BIOACTIVE COMPOUNDS IN CANOLA MEAL

SAIRA HUSSAIN

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Faculty of Science
School of Biomedical Sciences

Charles Sturt University

Australia

March 2015

© Saira Hussain, 2015
# TABLE OF CONTENTS

**BIOACTIVE COMPOUNDS IN CANOLA MEAL** ........................................................... i

DEDICATION .................................................................................................................. ii

CERTIFICATE OF AUTHORSHIP ................................................................................. viii

ACKNOWLEDGEMENTS .......................................................................................... ix

LIST OF TABLES ....................................................................................................... xii

LIST OF SYMBOLS AND ABBREVIATIONS ......................................................... xvi

LIST OF PUBLICATIONS ......................................................................................... xviii

ABSTRACT .................................................................................................................. 1

CHAPTER 1. Literature Review .............................................................................. 4

1.1. Introduction ........................................................................................................ 4

1.2. Bioactive compounds ..................................................................................... 13

1.2.1 Glucosinolates ......................................................................................... 16

1.2.2 Brassinosteroids ...................................................................................... 20

1.2.3 Phytates .................................................................................................... 21

1.2.4. Vitamins .................................................................................................. 22

1.2.5. Phenolic Compounds ........................................................................... 24

1.2.6 Proteinaceous compounds ....................................................................... 30

1.2.6.1 Bioactive Proteins ........................................................................... 31

1.2.6.2 Bioactive Peptides ........................................................................... 33

1.2.6.3 Protease Inhibitors (PIs) ................................................................. 35

1.3 Bioactive Properties/Activities of Bioactive compounds .............................. 41

1.3.1 Antioxidant .............................................................................................. 43

1.3.2 Topoisomerase Inhibition ....................................................................... 44

1.3.3 Antidiabetic activities .............................................................................. 49

1.3.4 ACE inhibition ........................................................................................ 51

1.3.5 Antiobesity activity ................................................................................ 53

1.3.5.1 Lipase Inhibition .............................................................................. 54
1.3.5.2 Antiadipogenesis ................................................................. 55
1.4 Extraction of bioactive compounds........................................... 56
1.5 Research Aims ......................................................................... 59

CHAPTER 2. Extraction, chemical characterisation, and in vitro antioxidant activity of canola meal ......................................................... 62
2.1. Introduction ............................................................................ 62
2.2 Materials and methods ............................................................ 64
  2.2.1 Materials ........................................................................... 64
  2.2.2 Preparation of canola meal .................................................. 65
  2.2.3 Preparation of canola meal extracts (CMEs) ......................... 66
  2.2.4 Total phenolic contents (TPC) ............................................. 67
  2.2.5 High performance liquid chromatography-diode array detection with online ABTS scavenging (HPLC-DAD-online ABTS) ............. 67
  2.2.6 Liquid chromatography-mass spectroscopy (LC-MS) for identification of canola extracts ......................................................... 69
  2.2.7 DPPH radical scavenging activity ....................................... 71
  2.2.8 Ferric reducing antioxidant power (FRAP) assay .................. 72
  2.2.9 Statistical analysis .............................................................. 73
2.3 Results ................................................................................... 74
  2.3.1 Preparation of canola extracts (CMEs) .................................. 74
  2.3.2 Total phenolic content (TPC) .............................................. 75
  2.3.3 Characterisation of phenolic composition and free radical scavenging activity of individual compounds ............................ 76
  2.3.4. DPPH radical scavenging activity ...................................... 84
  2.3.5. FRAP assay .................................................................... 84
2.4 Discussion .............................................................................. 87
2.5 Conclusion ............................................................................. 91

CHAPTER 3. Identification, purification and characterisation of protease inhibitors from canola meal ............................................................... 92
3.1. Introduction ........................................................................... 92
3.2. Materials and methods .............................................................. 93

3.2.1 Material ................................................................................. 93

3.2.2. Sample preparation for identification of protease inhibitors (PIs) in canola meal (CM) ................................................................. 94

3.2.3 Isolation and purification of canola meal protease inhibitors .... 94

3.2.3.1. Protein purification using Fast Performance Liquid chromatography (FPLC) .............................................................. 95

3.2.3.2. Reverse phase-high performance liquid chromatography .......... 96

3.2.3.3. Protein concentration estimation ............................................. 97

3.2.3.4. Spectrophotometric assay ....................................................... 97

3.2.3.5. PI activity staining of PAGE gels ............................................. 100

3.2.3.6. SDS-PAGE under non-reducing and reducing conditions ...... 102

3.2.4. N-terminal amino acid analysis (sequencing) ...................... 103

3.2.5. Data Analysis ....................................................................... 104

3.3. Results .................................................................................... 105

3.3.1. Protein Extraction ................................................................. 105

3.3.2. Protease inhibitor (PI) Purification .......................................... 111

3.3.2.1. Ion exchange chromatography ............................................. 111

3.3.2.2. Gel filtration chromatography .............................................. 111

3.3.3. Molecular properties and characterisation of purified PI ... 118

3.3.3.1. N-terminal amino acid sequencing of purified PI .................. 129

3.4 Discussion .............................................................................. 132

3.5 Conclusion .............................................................................. 137

CHAPTER 4. Measurement of bioactive properties using enzyme assays .... 138

4.1 Introduction ............................................................................ 138

4.1.1 In vitro topoisomerase-1 inhibition .......................................... 139

4.1.2 Antidiabetic assay ................................................................. 139

4.1.3 Angiotensin-converting enzyme (ACE) inhibitory activity .... 140

4.1.4 Lipase Inhibition ................................................................. 141
4.2 Material and methods .......................................................................................................................... 141

4.2.1 In vitro topoisomerase-1 inhibition ................................................................................................. 141

4.2.1.1 Materials ...................................................................................................................................... 141

4.2.1.2 Preparation of reagents ............................................................................................................... 142

4.2.1.3 Topoisomerase inhibition ............................................................................................................. 143

4.2.1.4 Poisoning assay ........................................................................................................................... 143

4.2.1.5 Suppression Assay ...................................................................................................................... 144

4.2.2 Antidiabetic assay ............................................................................................................................. 145

4.2.2.1 Materials ...................................................................................................................................... 145

4.2.2.2 Antidiabetic Assay ....................................................................................................................... 145

4.2.3 Angiotensin-converting enzyme (ACE) inhibitory activity ............................................................ 147

4.2.3.1 Materials ...................................................................................................................................... 147

4.2.3.2 ACE-inhibition activity (in-vitro) ................................................................................................. 147

4.2.4 Lipase Inhibition ............................................................................................................................... 148

4.4.2.1 Materials ...................................................................................................................................... 148

4.4.2.2 Pancreatic lipase inhibition assay (In-vitro) ................................................................................. 149

4.3. Data analysis ......................................................................................................................................... 149

4.4 Results .................................................................................................................................................. 150

4.4.1 In vitro topoisomerase-1 inhibition ................................................................................................. 150

4.4.2. Antidiabetic assay .......................................................................................................................... 161

4.4.3 Angiotensin-converting enzyme (ACE) inhibitory activity ............................................................ 164

4.4.4 Pancreatic lipase inhibition assay (In-vitro) ..................................................................................... 167

4.5 Discussion ............................................................................................................................................ 169

4.5.1 In vitro topoisomerase-1 inhibition ................................................................................................. 169

4.5.2 Antidiabetics assay .......................................................................................................................... 174

4.5.3 Angiotensin-converting enzyme (ACE) inhibitory activity ............................................................ 176

4.5.4 Lipase Assay ..................................................................................................................................... 178

4.6 Conclusion ............................................................................................................................................. 179
CHAPTER 5. In vitro inhibition of adipogenesis by canola meal extract......... 181

5.1 Introduction.................................................................................................................. 181

5.2. Material and Methods................................................................................................. 183

5.2.1 Material ..................................................................................................................... 183

5.2.2. Sample solubility, cell culture, viability and adipocyte differentiation................................. 184

5.2.2.1 Sample solubility .................................................................................................. 184

5.2.2.2 Cell Culture ......................................................................................................... 184

5.2.2.3 Cell Viability ........................................................................................................ 185

5.2.2.4 Adipogenic differentiation .................................................................................... 185

5.2.3 Oil–Red O staining and quantification of Intracellular Lipid Droplets ..................................... 186

5.2.3.1 Oil–Red O staining .............................................................................................. 186

5.2.3.2 Immunofluorescence staining ............................................................................. 187

5.2.4 RNA extraction, reverse transcription polymerase chain reaction (RT-PCR) and real time quantitative PCR (qPCR).......................................................... 187

5.2.4.1 RNA extraction and reverse transcription polymerase chain reaction (RT-PCR).......................... 187

5.2.4.2 Real time quantitative PCR (qPCR) ....................................................................... 189

5.2.5 Statistical analysis ...................................................................................................... 190

5.3 Result ............................................................................................................................. 191

5.3.1 Extract solubility, and measurement of cell viability.................................. 191

5.3.2 Oil–Red O staining staining and quantification of Intracellular Lipid Droplets ........................................ 197

5.3.2.1 Effect of CME on intracellular lipid accumulation........................................... 197

5.3.3 Immunoflourescence staining of PPARγ in C3H10T1/2 Cells.200

5.3.4 Effect of CMEs on the gene expression of PPARγ in C3H10T1/2 ........................................ 203

5.3.4.1 Qualitative analysis............................................................................................ 203

5.3.4.2 Quantitative analysis ......................................................................................... 203
5.4 Discussion .............................................................................................................. 207

5.5 Conclusion ........................................................................................................... 213

CHAPTER 6. General Discussion ............................................................................. 214

REFERENCES............................................................................................................ 220

APPENDIX 1. Antioxidant properties by FRAP activity showing linear regression line for Trolox dilution with concentration (Con) in μM/ml ........................................ 245

APPENDIX 2. Phenolic and antioxidant activity profile in AE and WE (A) RP-HPLC (absorbance at 280 nm), (a) PCD (absorbance at 414 nm) ........................................ 246

APPENDIX 3. Canola meal extracts representing topoisomerase Inhibition activity ................................................................................................................................. 248
DEDICATION

To my parents and family,

For their unconditional love and encouragement,

Who made me what I am today
CERTIFICATE OF AUTHORSHIP

I, Saira Hussain, hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma at Charles Sturt University or any other educational institution, except where due acknowledgment is made in the thesis. Any contribution made to the research by colleagues with whom I have worked at Charles Sturt University or elsewhere during my candidature is fully acknowledged.

I agree that this thesis be accessible for the purpose of study and research in accordance with the normal conditions established by the Executive Director, Library Services or nominee, for the care, loan and reproduction of theses*.

Name: Saira Hussain

Signature

Date 30/11/15

* Subject to confidentiality provisions as approved by the University.
ACKNOWLEDGEMENTS

First and foremost I praise and acknowledge Allah (God-the Almighty) the most gracious and merciful who gave me courage and patience to carry out this work. I like to express my sincere thanks and gratitude to Allah who made me the follower of Prophet Muhammad (May the peace and blessings of Allah be upon him) whose way of life has been a continuous guidance for me.

During my doctorate study, I received support and encouragement from many people. Their contribution values an extraordinary statement in this research and thesis. I would like to express my gratitude to them all in my humble acknowledgment. It is a pleasure to convey my gratitude to Faculty of Science Research Higher Degree Scholarship (FOSHDS) at Charles Sturt University (CSU), Australia for awarding me a PhD scholarship which enabled me to have overseas study to attain a doctorate degree and to have a very interesting life so far.

My deepest and most sincere appreciation goes to my supervisors, Professor Christopher Blanchard (School of Biomedical Science, CSU), Dr Ata Ur Rehman (CSU), Dr David Luckett (CSU&DPI) and Dr. Hassan Obied for their continuing supervision, valuable advice, crucial contribution, and precise guidance from the very initial times of this research to valued insight and understandings throughout the course of study. Thanks especially to Dr. Padraig Strappe for their help with the cell culture work in this thesis. Without support of anyone of you nothing would have been possible as you were the backbone of this research and the thesis. I also thank you for your precious
time to review this thesis and constructive comments. Special thanks are extended to Dr. Phillip Bwititi, Dr. Bruce Graham and Professor Jade Forwood, for your kind support during PhD study. Thank you for giving me an opportunity to learn from you in developing knowledge and skills not limited to science, as well as imparting the insightful wisdom in life experiences.

My appreciation goes to the School of Biomedical Sciences for providing me an opportunity to carry out research work at National Life Science Hub (NALSH), with assistance of many highly skilled and friendly technicians. Special thanks to Mr Michael Laughlin, Miss Lynette Matthews, Mrs Therese Moon, for assisting in a range of laboratory issues. I also appreciate my friends for their advices, encouragement, and support from Dr Adeola Alashi, Dr. Kah Yaw Ee, Dr Randy Adjonu, Saliya Gurusinghe, and Yogesh Khandokar. I wish to extend my appreciation to Gul Afsheen, Shumaila Arif, Dr. Siong Tang, Christina Chin, Soumi Mukhopadhyay, Nusrat Shahani, Saba Nabi, Harris Omer, Muhammad Kamran, Muhammad Asaduzzaman, Nusrat Subhan, Partho Adhikary, Md Ghani, Kare Maihemuti, Parul Srivastava, and Gayle Petersen. I would like to acknowledge whoever have helped me throughout the study, the staffs from the School of Biomedical Sciences, Lab Store, Research Office, Student Services and Library in CSU, and the Graham Centre.

Finally, I extend my acknowledgement to my precious family, especially my parents, Ahmad Hussain and Hameeda Begum. Thank you for your trust in me and your infinite love, encouragement, prayers and care. I am grateful to
my parents for their willingness for my overseas education, and to share their bright thoughts with me, which were very fruitful for shaping up my ideas and research. I am also grateful to my brother Ali Hussain, sisters, Tahira Hussain, Saima Hussain, Tayyaba Hussain and Atia Bano for your appreciation, continuous assistance, and enthusiasm. What I am today is only because of you all.
LIST OF TABLES

Table 1.1. Chemical composition of canola meal ................................................. 12
Table 1.2. Flavonoids present in various sources of foods .................................. 27
Table 2.1. HPLC peaks in canola meal extracts (CMEs) ..................................... 78
Table 3.1. Comparison of trypsin inhibitor (TI) activity of the two genotypes at each purification step ........................................................................................................ 115
Table 3.2. Comparison of the amino acid sequences of canola protease inhibitors (CPIs) from genotype-1 with other plant inhibitors ......................... 130
Table 3.3. Comparison of the amino acid sequences of canola protease inhibitors (CPIs) from genotype-2 with other plant inhibitors ......................... 131
Table 5.1. Analysis of canola meal extracts (CME) solubility ............................... 193
LIST OF FIGURES

Figure 1.1. Triangle of U, representing the genomic relationships between the three diploid and three amphidiploid species in the *Brassica* genus. .......................... 6
Figure 1.2. Canola and its uses ........................................................................... 8
Figure 1.3. Canola production (t) in Australia ..................................................... 9
Figure 1.4. Canola Production in tonnes (t) in Australia for 2012-2013 .... 10
Figure 1.5. Bioactive compounds in canola meal .............................................. 15
Figure 1.6. Hydrolysis of glucosinolates and formation of indole-3-carbinol in plants. .................................................................................................................... 18
Figure 1.7. Phenol and other bioactive compounds present in canola meal. .... 25
Figure 1.8. Major classification of Protease Inhibitors (PIs) ....................... 38
Figure 1.9. Some known potential health benefits of bioactive compounds. .... 42
Figure 1.10. Topoisomerase enzyme showing replication of DNA .................. 46
Figure 1.11. Topoisomerase enzyme showing poisoning and suppression. .... 47
Figure 1.12. Regulatory hormonal mechanism of renin angiotensin system (RAS) .................................................................................................................. 52
Figure 1.13. A flow chart highlighting the steps involved in the analysis of canola meal extractions for their biological activity .............................................. 60
Figure 2.1. Comparison of extraction yields (in percentage) of CMEs from two canola genotypes ........................................................................................................ 74
Figure 2.2. Comparison of total phenolic contents represented as milligram of gallic acid equivalent per gram dry weight (mg GAE/g DW) in CMEs from two canola genotypes .................................................................................................. 75
Figure 2.3. Typical HPLC-DAD chromatograms of CMEs ......................... 81
Figure 2.4. Comparison of antioxidant properties as measured by DPPH assay. ............................................................................................................................... 85
Figure 2.5. Comparison of antioxidant properties as measured by FRAP assay. ............................................................................................................................... 86
Figure 3.1. (A) Native-PAGE of canola meal crude extracts after staining with coomassie brilliant blue and (B) corresponding native-PAGE gel showing trypsin inhibitor activity (enzymatic staining) indicated by arrows. ................................................................. 106
Figure 3.2. Flow chart for the extraction of crude protein fraction ......... 108
Figure 3.3. (A) Native-PAGE view of canola meal extracts after staining with coomassie brilliant blue and (B) corresponding native-PAGE gel showing trypsin inhibitor activity (enzymatic staining) indicated by arrows ......................................................................................................................... 109
Figure 3.4. Native-PAGE profile of canola meal extracts of two genotypes, genotype-1 and 2; fractionated with 80% ammonium sulphate. (A) Coomassie brilliant blue stained gel and (B) corresponding native-PAGE gel exhibiting trypsin inhibitor activity (enzymatic staining). ........................................ 110
Figure 3.5. FPLC chromatogram of canola meal extracts from genotype-1 for the analysis PI ................................................................. 112
Figure 3.6. FPLC chromatogram of canola meal extracts from genotype-2 used for the analysis PI ................................................................. 113
Figure 3.7. Elution profiles for RP-HPLC (214 nm) analysis of canola protease inhibitor.

Figure 3.8. (A) Native-PAGE of canola meal anion exchanged fractions from genotype-1 after staining with coomassie brilliant blue and (B) correspondent native-PAGE gel showing trypsin inhibitor activity (enzymatic staining).

Figure 3.9. (A) Native-PAGE of canola meal gel filtration fractions of genotype-1 after staining with Coomassie brilliant blue and (B) corresponding native-PAGE gel having trypsin inhibitor activity (enzymatic staining).

Figure 3.10. Native-PAGE of re-chromatographed anion exchange peak PIBI with fractions 3-12.

Figure 3.11. SDS-PAGE profiles of rechromatographed anion exchange fractions of genotype-1 collected under peak PIBI.

Figure 3.12. (A) Native-PAGE view of canola meal anion exchanged fractions after staining with Coomassie brilliant blue and (B) corresponding native-PAGE gel showing trypsin inhibitor activity (enzymatic staining) in genotype-2.

Figure 3.13. (A) Native-PAGE view of canola meal gel filtration fractions after staining with Coomassie brilliant blue and (B) corresponding native-PAGE gel showing trypsin inhibitor activity (enzymatic staining) in genotype-2.

Figure 3.14. (A) Native-PAGE view of re-chromatographed anion exchange fractions PIRI from genotype-2 after staining with Coomassie brilliant blue and (B) corresponding native-PAGE gel showing trypsin inhibitor activity (enzymatic staining), confirming both the proteins as protease inhibitors.

Figure 3.15. SDS-PAGE profiles of pooled fractions and concentrated AEC eluted fractions 3-9 under peak PIR1 from genotype-2.

Figure 3.16. Isoelectric focusing gel (IEF gels 3-9 [pH range 3-9, Pharmacia]) were used for re-chromatographed anion exchange of purified protease inhibitors.

Figure 4.1. Electrophoresis gel showing topological position.

Figure 4.2. A). Electrophoresis gel showing poisoning result by topoisomerase I (Topo I)-induced cleavage by Canola meal extracts.

Figure 4.3. A) Electrophoresis gel showing poisoning result by topoisomerase I (Topo I)-induced cleavage by Canola meal extracts.

Figure 4.4. A) Electrophoresis gel showing poisoning result by topoisomerase-I (Topo I)-induced cleavage by purified protease Inhibitor.

Figure 4.5. Electrophoresis gel showing topoisomerase suppression activity.

Figure 4.6. Electrophoresis gel showing topoisomerase suppression activity.

Figure 4.7. Electrophoresis gel showing topoisomerase suppression activity.

Figure 4.8. Percentage of topoisomerase-I suppression activity.
Figure 4.9. DPP-IV inhibition activity of protease inhibitors (PIs) from two different genotypes (from canola meal) represented as IC\textsubscript{50} value.

Figure 4.10. Percentage (%) of DPP-IV inhibition activity of canola extracts from two different genotypes.

Figure 4.11. The ACE inhibition activity (IC\textsubscript{50}) of protease inhibitors obtained from canola meals of genotype-1 and genotype-2.

Figure 4.12. ACE inhibition activity (%) of canola meal extracts.

Figure 4.13. Inhibitory effect of different solvents (IC\textsubscript{50}) extracts from canola meal on pancreatic lipase inhibition.

Figure 5.1. Analysis of canola meal extracts (CME) solubility.

Figure 5.2. The effect of DMSO on C3H10T1/2 Cell Viability.

Figure 5.3. The effect of canola meal extract on C3H10T1/2 cell (CME) for cell viability.

Figure 5.4. Quantitative analysis of Oil red staining for the effect of adipogenic inhibition by CMEs.

Figure 5.5. Qualitative analysis for the effect of CME on adipogenic differentiation visualized at 100 µm.

Figure 5.6. Immunofluorescence staining of PPAR\textgamma in of CMEs in mesenchymal stem cells (CH310T1/2) visualized at 10 µm.

Figure 5.7. The effect of CMEs detected by qualitative PCR on PPAR\textgamma and \beta-actin expression during adipogenic differentiation.

Figure 5.8. Quantitative gene expression analysis of PPAR\textgamma in C3H10T1/2 cells treated with CMEs.
### LIST OF SYMBOLS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda )</td>
<td>absorbance wavelength</td>
</tr>
<tr>
<td>ABTS 2,</td>
<td>2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
</tr>
<tr>
<td>DPPH</td>
<td>2, 2′-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>FAPGG</td>
<td>N-[3-(2-furyl) acryloyl]-L-phenylalanyl-glycyl-glycine</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast-performance liquid chromatography</td>
</tr>
<tr>
<td>FRAP</td>
<td>ferric ion reducing antioxidant power</td>
</tr>
<tr>
<td>GAE</td>
<td>gallic acid equivalent</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>i.d.</td>
<td>internal diameter</td>
</tr>
<tr>
<td>IC(_{50})</td>
<td>50% of inhibition concentration</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>M</td>
<td>Molarity</td>
</tr>
<tr>
<td>ME</td>
<td>( \beta )-mercaptoethanol</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
</tbody>
</table>
PCD post column derivatisation
pI isoelectric point
PIs protease inhibitors
PVDF polyvinylidene fluoride
r correlation
RP-HPLC reverse phase-high performance liquid chromatography
rpm revolutions per minute
s second
SBTI soybean trypsin inhibitor
SDS sodium dodecyl sulphate
TIU trypsin inhibition units
mL mililitre
ng nanogram
t time
RT room temperature
TAME p-toluenesulphonyl-L-arginine methyl ester
TE Trolox equivalent
TFA trifluoroacetic acid
TU trypsin unit
UV ultraviolet
vis visible
µm micromolar
LIST OF PUBLICATIONS

Journal Article

Industry Paper

Conference Paper

Conference oral presentation

ABSTRACT

Canola meal, is a by-product of oil extraction and is of comparatively low value. This meal may have additional value in the biotechnology, food and biomedical fields, if potential health beneficial bioactive compounds with the ability to combat several modern day ailments can be identified.

Meal extracts produced using water, and aqueous 80% concentrations of solvents including methanol, ethanol, acetone, butanol, chloroform and hexane were assessed for the presence of phenolic compounds as well as their total antioxidant activity. These extracts were further characterised for the presence of phytochemical compounds and the antioxidant activity of these compounds was assessed. Acetone extracts had higher total phenolic contents and antioxidant activities. However, methanol extracts were found to have higher numbers of compounds than other extracts. A major peak corresponding to sinapine was present in all extracts except water and chloroform extracts. HPLC analysis of chloroforms extracts showed the presence of three unknown peaks not present in other extracts.

