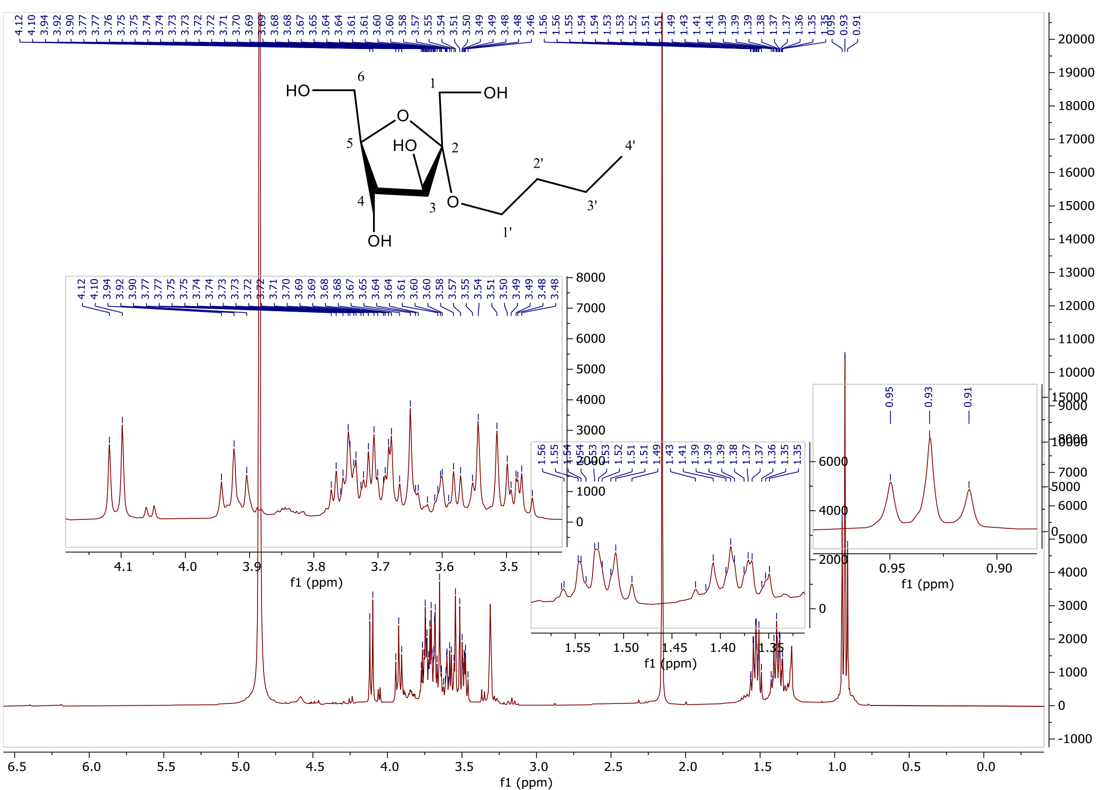


HSQC NMR spectra of compound **12**

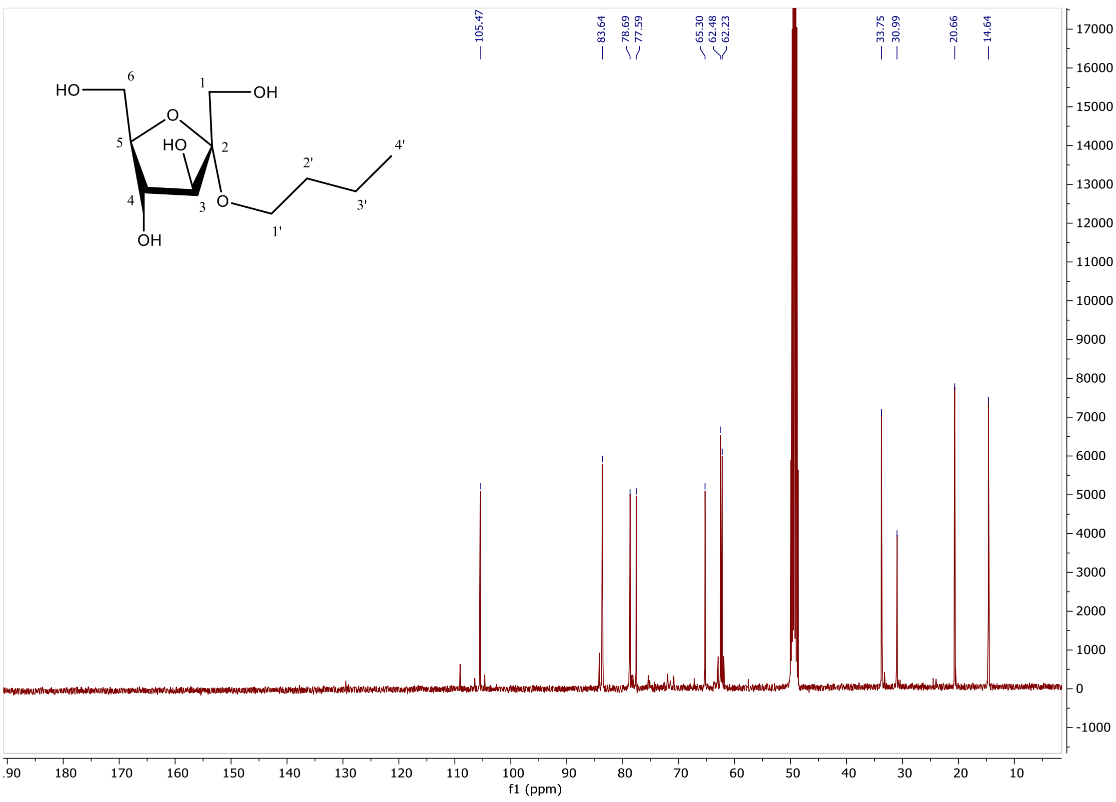


HMBC NMR spectra of compound **12**

Compound **13**

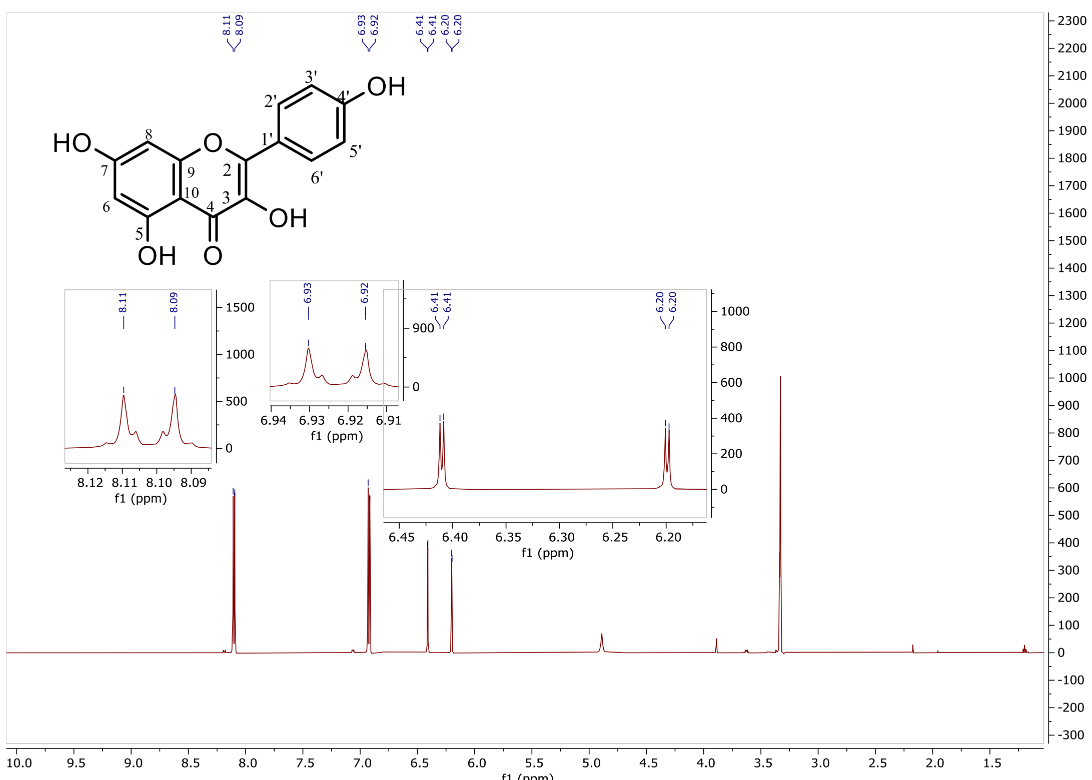


^{1}H NMR spectrum of compound **13**

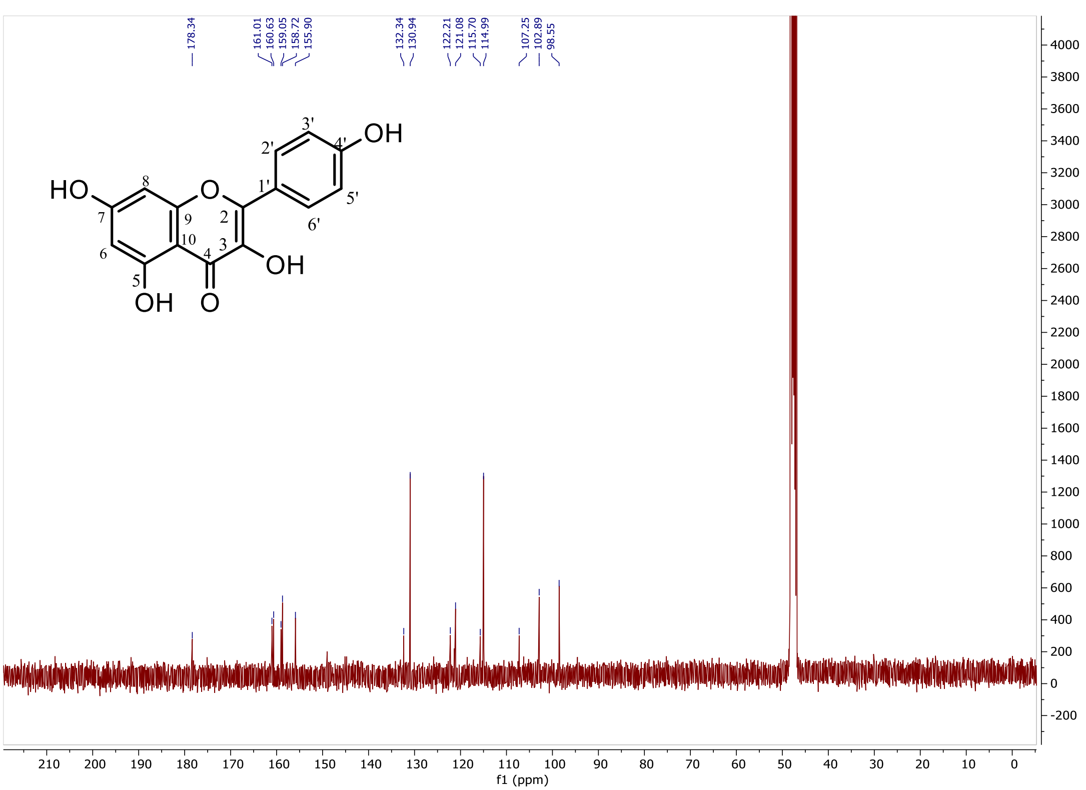


^{13}C NMR spectrum of compound **13**

Compound **15**

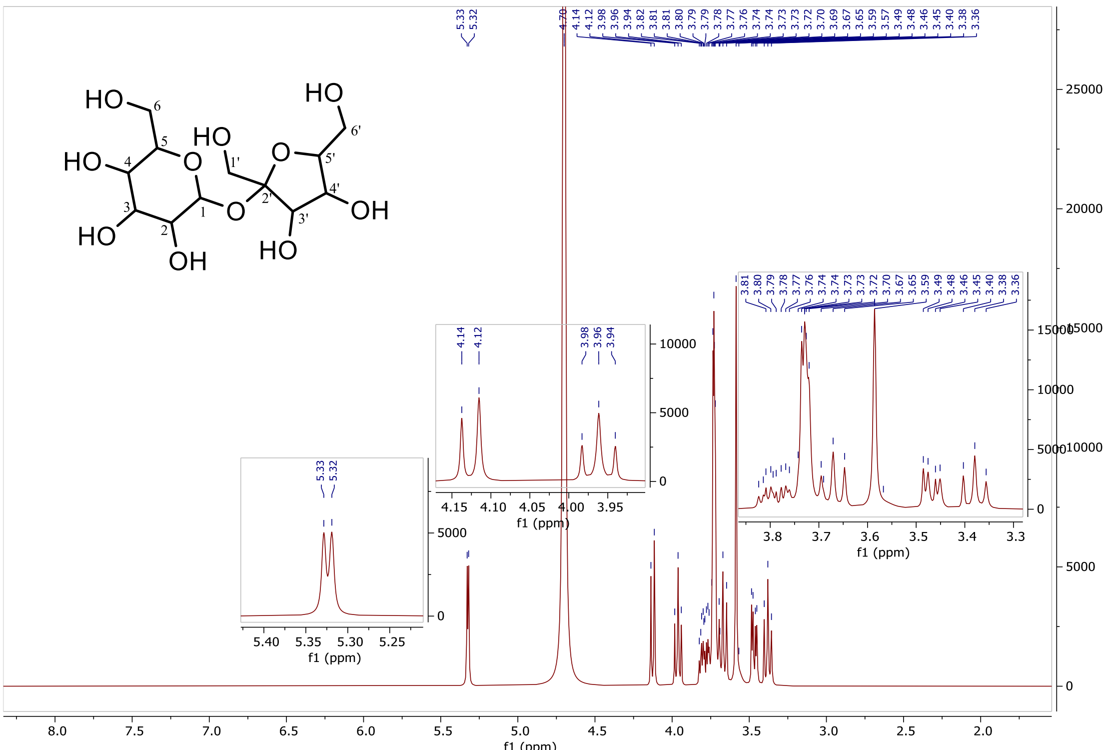


^{1}H NMR spectrum of compound **15**