Protease inhibitors (PIs) were purified from canola meal using two genotype of canola with ammonium sulphate precipitation followed by ion exchange and gel filtration chromatography. These PIs were then characterised using polyacrylamide gel electrophoresis and N-terminal amino acid sequencing. PIs from genotypes-1 were termed PIB1 and PIs from genotype-2 as PIR1,
which were different from each other as confirmed by their amino acid sequences. PIB1 had a specific activity of 72.23 trypsin inhibitor units per milligram (TIU mg\(^{-1}\)), with 0.21% recovery while PIR1 had a specific activity of 17.7 trypsin inhibitor units per milligram (TIU mg\(^{-1}\)), with 2.49% recovery from crude extract. PIs from PIB1 contained molecular masses of 13, 16 and 30 KDa, and amino acid sequence analysis suggested they have not been previously been identified. However the PIB1 amino acid sequences have some homology to serine and cysteine protease inhibitors and also Kunitz-type Kallikrein inhibitors. PIs from PIR1 had molecular masses of 11, 12 and 16 KDa, and amino acid sequence analysis suggested they were serine trypsin inhibitors, similar to those already identified in other genotypes of the Brassicaceae family.

All extracts were analyzed for the presence of bioactive properties using \textit{in vitro} assays. A high level of topoisomerase is found in cancer cells and poisoning or suppression of topoisomerase caused by bioactive compounds or drugs can result in anticancer activity. Acetone, ethanol and methanol extracts were found to contain potent topoisomerase inhibitors with both poisoning and inhibition activities. Also, other extracts including crude protein fractions and purified PIs also exhibited poisoning and suppression activities. These results demonstrated that topoisomerase inhibitors are present in canola meal suggesting canola may be a possible target for the isolation of compounds with anti-cancer properties.
Butanol, and water extracts showed high antidiabetic activity by inhibiting the dipeptidyl peptidase IV enzyme (DPP-IV) that plays a major role in glucose metabolism. Acetone, methanol and water extracts also showed higher antihypertension properties that other extracts by inhibiting the angiotensin-converting-enzyme (ACE). PIB1 displayed higher antihypertension activity and antidiabetic activities than PIR1. Acetone and methanol extracts showed potential antiobesity activity by inhibiting the enzyme lipase. These results were further employed in a cellular assay that involved inhibiting adipocyte differentiation in the absence of cell toxicity. Both quantitative and qualitative assays were employed using oil Red O staining, and measurement of the expression of a key transcription factor called peroxisome proliferator-activated receptor gamma (PPAR-γ) by immunofluorescence staining, and real time PCR. All results confirmed that the acetone extracts were superior compared to other extracts in the inhibition of adipocyte differentiation.

The demonstration of potential bioactive and health-functional properties of canola meal extracts may lead to the development of high value canola meal derived products that could return value to farmers, processors, food manufacturers, and the pharmaceutical industry as well as provide improved health outcomes for those suffering a range of diseases.
CHAPTER 1. Literature Review

1.1. Introduction

Canola is a term that refers to Brassica species (typically Brassica napus L.) specifically bred to meet a specific oil quality specification. The canola standard requires cultivars to have a low content of both glucosinolates and erucic acid. These cultivars are also referred to as ‘double low’ or ‘double zero’ rapeseed cultivars (Cumby, Zhong, Naczk, & Shahidi, 2008; Fang, Reichelt, Hidalgo, Agnolet, & Schneider, 2012; M. Yang et al., 2014). In high concentrations, erucic acid and glucosinolates can cause toxicity in both humans and animals (Xin, Khan, Falk, & Yu, 2014). Typically, canola seeds have less than 2% erucic acid in the oil, and less than 30 micromoles (µmol) of glucosinolates in the meal (Alashi, Blanchard, Mailer, & Agboola, 2013.)

As shown in Figure 1.1., the genus Brassica has approximately 100 species (Aachary & Thiyam, 2012). Genetically, Brassica napus L. (canola, an allotetraploid, AACC, 2n=20) is closely related to B. rapa L. (turnip, Chinese cabbage a diploid, AA, 2n=18), B. Juncea (an allotetraploid, 2n=36, AABB) and B. nigra. (Black mustard a diploid, BB, 2n=16), B. carinata (Ethopian mustard, an allotetraploid 2n=34, BBCC) and B. oleracea L. (cabbage, cauliflower, broccoli, a diploid, CC, 2n=18) (Nagaharu, 1935). Currently, Brassica juncea (Indian mustard, an allotetraploid, 2n=36) is also grown as a ‘canola-quality’ product, with a low glucosinolates content, more commonly in low-rainfall areas in Australia, where it has a potential for good yield,
shattering tolerance, early vigour, and disease resistance (Burton, Salisbury, & Potts, 2003).
**Figure 1.1.** Triangle of U, representing the genomic relationships between the three diploid and three amphidiploid species in the *Brassica* genus.

Source: (Nagaharu, 1935).
Canola is widely grown for its high oil content (for human consumption or biodiesel) and the by-product (termed meal), which is currently used as an animal feed (Figure 1.2). Canola is the second most important oilseed crop in the world (Alashi et al., 2013). The canola industry is global, and production in million metric tons (MT) for various countries includes Canada (15.4), China (14), India (6.7), France (5.4), Germany (4.8) and Australia (3.4). The worldwide production of canola in 2012/13 was 61.54 million metric tons (Food and Agricultural Organization of United Nations, 2013). The production of canola is increasing, which reflects the crop's ability to meet the large global demand for high-quality, edible oil, with the added benefit of a high-protein feed supplement (meal) for animals (Ghodvali, Khodaparast, Vosoughi, & Diosady, 2005).

In Australia, canola constitutes 57% of total oilseed production (Pillay, Polya, & Spangenberg, 2011). Canola production increased to 3.2 million tonnes in the year 2012-2013 (Figure 1.3.). The states of New South Wales, Western Australia, Victoria and South Australia contain major canola growing areas. A small percentage of canola is also produced in Tasmania and Queensland as shown in Figure 1.4. (Australian Bureau of Statistics, 2014).
Figure 1.2. Canola and its uses: (a) Flowering canola plant, (b) Canola pods showing at different stages of maturation, (c) Canola seeds, (d) Canola oil, (e) Canola meal, (f) Canola oil in the food market, (g) World’s first canola oil biodiesel car, (h) Canola meal as chicken feed, (i) Canola meal fed cattle.
Figure 1.3. Canola production (t) in Australia
Figure 1.4. Canola Production in tonnes (t) in Australia for 2012-2013
When canola seeds are crushed, they yield approximately 40% oil (Aider & Barbana, 2011) and the remaining 60% is termed meal (Newkirk, Classen, Scott, & Edney, 2003). With the expansion of canola oil production, there is a parallel increase in the global production of canola meal, prompting the need for a diverse use of this by-product.

The typical chemical composition of canola meal is shown in Table 1.1.
Table 1.1. Chemical composition of canola meal.

<table>
<thead>
<tr>
<th>Composition of Canola meal</th>
<th>Average (%)</th>
<th>Vitamins</th>
<th>Amount (mg/kg)</th>
<th>Amino Acids</th>
<th>Average %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein (%)</td>
<td>36</td>
<td>Biotin</td>
<td>0.96</td>
<td>Alanine</td>
<td>1.57</td>
</tr>
<tr>
<td>Rumen bypass protein (%)</td>
<td>35</td>
<td>Choline</td>
<td>6500</td>
<td>Arginine</td>
<td>2.08</td>
</tr>
<tr>
<td>Oil (%)</td>
<td>3.5</td>
<td>Folic Acid</td>
<td>0.8</td>
<td>Aspartate + asparagine</td>
<td>2.61</td>
</tr>
<tr>
<td>Linoleic acid (%)</td>
<td>0.6</td>
<td>Niacin</td>
<td>156</td>
<td>Cystine</td>
<td>0.86</td>
</tr>
<tr>
<td>Ash</td>
<td>6.1</td>
<td>Pantothenic Acid</td>
<td>9.3</td>
<td>Glutamate + glutamine</td>
<td>6.53</td>
</tr>
<tr>
<td>Crude fibre (%)</td>
<td>12</td>
<td>Pyridoxine</td>
<td>7</td>
<td>Glycine</td>
<td>1.77</td>
</tr>
<tr>
<td>Tannins (%)</td>
<td>1.5</td>
<td>Riboflavin</td>
<td>5.7</td>
<td>Histidine</td>
<td>1.12</td>
</tr>
<tr>
<td>Phytic acid (%)</td>
<td>3.3</td>
<td>Thiamin</td>
<td>5.1</td>
<td>Isoleucine</td>
<td>1.56</td>
</tr>
<tr>
<td>Glucosinolates (μMol/g)</td>
<td>7.2</td>
<td>Tocopherol</td>
<td>13</td>
<td>Leucine</td>
<td>2.54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biotin</td>
<td>0.96</td>
<td>Lysine</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Choline</td>
<td>6500</td>
<td>Methionine</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Folic Acid</td>
<td>0.8</td>
<td>Methionine + cystine</td>
<td>1.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Niacin</td>
<td>156</td>
<td>Phenylalanine</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Proline</td>
<td>2.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Serine</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Threonine</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tryptophan</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tyrosine</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Valine</td>
<td>1.97</td>
</tr>
</tbody>
</table>

* Part of the 36% crude protein from canola whole seed.
Canola meal contains certain compounds known as antinutritional agents that make it unsuitable for human consumption and limits its use in animal feed. Some of the known anti-nutritional compounds in meal, acting to restrict its use, include crude fiber, glucosinolates, tannins, sinapine, and phytic acid (Table 1.1). These compounds can be either heat stable or heat labile (Aider & Barbana, 2011; Newkirk et al., 2003). However, the same compounds have been shown to have therapeutic value in some circumstances and have attracted researchers to evaluate their pharmaceutical potential (Grabley & Sattler, 2003). It would be beneficial to find ways to modify and process the meal to render it more usable either in the pharmaceutical or food industry, by improving its nutritive value and/or its chemical properties.

1.2. Bioactive compounds

Compounds that influence cellular and physiological activities are called ‘bioactive compounds’ (Kris-Etherton et al., 2004). Currently there is a high demand for bio-screening program for the identification of bioactive molecules from natural products, particularly edible plants, due to their well-documented safety profile. The compounds that may have health benefits can broadly be classified as proteinaceous or non-proteinaceous (Figure 1.5). Non-proteinaceous compounds include phytochemical related compounds such as gluconsinolates, brassinosteroids, phytates, vitamins and phenolic compounds. Proteinaceous compounds are protein related compounds and may include peptide and various types of proteins including enzymes and protein-based protease inhibitors.
The assessment of commonly available by-products of agricultural-based industries is useful, in particular for their role in medical and food industries (Oskoueian, Abdullah, Hendra, & Karimi, 2011). Bioactive compounds isolated from agricultural waste of different plant sources have shown useful industrial applications in pest management, food preservation and promoting human and animal health health (Mirabella, Castellani, & Sala, 2013). Plant bioactive compounds have been shown to be beneficial both as adjunct therapies for the treatment of illnesses and also as a source for future new pharmaceuticals (Kris-Etherton et al., 2004). Several techniques such as aqueous and apolar partitioning, ultrafiltration, and chromatographic approaches (e.g. reverse phase, ion-exchange, affinity, gel permeation, and gas chromatography) have been used for fractionation and purification of these bioactive molecules.
Figure 1.5. Bioactive compounds in canola meal.
A. Representative bioactive compounds; B. Structure of bioactive compounds; C. Structure of glucosinolate and its derivatives.
Brassicaceae vegetables are well-known for their diverse range of bioactive compounds such as folic acids, phenols, carotenoids, selenium, glucosinolates, and vitamin C and E. These compounds can affect apoptosis, scavenge free radicals, modulate enzyme activities, reduce inflammation and control the cell cycle (Podsedek, 2007). Brassica plants have been associated in the treatment of cardiovascular, cancer and inflammatory diseases (Velasco et al., 2010).

Canola meal which is an abundant and cheap by-product from the canola/rapeseed biodiesel and edible oil industries can be used for the discovery and isolation of bioactive compounds. The presence of high amounts of phenolic acids in canola compared to other oilseed crops (Rezaeizad et al., 2011), also provides a reason to further explore canola as a potential source of health beneficial phenolic compounds possessing therapeutic properties for the treatment of chronic diseases (Cartea et al., 2010).

1.2.1 Glucosinolates

Glucosinolates (GLS) are a thioether anionic class of plant secondary metabolite (Bilia, 2014) which are precursors of isothiocyanates. They are S-glucopyranosyl thiohydroximates with a side chain (R) and a sulphur-linked β-D-glucopyranose moiety (Figure 1.5-C). GLS are also described as β-thioglucoside N-hydroxysulfates or (Z)-N-hydroximinosulfate esters. GLS are present in a range of plant tissues in various families of dicotyledonous
angiosperms (Fahey, Zalcmann, & Talalay, 2001). Multiple factors such as genotype, plant age and environmental conditions can affect GLS composition and concentration in plant tissues (Aires, Carvalho, & Rosa, 2012).

Generally, GLS constitute approximately 1% of the dry weight (DW) in various tissues and sometimes reaching close to 10% w/w in some *Brassicaceae* species (Fahey et al., 2001). GLS are major secondary metabolites in seeds of *B. napus*, reaching up to 30 µmol/g in canola meal, and much higher in the meal of rapeseed cultivars (Aider & Barbana, 2011). When canola seeds are crushed, GLS comes into contact with the myrosinase enzyme which rapidly hydrolyses the GLS to liberate a wide range of degradation products such as nitriles, thiocyanates, and isothiocyanates (Figure 1.6-i).

GLS degradation products not only impart an unpleasant taste and odor to canola meal, but also pass on anti-nutritional, hepatotoxic and goitrogenic (thyrotoxic) properties. There are different types of GLS exhibiting differences in their molecular structure such as chain length, and addition or removal of aromatic, mercapto or thio groups such as aliphatic, aromatic or indolyl GLSs. More than 130 GLS have been identified in plants (Fahey et al., 2001; Wiesner, Schreiner, & Glatt, 2014). Twelve intact glucosinolates were found in broccoli leaves (Ares, Nozal, Bernal, & Bernal, 2014), whereas Fang et al., (2012) identified eleven GLS from canola extract, including four major GLS, progoitrin, gluconapin, 8, 4-hydroxyglucobrassicin, and glucobrassicanapin.
Figure 1.6. Hydrolysis of glucosinolates and formation of indole-3-carbinol in plants.

(i) Hydrolysis of glucosinolates (ii) Synthesis of indole-3-carbinol
At sub-toxic doses, GLS can have some useful pharmacological applications including a potential role in the treatment of cancer (Herr, Lozanovski, Houben, Schemmer, & Buchler, 2013). Plants have developed some effective intrinsic defense mechanisms against invading pests and GLS are also a well-known natural pesticides (Fahey et al., 2001). GLS and myrosinase are stored in separate cellular compartments to prevent damage to the plant. This occurs when pest invasion prompts de-compartmentalization, allowing myrosinase to come in contact with glucosinolates, thereby releasing a library of bio-pesticides with demonstrated antimicrobial, insecticidal, nematocidal, herbicidal (allelopathic) and anti-cancer functionalities (Chuanphongpanich, Phanichphant, Bhuddasukh, Suttajit, & Sirithunyalug, 2006; Spalding et al., 2008). GLS such as sinigrin shows insecticidal, bactericidal and nematocidal properties, while other GLSs such as sinalbin and glucotropaeolin are antibacterial, antifungal and antiviral in nature.

Indolyl glucosinolates, such as glucobrassicin, undergoes hydrolysis to produce indole-3-carbinol (I3C) derivatives (Fahey et al., 2001) as shown in Figure 1.6-ii. The I3C compounds are effective in maintaining estrogen levels in the body, they are useful in the chemoprevention of breast, prostate, colon, and cervical cancer and some types of leukemia (Kumar & Andy 2012). GLS can also act as an indirect antioxidant by modifying the properties of xenobiotic metabolizing enzymes both in phase I and phase-II for detoxification. Phase-I enzymes usually enhance the solubility of lipophilic components, while phase-II enzymes enhance water reactivity to assist for the removal metabolites from the cell. Cells could be protected from cancer and oxygen radicals by phase-I inhibition and phase-II stimulation. A particular
sequences of DNA known as antioxidant response elements (ARE) are present in the genes of phase-II enzymes (Fahey et al., 2001).

1.2.2 Brassinosteroids

Brassinosteroids are commonly found in the stem, leaves, pollen and seeds of Brassica species. They are a class of natural polyhydroxysteroids that play a role as plant growth regulators (Figure 1.5B-iii). They are sometimes referred to as the sixth class of plant hormones (Kaplan & Gokbayrak, 2012). Brassinosteroids have been investigated as plant growth promoters and to enhance tolerance towards a number of biotic and abiotic stress conditions such as drought, salt, cold, water and oxidation (Kumar & Andy, 2012).

Brassinolide was the first isolated brassinosteroid (Figure 1.5B-iv); campesterol and its derivatives are the precursors for brassinolide (Zullo & Adam, 2002). Brassinosteroids have antiviral properties against Herpes Simplex Virus (HSV-1), retroviruses, and virus causing measles (Wachsman & Castilla, 2012). They possess potent antiproliferative and antiangiogenic activities (Oklest, Rarova, & Strnad, 2013).
1.2.3 Phytates

Phytates are phytic acid salts and esters (Figure 1.5B-v), including inositol penta (IP5), tetra- (IP4) and triphosphate (IP3). The main stored forms of phosphorus in plant seeds are phytic acid and myo-inositol hexaphosphate (IP6). They have a molecular formula, C₆H₁₈O₂₄P₆ with a molecular mass of 660.04 g/mol (Kumar et al., 2010). Due to their polyanionic nature they can chelate positively charged species such as di- and tri-valent metal ions and proteins to form insoluble complexes, preventing their absorption from the intestine. Binding of phytic acid with minerals and proteins, including several important digestive enzymes, has been known to cause a lower level of physiological and functional availability of nutrients to humans (Raboy, 2001).

Phytic acid is usually present at high concentration in mature seeds and constitutes 1.0 to 5.0% by weight of edible cereal seeds, oilseeds, legumes, pollens and nuts; and 3.0–7.0 g/100 g in canola meal. It is also known for its antioxidant activity when it binds with iron to form iron chelate which increases the rate of Fe²⁺-mediated oxygen reduction to inhibit iron-driven hydroxyl radical formation, thus suppressing lipid peroxidation (Khattab, Goldberg, Lin, & Thiyam, 2010). A high amount of phytic acid is beneficial in blocking polyphenoloxidase formation by blocking browning and putrefaction of various fruits and vegetables (Ioannou, 2013). It also forms interactions with protein and carbohydrate, which results in reduced absorption of glucose in the body, suggesting that phytate-based plant food
could be used for the reduction of blood glucose in diabetic patients (Lee et al., 2006).

Phytates have been shown to have anticancer activity in various organs and diseased tissues of colon, prostate, breast, rhabdomyosarcoma (RMS), hepatocellular carcinoma (HCC), and pancreatic, blood and bone marrow cancers. They also have activities against coronary heart disease, reduction in total lipids and triglycerides, dental caries, and renal lithiasis. In addition, it has antiviral, antiplatelet (Kumar et al., 2010) and neuroprotective properties (Xu, Kanthasamy, & Reddy, 2008).

1.2.4. Vitamins

Vitamins are essential organic chemical compounds, which cannot be synthesized in sufficient quantities by animals, and must be obtained from the diet. Canola meal has a higher content of B vitamins and essential minerals when compared to soybean meal (the commercial food processing ‘standard’) (Bell, 1993). Canola meal contains choline, niacin, pantothenic acid, riboflavin, biotin, folic acids, pyridoxine and thiamin as well as vitamin E, as shown in Table 1.2 (Newkirk, 2009).

The term “vitamin E” describes a group of structurally related compounds, the tocochromanols [tocopherols (α, β, γ and δ) and tocotrienols], which are essential lipophilic antioxidants for animals and humans (Association of Official Analytical Chemists, 1990; IUPAC–IUP Joint Commission on
Biochemical Nomenclature, 1982). Canola seed contains tocochromanols, but with low levels of δ-tocopherol (Valentin & Qi, 2005). Tocopherols have been reported to be an antioxidant with oxygen radical scavenging singlet oxygen quenching properties (El-Beltagi & Mohamed, 2010).
1.2.5. Phenolic Compounds

Phenolic compounds are an important component of the human diet and they are ubiquitous in all plants (Dobes et al., 2013) with more than 8000 known structures (Azmir et al., 2013) but some phenolic compounds are restricted to particular species (Dobes et al., 2013). Phenolic compounds give plant products flavor, taste, color, technological properties and potential health benefits (Boudet, 2007). Phenolic compounds possess an aromatic ring attached to one or more hydroxyl groups (Figure 1.5B-i). Their structure may range from a simple phenolic molecule (C6) to a complex high-molecular weight polymer (C6-C1) (Balasundram, Sundram, & Samman, 2006). Phenolic compounds are biosynthesized through the shikimate and acetate pathways. Although, they are commonly found in the bound form such as esters and glycosides they can also exist in their free forms (Azmir et al., 2013). Phenolic compounds can be classified into phenolic acids, phenolic alcohols, flavonoids, stilbenes, lignans and tannins (Archivio et al., 2007).

Biophenols (phenols or polyphenols) was a term devised by Romeo and Uccella (1996) to describe phenols having bioactive properties. Plant foods rich in phenolic compounds are usually favourable for human health (Kris-Etherton et al., 2002). Phenolics are present in larger quantities in rapeseed as compared to other oilseed species (Yang et al., 2014). Phenolics from Brassica have been reported to have protective properties for human health; increased levels of antioxidants such as carotenoids, ascorbic acid and tocopherols. Kaempferol from Brassica species is often acylated with caffeic acid resulting in strong antioxidant activity (Cartea et al., 2010).
Figure 1.7. Phenol and other bioactive compounds present in canola meal. (i-vi) Hydroxycinnamic acids found in canola, (vii-viii) flavonoid aglycones, (ix) lignan, (x) sinapine (xi) canolol
Flavonoids, through their antioxidant activity, impart protection against several human diseases such as prevention of coronary heart diseases and inhibitory effect in various stages of tumor/cancer (Dorta, Gonzalez, Lobo, Sanchez-Moreno, & Ancos de, 2014; Kuntz, Wenzel, & Daniel, 1999), inhibition of fat oxidation, and inhibition of human immunodeficiency virus (HIV) (Yao et al., 2004) Phenolic fractions from *Brassica oleraceae* are high in quercetin and kaempferol (Figure 1.7, vii-viii) and have been found to stop the growth of gram-positive and gram-negative bacteria (Ayaz et al., 2008). Phenolic compounds have also been found to have health benefits including antioxidant activity, anti-atherosclerosis activity, and cardioprotective effects (Cartea et al., 2010). Overall, the higher level of antioxidant activity in canola results from the flavonoids rather than the vitamins and carotenoids. The major phenols in canola are phenolic acids and condensed tannins (Kumar & Andy 2012).

Phenolic compounds, such as flavonol glycosides, are good fungistatic and fungitoxic chemicals (Shahidi & Naczk, 2004), while some of them are also known to provide a defense mechanisms against insects and pathogens (Balasundram et al., 2006). The potential of plant phenolics to deliver useful health benefits has encouraged scientists to characterise in detail the effects of certain compounds such as antioxidants against atherosclerosis (Cartea et al., 2010), tumors, heart diseases, and neural disorders (Boudet, 2007).

Canola meal contains phenolic acids in free and esterified forms. The total content of phenol in canola meal lies between 12.8 and 15.4 mg milligrams of gallic acid equivalents per gram dry weight (mg GAE/g DW) (Obied et al.,
Canola meal has higher amounts of phenolic compounds (phenolic acids and sinapine) than other oilseed crops, such as soybean, cottonseed, and peanut. They are present as free, esterified and insoluble bound forms, constituting 15% of free, and 80% of esterified form of total phenolic acids (Rezaeizad et al., 2011). Studies have reported absorbance of phenolic compounds such as hydroxycinnamates and flavonoids into the human body in a concentration sufficient for them to act as an antioxidant or exhibit activities such as antiobesity, anti-diabetic and anti-topoisomerase activity in cancer patients (Olthof et al., 2001; Scalbert & Williamson, 2000).