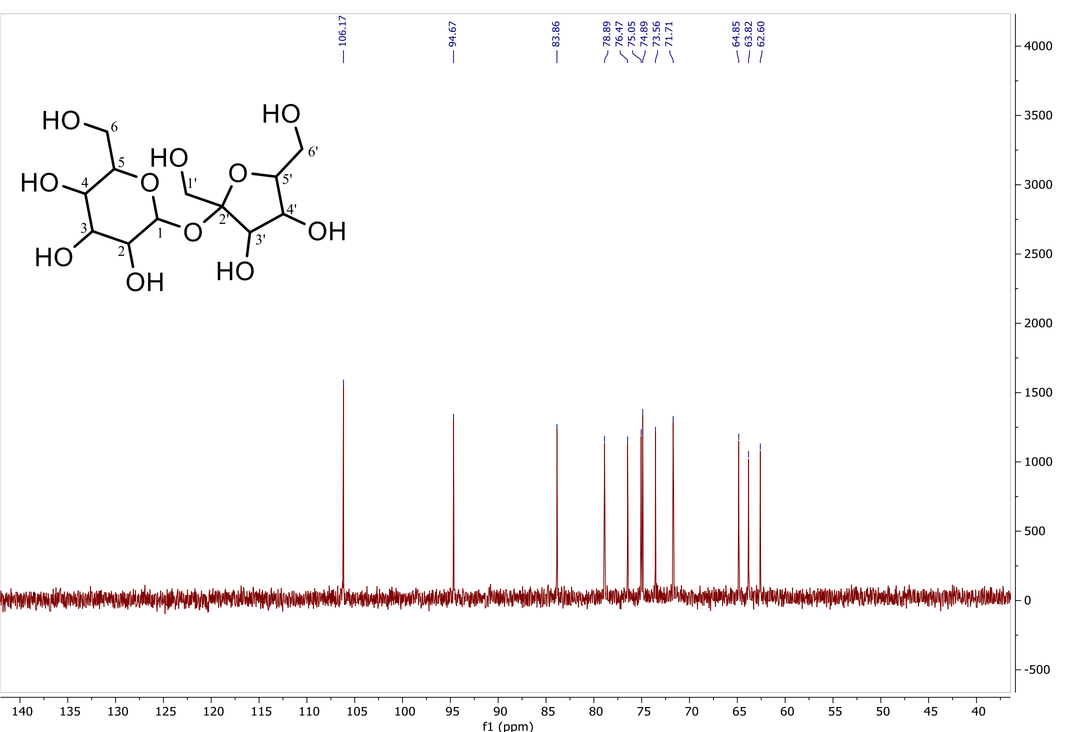


^{13}C NMR spectrum of compound **15**

Compound **17**



^{1}H NMR spectrum of compound **17**

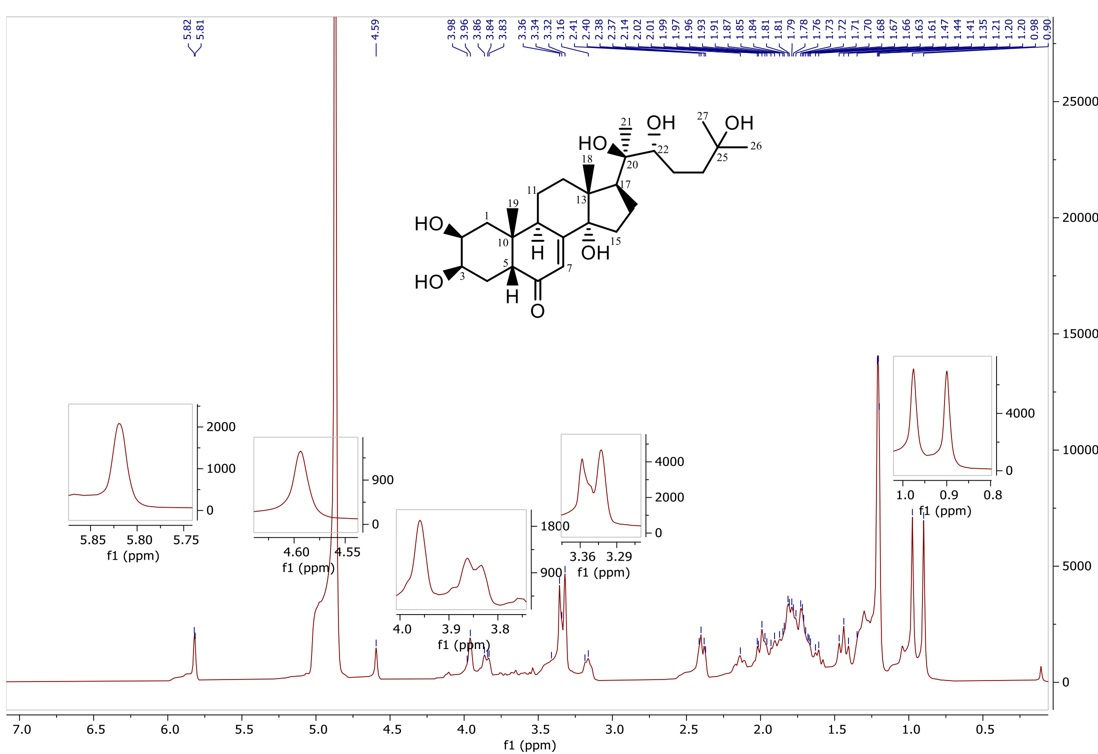


^{13}C NMR spectrum of compound **17**

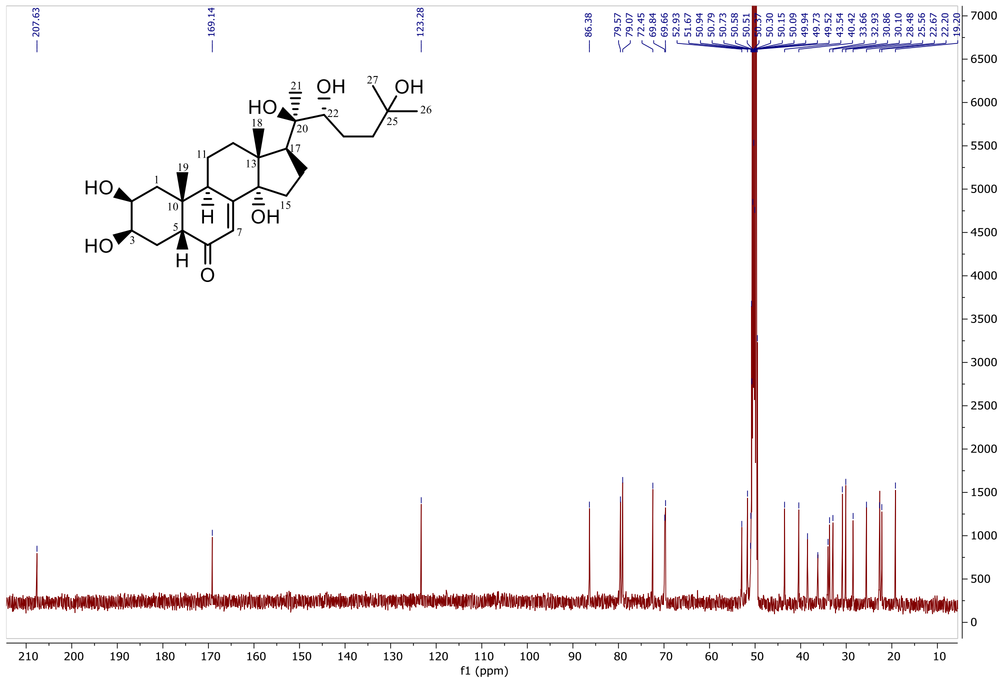
Appendix 1C

NMR spectra of isolated compounds from *Asparagus lignosus*

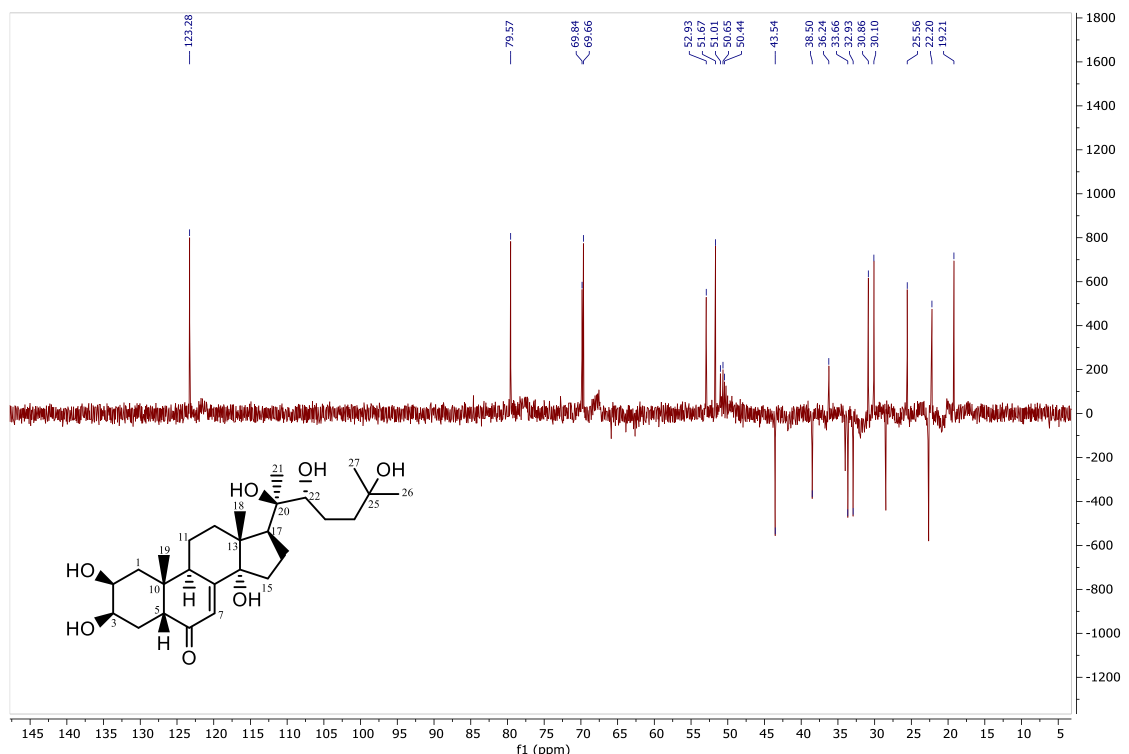
Compound **19**



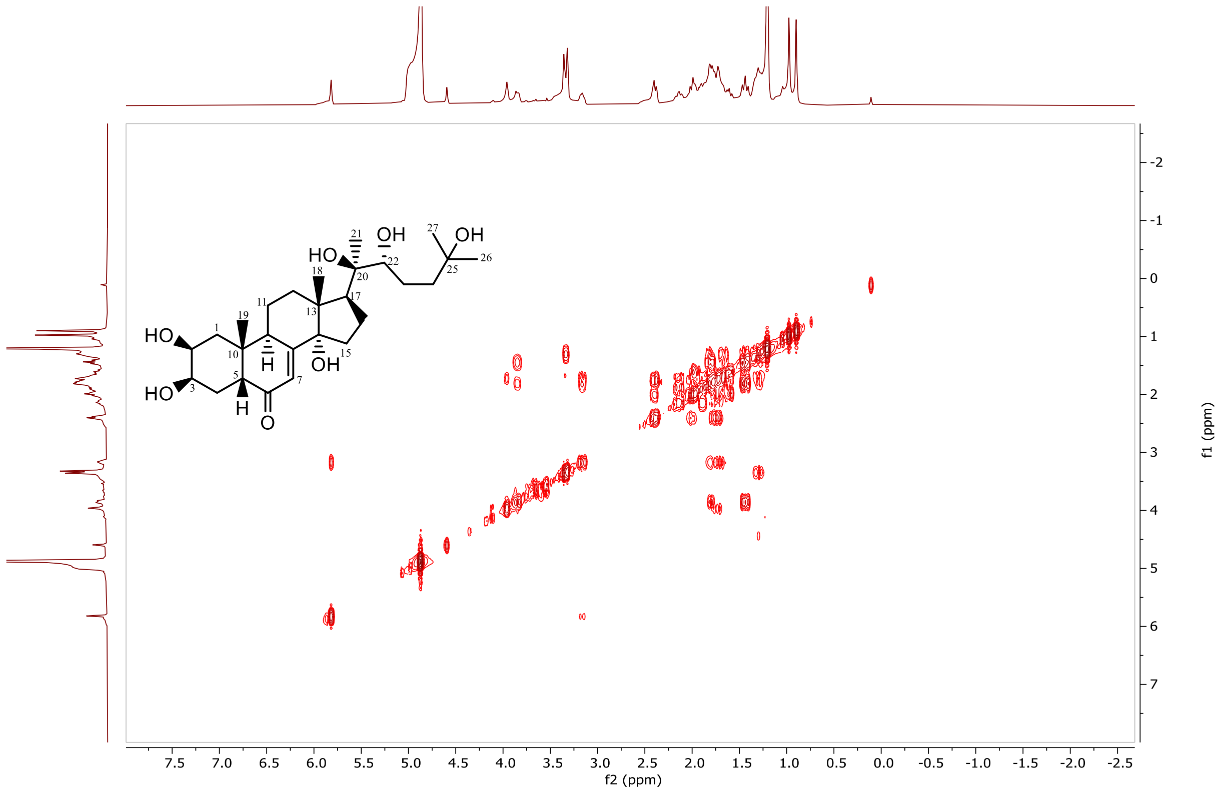