The most common polyphenols in canola are hydroxycinnamic acids and flavonoids (flavonols and anthocyanins). The main flavonols are quercetin, isorhamnetin, and kaempferol (Shao et al., 2014), while hydroxycinnamic acids are p-coumaric, ferulic acids, and sinapic (Hsu & Yen, 2007). Total phenolic content (TPC) and total tannin content (TTC) are reported to be high in canola meal. Anthocyanins are also present in Brassica as delphinidin, malvidin, pelargonidin, cyanidin, peonidin and petunidin. However, cyanidins are more frequently reported (Cartea et al., 2010). Major components of flavonoids are shown in Table 1.2.
Table 1.2. Flavonoids present in various sources of foods.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Color</th>
<th>Known flavonoids</th>
<th>Food sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthocyanins</td>
<td>Blue, red, and violet</td>
<td>Cyanidin</td>
<td>Fruits and flowers</td>
</tr>
<tr>
<td>Flavonols</td>
<td>Colorless</td>
<td>Catechin, gallacatechin, epicatechin, epicatechin, epigallocatechin galate</td>
<td>Apples, hops and tea</td>
</tr>
<tr>
<td></td>
<td>Yellow</td>
<td>Procyanidin, Theaflavins</td>
<td>Wine, fruits juice, black tea</td>
</tr>
<tr>
<td>Flavonons</td>
<td>Colorless</td>
<td>Hesperidin</td>
<td>Citrus fruits</td>
</tr>
<tr>
<td></td>
<td>Pale yellow</td>
<td>Naringenin, eriodictyol, Neohesperidin</td>
<td>cumin, oranges, grapefruits, peppermint</td>
</tr>
<tr>
<td>Flavones</td>
<td>Pale yellow</td>
<td>Apigenin, Chrysin, luteolin, diosmetin</td>
<td>herbs, cereals, fruits, parsley, thyme, vegetables</td>
</tr>
<tr>
<td>Flavonols</td>
<td>Pale yellow</td>
<td>Isorhamentin, kaempherol. Quercetin, myrcetin, rutin</td>
<td>onions, cherries, apples, broccoli, kale, tomatoes, berries, tea</td>
</tr>
<tr>
<td>Flavanonols</td>
<td></td>
<td>Taxifolin</td>
<td>limon, aurantium</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Colorless</td>
<td>Daizein, genistein, glycine, formononetin</td>
<td>Legumes (soyabean)</td>
</tr>
</tbody>
</table>

Source: (Yao et al., 2004)
Phenol and their antioxidant activity in canola meal from various Australian canola cultivars (ACM) have been determined using various extraction techniques. The major phenol sinapine (choline ester of sinapic acid) as shown in Figure 1.7-xi, was present in all canola meal extracts (Obied et al., 2013), and constitutes nearly 90% of free canola phenolic acids, and has been shown to reach up to 94.8% of the total phenolic acid content after hydrolysis (Khattab et al., 2014).

Canola meal contains 1-2% (w/w) sinapine (Yang et al., 2014). This reduces its palatability for animals due to its bitterness, and causes a fishy taste in eggs of brown-egged chicken layers. These chickens do not have the enzymes required for the conversion of methylated sinapine to trimethylamine (Wang et al., 2013). One novel approach to this problem was to select a new chicken breed from the standard “Rhode Island Red” that was able to metabolize the sinapine and produce acceptable eggs (Khajali & Slominski, 2012). In order to raise the value of canola apart from breeding varieties with reducing sinapine, plant breeders have also aimed at reduced the level of sinapate ester through gene silencing techniques (Wei et al., 2009).

Microwave assisted decarboxylation of an alkaline hydrolyzed product of canola meal produced a high yield of the bioactive compound canolol, which has the capacity to act as an antioxidant, anti-inflammatory and antimutagen agent (Khattab et al., 2014). Canola meal extracts are known for their lipid peroxidation inhibition properties, which is determined by the hydroxycinnamic acids, sinapic, ferulic and $p$-hydroxybenzoic acids (Mann & Khanna, 2013), as shown in (Figure 1.7).
Sinapate esters (lignin precursors) in combination with phenylpropanoid have been reported to be useful for ultraviolet protection (Mpoloka, 2010) The 4-Vinylsyringol is a decarboxylation product of sinapic acid that can suppress cancer and inflammatory cytokines. Purified mustard meal extract was reported to contain sinapic acid derivatives having antibacterial activity (Engels, Schieber, & Ganzle, 2012). Sinapine is also known to be an acetylcholinesterase inhibitor and, consequently, has applications in the treatment of neural diseases. In addition, its antioxidative activity has potential in food processing, cosmetics and the pharmaceutical industry (Niciforovic & Abramovic, 2014).

Lignans are another a class of phenolic compounds (Figure 1.8-ix) which are found in whole grains, including canola (Fang et al., 2012; Umezawa et al., 2013). They have been reported to exhibit biological and antioxidant activities against numerous ailments such as tumors, and cardiovascular diseases (Meija et al., 2013; Umezawa et al., 2013). Metabolic profiling of seven new lignans including those of three (±) thomasidioic acid and four (E,E)-dienolignan derivatives were also determined in Brassica napus by Fang et al., (2012) Sinapic acid is known to convert to a lignin called thomasidioic acid (Obied et al., 2013).

Plants also contain highly polymerized major secondary metabolites known as tannins (Barbehenn & Peter Constabel, 2011), categorized into three subgroups: hydrolyzable tannins, condensed tannins, and pseudo-tannins (Jain, Jain, Pareek, & Sharma, 2013). Condensed tannins are very complex...
polyphenols, classified more generally in the proanthocyanidin group. Major tannins in canola are proanthocyanidins which occur through the polymerization of flavan-3-ols or flavan-3,4-diols. Tannins constitute up to 0.2 to 3% (w/w) of canola meal; but the precise level may vary depending on genotype, extraction process, and age of seed. Acetone (70% v/v) was demonstrated to be a good solvent for the extraction of tannin with a higher amount of tannin in rapeseed dehulled flour (ground seed) (1.08mg/g) than canola meal (0.87mg/g) and hull (0.11mg/g), expressed as catechin (equivalents (CE) per gram dry mass (Khattab & Arntfield, 2009; Liu, Wu, Pu, Li, & Hu, 2012). Significant results with canola tannin were also observed when using TEAC (Trolox equivalent antioxidant capacity) for the determination of antioxidant properties.

1.2.6 Proteinaceous compounds

Proteins make up about 35–40% of canola meal as shown in Table 1.1, (Khattab & Arntfield, 2009). Protein is essentially required for the growth and development of numerous body functions (Yoshie-Stark & Wasche, 2004). Canola protein hydrolysates and peptide fractions have been separated based on enzymatic hydrolysis and studied for their antioxidant and functional food properties (Alashi et al., 2013). However, research on bioactive canola proteins has not attracted a lot of attention.
Bioactive proteins can be isolated and purified by subjecting an aqueous extract to ammonium sulphate precipitation, salting out, ultra membrane filtration, anion exchange & gel filtration chromatography, and preparative gel electrophoresis. All these steps progressively remove undesirable proteins, enzymes, and other components from the source material (Shahidi & Zhong, 2008).

The health benefit of canola meal in the diets of fish has been known since the 1990s. Now, it is clearly understood that concentrated protein produced from canola meal is of great value in fish diets (Enami, 2011). It is also used as a protein source in the dairy, swine, beef, and poultry industries (Cardoza & Stewart, 2007). In spite of the immense interest in their nutritional and functional properties, there is a paucity of available information about canola bioactive proteins. Canola meal is rich in protein, which has a complex composition. Canola protein may have a great potential for producing bioactive peptides (Aachary & Thiyam, 2012).

Certain fractions in canola, such as globulins and albumins, constitute the majority of the proteins present (Aider & Barbana, 2011). The separation and enrichment of peptides, however, is normally achieved by using ultrafiltration and nanofiltration techniques using specific molecular weight cut-off industrial membranes (Bazinet & Firdaous, 2009; Korhonen & Pihlanto, 2006). Canola protein extraction is often inefficient because of the wide
variety in isoelectric points and molecular weights of these proteins (Aachary & Thiyam, 2012).

Canola proteins demonstrate some very useful technological functional properties such as water absorption capacity, oil absorption, protein solubility, emulsification, and gelling ability. When steamed canola protein concentrates were used in the preparation of sausages the results was improved taste, texture and aroma (Yoshie-Stark, Wada, Schott, & Wasche, 2006). Canola meal has a key role to play in nutrition due to the composition of amino acids making it a valuable product for human consumption (Chabanon, Chevalot, Framboisier, Chenu, & Marc, 2007). Canola proteins are richer in sulphur containing amino acids compared to soybean meal which the industry standard for protein isolates (Rezaeizad et al., 2011). Excessive heating during canola processing is the main reason for the lower amino acid availability in meal resulting in reduced digestibility and availability of lysine for animals. Processes with reduced overheating may allow increased recovery of amino acids, improving the nutritional value of the meal (Table 1.1).

Canola proteins also have interesting pharmacological activities such as bile acid-binding, anti-hypertension activity via the inhibition of angiotensin converting enzyme (ACE), antithrombotic activity, anti-proliferative and antioxidant activities (Aider & Barbana, 2011). Peptides obtained from B. carinata have been shown to have antioxidant properties and are also effective against hypercholesterolemia (Pedroche et al., 2007).
1.2.6.2 Bioactive Peptides

Bioactive peptides generally contain between 3 and 20 amino acids and are derived from the enzymatic digestion of larger proteins, usually in the gut of animals and humans. In addition to their intrinsic nutritional value, they may also have the ability to exert multiple physiological effects in the body (Bucci & Unlu, 2000; Vermeirssen, Van Camp, & Verstraete, 2004). Bioactive peptides are intercellular signaling molecules. They are generally released in vivo during digestion by microbial or host enzymes. Purified proteins either heated in the presence of acid, or subjected to proteolytic enzymes under controlled conditions, release bioactive peptides (Moller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008).

Bioactive peptides are cleaved at basic amino acids like lysine (Lys) and arginine (Arg) but the most common are dibasic cleavage sites which are present as Lys–Arg and Arg–Arg (Fricker, 2005). Some peptides are active after post-translational modifications (PTM). The most frequently observed PTM of bioactive peptides are amidation, acetylation, and pyroglutamate or sulphate formation (Nemere & Hintze, 2008). Bioactive peptides can have different functions within the body such as modulating opioid receptors (Pihlanto-Leppala, 2000), stimulation of the immune system, antioxidant effects through heavy metals binding, antithrombotic (Zhang, Wang, & Xu, 2008), anticancer, antibacterial (Udenigwe & Aluko, 2012), and ACE inhibition (Murray & FitzGerald, 2007).
In order to act as bioactive agents, peptides must reach their target sites in an intact form. Modern techniques such as nano and microencapsulation, liposomes, and bio (muco) adhesion offer an easy way for targeted delivery of sensitive peptide constituents (Segura-Campos et al., 2011). However, considering that most functional peptides are present in complex matrices, the initial hydrolysis of their protein fractions, and the optimal separation and purification of subsequent peptides, is achieved using different industrial processes. In most cases, protein hydrolysis is carried out using immobilized enzymes, as they are not required to be inactivated and can be easily recovered and reused under highly controlled conditions.

Bioactive peptides are important constituents of functional foods mainly for their therapeutic importance and value in the food industry. There is an intimate relationship between diet and chronic degenerative diseases (Moller et al., 2008). Complete insight into the mechanism of action of bioactive peptides in foods can provide significant opportunities for better health (Segura-Campos et al., 2011). Different bioactive peptides have different mode of action. For example peptide having antimicrobial activity disrupt the required ionic strength and pH of the pathogen (Berrocal-Lobo, Molina, Rodriguez-Palenzuela, Garcia-Olmedo, & Rivas, 2009). Peptides generated from canola meal could also be evaluated for their antimicrobial properties.

Composition of canola protein hydrolysates determines their functional properties and their potential application in the food industry (Cumby et al., 2008). Industrially defatted meal is used for the isolation of canola protein peptides of different molecular sizes, obtained through hydrolysis with
specific enzymes. These peptides may confer more than one beneficial effect on human health as they have peptide ontained have shown anti-hypertensive activity, increased cell density of Chinese Hamster Ovary (CHO) cells and also as growth supplement in serum-free medium (Chabanon et al., 2008; Chabanon et al., 2007). Inhibitory activity of rapeseed hydrolysate against HIV proteases was also observed (Yust et al., 2004), making them a potential candidate for the treatment against HIV.

1.2.6.3 Protease Inhibitors (PIs)

Protease Inhibitors (PIs) from plants are usually small proteins that have a function in the regulation of endogenous proteases and may have functions including including antimicrobial, anticancer, and anti-inflammatory activities (Kuhar, Mittal, Kansal, & Gupta, 2014). These molecules act by inhibiting the function of specific proteolytic enzymes (Liener, 1980). Plant PIs were firstly described in 1938, by Read and Haas in soybean (Birk, 1961). This functional protein was partially purified by Bowman (1946), and then isolated by Kunitz in crystalline form (Kunitz, 1947). PIs are mostly present in plant seeds, but most widely spread among leguminosae family (Mosolov & Valueva, 2005).

PIs are commonly used for the management of unwanted protein degradation. They have a wide range of therapeutic properties in the fields of medicine, agriculture, food processing and biotechnology. PIs, in addition to having antimicrobial activity, are of great interest to the food industry for improving product quality by decreasing food deterioration. The food industry employs
PIs to stop protein hydrolysis, and slow down absorption of free amino acid (Mandal, Kundu, Roy, & Mandal, 2002). However, recent studies have shown that they may have important practical applications as PIs been shown to be involved in plant defence mechanisms as insecticides.

PIs have also been implicated in signalling interactions with receptors in mammals and may act as therapeutic agents against modern day human medical ailments such as cancer, dengue fever, allergic reactions, cardiovascular and inflammatory diseases (Kuhar et al., 2014). PIs can prevent the development of active oxygen species by stimulated neutrophils, stop tumor formation and halt protein digestion to amino acids resulting in the starvation of proliferating cells. Currently, cocktails of synthetic PIs are used for the early treatment of HIV infected patients. Polyprotein gag-pol are involved in the structure and formation of RNA required for HIV. Cleavage of gag and gag–pol protein precursors in infected cells is avoided by HIV-protease inhibitors by arresting maturation, thereby blocking the infection of nascent virions (Yust et al., 2004).

PIs are classified into families of associated proteins depending on the primary spatial structure including the number and position of disulfide bonds, and the active sites. They have been classified into four groups based on the active amino acid sequences at their reactive site and the class of proteinases they inhibit (Rawlings & Barrett, 1993). The four groups of PIs are described as serine, cysteine, asparatic and metalloprotease PIs (Lawrence & Koundal, 2002), as shown in Figure 1.8.
The families of PIs have been well characterised (Habib & Fazili, 2007). Serine PIs constitute a large group of recognized compounds e.g soybean protease inhibitor (Bowman-Birk) family (trypsin/chymotrypsin), soybean trypsin inhibitor (Kunitz) family (trypsin; others), potato I inhibitor family (chymotrypsin; trypsin), potato II inhibitor family (trypsin; chymotrypsin), cucurbit (squash) inhibitor family (trypsin), cereal inhibitor family (amylase; trypsin), ragi I-2 inhibitor family (amylase; protease), and thaumatin-related inhibitor family (amylase; trypsin) (Deshpande, 2002).

Two of the main types of PIs widely discussed in literature are the Kunitz and Bowman-Birk types. PIs with a molecular mass of approximately 21 KDa, with 121 amino acids and four cysteine residues making two disulphide bonds within one chain and a single reactive site are known as Kunitz PIs. Bowman-Birk type PIs have a molecular mass of 8 KDa with 60 to 80 amino acids and fourteen residues for the formation of seven disulphide bonds. Both of these inhibitors are involved with serine proteases (trypsin, chymotrypsin and elastase). Kunitz inhibitors mainly inhibit trypsin, while chymotrypsin is weakly inhibited (Kuhar et al., 2014). Inhibitors of trypsin, serine proteases and chymotrypsin are normally present in plant foods and tissues (Richardson, 1991).
Figure 1.8. Major classification of Protease Inhibitors (PIs)
Serine PIs are active in inhibiting serine enzymes *in vitro* within a pH range of 9 to 11. All serine inhibitor families from plants are competitive inhibitors (Lawrence & Koundal, 2002). They are known for regulating endogenous plant proteases and therefore act as biopesticides (Abdeen et al., 2005) and antifungal agents (Park et al., 2005), and also have potential applications in treatment of cancer (Kennedy, 1998), blood clotting (Oliva et al., 2000) and treatment of obesity (Duranti, 2006).

Cysteine PIs include papain, calpain, and lysome cathepsin. They are active within a pH range of 5 to 7 are commonly found in rice. Their thermostable activities and have been implicated in the defence against insects of the Bruchidae family (Lawrence & Koundal, 2002). Cysteine PIs have we characterised families incuding the cystatin superfamily, cystatin, stefin, and fitocystatin families (Deshpande, 2002). Plant proteinases that cleave seed storage proteins during germination were found to be cysteine proteinases of papain (Palma et al., 2002).. The activity of plant PIs is increased under conditions of abiotic or biotic stresses (Downing et al., 1992).

There is limited information on aspartic protease inhibitors. They are known to exist in potato tubers, sunflowers and barley (Lawrence & Koundal, 2002). They have been classified into families of human immunodeficiency virus (HIV), pepsin, renin, and cathepsin protease inhibitors (Deshpande, 2002). These PIs have been associated with antifungal properties (Braga-Silva & Santos, 2011) and HIV prevention (Mastrolorenzo, Rusconi, Scozzafava, Barbaro, & Supuran, 2007).
Metallo-protease inhibitors consist of two families named carboxypeptidase inhibitors (4 kDa), and cathepsin D inhibitors (27 kDa). Carboxypeptidase inhibitors are mostly present in plants such as potatoes and tomatoes. They are reported to inhibit carboxypeptidases from microorganisms, and they can also inhibit five proteases including trypsin, chymotrypsin, elastase and carboxypeptidase A and carboxypeptidase B in higher animals and insects (Lawrence & Koundal, 2002).

PIs have been previously identified in the rapeseed family and are known to be low molecular weight thermostable trypsin-type inhibitors (Visentin, Iori, Valdicelli, & Palmieri, 1992). Mustard trypsin inhibitor (MTI-2) was the first protease inhibitor to be purified from white mustard (Sinapis alba L.) seeds (Menegatti et al., 1992).

Another rapeseed PI named RTI with similar properties was isolated from Brassica napus L. var. Oleifera (Ceciliani et al., 1994). The protease inhibitors already known in the rapeseed family have been reported for their vital role in protecting the plant against environmental stresses such as high salinity and drought conditions (Mosolov, Grigoreva, & Valueva, 2001). Trypsin inhibitors from Indian mustard Brassica juncea (BjTI) were found to be a thermostable Kunitz-type PI having insect resistance properties (Mandal et al., 2002). PIs from under-utilized canola meal have rarely been exploited commercially for their use in the food or pharmaceutical industries and thus warrants further research, that may lead to the formulation of value added functional foods and new pharmaceutical products.
1.3 Bioactive Properties/Activities of Bioactive compounds

Bioactive compounds can be classified by their underlying biological modes of action, such as antioxidants, enzyme inhibitors and inducers, inhibitors and inducers of receptor activities, and inhibitors of gene expression (Kris-Etherton et al., 2004). Biological functional properties can also be categorized according to their specific activity against cells and tissues in the body (Figure 1.9). For example, anticarcinogenic, cardioprotective, antithrombotic, and bile acid-binding capacity (Aider & Barbana, 2011; Kobayashi, Suzuki, Kanayama, & Terao, 2004; Wang & Ng, 2006; Xiu Juan & Tzi Bun, 2011), osteoprotective, antilipemic, immunomodulatory, antimicrobial (fungus, bacteria and virus), antioxidant, opiate (Moller et al., 2008), and insecticidal (Zhao et al., 2010).

Bioactive compounds have different mechanisms of action for different biological activities. They may act as an antioxidant for the reduction of oxidation that decreases DNA damage; enzymes inhibition of angiotensin converting enzyme, reducing blood pressure; DNA Binding by inhibiting topoisomerase activity for cancer cell death; modification of gene expression by inhibition of fat cell differentiation for the reduction of obesity.
Figure 1.9. Some known potential health benefits of bioactive compounds.
1.3.1 Antioxidant

Oxidation in the human body produces chemicals called 'free radicals' which are linked to diseases such as heart, liver disease and cancer etc. These free radicals are harmful to health and environment and causes degenerative conditions such as deterioration of the eye lens, Parkinson’s/Alzheimer’s disease, cardiovascular diseases, and certain cancers. Antioxidants in food help neutralize free radicals. These antioxidants include vitamins A, C, E, and the minerals (copper, zinc and selenium). Antioxidants can help to protect the body from free radicals, reactive oxygen species and reactive nitrogen species (Wu and Hansen, 2008). Antioxidant compounds can originate from natural and synthetic sources. Synthetic antioxidant compounds are associated with the risk for toxicity or mutagenic effect, which has led scientists towards sources of natural antioxidants (Thangam et al., 2013).

Some of the antioxidant compounds present in oilseeds are fat soluble, as they are present in conjugate form with sugar compounds. Hence, canola meal could be a good source for the extraction of antioxidant compounds (Shahidi & Zhong, 2010). Natural occurring antioxidant classes include flavonoids, tocopherols, and phenolic acids (Naczk & Shahidi, 2006). Current research on natural antioxidants is focused on finding newer molecules with increased ability to inhibit free radicals of oxidative stress. High levels of antioxidant activity have been reported in extracts from legumes such as peas, navy beans, lentils, broad beans, faba beans, and adzuki beans (Amarowicz, Karamac, & Shahidi, 2003). Conventional extraction techniques based on organic solvents have been applied for the extraction of natural antioxidants from canola.
(Amarowicz et al., 2003; Cumby et al., 2008). Various phenolic compounds are present in canola meal or meal extracts, have demonstrated antioxidant properties (Vuorela, Meyer, & Heinonen, 2003).

DPPH (α, α diphenyl-1-picrylhydrazyl) is an organic chemical compound that is composed of stable free radical molecules. It is used in laboratory research to monitor chemical reactions involving radicals. The ferric reducing antioxidant power (FRAP) assay is based on the reduction of ferric tripyridyltriazine to its ferrous coloured form for the amount of antioxidants based on FRAP reagents. Rapeseed protein isolates were shown to have higher DPPH radical scavenging activity compared to soybean (Yoshie-Stark, Wada, & Wasche, 2008).

1.3.2 Topoisomerase Inhibition

Topoisomerase-I (topo-I) is an important enzyme that helps to catalyze the relaxation of DNA involved in cellular processes such as DNA replication, condensation of chromosomes, transcription, recombination and maintenance of genetic stability. Topoisomerase involves the transient breakage of one (topoisomerase-I) or both (topoisomerase-II) strands of DNA in a single or double strand of DNA (Pommier, Leo, Zhang, & Marchand, 2010).

DNA topoisomerase-I inhibitors have been shown to have anticancer activity (Pommier et al., 2010). Anticancer and antibacterial activities have been associated with topoisomerase inhibitors as they lead to a high level of
intermediate DNA cleavage, resulting in apoptosis (Cheng et al., 2013). This effectively kills cells that are dividing rapidly in an uncontrolled manner.

Topoisomerases affect the closed circular supercoiled DNA and double stranded DNA by creating a nick in one strand in the phosphodiester backbone (Figure 1.10 b). Usually a catalytic intermediate is formed when a covalent linkage is formed between the enzyme and the 3’ end of the nicked DNA strand (Figure 1.10 c). This nick in the DNA allows the rotation of the cleaved strand around the double helix axis, which changes the conformation of the DNA to a relaxed DNA (Figure 1.10 d). Topo-I can also religate the cleaved strand of DNA back to the double stranded form, which allows the DNA to keep replicating (Figure 1.10.e). The nicked stage in DNA replication has been confirmed to be an effective step with cell division.

Compounds that do not allow the nicked strand to relegate back to the double stranded position, therefore, have the capacity to interfere with cell division; and leads to apoptosis and cell death (Figure 1.11). There are two known mechanism to stop this cell division called topoisomerase poisoning and topoisomerase suppression (Webb & Ebeler, 2003). Topoisomerase poisoning is caused by irreversible binding of compounds to the enzyme resulting in the cell cycle proceeding towards apoptosis by p53-dependent cell death. (Pommier et al., 2010). The topoisomerase poisoning mechanism is shown in Figure 1.11.a-e.
Figure 1.10. Topoisomerase enzyme showing replication of DNA;
(a) Supercoiled DNA, (b) Supercoiled DNA+ topoisomerase, (c) Nicked DNA, (d) Relaxed DNA and (e) Supercoiled DNA
Figure 1.11. Topoisomerase enzyme showing poisoning and suppression.
(a-e) Topoisomerase Poisoning; (f-g) Topoisomerase suppression
Camptothecin (CPT) is a natural alkaloid extracted from the stem tissue of the Chinese tree *camptotheca acuminata* and has the ability to inhibit topo-I. It is currently used as an anticancer agent in clinical research, suggesting other topo-I inhibitors could also function as anti-cancer agents (Webb & Ebeler, 2003). The family of camptothecin (CPT) compounds which target and block DNA topo-I activity, have now been reported to be used in medical practice (Tabassum, Al-Asbahy, Afzal, Arjmand, & Bagchi, 2012). The use of non-camptothecin top-1 inhibitors, such as edotecarin, is being investigated in clinical trials (Pommier et al., 2010). It would be useful to investigate the activity of topo-I inhibiting compound in human diet.