^{1}H NMR spectrum of compound **19**



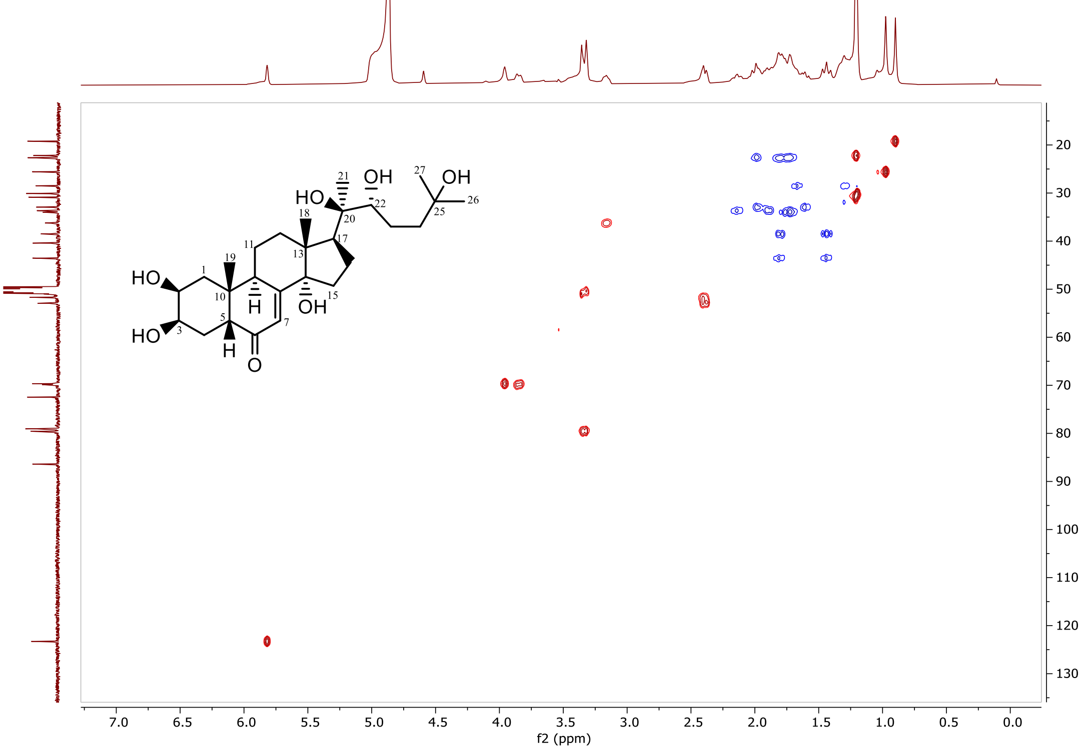
^{13}C NMR spectrum of compound **19**



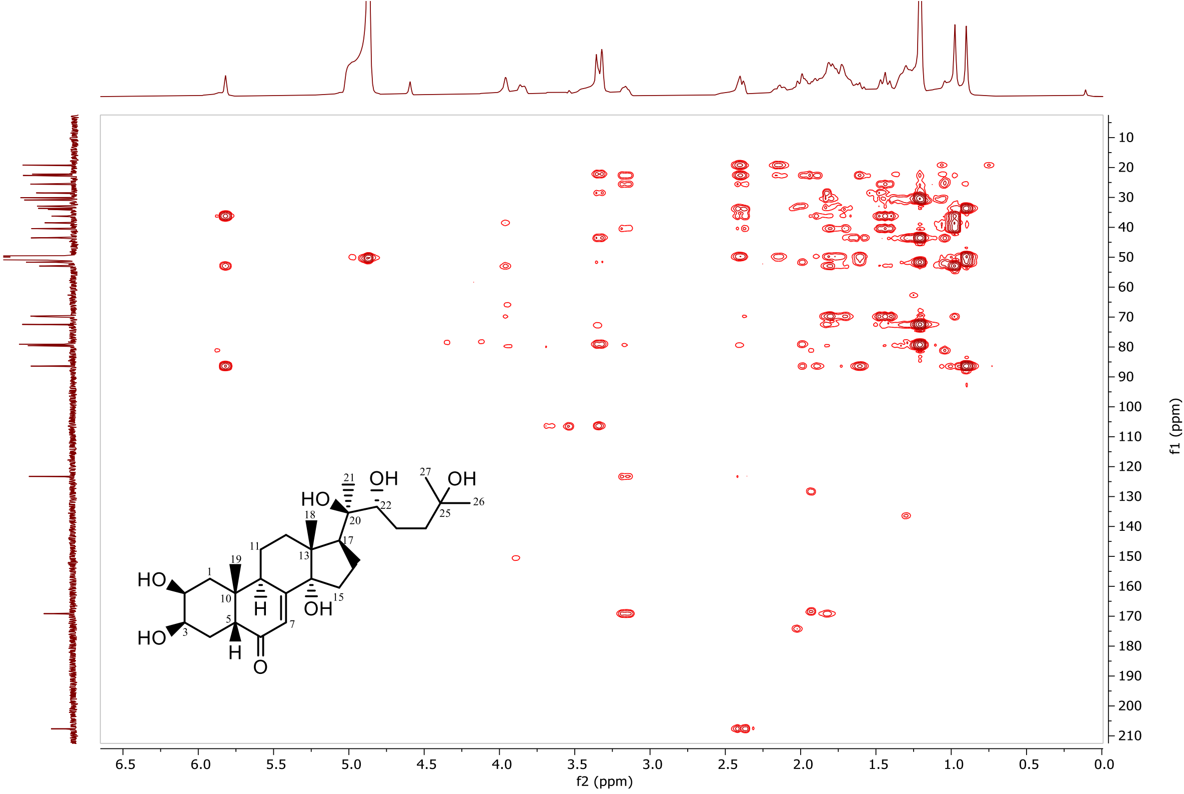
DEPT-135 NMR spectrum of compound **19**



^{1}H-^{1}H COSY NMR spectra of compound **19**

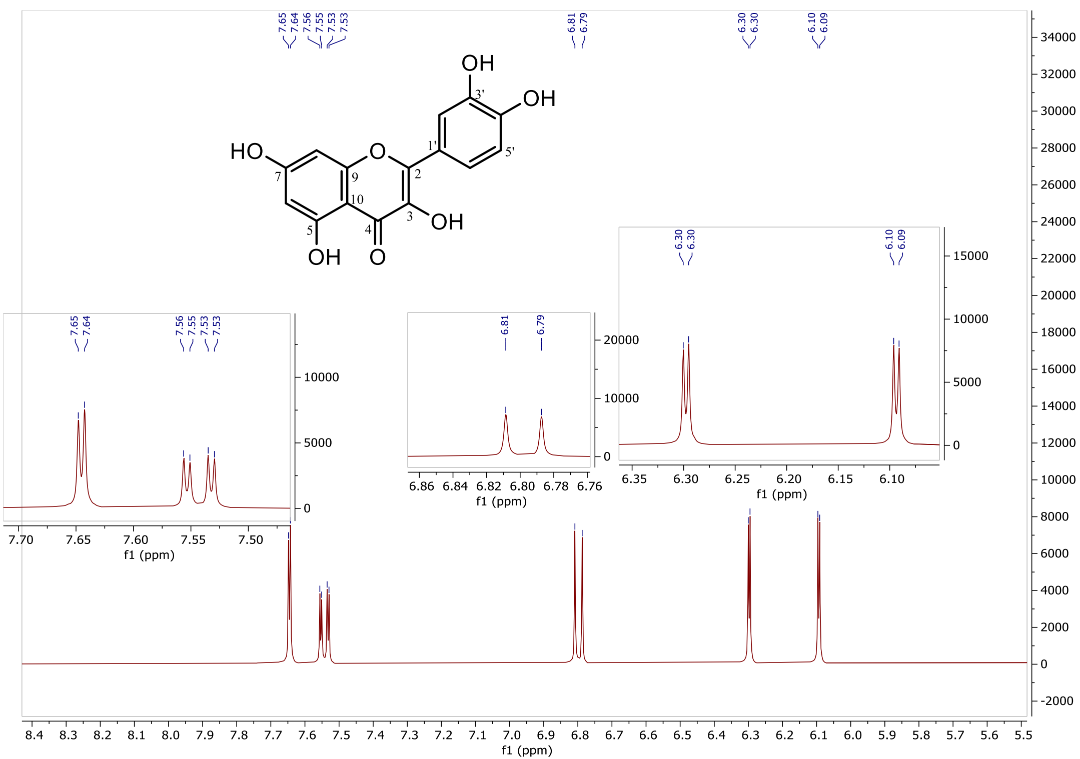


HSQC NMR spectra of compound **19**

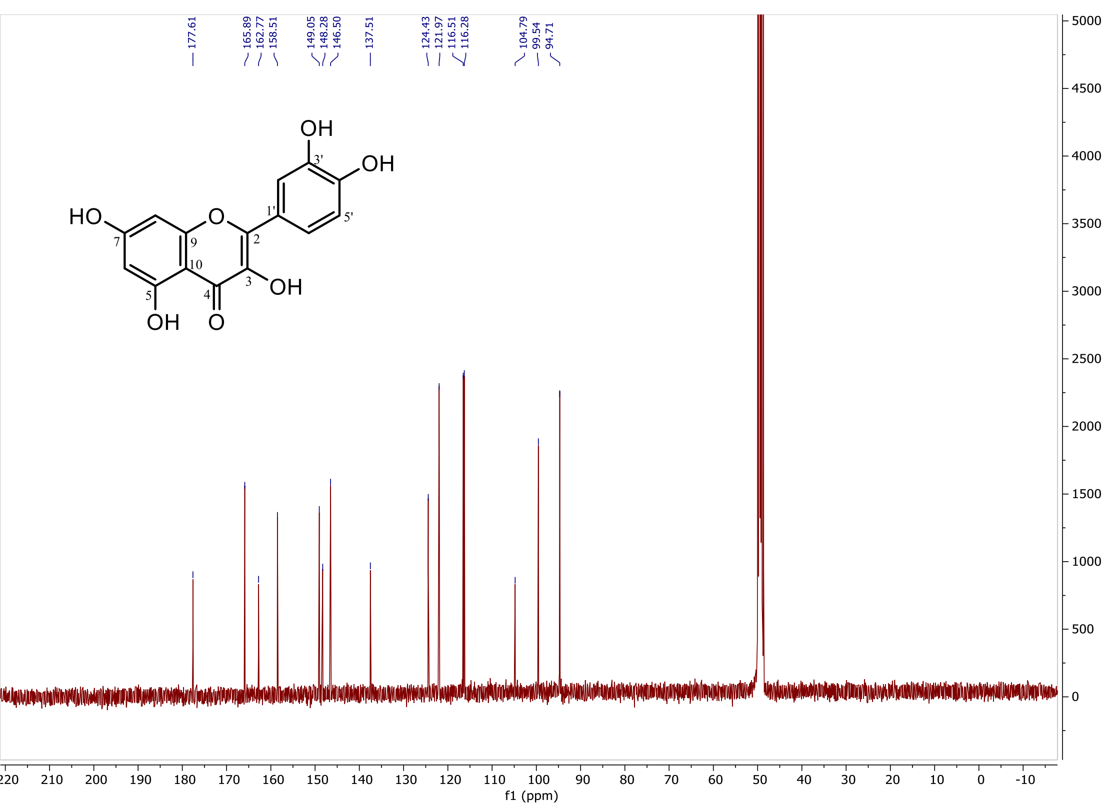


HMBC NMR spectra of compound **19**

Compound **20**

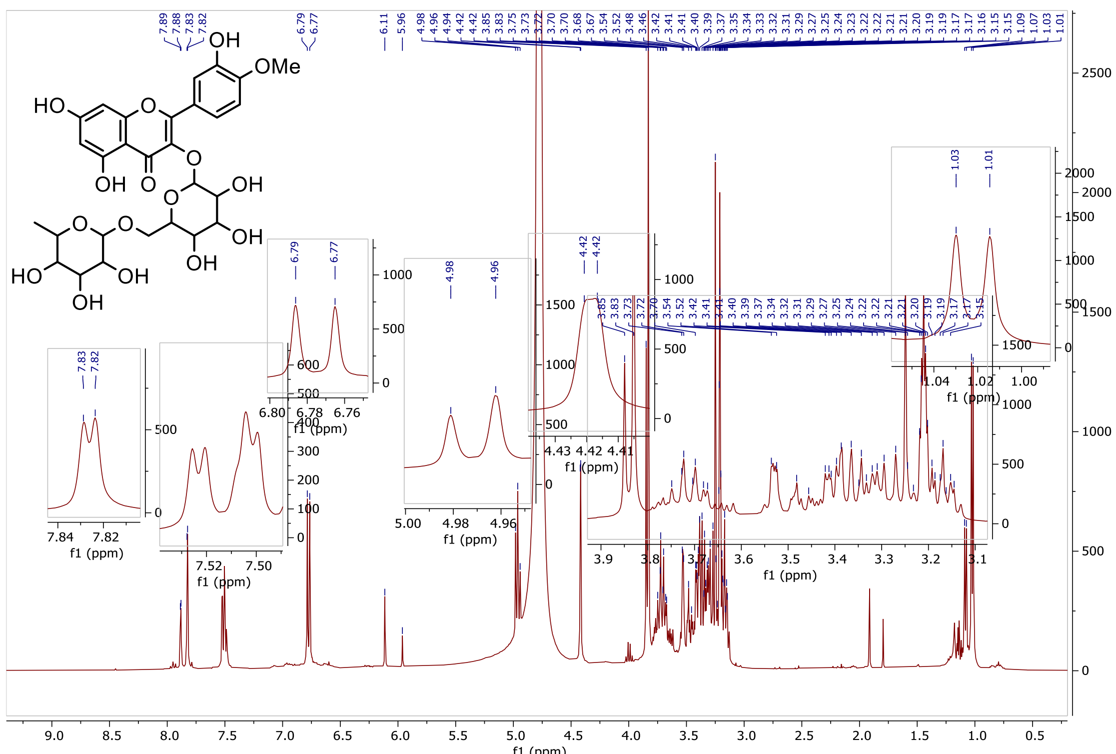