Another mechanism to reduce topoisomerase activity is known as topoisomerase suppression where compounds stop or reverse topo-I complex formation. Topoisomerase suppression results from interference with the supercoil relaxation activity of the enzyme. In this situation, a strong bond is formed between the compound and the top-1 enzyme, so topo-I is unable to cause a nick in the supercoiled DNA (Figure 1.11 f-h).

Some compounds found in natural products have been shown to be topoisomerase suppressors that interact directly with the enzyme. More than 60% of anticancer drugs are obtained from natural products (Stankovic et al., 2011). Compounds from natural products such as topostin, β-lapachone, topostatin, diospyrin have been shown to be topo-I inhibitors (Bailly, January 2000). Flavonoids derivatives have been shown to stop the binding of DNA to enzymes or inhibit the binding of ATP to topo-I (Hwangbo et al., 2012; Palchaudhuri & Hergenrother, 2007).
Different stages that represent interaction between supercolied DNA and enzyme topo-I are called as topoisomers. These specific confirmations of DNA which represent degrees of interactions between compounds and enzyme are used to assess ability of compounds to interfere with topo-I. These different topoisomers can be visualized on agarose gels (Webb & Ebeler, 2003).

1.3.3 Antidiabetic activities

Diabetes mellitus (DM) is a metabolic syndrome caused by high level of blood glucose following deficiencies in insulin secretion, insulin action or both (Ashcroft & Rorsman, 2012). DM is widely spread worldwide (Ginter & Simko, 2013), with an increasing rate of occurrence in Australia (Chamberlain et al., 2013). A range of health problems such as cardiovascular diseases (CVD), peripheral vascular disease (PVD), coronary artery disease (CAD), stroke (Pontiroli, Alberto, Paganelli, Saibene, & Busetto, 2013), neuropathy, renal failure, retinopathy amputations, and blindness, are recognized to be linked with DM DM (American Diabetes Association, 2013).

Dipeptidyl peptidase-IV (DPP-IV) is a membrane bound leukocyte antigen CD26 (cluster of differentiation 26 or T-cell activation antigen CD26), and is soluble in body fluids and various tissues (Singh et al., 2011). It is a serine protease (Silveira, Martinez-Maqueda, Recio, & Hernandez-Ledesma, 2013) which remove dipeptides from the N-terminus of proteins by cleavage of two amino acids (proline or alanine residues) of glucose dependent insulinotropic
polypeptide (GIP) and glucose like peptide (GLP-1) making them inactive (Jadav et al., 2012). This also results in a short half-life of active GLP-1 and GIP of 1-2 minutes (Nongonierma & FitzGerald, 2013). Hence, there is a need to identify inhibitors for DPP-IV, which may be able to enhance the amount of endogenous active GLP-1, GIP and insulin secretion.

Previous research on animals with a genetic loss of DPP-IV showed better tolerance of glucose and high rates of insulin secretion (Yogisha & Ravisha, 2010). Current research for type-II diabetes has also revealed that DPP-IV inhibition for a prolonged period of one year stops diabetes due to a reduction of fasting and postprandial glucose and glycated hemoglobin (HbA1c) levels (Deacon & Holst, 2006). It would be useful to identify a natural DPP-IV inhibitor which has minimal side effects (Ali et al., 2012).
1.3.4 ACE inhibition

Angiotensin-converting enzyme (ACE) is a carboxydipeptidase (EC. 3.4.1.5.1) regulated by renin-angiotensin-aldosterone system (RAAS) and kinin nitric oxide system (KNOS). RAAS has a major role of controlling blood pressure and salt-water in a balance, whereas in KNOS, ACE deactivates a hypotensive peptide called bradykinin (Connolly, O’Keeffe, Piggott, Nongonierma, & FitzGerald, 2014). This regulatory mechanism occurs in the kidneys, where the catalytic enzyme renin is produced.

ACE hydrolyses is the mechanism for the conversion of angiotensin-I (decapeptide) to angiotensin-II (octapeptide). The Angiotensin II is a vasoconstricting octapeptide that increases the blood pressure by degradation of a vasodilator called bradykinin (Chen et al., 2009). The mechanism of action for ACE is shown in Figure 1.12. The inhibition of ACE may be used as a therapeutic tool for the treatment of high blood pressure (Lacaille-Dubois, Franck, & Wagner, 2001).

ACE inhibitory drugs reduce the risk of heart related chronic diseases such as cardiovascular death (CVD) (Yusuf et al., 2011), nonfatal myocardial infarction (Kober et al., 1995) or cardiac arrest in stable coronary heart disease (White & Greene, 2011), heart failure (Krum et al., 2011), prevent left ventricular remodelling (Yokota et al., 2014), delay the frequency of microalbuminuria, the threat of diabetic nephropathy in type-II diabetes, and the probability of newly diagnosed diabetes mellitus (Haller et al., 2011).
Figure 1.12. Regulatory hormonal mechanism of renin angiotensin system (RAS)
Qian et al., (2012) demonstrated ACE activity in extracts from wild rice (Zizania latifolia Turcz.). It was also found that peptides derived from freshwater Zooplankton have the potential to act as antihypertension compounds in foods and pharmaceutical industry through the inhibition of ACE (Lee, Lee, Park, Kim, & Byun., 2010). Ojeda et al., (2010) reported that anthocyanins inhibit the ACE enzyme activity by competing with the active site, and also identified ACE inhibitory activity of anthocyanins from aqueous extract of the medicinal plant Hibiscus sabdariffa.

1.3.5 Antiobesity activity

Obesity has spread at a high rate worldwide due to life style changes; less physical exercise and unhealthy eating habits (Yang, Chen, Zhao, Ge, & Liu, 2014). Obesity is a health condition that also leads to several other chronic diseases such as high blood pressure, arteriosclerosis, type-II diabetes, respiratory problems and coronary heart diseases (Yang et al., 2014).

Plants derived compounds have been demonstrated to show anti-obesity properties (D'Mello, Darji, & Shetgiri, 2011). Plant derived foods, vegetables and nuts have compounds such as polyphenols and carotenoids, which are nutritional compounds that function as signalling molecules for the inhibition of adipogenic differentiation (Warnke, Goralczyk, Fuhrer, & Schwager, 2011). It is also known that flavonoids play significant roles in the process of food assimilation and other phases of energy metabolism and also play a role in prevention of diseases such as type-II diabetes and obesity (Hussain & Marouf, 2013).
Screening botanical extracts using human-derived cell culture-based screening systems is an economic and efficient method of identifying natural products with potential therapeutic effects such as anti-adipogenic properties (Buehrer et al., 2011). The ability to inhibit enzymes involved in lipid absorption is another approach to studying the impact of natural compounds. Inhibition of adipogenesis and inhibition of lipase activity are two methods for studying the potential anti-obesity properties of natural compounds.

1.3.5.1 Lipase Inhibition

Pancreatic lipases (PL) regulate lipid metabolism, thereby reducing serum cholesterol, obesity, hypertension and diabetes (Zhu et al., 2015). Recent reports in the literature suggest that compounds from tea (Zhu et al., 2015), Walnut Shell (Juglans regia L.) (Yang et al., 2014) berries, grape seeds, black soybeans, pomegranate (Sergent, Vanderstraeten, Winand, Beguin, & Schneider, 2012), and apples (McGhie, Hudault, Lunken, & Christeller, 2011) have lipase inhibition properties (Conforti et al., 2012).

There two main types of drugs used for the treatment of obesity. Orlistat (Xenical) decreases intestinal fat absorption through inhibition of pancreatic lipase (PL) and sibutramine (Reductil) is an anorectic (appetite suppressant). However, both drugs cause side effects such as hypertension, a dry mouth, constipation, and headaches. Sibutramine has been withdrawn from sale due to the severe adverse side effects (Ado, Abas, Mohammed, & Ghazali, 2013).
1.3.5.2 Antiadipogenesis

Adipocytes originate from progenitor cells called preadipocytes, and the process of differentiation from preadipocytes to adipocytes is termed adipogenesis (Stephens, 2012). The size of adipocytes is regulated by utilization of triglycerides, however, their number is not reduced with fat utilization but controlled by the rate of adipogenesis (Carter et al., 2013). Inhibition of adipogenic differentiation pathways is commonly assessed using the mouse preadipose 3T3 L1 clonal cell line to determine the impact of compounds on the regulation of adipogenesis (Kowalska, Olejnik, Rychlik, & Grajek, 2014).

The C3H10T1/2 cell line is another multipotent embryonic fibroblast cell line used to demonstrate adipogenic differentiation and could be employed to assess antiadipogenic properties (Warnke et al., 2011). These murine embryonic mesenchymal stem cells (C3H10T1/2) are induced by hormones to differentiate into adipogenic cells. During differentiation, cells undergo mitotic division complemented by wide-ranging transcription factors with peroxisome proliferators-activated receptor gamma (PPARγ) as a master regulator along with other chromatin regulators (Lowe, O'Rahilly, & Rochford, 2011).
1.4 Extraction of bioactive compounds

Some of the antinutritional agents in canola may be classed as bioactive compounds if they can be shown to have a physiological effect. These compounds may fall into various chemical classes, which would require targeted extraction procedures for each type of compound.

Some conventional chemical extraction methods using plant (botanical) material include soxhlet, maceration and hydro-distillation, while more recent and advanced methods include ultrasonic disruption, microwave pulsed electric field assisted technique, supercritical fluid and pressurized liquid extraction (Azmir et al., 2013). When a new chemical is under investigation it is common for the various extraction methods to be tested for their applicability, and then modified to optimize the yield.

The total yield and activity of any extract depends on the method of handling the specimen during the process of extraction and the specific solvent used (Boulekbache-Makhlouf, Medouni, Medouni-Adrar, Arkoub, & Madani, 2013). Separation and identification of compounds from plant sources is often difficult as the source plant material is variable (due to tissue source, season, soil type, storage conditions and duration) and consists of a complex mixture of many different compounds which may chemically interact, or bind to non-reactive components. Researchers have developed various methods for compound extraction using aqueous mixtures and solvents such as water, methanol, ethanol, acetone, ethyl acetate and to a lesser extent propanol and dimethylformamide, both individually and in combination. A combination of
alcohols with water appeared to be more efficient than using a pure solvent for most plant extractions (Chavan, Shahidi, & Naczk, 2001; Zhang, Wang, & Xu, 2007), possibly because a combination of solvents are capable of extracting a range of less polar aglycones and sugar attached glycosides (Escribano-Bailon & Santos-Buelga, 2003). Aqueous mixtures of ethanol, methanol and acetone, are commonly used at a concentration of 70% or 80% for extraction purposes (Naczk & Shahidi, 2004).

Compound identification and quantification is usually performed using high performance liquid chromatography (HPLC) with diode array detection (HPLC-DAD) and liquid chromatography mass spectrophotometry (LCMS) (Obied et al., 2013). Mustard meal purified extract has been characterised and identified using ultra-high-performance liquid chromatography with diode array and electrospray ionization-mass spectrometric detection (UHPLC-DAD-ESI-MSn) (Engels et al., 2012). Commonly, unknown compounds are identified by nuclear magnetic resonance (NMR) spectroscopy (Thiyam-Hollander, Aladedunye, Logan, Yang, & Diehl, 2014).

This literature is a compilation of recent information on canola meal bioactive compounds and their biological properties. Recent findings are discussed to identify gaps in research on canola meal biologically active photochemical and proteinaceous compounds. Future work on economic production of novel bioactive compounds is required to expand the spectrum of canola meal industrial application in food, medicine and agriculture. Canola meal research on bioactivities may have the capacity to expand for improved human health and profitability.
Previous study about canola meal bioactivities relates to sinapine, peptides and hydrolysate with virtually no bioactivity carried out with canola meal generated different solvents and protease inhibitor. Hence studies are required to identify compounds using different solvents and analyze them for their bioactive properties such as antioxidant, antiobesity, antitopoisomerase, and angiotensin converting enzyme (ACE) inhibition.
1.5 Research Aims

As described above, there have been numerous examples for the isolation and characterisation of plant derived bioactive compounds. This characterisation has varied from the determination of the chemical composition of extracts, assessment of their activities using reagent based assay, enzyme-based assays and cellular based assays. Ultimately the goal of this research is to demonstrate physiological activities of bioactive compounds in whole animal or human systems. However, there are very few studies in this area due to the complexity and cost of measuring bioactive properties in whole animal systems. Until the cost and complexity of whole animal studies can be reduced, it is useful to pursue reagent, enzyme and cellular based assays to measure bioactivity as a way of predicting the potential value of bioactive compounds. This study focuses on the isolation and characterisation of potential bioactive compounds from canola meal including protease inhibitors (PIs) and phytochemicals. Figure 1.12 shows a flow chart illustrating the steps in involved in extraction, purification and characterisation of phenols and protease inhibitors from canola meal and their effect for bioactive assays.
Figure 1.13. A flow chart highlighting the steps involved in the analysis of canola meal extractions for their biological activity
The objectives of this study are:

i. To investigate the use of different solvents to generate extracts which potentially contain bioactive compounds

ii. Characterise the chemical composition of extracts to assess the likely bioactivity potential.

iii. To determine antioxidant properties in the different solvent extracts from canola meal using reagent-based assays as a way of predicting potential in-vivo bioactivity.

iv. To extract and characterise novel protease inhibitors (PIs) from canola meal.

v. To determine the impact extracts on a range enzymes including topoisomerase, angiotensin converting enzyme (ACE), dipeptidyle peptidase-IV (DPP-IV) and pancreatic lipase as a way of predicting potential in-vivo bioactivity.

vi. To assess the potential in-vivo bioactivity of canola meal extracts using a cellular based assay (inhibition of adipogenesis in stem cells)

By using a range of techniques including chemical characterisation, purification, reagent based-assays and cellular based assays, it is hoped that a better understanding of the potential bioactivity of canola meal extracts can be determined.
CHAPTER 2. Extraction, chemical characterisation, and in vitro antioxidant activity of canola meal

2.1. Introduction

An ideal extraction procedure is one that has been optimized to deliver a high yield of chemical(s) in a relatively pure form using a simple, cheap, safe, reliable and repeatable method (Hernández, Lobo, & González, 2009; Spigno, Tramelli, & De Faveri, 2007). Phenolic compounds are key secondary metabolites in all plants, and are reported for a potential health effect. Therefore, optimal extraction is a prerequisite in laboratory-based research for the subsequent identification and classification of individual chemicals; it is also required in industry where the combined extraction and purification process must be financially viable (Azmir et al., 2013).

The selection of a suitable solvent for extraction of phytochemicals from plant material is a crucial step in any subsequent chemical analysis, as it determines the type and the quantity of compounds that was recovered. For extraction of plant phenols a number of solvents have been tried including neat solvents or solvent mixtures. Hydro-organic solvents –water/organic solvent mixtures– revealed superior extraction characteristics that exceed either solvent alone (Tomsone, Kruma, & Galoburda, 2012; Turkmen, Sari, & Velioglu, 2006). The organic solvent choice and its percentage are largely dependent on the phenolic composition and the nature of plant matrix. Major phenolic components of canola meal were found to be sinapic acid conjugates (Siger, Czubinski, Dwiecki, Kachlicki, & Nogala-Kalucka, 2013). Different extraction solvents have been used to extract canola meal
phenols (Obied et al., 2013). Water-alcohol mixtures seem the most common, while water-acetone mixtures are the most effective (Obied et al., 2013).

No doubt, the prime characteristic of plant phenols is their outstanding antioxidant properties. The need for natural antioxidants is necessary in food, cosmetic and pharmaceutical industries. Antioxidants can protect food and cosmetics from oxidative deterioration and can fight oxidative stress which is involved in the pathophysiology of aging and many chronic diseases. Antioxidants may exert their effects by prevention of free radicals formation, scavenging of formed free radicals, reduction of oxidizing agents, and/or metal chelating activity. A compound may demonstrate antioxidant properties in one setting, no activity, or even, prooxidant activity in other settings. Therefore, antioxidant activity is considered a multidimensional property that should be assessed using multiple assays for a more accurate evaluation (Frankel & Meyer, 2000). In vitro antioxidant activity of canola phenols has been investigated in a number of model systems (Obied et al., 2013).

The objective of this study is to examine the power of hydro-organic solvents in extracting canola meal phenols. In addition to aqueous-acetone, aqueous-methanol and aqueous-ethanol mixtures, water, and aqueous mixtures with butanol, chloroform, and hexane were also investigated for the first time. Phenolic content was studied by Folin-Ciocalteu assay and chromatographic analysis. Antioxidant activity was assessed by online ABTS radical scavenging assay, offline DPPH radical scavenging assay, and ferric reducing antioxidant power (FRAP) assay.
2.2 Materials and methods

2.2.1 Materials

Canola seed samples from two different genotypes: genotype-1 and genotype-2 were obtained from the Department of Primary Industries (DPI), Wagga Wagga, Australia. Genotype-1 is a local, open-pollinated breeding line with high-yielding, well-adapted, early-flowering with high blackleg resistance. Genotype-2 is an open pollinated commercial cultivar with a typical, local cultivar with mid-flowering, high blackleg resistance, and good adaptation (high yield and canola quality).

HPLC-grade methanol, \( n \)-hexane, \( n \)-butanol, acetone were from Fisher Scientific (New Jersey, USA). Glacial acetic acid and concentrated hydrochloric acid were obtained from Merck (Melbourne, Australia). Anhydrous acetonitrile, chloroform and ethanol were from UNICHROME (Sydney, Australia). Sodium carbonate (\( \text{Na}_2\text{CO}_3 \)), ferric chloride, Folin-Ciocalteu’s reagent, 2,2’-diphenyl-1-picrylhydrazyl (DPPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2’-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammmonium salt (ABTS), 2-4-6-tripyridyl-s-triazine (TPTZ), potassium persulphate, naringenin, gallic acid, catechin hydrate, caffeic acid, chlorogenic acid, ferulic acid, rutin, luteolin, sinapic acid, \textit{trans}-cinnamic acid, 4-hydroxybenzoic acid, phenylisothiocyanate, quercetin, ellagic acid, tyramine, pyrogallol, and vanillic acid were purchased from Sigma Aldrich (Sydney, Australia). Epicatechin, kaempferol, were obtained from Extrasynthese (Genay, France). Membrane nylon filters (0.22 μm) were purchased from Sigma Aldrich (Sydney, Australia). Syringe
filters (0.22 μm) were obtained from Millex GP (Sydney, Australia). Ultrapure water (UPW) was prepared by GenPure water purification system (0.22 μm filter) Thermofisher Scientific (Melbourne, Australia).

2.2.2 Preparation of canola meal

Approximately 200 g of seeds were used for canola meal (CM) preparation by grinding seed four times using a mechanical grinder (Foss Knifetec™ 1095, Slangerupgade, Denmark) at the Australian Oilseed Laboratory, Department of Primary industries, NSW, Wagga Wagga, Australia. The oil was removed from the ground seeds using soxhlet (soxtec™ 2050, Tector™ Technology, Slangerupgade, Denmark) with Buchi B-811 Extraction System (BUCHI Labortechnik, Flawil, Switzerland) using absolute n-hexane for 16 h. The oil was discarded. The meal remaining after oil extraction was air dried, mixed to remove lumps, and left overnight in a fume hood at room temperature (RT) in order to remove all residual hexane. The resultant material was base meal used for extraction purposes in all subsequent analyses.
2.2.3 Preparation of canola meal extracts (CMEs)

Exactly, 5 g samples of CM were dissolved in either 100 mL in ultra-pure water UPW, or 80% organic solvent in UPW (v/v). Organic solvents used were methanol, ethanol, acetone, butanol, chloroform and hexane. These solvents were mixed with CM using a vortex (Super-Mixer Lab-Line Instrument. USA) and then continuously mixed in a suspension mixer (Ratex, Rowe Scientific Pty Ltd) for 12 h at room temperature, then centrifuged (Eppendorf centrifuge 5810R) at 3000 rpm for 30 min to produce a supernatant extract. Samples of supernatant were filtered using a syringe filter for maximum recovery of the soluble extract. These extracts were transferred to a round bottom flask and passed through a rotary evaporator called Rotavapor R-210 (BUCHI Labortechnik, Flawil, Switzerland) for solvent removal. The samples were then dissolved in a minimum amount of UPW, after which whole extract was stored at −80°C for approximately 12 h, before being freeze dried using a Christ-Alpha 2-4 LD Plus freeze dryer (Biotech International, Germany). The freeze drying was done for 72 h, after which the samples were stored at −20°C until required.

This freeze dried material will be called “extract” and is used frequently in all assays and experiments of this thesis. All canola meal extracts (CMEs) were named according to solvent used for extraction with meal such as water extract (WE), methanol extract (ME), ethanol extract (EE), acetone extract (AE), butanol extract (BE), chloroform extract (CE) and hexane extract (HE).
2.2.4 Total phenolic contents (TPC)

All freeze dried extracts were mixed with 50 % methanol, and filtered for measuring total phenolic content using Folin-Ciocalteu reagent. Gallic acid stock solution with 1 mg/ mL in 50% aqueous methanol was used for the preparation of the following dilution series: 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/mL. Gallic acid was used as the standard to produce a calibration curve for the determination of the total phenolic content of the sample (CMEs). Blank (50% aqueous methanol) and different gallic acid concentrations (100 µL) were added to 10 mL volumetric flask, containing 7 mL of UPW. The mixture was then mixed with 500 µL Folin-Ciocalteu’s reagent and 1.5 mL of 20 % sodium carbonate. The reaction was incubated for 1 h at room temperature. Samples were then measured by spectrophotometer (Cary 50 spectrophotometer, with Cary WinUV version 3 software, Varian, Australia at 760 nm. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram dry weight (mg GAE/g DW).

2.2.5 High performance liquid chromatography-diode array detection with online ABTS scavenging (HPLC-DAD-online ABTS)

Phenolic compounds were characterised using HPLC-DAD and LC-MS where all CMEs were prepared as 15 mg/10 mL in 50 % of methanol water mixture and filtered. All samples were processed in the vortex and/or underwent sonication where required for complete solubilisation. For the HPLC, same conditions were maintained as described by Obied et al., (2013) with modification of time used for each run.
All samples were analysed by a reversed phase column (150 mm × 4.6 mm (i.d.), 3 μm) Gemini C-18 (Phenomenex, Sydney, Australia). The HPLC-DAD online ABTS•⁺ scavenging system consists of a Varian Prostar 240 solvent delivery process, comprised of a Varian Prostar 335 diode array detector and a Varian Prostar 410 autosampler. Blank (50% aqueous methanol) and standards were also analysed for quality control and identification purposes. The whole HPLC system was controlled by a Star Chromatography Workstation, version 6.41 (Varian, Inc. California, USA). Samples were analysed by gradient elution. Mobile phases were solvent A (0.2 % formic acid solution in water), and solvent B (0.2 % formic acid solution in methanol). The system was initiated and equilibrated with 5 % solvent B and then solvent B increased to 80 % over 70 min. HPLC system was rinsed equilibrated for 15 min at initial condition composition after each run. All solvents gradient were filtered (Nylon filter, 0.22 micron). The HPLC-DAD flow rate was maintained at 0.7 mL/min, while the injection volume was 10 μL. Data analysis was performed using Star Chromatography workstation version 6.41 2004 (Varian, Inc. California, USA). ABTS stock solution was prepared by reacting 7 mM ABTS and 2.45mM potassium persulphate. ABTS•⁺ working solution (A₇₃₄=0.70) was always prepared fresh from the stock solution.

Online ABTS•⁺ with HPLC-DAD was performed on a Varian Prostar 240 solvent delivery system attached with a Varian Prostar 410 autosampler. The HPLC-DAD system was controlled by Star Chromatography workstation version 6.41 (Varian, Australia), as mentioned previously in the materials and methods section of Chapter-2 for determination of total phenolic compounds. In addition, outflow from HPLC-DAD was joined to reaction coil (PEEK; 3.4 m x 0.178 mm, maintained at
37°C) attached to a Perkin-Elmer series 10 HPLC pump (Varian 2401 pump). The change in ABTS** absorbance was monitored at 414 nm using a Varian 9050 UV-VIS detector, where only peaks showing positive results for antioxidant activity were detected.

2.2.6 Liquid chromatography-mass spectroscopy (LC-MS) for identification of canola extracts

For the HPLC-DAD-MS/MS, all conditions were maintained as described by Obied et al., (2013). All samples were analysed by Agilent 1200 series liquid chromatograph (Agilent technologies, Germany) using gradient elution and a Gemini C-18 column [50 mm × 4.6 mm (i.d.), 3 μm], (Phenomenex, Sydney, Australia).

The flow rate was kept at 0.7 mL/min, and the injection volume was 5 μL. The mobile phase used was the same as for the HPLC-DAD. The total run time was 70 min. A gradient consisting of five steps was as follows: initial condition, 5% solvent B; followed by solvent B increased to 80% over 65 min; then solvent B increased to 100% over 2 min; back to initial conditions in 3 min and finally the system was isocratic for 15 min. The effluent from the DAD was connected to a 6410 triple-quadrupole LC-MS (Agilent Technologies, California, and USA) equipped with an electrospray ionization (ESI) interface.

The MS procedure was performed in both the negative and positive ion mode (m/z 100–1200) under the following conditions: nitrogen gas; at a temperature of 350°C;
with a gas flow rate of 9 L/min and a nebulizer pressure, 40 pounds per square inch (psi). The capillary voltage was 4 kV and the cone voltage, 100 V. Results were analysed using an Agilent Mass Hunter workstation version B.01.04 2008 (Agilent Technologies, Waldbronn, Germany).
2.2.7 *DPPH-* radical scavenging activity

The scavenging activity of seven different extracts against the DPPH radical was measured as described by Alashi et al., (2014) with slight modifications. Briefly, 100 µL of phenolic extracts (0.75 to 6 mg/mL) or trolox standard (0.3 to 5 mg/mL) in 80% methanol were reacted with 100 µL of DPPH (100 µM in 80% methanol) in a 96 well microplate (Greiner, VWR, USA). The microplate was then incubated at room temperature in the dark for 30 min. The scavenging activities of the extracts or trolox against DPPH free radicals was measured at 517 nm using FLUOstar omega microplate reader (BMG Labtech, Offenburg, Germany). The scavenging power of the extracts against DPPH radicals was expressed in percentage according to the formula:

\[
\text{DPPH radical scavenging (\%) = } \frac{A_c - A_s}{A_c} \times 100
\]

Where \( A_c \) is the absorbance of the control and \( A_s \) is absorbance of the sample respectively. The results were expressed as EC\(_{50}\) (amount of maximum concentration in a percentage that scavenges 50% of the free radicals for each sample).
2.2.8 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out as described by Benzie & Strain (1996) and modified to a 96-well microplate (Merola, Alonso, Ros, & Castón, 2013). Three reagents were prepared for this assay named reagent A, B and C. Reagent A consisted of 300 mM acetate buffer at pH 3.6 (16 mL of glacial acetic acid in 3.1 g of sodium acetate trihydrate, made to a total volume of 1L with UPW). Reagent B was prepared by using 5 mM of TPTZ in 10 mL of 40 mM of HCl. Reagent C was prepared using 5mM of ferric chloride in 10 mL of UPW. Reagent B and C were always prepared fresh. Finally, FRAP reaction mixture was prepared mixing 2.5 mL of each reagents B and C, and 25 mL of reagent A, making total volume 30 mL for FRAP reagent. This reagent mixture was heated at 37°C for 10 min.

Samples and standard solution were prepared in 50% methanol. For the FRAP assay 10 µL of sample (1mg/mL) was mixed with 40 µL of water and mixed to which 200 µL of FRAP reagent assay mixture was added. Change in absorbance was measured at 593 using FLUOstar omega UV-VIS spectrophotometer (BMG Labtech, Offenburg, Germany). Trolox (1000 µM/ml) was used as a standard. Results for FRAP assays were expressed as trolox equivalents per 100 g of dry weight (mM TE/100 g DW).
2.2.9 Statistical analysis

Experiments were done in triplicates. Data are presented as the mean ± standard deviation (SD). All results were analyzed using Graph pad prism 5, Microsoft Excel 2013 and one way analysis of variance (ANOVA) using SAS® system for Window V8 (SAS institute, USA). Comparison between sample means were calculated using the Duncan multiple range test at a 5% probability level ($p<0.05$).
2.3 Results

2.3.1 Preparation of canola extracts (CMEs)

In the current study, seven different solvents with different polarities were used for the generation of different extracts from canola meal. Figure 2.1 compare extraction yield for genotype-1 using different solvents.

![Figure 2.1](image)

**Figure 2.1.** Comparison of extraction yields (in percentage) of CMEs from two canola genotypes.

Different letters on the top of each bar on graphs represent mean values (n=3), which are significantly different \((p<0.05)\).

Extracts can be ranked according to their extracting capacity in the following descending order: \(\text{WE} > \text{ME} \geq \text{EE} \geq \text{AA} > \text{BE} > \text{CE} > \text{HE}\). The yield obtained for all extracts from genotype-1 meal were consistently higher than for genotype-2. Water appeared to extract more materials (50% yield) from canola meal than any other solvent. Following water, methanol, ethanol and acetone have very close results.
(20-30%). Less polar solvents had less than 20% yield. Irrespective of the solvent used, genotype-1 had higher yield than genotype-2.

2.3.2 Total phenolic content (TPC)

A calibration curve was produced by linear regression for the determination of TPC from CM extracts ($R^2 = 0.9998$) using gallic acid.

![Diagram showing comparison of total phenolic contents](image)

**Figure 2.2.** Comparison of total phenolic contents represented as milligram of gallic acid equivalent per gram dry weight (mg GAE/g DW) in CMEs from two canola genotypes.

Different letters on the top of each bar on graphs represent mean values (n=3), which are significantly different ($p<0.05$).

Regardless of genotype, acetone stands out amongst other solvents with more than 4-fold higher phenol recovery. Extracts with other solvents can be arranged in decreasing order of TPC as follows: ME>WE>BE >EE>CE>HE. Genotype-1
showed higher TPC than genotype-2 in all extracts apart from water and acetone extracts (Figure 2.2).

2.3.3 Characterisation of phenolic composition and free radical scavenging activity of individual compounds

The phenolic composition in different canola extracts was analyzed by reverse phase-HPLC-DAD with online detection of ABTS scavenging activity (Figure 2.3). The m/z data and molecular masses were detected by LC-MS (Table 2.1). The data gathered from UV-Vis spectra, ABTS scavenging activity, relative retention times, and mass spectra were utilized in combination with reference standards and literature data to characterise the chemical composition of CMEs. Polar compounds (charged or glycosides) elute first on RP-HPLC system, while less polar, more hydrophobic derivatives come later in the chromatogram.

A large number of peaks (close to 100) were detected in DAD chromatogram at 280 nm and total ion chromatogram (TIC). Only prominent peaks –as appearing at 280 nm or in the TIC– were selected for further identification. Table 2.1 provides spectral data of 47 peaks (Figure 2.3) from all the CMEs. Thirty two compounds were fully identified using standards or tentatively identified based on their spectral data. Fifteen compounds could not be identified. Early eluting peaks (highly polar compounds) were prominent in WE (Figure 2.4). Compounds detected in CMEs were mostly glucosinolates or phenols. Glucosinolates identified were progitrin, sinigrin, glucalyssin, Glucoraphanin, gluconapoleiferin, gluconapin, 4-hydroxyglucobrassicin, and gluconasturtin. All glucosinolates came out within the
first 18 min excluding gluconasturtin which came out at 29.4 min. WE appeared as the best solvent for the recovery of glucosinolates.
Table 2.1. HPLC peaks in canola meal extracts (CMEs).

<table>
<thead>
<tr>
<th>P</th>
<th>Identity</th>
<th>GN</th>
<th>HE</th>
<th>CE</th>
<th>BE</th>
<th>AE</th>
<th>EE</th>
<th>ME</th>
<th>WE</th>
<th>RT</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt;</th>
<th>ABTS</th>
<th>ESI&lt;sup&gt;+&lt;/sup&gt;</th>
<th>ESI&lt;sup&gt;-&lt;/sup&gt;</th>
<th>MW</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Unknown</td>
<td>GN2</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>4.1</td>
<td>258</td>
<td>–</td>
<td>124</td>
<td>122</td>
<td>123</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>Unknown</td>
<td>GN1</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>5.0</td>
<td>275</td>
<td>–</td>
<td>613</td>
<td>611</td>
<td>612</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>Unknown</td>
<td>GN2</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>5.5</td>
<td>249</td>
<td>–</td>
<td>137</td>
<td>135</td>
<td>136</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>Unknown</td>
<td>Both</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>6.0</td>
<td>262</td>
<td>–</td>
<td>268</td>
<td>243</td>
<td>244</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>Unknown</td>
<td>Both</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>6.2</td>
<td>256</td>
<td>–</td>
<td>233</td>
<td>231</td>
<td>232</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>Progoitrin</td>
<td>Both</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>6.7</td>
<td>220</td>
<td>–</td>
<td>NI</td>
<td>388</td>
<td>389</td>
<td>(Kusznierewicz, Iori, Piekarska, Namiesnik, &amp; Bartoszek, 2013)</td>
</tr>
<tr>
<td>7</td>
<td>Sinigrin</td>
<td>Both</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>7.8</td>
<td>285</td>
<td>–</td>
<td>NI</td>
<td>358</td>
<td>359</td>
<td>(Kusznierewicz et al., 2013)</td>
</tr>
<tr>
<td>8</td>
<td>Unknown</td>
<td>Both</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>8.8</td>
<td>252, 270s</td>
<td>–</td>
<td>277</td>
<td>NI</td>
<td>276</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>Glucoalyssin</td>
<td>Both</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>9.5</td>
<td>225</td>
<td>–</td>
<td>452</td>
<td>450</td>
<td>451</td>
<td>(Lelario, Bianco, Bufo, &amp; Cataldi, 2012)</td>
</tr>
<tr>
<td>10</td>
<td>Glucoraphanin</td>
<td>Both</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>10.5</td>
<td>230</td>
<td>–</td>
<td>NI</td>
<td>435</td>
<td>436</td>
<td>(L. Lee &amp; Boyce, 2011)</td>
</tr>
<tr>
<td>11</td>
<td>Gluconapoleiferin</td>
<td>Both</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>11.1</td>
<td>275</td>
<td>–</td>
<td>NI</td>
<td>402</td>
<td>403</td>
<td>(Velasco et al., 2010)</td>
</tr>
<tr>
<td>12</td>
<td>Gluconapin</td>
<td>Both</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>11.3</td>
<td>220</td>
<td>–</td>
<td>NI</td>
<td>372</td>
<td>373</td>
<td>(Obied et al., 2013)</td>
</tr>
<tr>
<td>13</td>
<td>Unknown</td>
<td>Both</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>13.7</td>
<td>278</td>
<td>–</td>
<td>451</td>
<td>449</td>
<td>450</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>Unknown</td>
<td>Both</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>13.7</td>
<td>288</td>
<td>–</td>
<td>NI</td>
<td>315</td>
<td>316</td>
<td>–</td>
</tr>
<tr>
<td>15</td>
<td>Sinapine</td>
<td>Both</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>15.7</td>
<td>237, 328</td>
<td>–</td>
<td>310</td>
<td>294, 663</td>
<td>310</td>
<td>(Thiyam-Hollander et al., 2014)</td>
</tr>
<tr>
<td>16</td>
<td>Caffeoyl dihexoside</td>
<td>Both</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>17.2</td>
<td>297a, 327</td>
<td>++</td>
<td>NI</td>
<td>503</td>
<td>504</td>
<td>(Obied et al., 2013)</td>
</tr>
<tr>
<td>17</td>
<td>4-hydroxyglucobrassicin</td>
<td>Both</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>17.6</td>
<td>292</td>
<td>–</td>
<td>NI</td>
<td>463</td>
<td>464</td>
<td>(Lelario et al., 2012)</td>
</tr>
<tr>
<td>18</td>
<td>Ferrolyl choline(4-0-8')guiacyl</td>
<td>Both</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>v</td>
<td>17.7</td>
<td>270b, 325b</td>
<td>–</td>
<td>476</td>
<td>NI</td>
<td>476</td>
<td>(S.-C. Yang et al., 2015a)</td>
</tr>
<tr>
<td></td>
<td>Compound</td>
<td>19</td>
<td>20</td>
<td>21</td>
<td>22</td>
<td>23</td>
<td>24</td>
<td>25</td>
<td>26</td>
<td>27</td>
<td>28</td>
<td>29</td>
<td>30</td>
<td>31</td>
<td>32</td>
<td>33</td>
</tr>
<tr>
<td>---</td>
<td>----------------------------------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>Ferroyl choline guaiacyl isomer</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>18.3</td>
<td>270b,325b</td>
<td>++</td>
<td>476</td>
<td>NI</td>
<td>476</td>
<td>(S.-C. Yang et al., 2015a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4'-glucosylsinapic acid</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>20.6</td>
<td>317b</td>
<td>−</td>
<td>NI</td>
<td>385</td>
<td>386</td>
<td>(Obied et al., 2013)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sinapoyl dihexoside</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>19.0</td>
<td>328.54</td>
<td>+</td>
<td>NI</td>
<td>547</td>
<td>548</td>
<td>(Obied et al., 2013)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclic spermidin derivative</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>20.8</td>
<td>316</td>
<td>−</td>
<td>496</td>
<td>494</td>
<td>495</td>
<td>(Fang et al., 2012)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclic spermidin derivative</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>21.7</td>
<td>320</td>
<td>−</td>
<td>496</td>
<td>494</td>
<td>495</td>
<td>(Fang et al., 2012)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sinapoyl hexoside</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>22.7</td>
<td>330</td>
<td>++</td>
<td>NI</td>
<td>385</td>
<td>386</td>
<td>(Obied et al., 2013)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sinapoyl hexoside isomer</td>
<td>GN1</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>23.1</td>
<td>330</td>
<td>++</td>
<td>NI</td>
<td>385</td>
<td>386</td>
<td>(Obied et al., 2013)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>GN1</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>23.5</td>
<td>305b</td>
<td>−</td>
<td>533</td>
<td>531</td>
<td>532</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>Both</td>
<td>✗</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>23.6</td>
<td>310b</td>
<td>−</td>
<td>449</td>
<td>447</td>
<td>448</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Feruloyl choline(5-8')guaiacyl</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>24.4</td>
<td>328,280s</td>
<td>++</td>
<td>457</td>
<td>458</td>
<td>459</td>
<td>(S.-C. Yang et al., 2015a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
<td>Both</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>24.6</td>
<td>260s,280s,290s,268,333</td>
<td>++</td>
<td>429</td>
<td>427</td>
<td>428</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kaempherol-sinapoyl-trihexoside</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>26.3</td>
<td>979</td>
<td>978</td>
<td>977</td>
<td>(Siger et al., 2013)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gluconasturtiin</td>
<td>Both</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✓</td>
<td>✓</td>
<td>✗</td>
<td>29.4</td>
<td>230</td>
<td>+</td>
<td>NI</td>
<td>422</td>
<td>423</td>
<td>(Lelario et al., 2012)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trans-Sinapic acid</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>32.6</td>
<td>324</td>
<td>++</td>
<td>NI</td>
<td>223</td>
<td>224</td>
<td>(Obied et al., 2013)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kaempherol 3-dihexoside-7-sinapoyl-hexoside</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>33.0</td>
<td>266,323</td>
<td>++</td>
<td>979</td>
<td>978</td>
<td>977</td>
<td>(Obied et al., 2013)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cis-sinapic acid</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>35.3</td>
<td>266,323</td>
<td>++</td>
<td>NI</td>
<td>223</td>
<td>224</td>
<td>(Obied et al., 2013)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Feruloyl choline (4-O-8') guaiacyl-di-sinapoyl</td>
<td>GN1</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>36.1</td>
<td>323</td>
<td>++</td>
<td>682</td>
<td>NI</td>
<td>682</td>
<td>(S.-C. Yang et al., 2015a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unknown (methyl sinapate dihexoside)</td>
<td>Both</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✓</td>
<td>✓</td>
<td>✗</td>
<td>36.4</td>
<td>329</td>
<td>++</td>
<td>NI</td>
<td>561</td>
<td>562</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Disinapoyl dihexoside</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>38.6</td>
<td>230,330</td>
<td>++</td>
<td>NI</td>
<td>753</td>
<td>754</td>
<td>(Obied et al., 2013)</td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>Compound</td>
<td>Appearance</td>
<td>HE</td>
<td>CE</td>
<td>BE</td>
<td>AE</td>
<td>EE</td>
<td>ME</td>
<td>WE</td>
<td>RT (min)</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt;</td>
<td>ESI&lt;sup&gt;+&lt;/sup&gt;</td>
<td>ESI&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Scavenging Activity</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>-----</td>
<td>--------------------------------</td>
<td>------------</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>Trisinapoyl dihexoside 1</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>39.7</td>
<td>327</td>
<td>++</td>
<td>NI</td>
<td>959</td>
<td>960</td>
<td>(Obied et al., 2013)</td>
</tr>
<tr>
<td>39</td>
<td>Disinapoyl hexoside</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>40.7</td>
<td>330</td>
<td>++</td>
<td>NI</td>
<td>591</td>
<td>592</td>
<td>(Obied et al., 2013)</td>
</tr>
<tr>
<td>40</td>
<td>Tetrasinapoyl dihexoside</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>41.9</td>
<td>326</td>
<td>++</td>
<td>NI</td>
<td>1183</td>
<td>1184</td>
<td>(Obied et al., 2013)</td>
</tr>
<tr>
<td>41</td>
<td>Methyl sinapate</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>42.5</td>
<td>325</td>
<td>++</td>
<td>NI</td>
<td>237</td>
<td>238</td>
<td>(Obied et al., 2013)</td>
</tr>
<tr>
<td>42</td>
<td>Unknown</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>43.1</td>
<td>325</td>
<td>+</td>
<td>NI</td>
<td>545</td>
<td>546</td>
<td>(Fang et al., 2012)</td>
</tr>
<tr>
<td>43</td>
<td>Disinapoyl hexoside isomer</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>45</td>
<td>326</td>
<td>+</td>
<td>NI</td>
<td>591</td>
<td>592</td>
<td>(Obied et al., 2013)</td>
</tr>
<tr>
<td>44</td>
<td>Unknown</td>
<td>Both</td>
<td>✗</td>
<td>✓</td>
<td>✓</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>47.2</td>
<td>320</td>
<td>++</td>
<td>275</td>
<td>NI</td>
<td>274</td>
<td>–</td>
</tr>
<tr>
<td>45</td>
<td>Unknown</td>
<td>GN1</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>47.6</td>
<td>325</td>
<td>++</td>
<td>454</td>
<td>NI</td>
<td>453</td>
<td>–</td>
</tr>
<tr>
<td>46</td>
<td>Unknown</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>48.7</td>
<td>325</td>
<td>+</td>
<td>245</td>
<td>NI</td>
<td>244</td>
<td>–</td>
</tr>
<tr>
<td>47</td>
<td>Unknown</td>
<td>Both</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
<td>58.2</td>
<td>327</td>
<td>+</td>
<td>303</td>
<td>279</td>
<td>280</td>
<td>–</td>
</tr>
</tbody>
</table>

P, peak number from Figure 2.4; GN, genotypes; GN-1, only in genotype-1; GN-2, only in genotype-2; Both, genotype-1 & 2; HE, hexane extract; CE, chloroform extract; BE, butanol extract; AE, acetone extract; EE, ethanol extract; ME, methanol extract; WE, water extract; ✓, traces; ✓✓, present; ✓✓✓✓, major peak; ✗, could not be detected; RT, retention time; λ<sub>max</sub>, UV-Vis spectra; ESI<sup>+</sup>, electrospray ionization peaks in positive mode; ESI<sup>−</sup>, electrospray ionization peaks in negative mode; MW, molecular weight; NI, did not ionize under ESI modes; b, broad peak; s, peak shoulder. ; ABTS, ABTS<sup>•</sup>+ scavenging activity in online assay (antioxidant); +, low ABTS scavenging activity; ++, good ABTS scavenging activity; ++++, high ABTS scavenging activity.
Figure 2.3. Typical HPLC-DAD chromatograms of CMEs. (Continued)
Figure 2.3. (Continued). Typical HPLC-DAD chromatograms of CMEs. Chromatogram 1, 2, 3 and 4 A detected at 280 nm shows presence of compounds while chromatogram 1, 2, 3 and 4 B detected at 4114 nm shows their ABTS+ scavenging activity (antioxidant activity). 1A) WE at 280 nm; 1B) WE at 414 nm; 1A) WE at 280 nm; 1B) WE at 414 nm; 2A) ME at 280 nm; 2B) ME at 414 nm; 3A) CE at 280 nm; 3B) CE at 414 nm; 4A) BE at 280 nm; 4B) BE at 414 nm. All identified peaks with number are given in Table 2.1.
Phenolic compounds were sinapine, caffeoyl dihexoside, ferroyl choline guiacyl, ferroyl choline guiacyl isomer, sinapoyl dihexoside, 4'-glucosyl sinapic acid, cyclic, sinapoyl hexoside, sinapoyl hexoside isomer, feruloyl choline (5-8') guaiacyl, kaempherol-sinapoyl-trihexoside, feruloyl choline (4-O-8') guaiacyl-di-sinapoyl, disinapoyl dihexoside, trisinapoyl dihexoside, disinapoyl hexoside, methyl sinapate, and disinapoyl hexoside isomer. Two alkaloids, spermidine derivatives, were also identified.

Overall, ME had the largest qualitative recovery from CM (number of peaks), though AE showed much higher quantitative recovery of phenols (peak area). Sinapine (peak-15) appeared as the major phenol in all CMEs (Figures 2.3), with highest recovery in AE. It also appeared that ME, EE, AA, BE and HE had trans-sinapic acid as a dominant peak, with its highest recovery in AE again. Surprisingly, water extracts had higher recovery of trans-sinapic acid (peak-32) compared with sinapine. Sinapine is more polar than trans-sinapic acid as it is a positively charged molecule. Thus, it should be more soluble in water. The lower recovery of sinapine can be explained by potential hydrolysis of sinapine in water to produce trans-sinapic acid and choline base.

Comparing both genotypes, WE of genotype-2 showed more trans-sinapic acid compared to sinapine suggesting more hydrolysis. The cis-isomer of sinapic acid (peak-34) was detected in all extracts. Peak-40 identified as tetra-sinapoyl dihexoside was prominent peak in ME, while other extracts have only few traces of this compound. A feruloyl choline (4-O-8’) guaiacyl (peak-18) and its isomers (peak-19), including Feruloyl choline (5-8’) guaiacyl (peak-28) were observed at in ME in both genotypes, while traces were observed only in other extracts. Meanwhile, feruloyl choline (4-O-8’) guaiacyl-di-sinapoyl (peak-35) was detected in genotype-1 only with the highest recovery in ME. Though there
was no free kaempferol identified in canola meal extracts, there were two glycoside derivatives of kaempferol namely, kaempferol-sinapoyl-trihexoside (peak-30), and kaempherol 3-dihexoside-7-sinapoyl-hexoside (peak-33).

2.3.4. DPPH radical scavenging activity

The DPPH radical scavenging activity of the canola meal extracts (CMEs) expressed as EC$_{50}$ value for antioxidant properties and is shown in Figure 2.4. It worth noting that the potent the antioxidant activity is the lower the EC$_{50}$. The trend for the scavenging activity for both genotypes was the same and following the descending order AE > ME > BE ≥ WE > HE > EE > CE extracts. The AE exhibited the highest DPPH radical scavenging activity with similar potency to the water soluble derivative of vitamin E, trolox. WE and BE had similar DPPH scavenging activities. CE had the least DPPH scavenging activity.