^{1}H NMR spectrum of compound **20**

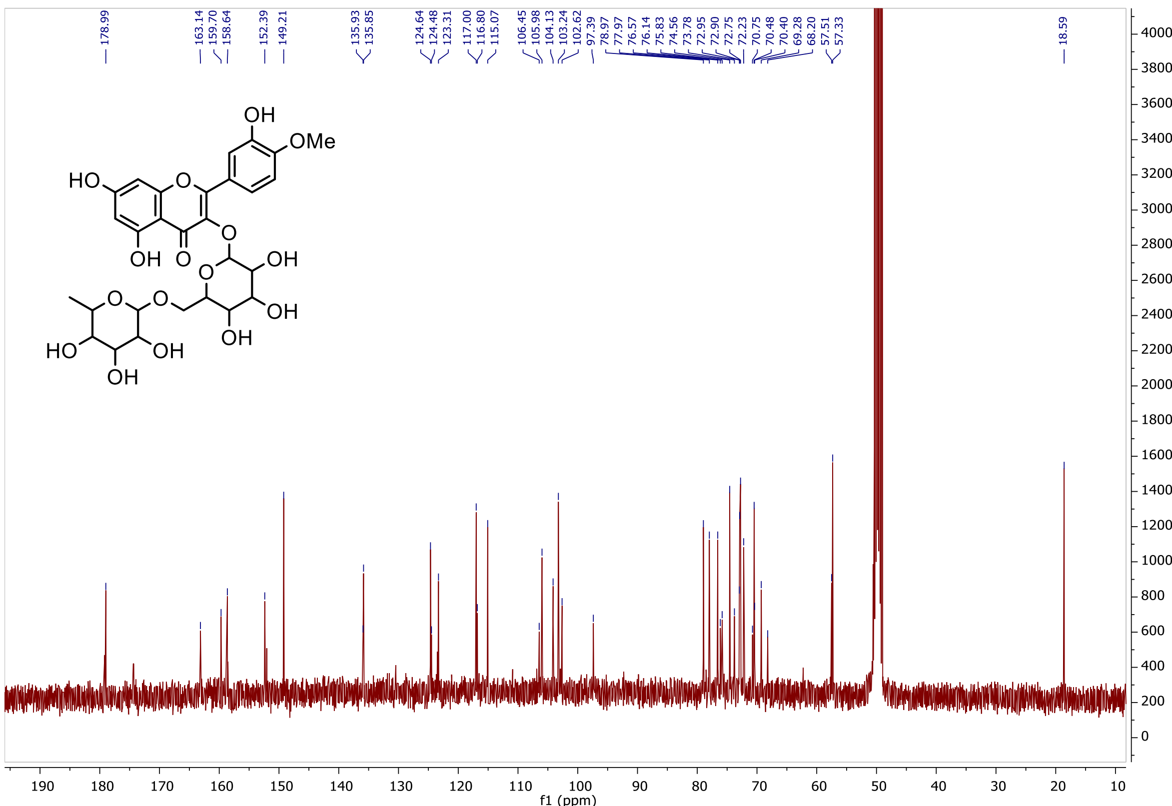


^{13}C NMR spectrum of compound **20**

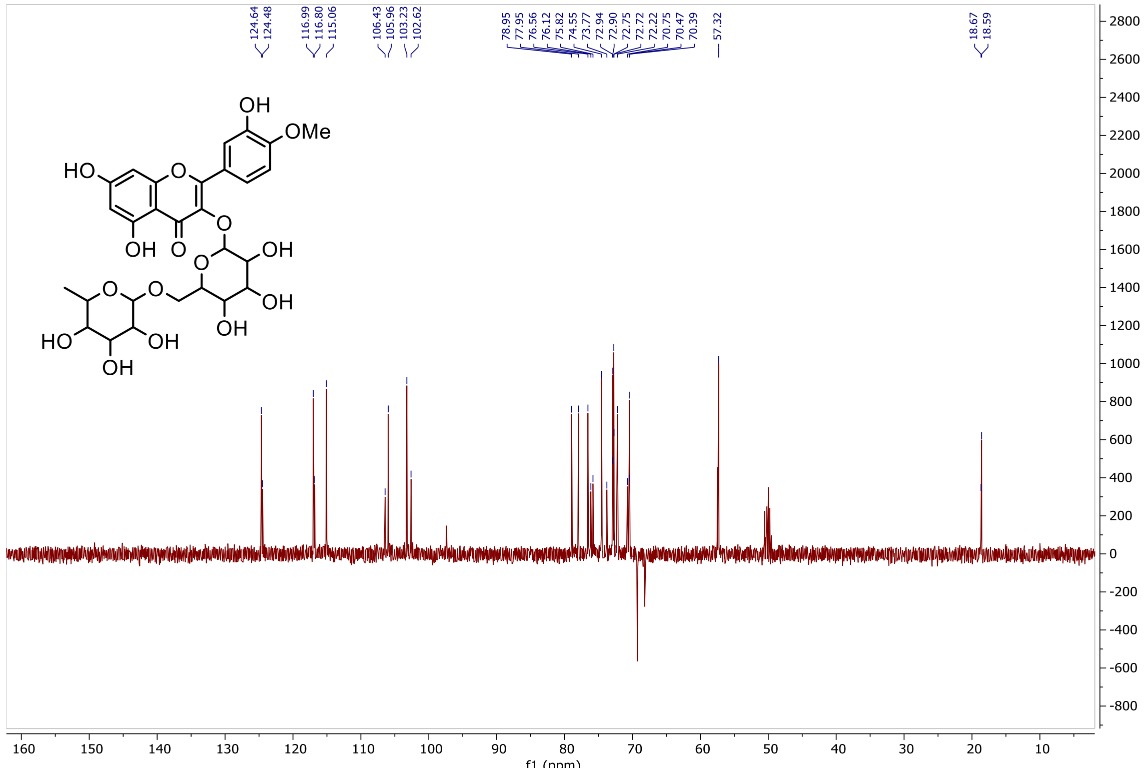
Compound **21**



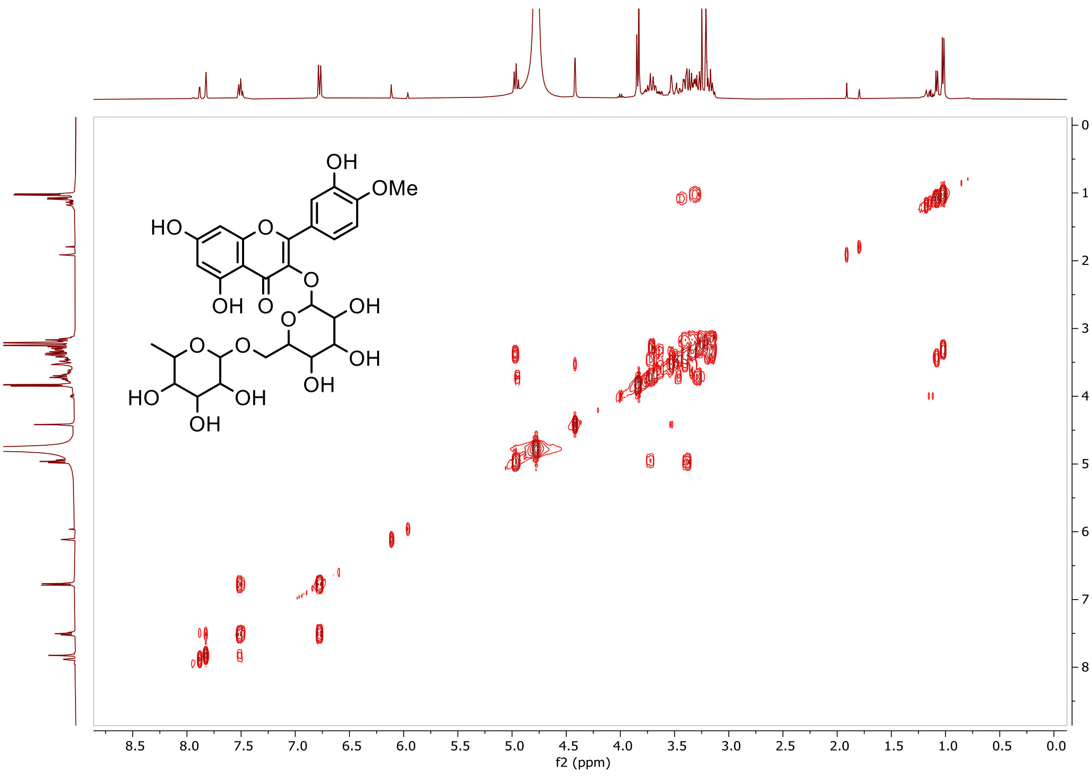
^{1}H-NMR spectrum of compound **21**