2.3.5. FRAP assay

The FRAP activity of CMEs are shown in Figure 2.5. The reducing power activity for the extract in both genotypes is in following orders: AE ≥ ME ≥ EE > HE > WE > BE > CE. Parallel to their radical scavenging activities, AE showed the highest, while CE showed the least potential to reduce Fe (III). On the other hand, ME and EE showed similar reducing power abilities to AE.
Figure 2.4. Comparison of antioxidant properties as measured by DPPH assay. Different letters on the top of bars in graphs are mean values (n=3) that are significantly different.
Figure 2.5. Comparison of antioxidant properties as measured by FRAP assay. Different letters on the top of bars in graphs are mean values (n=3) that are significantly different.
2.4 Discussion

Two genotypes of canola were used to compare the extractability of plant phenols from canola meal using seven different solvents: water and different water-solvent mixtures (methanol, ethanol, acetone, butanol, chloroform and hexane). Different solvents showed different extraction properties. The extraction yield varied widely (5-55%) among different solvents and genotypes (Figure 2.1). The polarity of the solvent was directly proportional to the yield. Water extracted far more material than any other solvent, in genotype-2, WE showed double the recovery from methanol and ethanol. This suggests the polar nature of canola meal components. After Soxhelt exhaustive hexane extraction of lipids from crushed canola seeds, it is logical to expect that there is nothing left to recover with a room temperature short time extraction with hexane. However, adding water to hexane had pulled out more hydrophilic components.

For extraction of plant constituents, water has unique dissolving properties and their extracts usually contain a mixture of low molecular weight polar compounds such as sugars amino acids, organic acids, simple phenols, anthocyanins, saponins, glycosides, as well as carbohydrates, proteins, nucleic acids and tannins. Furthermore, a mixture of water with organic solvents gives broader range of extraction ability compared to neat organic solvents (Spigno et al., 2007). Different organic solvents have different extracting properties based on their physicochemical properties and the phytochemical composition of the plant. Ethanol and methanol extracts usually contain tannins, simple phenols, flavonoids, terpenoids, anthocyanin, terpenoid, and alkaloids; chloroform extracts mostly extract alkaloids,
terpenoids and flavonoids. While hydro-alcoholic (water-methanol/ethanol) mixtures are typical for extraction of plant phenols (Obied, 2013), acetone extracts are superior for extracting flavonoids (Azmir et al., 2013).

With regard to total phenol recovery, the current results for TPC (genotype-1 AE= 94.5 ± 2.5 mg GAE/g DW; genotype-2 AE=103.7 ± 5.0 mg GAE/g DW) were higher than literature values, 39.2 mg GAE/g (Obied et al., 2013). This can be explained by genetic variability as both genotype-1 and 2 have not been studied before for their phenol content and experimental factors such as freeze-drying which is known to increase phenol recovery from plants (Azmir et al., 2013). Different genotypes demonstrated different TPC. This has been frequently reported in canola and in other plants (Beynon & Bond, 2001; Obied et al., 2013). Genotype-1 has higher TPC than genotype-2 in all extracts apart from AE and WE. This indicates that the TPC data depend not only on the genotype but also on the solvent used.

The highest TPC was achieved by AE and ME, which is in agreement with previous findings in canola meal (Obied et al., 2013) and other plants (Liu & Yao., 2007). ME had higher recovery than EE. Ethanol is less polar than methanol, yet their extracting abilities for phenolic compounds differ based on the plant extracted (Liu & Yao, 2007; Mohammedi & Atik, 2011). Though WE had the highest yield, their TPC was not as high. It can be also concluded that WE contains mostly non-phenolic constituents on weight basis. In fact, BE showed higher TPC from genotype-1 than WE (Figure 2.2).
Due to the polar nature of plant phenols, HPLC is the most commonly used method for their isolation and identification. By means of UV-Vis and mass spectra, 32 compounds out of 47 studied peaks have been fully or tentatively identified. While AE showed the highest TPC, ME showed more peaks. Thus, for qualitative analysis of canola meal, methanol should be favored over acetone.

Sinapic acid derivatives (glycoside and choline conjugates) comprise the chief constituents of canola meal. This is a characteristic feature of *Brassica* plants including canola (Obied et al., 2013; S. Yang et al., 2015b). ABTS scavenging activities of extracts are exclusively due to their sinapic acid derivatives. Though sinapine was the main contributor to ABTS scavenging activity, some minor constituents demonstrated higher antioxidant activity that is not proportional to their concentration. This comes in accord with previous findings (Obied et al., 2013). Previous literature have reported kaempferol derivatives identified in tissues of *Brassica napus* L. in methanol extract (Farag et al., 2013). kaempferol-sinapoyl-trihexoside (peak-30) and kaempferol 3-dihexoside-7-sinapoyl-hexoside (peak-33) found in present study of CMEs were also reported by Siger et al., (2013) and Obied et al., (2013), respectively.

Glucosinolates were always co-extracted with phenols due to the water component in all solvents. WE showed the highest content and number of glucosinolates. A cyclic spermidine alkaloid and its isomers (peak, 22 & 23) were identified in CMEs.
Though syringic acid, epicatechin, chlorogenic acid, gallic acid, catechin hydrate, caffeic acid, naringin, ferulic acid, quercetin, luteolin, kaempferol, pyrogallol, 4-hydroxybenzoic acid, and vanillic acid were reported earlier in canola meal but their absence was confirmed in canola samples by means of standards (Obied et al., 2013; Shao et al., 2014).

Many assays have been established for qualitative and quantitative analysis of antioxidant compounds. But, there is no common assay that can calculate the antioxidant activity accurately for one sample as these assays works by different mechanism of action based on involved chemical reaction. Therefore, the current study has assessed the antioxidant activities of CMEs in DPPH and FRAP assays, in addition to online ABTS assay. The DPPH assay is simple and fast scavenging assay reported as accurate method for measuring antioxidant activity in extracts (Hassas-Roudsari, Chang, Pegg, & Tyler, 2009; Sanchez-Moreno, 2002). However, FRAP assay is a novel method for assessing antioxidant power which involves reduction of ferric to ferrous ion that causes a coloured ferrous-tripyridyltriazine complex to form (Changing yellow colour to green or blue) depending on antioxidant activity (Benzie & Strain, 1996).

AE proved to be the superior solvent with the highest DPPH radical scavenging (Figure 2.4) and reduction capacity (Figure 2.5) from CMEs. This is because AE appeared to recover more quantity of antioxidant compounds than other extracts. Similar findings were reported by Obied et al., (2013). Similarly, the extracts obtained by chloroform and hexane from both genotypes showed the lowest scavenging active. Both are non-polar organic
solvents compared to acetone, butanol, ethanol and water (polar solvents) making them unsuitable for extraction of antioxidant phenolic compounds from canola meal. Overall, DPPH free radical scavenging activity (Figure 2.4) and the FRAP activity (Figure 2.5) could be correlated with total phenolic content (TPC) for each extract as (Figure 2.2). Online-on line-ABTS HPLC data show that phenolic compounds (Figure 2.3) are important constituents of antioxidant activity in CMEs including major sinapine and sinapic acid.

2.5 Conclusion

Phenolic composition of extracts depends on the extraction solvent and the canola genotype. Genotype-2 provided higher contents of phenolic compounds than genotype-1. Higher recovery per weight do not go parallel to TPC. Water extracts many of the non-phenolic species and can result in hydrolysis of sinapine. Acetone extract showed the highest recovery of TPC, while methanol extract showed the largest number of peaks. Superior antioxidant activity was availed with maximum recovery for acetone extracts. Thus, acetone should be preferred for quantitative recovery, while methanol should be selected for qualitative investigations. By means of HPLC-DAD-ABTS and LC-MS, 47 compounds were detected where 32 were identified and 15 remain unknown. Similar to previous reports, sinapic acid derivatives were the major phenols and were the chief contributors to canola meal antioxidant activity. Water extracts appeared superior for the recovery of glucosinolates.
CHAPTER 3. Identification, purification and characterisation of protease inhibitors from canola meal

3.1. Introduction

Plant proteins are well known for their potential nutraceutical/pharmaceutical applications. Some of these proteins have been shown to impart inhibitory and/or suppressive activity against pests and diseases of agricultural crops, stored grain pests, and microbes (Kuhar et al., 2014). The process of extracting proteins from plants is important to achieve the highest yield of good quality protein isolates. Protein extraction often involves techniques such as ammonium sulphate fractionation, dialysis and ultrafiltration (Aider & Barbana, 2011). Chromatographic techniques such as ion exchange chromatography, and gel filtration chromatography are used to recover proteins with high purity. Purification cost is an important factor for the commercial protein isolation industry to achieve reasonably priced products that can meet customer demands.

The isolation of protease inhibitors (PIs) from plant sources could be a potential useful commercial pursuit, given their potential range of functionalities. (Alashi et al., 2013; Tan, Mailer, Blanchard, & Agboola, 2011). However, there are limited studies on canola proteins that exhibit protease inhibitor properties. This chapter focuses on the isolation, purification and characterisation of PIs from canola meal.
3.2. Materials and methods

3.2.1 Material

Canola seeds (Brassica napus) of two genotypes [Rivette (genotype-1), Bln-3347 (genotype-2)] were supplied by the NSW Department of Primary Industries (DPI), Wagga Wagga, Australia. Chemicals including ammonium sulphate, calcium chloride (CaCl₂), tris hydrochloride (Tris-HCl), glycerol, bromophenol, N-acetyl-DL-phenylalanine β-naphthyl ester (APNE), tetrazoitized (zinc chloride complex) o-dianisidine (Fast blue B salt), bovine trypsin, soybean trypsin inhibitor, p-toluenesulfonyl-L-arginine-methyl ester (TAME), sodium dodecyle sulphate (SDS), ammonium persulfate (APS), N,N,N',N'-tetramethylene diamine (TEMED), β-mercaptoethanol (ME), N, N-dimethylformamide, Trifluoroacetic acid (TFA), and colour marker were purchased from Sigma Aldrich, Australia. Polyacrylamide precast gels (PhastGel gradient 8-25, Homogeneous 20 and IEF), PhastGel buffer strips, PhastGel Blue R tablets, and PlusOne silver staining kit were purchased from GE Healthcare, Uppsala, Sweden.

Precision Plus Protein™ Dual Xtra Standard marker 2-250 KDa, acrylamide (40%), bisacrylamide (2%) were purchased from Biorad. BCS protein assay kit (Pierce™) was purchased from Thermo Fisher Scientific. Chromatographic media, Fast Protein Q-Sepharose (anion exchange) and Hi Load 26/60 Superdex 200 preparatory grade (gel filtration) were obtained from GE Healthcare Life Sciences, Uppsala, Sweden. Bio-Spin® 30 Columns
were purchased from Bio-Rad Laboratories, Australia. Ultrapure water (UPW) with 0.22 μm filter was supplied by Thermoscientific, Victoria, Australia.

3.2.2. Sample preparation for identification of protease inhibitors (PIs) in canola meal (CM)

Canola meal (180mg/mL) from two genotypes were dissolved in extraction buffer (0.023M CaCl₂, 0.092M Tris-HCl at pH8.1), centrifuged (HermLe; Gosheim, Germany) and filtered to get rid of debris. Nearly, 100 μL of the filtered sample was passed through Bio-Spin® chromatography columns for more purification of protein. The filtered sample and sample purified through Bio-Spin® chromatography columns was run on native gel in order to find out presence of PIs in each sample. Presence of PIs was identified by PI activity assays as described in section 3.2.4.

3.2.3 Isolation and purification of canola meal protease inhibitors

Once the presence of PIs were confirmed in the CM genotypes, four hundred grams of meal of each genotype were separately mixed for 5 h in ultrapure water (UPW) at a ratio of 1:10 ratio under constant stirring at 4°C. The debris in the mixture was centrifuged in 1 L centrifuge bottles (Beckman Coulter Avanti J E; California, United States) at 6300 rpm for 30 min, and the supernatant transferred to the sterile beaker. The proteins in the resultant supernatant were fractionated under constant stirring and sequential addition of 25%, 50%, and 80% of ammonium sulphate (NH₄SO₄). The precipitated
proteins (due to the addition of NH$_4$SO$_4$) at each step were separated by centrifugation (Beckman Coulter Avanti J E) at 6300 rpm for 30 min. Each fraction was dissolved in a minimal amount of UPW and dialyzed for 24 h. Each dialyzed fraction was measured for total protein and PI activity by spectrophotometric assay and native gel electrophoresis. Therefore, fraction, which showed more PI activity were used for further purification and characterisation. The protein fraction (CMI) obtained at 80% NH$_4$SO$_4$ saturation showed the highest PI activity, therefore, it was concentrated using Amicon Ultra-15 centrifuge filter tubes and was subsequently used for further purification.

3.2.3.1. Protein purification using Fast Performance Liquid chromatography (FPLC)

The concentrated CMI was loaded onto a HiPrep 16/10 Q-Sepharose Fast Flow anion exchange column (GE Healthcare, Life Sciences, Upppsala, Sweden), pre-equilibrated with Tris-HCl (50 mM) buffer at pH 7.0 at a flow rate of 1 mL/min. All buffers were filtered using SCGPT05RE steritop™ filter unit 0.22 µm PES 45 mm (Merck Millipore, Australia). Fractions of 5 mL were eluted with a linear gradient of NaCl (0.0-0.5 M) in the same buffer.

All fractions under each peak were pooled, dialysed and assayed for PI activity. Only the pooled fractions that exhibited PI activity were concentrated (CM2) using Amicon Ultra-15 Centrifuge tubes fitted with 10 KDa Cut-Off filter (Merck Millipore, Australia). The concentrated retentate (CM2) was loaded onto a gel filtration column (Hi Load 26/60 Superdex 200 preparatory grade from GE Healthcare Life Sciences, Uppsala, Sweden) pre-
equilibrated with 50 mM Tris-HCl buffer (pH 7.0) at a flow rate 1 mL/min. All the fractions eluted from the gel filtration column were tested for PI activity and the protein peak with the highest PI activity were concentrated (CM3) using Amicon Ultra-15 centrifuge filter tubes as above.

The concentrated fraction (CM3) was rechromatographed using the same anion exchange column but pre-equilibrated with 50 mM bis-Tris-HCl buffer at pH 6.0, and eluted with a linear gradient of 0.0–0.5 M NaCl at a flow rate of 1 mL/min. All the fractions under the highest PI activity protein peak were pooled (CM4) and concentrated using Amicon Ultra-15 centrifuge filter tubes as above. The purified fraction (CM4) was stored at 20°C until required for further analyses.

3.2.3.2. Reverse phase-high performance liquid chromatography

The pooled fraction (CM4) was directly injected onto a HPLC Luna analytical C18 RP column (i.d. = 3.0 mm, length = 150 mm with a 10 μm particle size) using Varian HPLC system pre-equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in UPW.

All buffers were filtered using nylon filters, while samples were prepared in 2 mL HPLC vials (Phenomenax). Protein separation was achieved using linear acetonitrile gradient of 1–100% in 0.1% (v/v) TFA for 70 min at a flow rate of 1 mL/min. The eluting protein fraction was analysed at a detection wavelength of 214 nm.
3.2.3.3. Protein concentration estimation

Protein concentration estimation was carried out using a Pierce™ BCA protein assay kit according to manufacturer’s protocol (Thermoscientific, Melbourne, Australia). Samples and the Bovine Serum Albumin (BSA) standard were prepared by diluting with 50 mM Tris-HCl buffer at pH 7.0.

A 25 μL aliquot of each sample and the BSA standards was dispensed into a Greiner 96 microplate well plate (VWR, USA). A 200 μL working solution was transferred to each well, mixed on a shaking platform and incubated in the dark at 37 °C for 2 h. The absorbance was measured after cooling to room temperature (RT) at 562 nm using FLUOstar omega UV-VIS spectrophotometer (BMG Labtech, Offenburg, Germany). Absorbance values versus protein concentration of BSA were plotted on a calibration curve to determine the concentration of protein in each sample.

3.2.4. Protease inhibitor (PI) activity assay

Protease inhibitor activity of different fractions generated during PI purification steps was ascertained spectrophotometrically and by visualizing trypsin inhibiting PI bands resolved on native PAGE gels.

3.2.3.4. Spectrophotometric assay

PI activity was determined using the modified method by Beynon & Bond (2001) and Kollipara & Hymowitz (1992). This assay involves the esterolytic activity of trypsin toward the substrate p-toluenesulphonyl-L-arginine methyl
ester (TAME). Since hydrolysed product was detected at 247 nm, the inhibition of enzymatic reaction could be observed from the low level of the ultraviolet (UV) light absorbance. One trypsin unit (TU) unit was defined as 1 μmol of substrate hydrolysed per minute, while, one inhibition unit was defined as a unit of enzyme inhibited in the reaction. The specific activity was determined at each step of purification, and was estimated as trypsin inhibition units (TIU) per milligram (TIU/mg) of protein.

The inhibitor assay buffer (10.3 mM CaCl2, 41.4 mM Tris-HCl) was prepared at pH 8.1. Substrate TAME (10 mM), prepared fresh for the assay was made up of 10 mL of assay buffer in 37.9 mg TAME. The bovine trypsin enzyme solution was prepared using 20 μg mL-1 of bovine trypsin in 1 mM HCl. The reaction mixture was made by mixing 2600 μL of assay buffer with 300 μL of TAME in a quartz cuvette (10 mm path length, 3500 μL) followed by addition of 100 μL of bovine trypsin. The contents were mixed quickly and absorbance was measured immediately at 247 nm using Cary 50 UV-Vis spectrophotometer (Varian, Inc., California). The slope of enzyme activity was recorded as change in absorbance over 3 min and the linear progression of the reaction curve was confirmed.

Canola meal samples were prepared in extraction buffer (0.023 M CaCl2, 0.092 M Tris-HCl with pH 8.1), while purified canola PI samples were prepared in 50mM Tris-HCL Buffer (pH 8). A mixture with the same volume of the assay buffer, TAME, and 1 mM HCl without enzyme was used as a reference blank. The inhibition assay was performed by mixing 6 μl canola
meal extract or purified canola PI sample into a solution of assay buffer and bovine trypsin in a cuvette and incubating at RT (25°C) for 6 min.

The TAME substrate was added to the reaction mixture after the incubation period and the absorbance reading was recorded spontaneously at 247 nm ($A_{247}$), continuing for 3 mins. A 6 µL canola meal extraction buffer (0.023M CaCl$_2$, 0.092M Tris-HCl with pH8.1) and or 6 µL of 50 mM Tris-HCl buffer were used as negative control. Purified soybean inhibitor or wattle seed crude extract were also used as a control in the assay.

The TAME substrate was mixed into the reaction mixture after the incubation period and the absorbance reading was recorded at 247 nm ($A_{247}$) spontaneously or 3 min. The negative control, depending on the solvent in which sample was dissolved, was either 6 µL canola meal extraction buffer (0.023M CaCl$_2$, 0.092M Tris-HCl with pH8.1), or 6 µL of 50 mM Tris-HCl buffer. A Soybean inhibitor or wattle seed crude extract was also used as a control in the assay.

The curve of the slope for inhibitor activity was measured as change in absorbance over 3 min. The trypsin inhibitor activity was estimated as trypsin inhibitor units per gram of extract/samples as follows:

\[
\text{TIU/g extract} = \frac{[(T\Delta A_{247}/\text{min} - I\Delta A_{247}/\text{min}) \times 3 \times 1000]}{540 \times \text{protein content (g) of canola meal}}
\]

Where, $T\Delta A_{247}/\text{min}$ is the trypsin activity and shows the change in absorbance as $A_{247}/\text{min}$ in the absence of inhibitor (substrate and trypsin
only), while \( \Delta A_{247}/\text{min} \) is the inhibition activity is the change in absorbance \( A_{247}/\text{min} \) in the presence of an inhibitor in the presence of inhibitor in the samples as inhibitor activity. The factor 540 is the molar extinction coefficient at \( A_{247} \), which is estimated empirically given the assay buffer mixture and light path length (10 mm) in a quartz cuvette.

Trypsin unit (TU) is the amount of trypsin required for the hydrolysis of 1 \( \mu \text{mol} \) of substrate per min, while trypsin inhibition unit (TIU) is the decrease in trypsin activity by one trypsin unit (TU).

### 3.2.3.5. PI activity staining of PAGE gels

Presence of PI was also confirmed by activity staining of PAGE gels using Biorad Protean (Biorad Laboratories) and Pharmacia Phast (GE Healthcare Life Sciences, Uppsala, Sweden) systems. Crude samples from canola meal extracts were run on a Biorad Protean electrophoresis unit (Bio-Rad Laboratories, Australia) under a discontinuous buffer system using 12% native PAGE gels. Initially, 100 \( \mu \text{L} \) of the crude canola meal extract sample was passed through Bio-Spin® chromatography columns for further clean-up of the proteins in the sample. The crude canola meal extract samples with and without Bio-Spin® cleaning step were run on the native PAGE gel in order to visualize resolved PI bands on the activity stained gel.

The native PAGE gels were prepared as follow. A mixture of separating gel comprising acrylamide (40%), bisacrylamide (2%), 1.5M Tris-HCL (pH 8), UPW, was degassed for \( \frac{1}{2} \) h, to which APS (2%) and TEMED were mixed.
and gently applied onto a Biorad Protean electrophoresis unit. A layer of a 70% ethanol was gently poured over the freshly poured separating gel to ensure a straight interface between the separating and stacking gel.

The stacking gel was prepared with same composition of mixture as separating gel but 1 M Tris-HCL (pH 6.8) was used instead of 1.5 M Tris-HCL (pH 8). The mixture was finally loaded on top of the separating gel after 3 h of setting (polymerization) of the separating gel. Sample buffer was prepared with glycerol (10%), bromophenol blue (0.03%) in extraction buffer for the analysis of trypsin inhibitor in CM. The 6x sample buffer for native gel was prepared using 48% of glycerol, 0.03% bromophenol blue in 375 mM Tris HCl (pH 6.8). Samples run on native PAGE were incubated for 30 min with sample buffer before loading onto the native gel. The native marker, High Molecular Weight (HMW) 66 to 669 KDa (GE Healthcare), was also loaded along with the samples and soybean trypsin inhibitor (positive control). For general analysis, gels were stained using PhastGel blue-R and coomassie brilliant blue.

The purified samples used in the PI inhibition assay, however, were resolved using native gel (PhastGel homogeneous 20), denaturing gel (PhastGel gradient 8-25) and isoelectric focusing gel termed as IEF 3-9, optimized for the pH range of 3–9, was used for the investigation. All native PAGE gels were run in duplicate, stained for protein using Coomassie brilliant blue, and PhastGel blue R and their corresponding gels stained to identify trypsin inhibiting bands. The resultant bands on gels were visualized using a Gel Doc
2000 Video Gel Documentation System (Bio-Rad Laboratories, Pty, Ltd, Sydney, NSW, Australia).

The native PAGE gels were rinsed three times with UPW for 1 min each before staining. The trypsin enzyme was prepared by dissolving 9 mg of trypsin in 18 mL of assay buffer. The staining solution was always prepared fresh before staining by dissolving 0.0175 g of N-acetyl-DL-phenylalanine β-naphthyl ester (APNE) in 5 mL of N,N-dimethylformamide, and 0.025 g of tetrazoitized (zinc chloride complex) o-dianisidine (Fast blue B salt) in 20 mL of trypsin assay buffer. Both solutions were mixed immediately before pouring onto the respective gel. The gel incubation was done for 15 to 20 min at RT. The Trypsin inhibiting PI bands appeared as white clear band against the dark pink background of the trypsin-substrate reaction product. The stained gels were washed in distilled water and stored in 7.5% (v/v) acetic acid before visualizing the gels on Gel Doc 2000 system.

3.2.3.6. SDS-PAGE under non-reducing and reducing conditions

Purified PI samples were further analyzed on SDS-PAGE gels (Phastgel gradient 8-25) on a Pharmacia Phast System (GE Healthcare Life Sciences, Uppsala, Sweden). Sample buffer under non-reducing conditions was prepared in Tris-CaCl\(_2\) extraction buffer (pH 8.1) containing 10% (v/v) glycerol, 4.6% (w/v) SDS, and 0.003% (v/v) bromphenol blue in Tris-CaCl\(_2\) extraction buffer (pH 8.1). Samples were mixed with buffer and incubated for 15 min at 95 °C.
However, for SDS-PAGE under reducing condition 2.5 % β-mercaptoethanol (β-ME) was added to the sample buffer. PI samples were incubated for 15 min at 100 °C, cooled at RT and centrifuged at 16,000 rpm for 5 min. The supernatant was used for direct loading, after mixing with gel loading dye, onto the gels. Precision Plus Protein™ Dual Xtra Standard marker 2-250 KDa, and color Marker low range, mol wt 6,500-45,000 Da were also loaded along with the samples. All gels were stained by using PhastGel blue R.

3.2.3.7. Isoelectric focusing (IEF)

Isoelectric points for the canola meal crude extracts and purified samples were determined by conducting IEF on the Pharmacia Phast System (GE Healthcare Life Sciences, Uppsala, Sweden). Isoelectric focusing gels termed as IEF 3-9 (which are optimized for the pH range of 3–9), were used to resolve proteins based on their iso electric points. The reference sample used was an Amersham Biosciences broad-range pI calibration kit having different proteins with well-known isoelectric points ranging from 3 to 10 was used. The protein bands were visualized using the silver staining method as per manufacturer’s protocol (GE Healthcare Life Sciences, Uppsala, Sweden).

3.2.4. N-terminal amino acid analysis (sequencing)

Purified samples of PIs were electrophoresed on SDS-PAGE gels under reducing conditions on a Biorad Protean electrophoresis unit. The coomassie blue stained visible PI band were excised using a scalpel blade and passively eluted from the gel matrix using SDS elution buffer overnight. The samples were then loaded onto a ProSorb filter cartridge (Applied Biosystems) and
washed with 0.1 % TFA (2 × 100 μL) to remove the SDS and reduce the background contamination.

The sample on the polyvinylidene difluoride (PVDF) membrane was subjected to 10 cycles of Edman N-terminal sequencing. Automated Edman degradation was carried out using an Applied Biosystems 494 Procise Protein Sequencing System. Performance of the sequencer was assessed routinely with 10 pmol β-Lactoglobulin standard. The initial ten amino acids from the N-terminal end of each protein were sequenced and analysed using the blast alignment software (http://www.ncbi.nlm.nih.gov.au) to determine the level of similarity to other proteins.

3.2.5. Data Analysis

Experiments were done in triplicate for precision with values reported as mean ± SD (standard deviation). All results were analyzed using Microsoft Excel 2013 and analyzed by One way analysis of variance (ANOVA) using SAS® system for Window V8 (SAS institute, USA). Comparison between sample means were calculated using the Duncan multiple range test at a 5% probability level (p<0.05). All gels were analyzed by image lab software (Bio-Rad; Australia).
3.3. Results

3.3.1. Protein Extraction

Staining of native-PAGE gels confirmed the presence of PI activity in the canola meal crude extracts. The additional step of passing crude extracts through a BioSpin® column increased the PI activity in the bands on the PAGE gels as shown in Figure 3.1 A& B. The gel conclusively revealed the presence of PI activity bands seen in lanes 1, and 3 (purified using BioSpin columns) and lanes 2 and 4 (crude extract) with genotype-1 (lane 1, 2) and genotype-2 (lane 3 and 4) respectively. The results confirmed the presence of trypsin inhibitors in both the genotypes (genotype-1 and genotype-2) of canola meal analysed (Figure 3.1B).
Figure 3.1. (A) Native-PAGE of canola meal crude extracts after staining with coomassie brilliant blue and (B) corresponding native-PAGE gel showing trypsin inhibitor activity (enzymatic staining) indicated by arrows. More bands were visible (Lane 1 and 3) when fractions were passed through Bio-Spin® 30 Columns as compared to crude samples (Lane 2 and 4) in both genotypes.
After confirmation of the presence of trypsin inhibitors in canola meal, crude fractions were subjected to ammonium sulphate (AS) fractionation as a first step of purification process. The process flow chart for the extraction of the PI is shown in Figure 3.2. All fractions of canola meal prepared using 25%, 50%, and 80% of AS were subjected to spectrophotometric trypsin inhibition activity assays and their corresponding PAGE activity gels were also used to confirm the relative concentration of PI in each fraction (Figure 3.3 A&B).

Fractions obtained at 80% Ammonium sulphate (AS) saturation contained the highest PI activity and were again analysed for the presence of PI activity using PAGE. Trypsin inhibition activity assays revealed maximum inhibition in the CM of both genotypes fractionated with 80% of ammonium sulphate (Figure 3.4 A&B), therefore, were further used for purification and characterisation of PI.
Figure 3.2. Flow chart for the extraction of crude protein fractions
Figure 3.3. (A) Native-PAGE view of canola meal extracts after staining with coomassie brilliant blue and (B) corresponding native-PAGE gel showing trypsin inhibitor activity (enzymatic staining) indicated by arrows. Lanes: 1, 2 and 3 show 25%, 50% and 80% (w/v) ammonium sulphate precipitated fractions (AS) from genotype-1; Lanes: 4, 5 and 6 shows 25%, 50% and 80% (w/v) AS fraction of genotype-2; respectively.
Figure 3.4. Native-PAGE profile of canola meal extracts of two genotypes, genotype-1 and 2; fractionated with 80% ammonium sulphate. (A) Coomassie brilliant blue stained gel and (B) corresponding native-PAGE gel exhibiting trypsin inhibitor activity (enzymatic staining). Lanes: 1, Native marker; Lanes: 2 PI Control from wattle seed extract; Lanes: 3. Genotype-1; Lanes: 4 genotype-2; respectively
3.3.2 Protease inhibitor (PI) Purification.

3.3.2.1 Ion exchange chromatography

Among the different protein peaks observed in anion exchange chromatography (AEC), only peak IB3, between fractions 42 to 53, exhibited PI activity in genotype-1 (Figure 3.5A). The fractions 42 to 53 were pooled. However, in genotype-2 the PI activity was only observed in the unbound eluted fractions ranging from 1 to 18, under the peak IR1. All 18 fractions were pooled together (Figure 3.6A).

3.3.2.2 Gel filtration chromatography

The active fractions resulting from anion exchange chromatography (AEC) were pooled and applied to a gel filtration chromatography (GFC) column in order to separate proteins according to their molecular weight (Mol.wt). The genotype-1 pooled fractions (42-53) from AEC upon elution revealed active fractions, 24 to 38, under the peak GB2 (Figure 3.6 B). The genotype-2, however, revealed a wider active PI peak GR2 between fractions 32 to 55 (Figure 3.6B).

3.3.2.3 Second Ion exchange chromatography

The active pooled fractions of genotype-1 and genotype-2 from gel filtration step, were reloaded onto the AEC column. The genotype-1 revealed a PI active peak PBI1 with active fractions collected from 3 to 12. (Figure 3.5C). Genotype-2 also showed one peak, PRI1, ranging from fraction 3 to 17 (Figure 3.6C).
Figure 3.5. FPLC chromatogram of canola meal extracts from genotype-1 for the analysis PI. Numbers in the bracket at the top of each peak represent fraction numbers obtained in respective peaks (A) Elution profiles of ammonium sulphate precipitate (AS) fractionated by ion exchange as IB1, IB2, IB3, showed that only peak IB3 was positive for PI. (B) Elution profile of IB3 (Ion exchange) in gel filtration shows two peaks GB1 and GB2, where only GB2 was the only peak active for PI (C) Peak GB2 re-chromatographed by ion exchange, shows the most active peak for PI lab
Figure 3.6. FPLC chromatogram of canola meal extracts from genotype-2 used for the analysis PI. Numbers shown in brackets on the top of each peak represent corresponding fraction numbers (A) Elution profile of ammonium sulphate precipitate (AS) fractionated by ion exchange as IR-1, IR2, IR3, and IR3, where only peak IR1 was positive for PI. (B) Elution profile of IR1 (Ion exchange) in gel filtration showing two peaks GB1 and GB2, where GB2 was the only peak with PI activity (C) GB2 peak re-chromatographed using ion exchange showing one peak with high PI activity, labelled PIR1.
Specific enzyme activity was measured in trypsin inhibition units (TIU). Specific activity for the crude water extract in genotype-1 was 0.01TIU mg-1, whereas for genotype-2 it was half the specific activity of genotype-1 (Table 3.1). The salt-precipitated fraction (AS) on the other hand had an activity of 0.59 TIU mg-1 for genotype-1 having 24% protein recovery, while 0.07TIU mg-1 activity was recorded for genotype-2 with 17.2% protein recovery. AEC eluted fractions had active peak fractions (Figure 3.5A & Figure 3.6A) corresponding to 5.8 TIU mg-1 with a purification factor of 387 for genotype-1, while a 0.2 TIU mg-1 activity was observed with a purification factor of 40 for genotype-2 respectively.

The AEC fractions separated by gel filtration showed two major protein peaks. However, only one peak of the two peaks was shown to have trypsin inhibition activity in each genotype (Figure 3.5B & Figure 3.6B). A trypsin inhibition activity of 13.01 TIU mg-1 and purification factor of 867 was seen for genotype-1. Genotype-2 showed a trypsin inhibition activity of 5.78 TIU mg-1 and a purification factor of 1156.
**Table 3.1. Comparison of trypsin inhibitor (TI) activity of the two genotypes at each purification step.**

<table>
<thead>
<tr>
<th>Steps/Characteristics</th>
<th>Total protein(^1) (mg)</th>
<th>Specific activity (TIU mg(^{-1}))(^2)</th>
<th>% Recovery(^3)</th>
<th>Purification factor(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GN-1</td>
<td>GN-2</td>
<td>GN-1</td>
<td>GN-2</td>
</tr>
<tr>
<td>Crude extract</td>
<td>4405±0.54</td>
<td>7560±0.24</td>
<td>0.015±0.02</td>
<td>0.005±0.002</td>
</tr>
<tr>
<td>Ammonium sulphate precipitate (AS)</td>
<td>1050±0.11</td>
<td>1300±0.2</td>
<td>0.59±0.30</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>Ion exchange fraction (IEF)</td>
<td>54±0.12</td>
<td>1065±0.11</td>
<td>5.8±0.87</td>
<td>0.2±0.02</td>
</tr>
<tr>
<td>Gel filtration fraction</td>
<td>44±0.07</td>
<td>205±0.27</td>
<td>13.01±0.96</td>
<td>5.78±0.11</td>
</tr>
<tr>
<td>Re-chromatographed IEF</td>
<td>9.36±0.02</td>
<td>188.6±0.01</td>
<td>72.23±0.92</td>
<td>17.7±0.04</td>
</tr>
</tbody>
</table>

GN-1 (Genotype-1); GN-2 (Genotype-2). \(^1\)Total protein was estimated by using Pierce™ BCA Protein Assay Kit (Thermo Scientific). One trypsin unit (TU) was expressed as one micromole substrate hydrolysed per minute of reaction, whereas one trypsin inhibitor unit (TIU) was expressed as unit of enzyme inhibited. \(^2\)Specific activity was expressed as inhibition units per mg of protein. \(^3\)Protein recovery was determined as percentage of purified inhibitor. \(^4\)Purification factor was measured by specific activity values.
Peaks containing fractions with PI activity were pooled and reapplied to AEC. This additional procedure increased the activity 12 fold for genotype-1 and 88 fold for genotype-2 with the purified fractions having PI activities in only one peak (Figure 3.5C & Figure 3.6C). Trypsin inhibition activity of 72.23 TIU mg\(^{-1}\) and purification factor of 4815 was observed for genotype-1 and a 5.78 TIU mg\(^{-1}\) trypsin inhibition activity and purification factor of 3540 was observed for genotype-2 (Table 3.1).

The protein recovery of active fractions peak indicated with red lines, as shown in Figure 3.5 & Figure 3.6 is illustrated in Table 3.1. Comparison of trypsin inhibition activity between the two genotypes revealed more trypsin inhibition activity for genotype-1 than genotype-2, but with 12 fold more recovery for genotype-2 than the genotype-1. These results confirm the initial observation of trypsin inhibition activity between the two genotypes before their selection for further purification.

The RP-HPLC analysis of the pooled fractions of genotype-1 eluted from the second AEC revealed only one peak (Figure 3.7A). However, analysis of genotype-2 showed two small peaks immediately followed by one major single peak, implying the presence of more than one type of PI that could not be completely separated based on their molecular size alone (Figure 3.7B).
Figure 3.7. Elution profiles for RP-HPLC (214 nm) analysis of canola protease inhibitor. 
3.3.3 Molecular properties and characterisation of purified PI

Native gel Electrophoresis was performed by duplicating all protein fractions and simultaneously running two PAGE gels. Each gel representing full set of all protein fractions. One gel was stained with Coomassie brilliant blue and the other one stained for trypsin inhibitor activity. The pooled fractions of genotype-1 collected under ion exchange peak IB1, IB2 & IB3 (Figure 3.5A) and genotype-2 collected under ion exchange peak IR1, IR2 & IR3 respectively (Figure 3.6A) were resolved on native-PAGE gel. Clear bands were seen showing the presence of trypsin inhibition under peak IB1 (42-53), and IR1 (1-18) with enzymatic staining (Figure 3.8; lane 4 & 3.12; lane 2).

Gel filtration fractions under peak GB2 (fraction number 24 to 38) for Genotype -1 and peak GR2 (fractions number 32 to 55) for genotype -2 showed clear bands for active fractions (Figure 3.9; lane 3 & 3.13; lane 2).

Subsequent anion exchange chromatography of the concentrated peak GB2 & GR2 revealed single peak PIB1 and PIR1 respectively. Each peak showed three active PI bands free from other contaminating proteins as revealed by native PAGE gel (Figure 3.10 & 3.14).
Figure 3.8. (A) Native-PAGE of canola meal anion exchanged fractions from genotype-1 after staining with coomassie brilliant blue and (B) correspondent native-PAGE gel showing trypsin inhibitor activity (enzymatic staining). Lanes-1: Native marker; Lanes-2: peak IB1 with 3-10 fractions; Lanes-3: peak IB2 with 24-36 fractions; Lanes-4: peak IB3 with 42-53 fractions.
Figure 3.9. (A) Native-PAGE of canola meal gel filtration fractions of genotype-1 after staining with Coomassie brilliant blue and (B) corresponding native-PAGE gel having trypsin inhibitor activity (enzymatic staining). Lanes: 1, shows Native marker; Lanes: 2 shows a positive control consisting of a protease inhibitor from crude wattle seed extract; Lanes: 3 shows peak GB2 with gel filtration fraction 24-37 respectively.
Figure 3.10. Native-PAGE of re-chromatographed anion exchange peak PIBI with fractions 3-12 (A) Gel stained with Coomassie brilliant blue and (B) corresponding native-PAGE gel showing trypsin inhibitor activity (enzymatic staining).
The purity of pooled fractions collected after AEC under peak PIB1 and PIR1 was analyzed by SDS-PAGE under reducing and non-reducing conditions (with and without β-mercaptoethanol) respectively as shown in Figure 3.11 (lane 3 for genotype-1) and Figure 3.15 (lane 2 for genotype-2). The fractions 3-12 under peak PIB1 yielded three different trypsin inhibitors having molecular weights of 8, 11 and 18 KDa for genotype-1 that could not be further separated on the basis of their net charge.

The pooled and concentrated fractions, 3-17, under peak PIR1 were also analyzed by SDS-PAGE under non-reducing (lane-2) and reducing conditions (lane-2) as showed in Figure 3.15 (A&B). Fractions 3 to 17 appeared to have three bands of 16, 27 and 35 KDa under non-reducing conditions. However under reducing condition these fractions were reduced to bands having molecular weights of 7, 15.5 and 19 KDa.

While, fractions from genotype-2 under peak PIR1 were eluted as an unbound fraction, the fractions from genotype-1 under peak PIB1 were eluted with the bound fraction using linear NaCl gradient. Furthermore, their isoelectric point (pI) was confirmed by running IEF gels. The pI values of the PIs in the unbound fractions (peak PIR1) were 9.30 for CRP2 and 8.65 CRPI and 6.55 CRPI3; while the bound fraction (peak PIB1) had pI values of 4 for CBPI, 4.6 for CBP2, and 4.90 for CBP3, respectively (Figure 3.16).
**Figure 3.11.** SDS-PAGE profiles of rechromatographed anion exchange fractions of genotype-1 collected under peak PIB1. Arrows shows numbers representing molecular weight in kilodalton (KDa) (A) non-reducing condition and (B) reducing condition with β-mercaptoethanol (ME). Lanes: 1 Precision Plus Protein™ Dual Xtra Standard marker 2-250 KDa as labelled; Lane 2 Soybean PI, Lane 3, PIB1 canola PIs.
Figure 3.12. (A) Native-PAGE view of canola meal anion exchanged fractions after staining with Coomassie brilliant blue and (B) corresponding native-PAGE gel showing trypsin inhibitor activity (enzymatic staining) in genotype-2. Lanes-1: Soybean trypsin inhibitor; Lanes-2: peak IR1 with 1-18 fractions; Lanes-3: peak IR2 with 22-65 fractions; Lanes-4: peak IR3 with 69-85 fractions, respectively.
Figure 3.13. (A) Native-PAGE view of canola meal gel filtration fractions after staining with Coomassie brilliant blue and (B) corresponding native-PAGE gel showing trypsin inhibitor activity (enzymatic staining). Lanes-1, shows Soybean trypsin inhibitor; Lanes-2 shows peak GR2 with gel filtration fraction 32-55, respectively.
Figure 3.14. (A) Native-PAGE view of re-chromatographed anion exchange fractions PIRI from genotype-2 after staining with Coomassie brilliant blue and (B) corresponding native-PAGE gel showing trypsin inhibitor activity (enzymatic staining), confirming both the proteins as protease inhibitors.
Figure 3.15. SDS-PAGE profiles of pooled fractions and concentrated AEC eluted fractions 3-9 under peak PIR1 from genotype-2. Arrows show numbers representing molecular weight in kilodalton (KDa) A) non-reducing condition (B) reducing condition with β-mercaptoethanol (β-ME). Lane-1 is a Precision Plus Protein™ Dual Xtra Standard marker 2-250 KDa as labelled; Lane-2 is the PIR1 fraction and PIR1 fraction.
Figure 3.16. Isoelectric focusing gel (IEF gels 3-9 [pH range 3-9, Pharmacia]) were used for re-chromatographed anion exchange of purified protease inhibitors. Lane-1: Markers with different isoelectric points (pI) ranging from 3 to 10. Lanes-2: genotype-2 PIs, Lanes-3: genotype-1 PIs
3.3.3.1 N-terminal amino acid sequencing of purified PI

The N-terminal amino acid sequences of purified proteins were determined for genotype-1 (Table 3.2) and genotype-2 (Table 3.3) and aligned to protease inhibitor sequences contained in public databases using BLAST software. Sequences with high homology are listed in Tables 3.2 and 3.3. For genotype-1 homologous sequences retrieved from the database had not previously been reported as a canola derived protease inhibitors. Sequences homologous to the genotype-2 sequences were a mix of a canola derived protease inhibitor and protease inhibitors from other organisms.

The canola sequence¹ (Genotype-1), (Table 3.2) with a Mol.wt 8 KDa showed homology to Kunitz type Kalikren inhibitor. Canola sequence² (Genotype-1) with a Mol.wt of 11 KDa is homologous to a cysteine PI found in a parasite and a shrub. Canola Sequence³ (Genotype-1) with a Mol.wt 18 KDa has sequence homology to a serine PI found in papaya.
Table 3.2. Comparison of the amino acid sequences of canola protease inhibitors (CPIs) from genotype-1 with other plant inhibitors

<table>
<thead>
<tr>
<th>Sources</th>
<th>Amino acid residue number</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Canola Sequence¹ (Genotype-1)</strong></td>
<td>A</td>
<td>P</td>
</tr>
<tr>
<td><strong>Carica papaya (papaya)</strong></td>
<td>T</td>
<td>L</td>
</tr>
<tr>
<td><strong>Serine Protease Inhibitor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Canola Sequence² (Genotype-1)</strong></td>
<td>A</td>
<td>D/E</td>
</tr>
<tr>
<td><strong>Trypanosoma cruzi</strong></td>
<td>E</td>
<td>L</td>
</tr>
<tr>
<td><strong>Tabernaemontana divaricata</strong></td>
<td>Q</td>
<td>L</td>
</tr>
<tr>
<td><strong>Cysteine Protease Inhibitor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Papain like Cysteine Protease Inhibitor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Antibacterial Inhibitor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Canola Sequence¹ (Genotype-1)</strong></td>
<td>G</td>
<td>Q</td>
</tr>
<tr>
<td><strong>Bauhinia bauhinioides</strong></td>
<td>H</td>
<td>R</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td><strong>Kunitz-Type Kallikrein Inhibitor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FPRL1 inhibitory protein</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Amino acid identical to those of CPIs are highlighted; Empty areas shows amino acid not determined. Amino acid abbreviation: A, Alanine; P, Proline; T, Threonine; Q, Glutamine; G, Glycine; E, Glutamine; C, Cystine; I, Isoleucine; K, Lysine; D, Aspartic acid; L, Leucine; V, Valine; H, Histidine; R, Arginine.
Table 3.3. Comparison of the amino acid sequences of canola protease inhibitors (CPIs) from genotype-2 with other plant inhibitors.

<table>
<thead>
<tr>
<th>Sources</th>
<th>Amino acid residue number</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Canola Sequence² (Genotype-2)</strong></td>
<td>A</td>
<td>A/E</td>
</tr>
<tr>
<td>Mouse Contrapsin</td>
<td>A</td>
<td>K</td>
</tr>
<tr>
<td>Bos taurus</td>
<td>A</td>
<td>K</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>Locusta migratoria</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>Melanoplus sanguinipes</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td>Schistocerca gregaria</td>
<td>A</td>
<td>E</td>
</tr>
<tr>
<td><strong>Canola Sequence³ (Genotype-2)</strong></td>
<td>P</td>
<td>Q</td>
</tr>
<tr>
<td>Sinapis arvensis</td>
<td>P</td>
<td>Q</td>
</tr>
<tr>
<td><strong>Canola Sequence¹ (Genotype-2)</strong></td>
<td>I</td>
<td>Y</td>
</tr>
<tr>
<td>B.napus seed var Oleifera</td>
<td>I</td>
<td>Y</td>
</tr>
<tr>
<td>Pseudomonas mesoacidophila</td>
<td>Y</td>
<td>P</td>
</tr>
</tbody>
</table>

Amino acids identical to those of CPIs are highlighted; Empty areas shows amino acid not determined. Amino acid abbreviation: I, Isoleucine; Y, Tyrosine; P, Proline; S, Serine; F, Phenylalanine; C, Cystine; V, Valine; E, Glutamine; R, Arginine; A, Alanine; K, Lysine; G, Glycine; M, Methionine; T, Threonine; Q, Glutamine; D, Aspartic acid; L, Leucine.

All canola sequences from genotype-2 are shown in Table 3.3. Canola Sequence¹ (Genotype-2) with a Mol.wt of 7 KDa showed 100% homology to a serine trypsin inhibitor from *B. napus* with variety Oleifera. Canola sequence² (Genotype-2) with a Mol.wt 15.5 KDa has 100% homology to a 100% trypsin inhibitor already identified in the seed of Charlock (*Sinapis arvensis*) a member of the napin family. Canola sequence³ (Genotype-2), with a Mol.wt of 19 KDa has homology to some sequences with serine trypsin inhibitor activity found in mice and insects.

### 3.4 Discussion

The success of initial attempts to detect the activity of isolated PIs was limited. Initially, 20 mg of canola meal was dispersed in 1 mL of extraction buffer and incubated at 37°C for 30 min. The sample was centrifuged for 15 minutes and supernatant was used to run on native PAGE gel to detect PI activity. The stained gel revealed only very faint PI bands. However, passing of the same crude sample through a Bio-Spin® 30 Column, to remove interfering impurities resulted in clear PI bands, confirming the presence of protease inhibitors in the canola meal samples (Figure 3.1 A&B).

The activity staining of the gels confirmed the presence of different inhibitor bands with several clear bands of varying intensity against a pink background, with the 80% AS fraction exhibiting the most intense bands. The corresponding gels were also stained with coomassie brilliant blue to detect the presence of protein.
Native-PAGE gel and PI activity staining of the gel confirmed the presence of protease inhibitors in the crude extracts of both the genotype examined (Figure 3.3 A&B). However, fractions of canola meal extract generated using ammonium sulphate at 80% saturation showed maximum activity using both spectrophotometric assays and native PAGE gels (Figure 3.4 A&B). Both the genotype showed higher trypsin activity and thus were selected for further purification of PIs by FPLC using ion exchange and gel filtration column chromatography methods.

Despite, fractions containing genotype-2 protease inhibitors always eluted as unbound during both AEC steps, the anion exchange resin was still retained as the medium of choice. All fractions with PI activity were concentrated with Amicon Ultra-15 filtration tubes and centrifugation. This method was quite efficient in eliminating salt and other contaminants. The pooled fraction were loaded onto a gel filtration and then reloaded onto AEC.