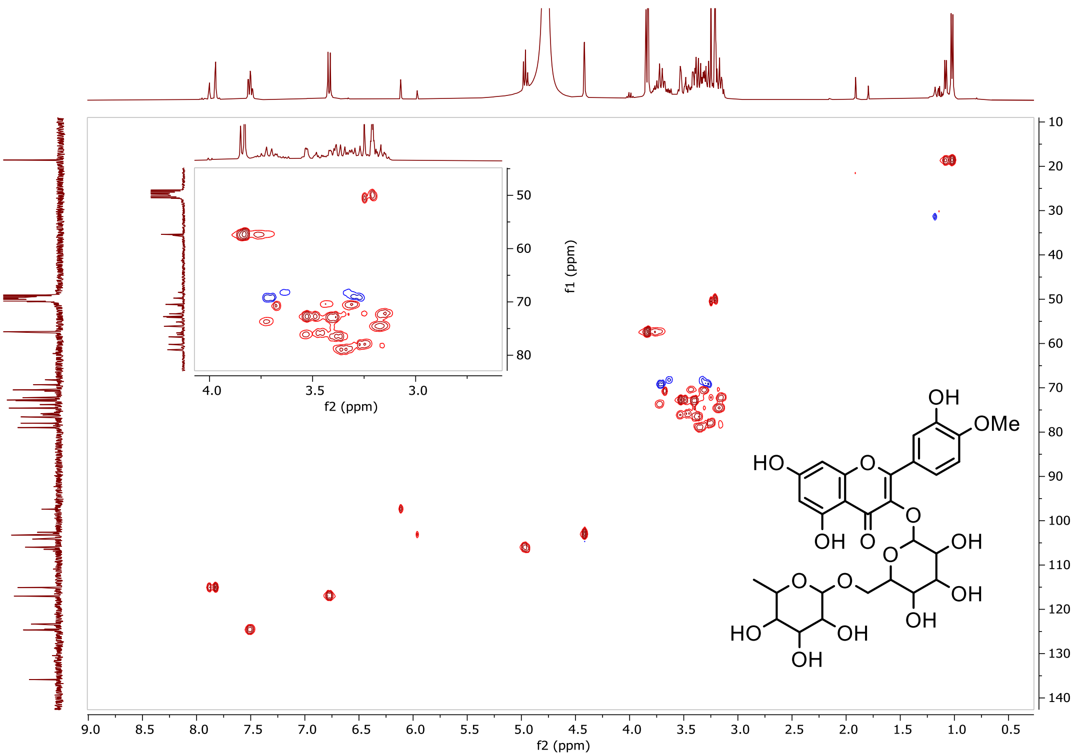
^{13}C-NMR spectrum of compound **21**



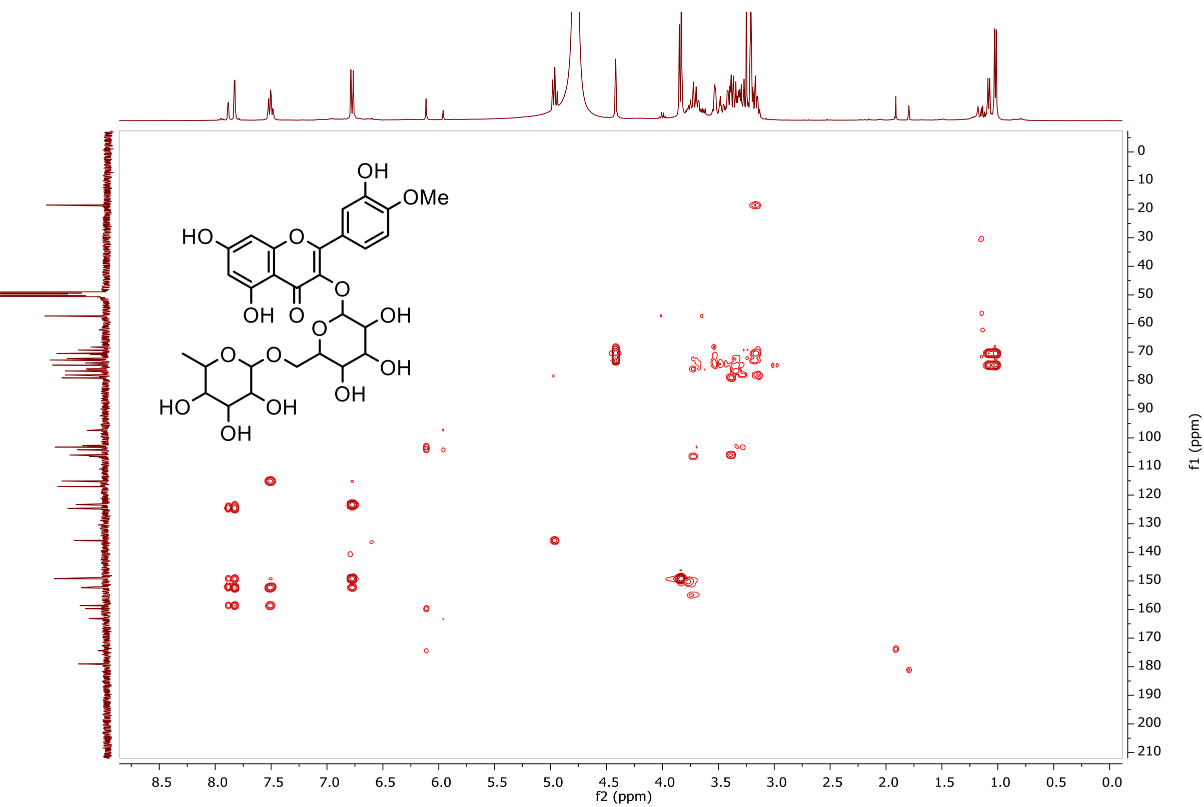
DEPT-135 NMR spectrum of compound **21**



^{1}H-^{1}H COSY NMR spectra of compound **21**



HSQC NMR spectra of compound **21**



HMBC NMR spectra of compound **21**