SDS-PAGE separates proteins according to their mass/charge (m/z) ratio. IEF gels resolve proteins according to the same mechanism and also by isoelectric points. Separation and charge of PIs in both the genotype of canola was confirmed by IEF gels (Figure 3.16.).

RP- HPLC was conducted to confirm the purity of PIs in canola genotypes from FPLC eluted fractions (Figure 3.7). The appearance of a single peak did not confirm the presence of a single protein with PI activity according to the results of SDS-PAGE. RP-HPLC of fractions from genotype-2 revealed two peaks appearing as shoulders to the third main peak indicates the presence of
more than one PI that cannot be separated solely on RP-HPLC the basis of their molecular size alone. The SDS-PAGE of the pooled fractions 3-17 under reduced condition and IEF revealed three bands confirming the presence of at least three kind of PIs. The presence of different protease inhibitors in the same plant species with different physiological role has been reported in the past (Ceciliani et al., 1994). These PIs could be further separated by more sensitive techniques such as preparative IEF separation that will in turn allow the understanding of the properties of individual PIs in more detail.

Table 3.2 shows N-terminal amino acid sequence comparisons of three PI subunits of canola genotype-1 (Genotype-1) with similar sequences from other species using a Blast search. The 8 KDa protein subunit designated as canola sequence¹ (Genotype-1) had some homology to Kunitz type Kalikren inhibitor from Bauhinia bauhioides (Navarro et al., 2005). This sequence also has some homology to a bacterium Staphylococcus aureus FPRL1 inhibitory protein, which act as chemoattractant receptor inhibitors (Prat et al., 2009).

Canola sequence² (Geotype-1) with a Mol.wt of 11 KDa has some homology to a cysteine PI found in parasites known as Trypanosoma cruzi (Monteiro, Abrahamson, Lima, Vannier-Santos, & Scharfstein, 2001), and also exists in a shrub called Tabernaemontana divaricate (Thakurta et al., 2004). This sequence also has homology to an inhibitor of activated Signal transducer and transcriptional activator (STAT) protein gamma in Mus musculus (Sturm, Koch, & White, 2001). It also has homology with a protein from Streptococcus pneumonia which has antibacterial activity (Green et al.,
2012). Canola Sequence\textsuperscript{3} (Genotype-1) with a Mol.wt of 18 KDa has some sequence homology to a serine PI found in papaya (Azarkan, Martinez-Rodriguez, Buts, Baeyens-Volant, & Garcia-Pino, 2011). These results suggest that the PIs from canola may potentially have similar activities to previously identified PIs.

Table 3.3 also shows the amino acid sequence for canola sequence\textsuperscript{1} of the 7 KDa subunit of genotype-2 (Geotype-2), which showed 100% homology to a previously reported serine trypsin inhibitor from \textit{B. napus}. Ceciliani et al., (1994) reported similar kind of PI of 6.7 KDa from \textit{Brassica napus} seed with trypsin and chymotrypsin inhibition activity and thermostability at 80°C for 3 min. This PI has been reported to be important for plant defense. This sequence also has some homology with a \textit{Pseudomonas mesoacidiphila} protein with antiviral activity (Ravaud et al., 2007). This result suggests that these putative PIs from canola may potentially have antiviral activity in plants and humans.

The 15.5 KDa subunit of genotype-2 labelled as Canola Sequence\textsuperscript{2} (Geotype-2) has part of its sequence showing 100% homology to a known inhibitor in the seed of Charlock (\textit{Sinapis arvensis}) in the napin family with a Mol.wt of 15.5 KDa and is known to be a part of the 2S storage albumins of \textit{Brassicaceae} (Table 3.3). This inhibitor has been reported to be containing an \textit{alpha} and a \textit{beta} chain. This low molecular weight inhibitor is a two chain trypsin inhibitor having storage properties (Svendsen, Nicolova, Goshev, & Genov, 1994).
Results from SDS-PAGE demonstrated that Canola sequence³ (Genotype-2) have N-terminal amino acid sequence³ of the 19 KDa subunit from genotype-2 (Geotype-2) and showed some sequence homology to protease inhibitors from several organisms with activities against disease causing organisms and human ailments. There was some sequences with homology to a serine trypsin inhibitor found in mice and insects (Suzuki, Yamamoto, & Sinohara, 1990). Some sequence homology was also observed with peptide sequences with demonstrated human and mouse leukemia (Boulanger, Bankovich, Kortemme, Baker, & Garcia, 2003), and plasminogen activator inhibition properties. These sequences have also been shown to have Fas apoptotic inhibitory molecule-1 properties which helps to protect neurons from cell death (Table 3.3).

The family of Kunitz type protease inhibitors is well known, existing in higher quantities in plant seeds (Birk, 2003) and have Mol.wt more than 8 KDa. Since all sequences have a Mol.wt more than 8 KDa except canola Sequence¹ (Genotype-2). It is likely that most of canola PIs also belong to the Kunitz type trypsin inhibitor family. PIs from Brassicaceae have also been known for their function under environmental stresses in plants such as salinity and drought (Mosolov et al., 2001). Canola protein extraction is less in recovery due to different isoelectric points and molecular weights as reported by Aachary & Thiyam (2012). Therefore large amount of meal was required to generate sufficient protein for analysis (Table 3.1).
3.5 Conclusion

Both genotypes were found to contain three PIs that co-eluted during gel filtration and ion exchange chromatography. The full sequences of the three PIs of Genotype-1 have not been previously reported in the literature. Canola sequences from genotype-2 shows 100% homology with other known PIs, storage and defense proteins. The blast search of Genotype-1; canola sequence\(^1\) and canola sequence\(^2\), Genotype-2 canola sequence\(^3\) revealed similarity with PIs with potential health benefits. Most of canola PI belong to serine PI and Kunitz-Type PI families. Further research is needed to separate these individual PIs to homogeneity and to ascertain their potential therapeutic properties by conducting appropriate assays.
CHAPTER 4. Measurement of bioactive properties using enzyme assays

4.1 Introduction

The measurement of bioactive compounds using enzyme-based assays provides an efficient way to assess the potential *in-vivo* activities of compounds. The ability to utilize enzymes found in important biological processes provides a cost-effective way to measure the potential bioactivities without having to conduct *in-vivo* experiments. This provides the ability to screen a large number of potential compounds before moving to more complex cellular or *in-vivo* systems. These assays also provide the opportunity to assess a number of different bioactives that may have roles in various disease states.

This chapter describes the measurement of bioactivies using enzyme based assays that assess the potential anti-cancer properties (*Topoisomerase-1* inhibition), antidiabetic properties (*dipeptidylpeptidase-IV* inhibition) and antiobesity properties (*lipase* inhibition). The objective of the current research was to screen i) various canola meal extracts (CMEs) for their *topoisomerase-1* (*topo-I*) poisoning and suppression activities ii) to investigate the potential antidiabetic activity of different extracts and protease inhibitors obtained from the meals of both genotypes, iii) to investigate the ACE-inhibitory activity of protease inhibitors (PIs) and CMEs iv) to investigate the inhibitory actions of canola meal extracts on pancreatic lipase activity.
4.1.1 In vitro topoisomerase-1 inhibition

Compounds with anticancer properties are of particular interest as cancer is the main cause of death worldwide (Tabassum et al., 2012). About 3 in 10 deaths are caused by cancer in Australia (Australian Institute of Health and Welfare & Australian Association of Cancer Registries, 2012). DNA topoisomerase inhibitors have been identified as potential anti-cancer agents (Pommier et al., 2010).

4.1.2 Antidiabetic assay

Type-II diabetes typically involves a gut hormone known as glucagon-like peptide-1 (GLP-1). GLP-1 is an insulinotropic gut hormone that stops diabetes by mutual role for the stimulation of insulin secretion (incretin), high rate of beta-cell, and reduction of glucagon secretion (Chakrabarti, Bhavtaran, Narendra, & Varghese, 2011). All these actions slow down the rate of stomach emptying and regulate the normal feedback mechanism (Fan, Johnson, Lila, Yousef, & de Mejia, 2013).

However, GLP-1 is instantly inhibited/degraded by the enzyme dipeptidylpeptidase-IV (DPP-IV). Inhibition of DPP-IV has therefore been reported to be an effective treatment for type-II diabetes (Singh et al., 2011). However, only a few drugs are known to inhibit DPP-IV and they have been associated with side effects including insulin resistance, atherosclerosis and
obesity (Parmar et al., 2012). Previous literature has not reported any DPP-IV inhibitory activity for canola meal extracts or protease inhibitors (PIs).

4.1.3 Angiotensin-converting enzyme (ACE) inhibitory activity

High blood pressure is related to chronic disease including stroke, coronary heart disease (CHD), kidney dysfunction, disability, and death (Chen et al., 2009). The fifth mostly prescribed medicine for lowering blood pressure are Angiotensin-converting enzyme (ACE) inhibitors (Fagyas et al., 2014). This has prompted a number of research projects aimed to identify compounds with the capacity to inhibit ACE.

Previous studies have indicated that proteins, hydrolysates and peptides obtained from canola meal have antihypertensive and ACE-inhibition activities (Alashi et al., Aider & Barbana, 2011; 2014b; Wu, Aluko, & Muir, 2008). This suggests that proteins and other compounds (protease inhibitors and polyphenols) from canola meal may be exploited in the management of high blood pressure.
4.1.4 Lipase Inhibition

Pancreatic lipase is the key enzyme secreted from the pancreas and has a major role in the hydrolysis of 50–70% of dietary triglycerides (TAG) into monoacylglycerides and free fatty acids before absorption by the enterocytes (Sergent et al., 2012). Therefore, the inhibition of this enzyme can result in a lower fat absorption and energy uptake, which is one of the major targets in the fight against obesity.

There have been no reports on the identification of phytochemicals from canola meal that have been shown to demonstrate inhibitory effects on lipases.

4.2 Material and methods

4.2.1 In vitro topoisomerase-1 inhibition

4.2.1.1 Materials

Canola breeding line Bln-3347 (Genotype-1) was supplied by the NSW Department of Primary Industries, Wagga Wagga, Australia and used for meal preparation. The following reagents were used: Topoisomerase-1 enzyme (Promega), Protease K (Biovisual), PBR 322 Plasmid DNA (0.5 µg/µL), and Gene ruler 1 Kb DNA Ladder (Fermentas).
Chemicals including Trishydroxymethylamino methane (Tris), glycerol, sodium dodecyl sulphahte (SDS), bovine serum albumin (BSA), camptothecin, xylene cyanol FF, ethylenediaminetetraacetic acid (EDTA), 36% hydrochloric acid (HCL), phosphate buffer saline (PBS) and potassium Chloride (KCl) were purchased from Sigma Aldrich (Sydney, Australia). Other chemicals used included Ficoll® 400 (Millipore, Australia), dithithretiol and hexane (Thermofisher Scientific), Ultrapure water (Wasser-Aufbereitungs system), Agarose (Biorad) and Gel star™ (Lonza).

Canola meal extracts (CMEs) named based on the solvent used in the extractions: water extract (WE), methanol extract (ME), ethanol extract (EE), acetone extract (AE), butanol extract (BE), chloroform extract (CE) and hexane extract (HE). Other protein extracts prepared with 25, 50 and 80% ammonium sulphate (PI25, P150 and PI80) including purified protease inhibitor (PI80p), were also used in these experiment (see chapter-3).

4.2.1.2 Preparation of reagents

Stock DNA relaxation buffer was prepared as 50 mM Tris-HCl (pH 7.5), 20 mM KCl, 1 mM EDTA in ultrapure water (UPW). Working relaxation buffer was always prepared fresh by taking a 1 mL aliquot of the stock relaxation buffer and 1mM Dithiothretol containing 0.3 mg/mL of BSA. This working solution was used to prepare 8.8 ng/μL PBR322 plasmid DNA and enzyme dilutions.
Relaxed DNA was prepared by adding 0.0375 µL of topo-I (Top-1X) to 75 ng of supercoiled and incubated at 37 °C for 1.5 hours. DNA loading Dye was prepared in a 10 mL solution containing 2 % SDS, 14% Ficoll®400, 0.1 % Bromophenol blue and 0.05 % Xylene Cyanol FF in 1X TAE buffer (pH 8). All the samples were prepared in PBS buffer except standard sample camptothecin (CT) and morin which were prepared in 80 % methanol with PBS. All samples and buffers were filtered through a 0.22 µM syringe filter before use.

4.2.1.3 Topoisomerase inhibition

Topoisomerase inhibition activity was conducted according to the method described by Webb & Ebeler (2003), with slight modification. This method measures both topoisomerase poisoning and topoisomerase suppression. The reaction mixture was prepared in a 96-well PCR plate (Sigma-Aldrich, Australia). The products of the reaction were then ran on to an agarose gel electrophoresis. Camptothecin was used as a standard standard and PBS was run as a blank control (no addition of relaxed or supercoil DNA). Images of gels were captured using a Bio-Rad Gel Doc, (Universal hood II using Quantity One Software)

4.2.1.4 Poisoning assay

The poisoning assay was performed by reacting together 10 µL of relaxed DNA (75 ng) and 5 µL of extract sample at varying concentrations (1.25, 5 and 20 µg/µL) or the standard sample (10 µM) and then, incubated at room
temperature for 20 min. After the incubation time, 1.5 µL of topo-I (10X) was added to the reaction mixture and further incubated at 45 °C in dark for 1 hour with gentle shaking. The reaction was stopped by the addition of 2 µL 10 % SDS and 2 µL Proteinase K (1mg/mL). Loading dye 6X was added in to the reaction product (in a ratio of 1: 5 (v/v). The reaction product was electrophoresed on an agarose gel using a horizontal gel apparatus (Pharmacia Biotech, Piscataway, New Jersey, USA) with 40 volts without pre-staining.

After 3 h, gels were post-stained in 50 mL of Tris buffer (pH 8.5) containing 15 µL of Gel star and 50 mL of glycerol. The gels were further electrophoresed for 2 h to improve band resolutions. Gels were then visualized as before.

4.2.1.5 Suppression Assay

The suppression assay was carried out by adding 10 µL supercoiled DNA (75 ng) to 5 µL of extract sample (1.25, 5 and 20 µg/µL) and incubated for 20 minutes. After the incubation time, 1.5 µL of topo-I (2X) was added to the reaction mixture and further incubated at 45 °C in dark for 1 hour with gentle shaking. The reaction was stopped by the addition of 2 µL 10 % SDS and 2 µL Proteinase K (1mg/mL). Loading dye 6X was added in to the reaction product (in a ratio of 1: 5 (v/v). Gel preparation, loading and analysis were carried out as described in section 4.2.4.1.
The percent suppression activity (SA) was calculated as follows:

\[
SA(\%) = \frac{S_s - S_r}{S_c - S_r} \times 100
\]

where, \(S_s\) = percent of supercoiled DNA used with sample and enzyme, \(S_c\) = percent of control (supercoiled DNA only) and \(S_r\) = percent of relaxed DNA only.

4.2.2 Antidiabetic assay

4.2.2.1 Materials

Dipeptidylpeptidase-IV (DPP-IV, CD26) from porcine kidney and Diprotin-A (Ile-pro-ile) were purchased from Merck (Darmstadt, Germany). Gly-pro-p-nitroanilide, and Tris hydrochloride (Tris-HCl) were purchased from Sigma Aldrich. Seven canola meal extracts (CMEs: WE, ME, EE, AE, BE, CE and HE) and purified protease inhibitors (PIs) prepared from meals from two different canola genotypes (genotype-1 and genotype-2) as previously described were used for the experiment.

4.2.2.2 Antidiabetic Assay

The antidiabetic activity was conducted as described by Lacroix & Li-Chan (2012) with slight modifications. All samples were prepared in 100 mM Tris-
HCl buffer. The assay was carried out in 96 micro well plates using Diprotin-A (Ile-Pro-Ile) as a standard inhibitor. A total reaction volume of 250 µL was prepared and consisted of 100 mM Tris-HCl buffer (pH 8.4), 7.5 µL of DPP-IV enzyme (0.2U/mL) and 232.5 µL of either test samples, standard inhibitor or the buffer as blank. This mixture was incubated at 37 °C for 30 mins, followed by the addition of 10 µL of 1.59 mM Gly-pro-pnitroanilide (substrate). The reaction mixture was mixed and further incubated at 37 °C for 30 mins. The absorbance at 410 nm was measured in a microplate (FLUOstar omega microplate reader; BMG Labtech, Offenburg, Germany).

The percentage inhibition of DPP-IV was calculated as:

\[
\text{Inhibition (\%)} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{inhibitor}}}{\text{Absorbance}_{\text{control}}} \times 100
\]

Where, the control corresponds to enzyme while, the inhibitor corresponds to sample absorbance. The results were expressed as the IC\textsubscript{50} value or concentration (the concentration of sample needed to inhibit DPP-IV by 50%). The IC\textsubscript{50} of each sample was calculated from the least squares regression line of the plot of logarithm of the sample concentration (log) versus the DPP-IV inhibition activity (\%).
4.2.3 Angiotensin-converting enzyme (ACE) inhibitory activity

4.2.3.1 Materials

Chemicals used in this study included angiotensin-converting enzyme (ACE; EC 3.4.15.1) from rabbit lung, N-[3-(2-furyl) acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG), Tris hydrochloride (Tris-HCl), and sodium chloride were purchased from Sigma-Aldrich, Castle Hill, NSW, Australia.

Protease inhibitors (PI) and seven extracts were produced from canola meals (CME) using two different canola genotypes (genotype-1 and genotype-2) as previously described.

4.2.3.2 ACE-inhibition activity (in-vitro)

The ACE-inhibition activity was performed as described by Adjonu et al., (2013). This involved taking 10 µL of ACE enzyme (0.25 units/mL in deionised water) and 30 µL of sample solution (0.625–10 µg/mL in 50 mM Tris–HCl buffer, pH 7.5, containing 300 mM NaCl) or or 30µL of Tris-HCl buffer (control solution) and loaded individually into a 96 well microplate. All samples were centrifuged (HermLe; Gosheim, Germany) at 5500 x g for 5 min to remove debris or solid matter before analysis. The sample plates were incubated at 37 °C for 5 min in a microplate reader (FLUOstar Omega plate reader; BMG, GmbH, Offenburg, Germany). After incubation, 150 µL of 0.88 mM FAPGG (substrate) was added into each well.
The changes in the absorbance at 340 nm due to the degradation of FAPGG by ACE and the inhibitory properties of the PIs and CMEs were monitored for 50 minutes. The ACE activity was evaluated by the slope of the decrease in the absorbance at 340 nm (ΔA) over a linear interval from the 1st to the 13th min, because within this time interval absorbance values were stable. The ACE inhibition activity (%) of the PIs and CMEs was calculated as:

\[
ACE \text{ inhibition (\%)} = \left(1 - \frac{\Delta A_{\text{inhibitor}}}{\Delta A_{\text{control}}}\right) \times 100
\]

where \(\Delta A_{\text{sample}}\) and \(\Delta A_{\text{control}}\) are the slopes of the samples (PIs and CMEs) and of the control, respectively. The results were expressed as the IC\textsubscript{50} value.

4.2.4 Lipase Inhibition

4.4.2.1 Materials

Chemicals including Orlistat, 4- methylumbelliferyl oleate (4-MU oleate), Tris hydrochloride (Tris-HCl), calcium chloride (CaCl\textsubscript{2}), and sodium citrate, sodium chloride (NaCl) were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Pancreas lipase (PL, from porcine) was purchased from Roche Diagonistic (Melbourne, Australia). Seven canola meal extracts (CMEs) were obtained as previously described.
4.4.2.2 Pancreatic lipase inhibition assay (In-vitro)

Inhibition of Pancreatic lipase (PL) was determined using the procedure as described by Nakai et al., (2005) using the fluorogenic substrate, 4-MU oleate (substrate) and Orlistat (control sample). CMEs and PIs were dissolved in Tris-HCl buffer (13 mM) containing 50 mM NaCl and 1.3 mM CaCl\textsubscript{2}. The reaction mixture was prepared in a 96-well microplate and included 25 μL of each sample (concentration), 50 μL of substrate (0.1 mM), and 25 μL of PL enzyme (50 U/mL). The plate containing the mixture was incubated at 25 °C for 30 min to start the enzymatic reaction. The reaction was stopped by the addition of 100 μL of sodium citrate (100 mM, pH 4.2). The relative fluorescence intensity was measured using a Cary Eclipse Fluorescence Spectrophotometer (Varian, Inc, Victoria, Australia) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The results were expressed as IC\textsubscript{50} value.

4.3. Data analysis

For topoisomerase inhibition assays all experiment were performed at least in triplicate. The gel band intensities were measured using software image J (1.48v, Wayne Rasband, National Institute of Health, USA).

For other assays all other experiments were also performed in triplicate and results were reported as mean ± standard deviation (SD) and analyzed by One way analysis of variance (ANOVA) using SAS\textsuperscript{®} system for Window V8 (SAS institute, USA). Comparison between sample means were calculated using the Duncan multiple range test at a 5% probability level ($p<0.05$).
4.4 Results

4.4.1 *In vitro* topoisomerase-1 inhibition

A marker was run in each gel in order to demonstrate all possible changes to the DNA. Figure 4.1 shows all the different topological forms of DNA identified: nicked (nick), supercoiled (SC), fully relaxed (Rx) and partially relaxed (Rp).

The results for the poisoning assay are depicted in Figures 4.2, 4.3 and 4.4, where samples showing a poisoning effect is indicated by a higher intensity in the nicked DNA band. Figure 4.2 (A and B) shows the poisoning activity in the five CMEs (ME, WE, CE, AE, and BE) in a concentration dependent manner. Poisoning activity varied as indicated by the different intensities when using 20, 5 and 1.25 µg/µL of ME (lanes 5 to 6). No DNA was observed when the WE was loaded at 5, 20 µg/µL (lanes 8 and 9). However, at the lowest concentration of 1.25 µg/µL (lane 10), a very thick band with high intensity was observed indicating the presence of poisoning activity. The CE on the other hand gave bands of higher intensities at all concentrations (lane 10 to 12). In addition, the AE and BE bands showed high intensities which decreased with decreasing concentration.
Figure 4.1. Electrophoresis gel showing topological position. lane 1) supercoiled (SC) DNA; lane 2) relaxed (Rx) DNA and partially relaxed (Rp).
Figure 4.2. A). Electrophoresis gel showing poisoning result by topoisomerase I (Topo I)-induced cleavage by Canola meal extracts. Lane 1, Marker; Lane 2, SC DNA; Lane 3, Rx DNA; Lane 4, Relaxed DNA+10µM CT+ Topo 1 (10X), Lane 5-7, Relaxed DNA+ME 20, 5 and 1.25 µg/µL+ Topo 1 (10X), Lane 8-10, Rx DNA+WE 20, 5 and 1.25 µg/µL + Topo 1 (10X); Lane11-13, DNA+CE 20, 5 and 1.25 µg/µL + Topo 1 (10X); Lane14-16, DNA+AE 20, 5 and 1.25 µg/µL + Topo 1 (10X); Lane17-19, DNA+BE 20, 5 and 1.25 µg/µL + Topo 1 (10X). B). Graphical representation of the cleavage data presented in figure 4.2 A. Column labels are the lane numbers of the gel shown in 4.2 A. Relaxed DNA is represented as Rel while cleavage is measured as band intensity.
Figure 4.3 A and B shows poisoning activity for CMEs (EE and HE) and protein fractions. The EE showed poisoning activity in all concentration but had higher intensities at the 1.25 and 20 µg/µL concentrations (lanes 6 and 7), for poisoning activity. Whereas, the HE showed poisoning activity at the 5 and 20 µg/µL (lanes 8, 9) only. All protein fractions (PI25, PI50 and PI80) had poisoning activity regardless of concentration used but the purified protease inhibitor fraction (P80p) showed activity at 20 µg/µL only (Figure 4.4 A and B).
Figure 4.3. A) Electrophoresis gel showing poisoning result by topoisomerase I (Topo I)-induced cleavage by Canola meal extracts. Lane 1, Marker; Lane 2, SC DNA; Lane 3, Rx DNA; Lane 4, SC DNA+10µM CT+ Topo 1 (10X); Lane 5-7, SC DNA+ EE 20, 5 and 1.25 µg/µL + Topo 1 (10X)+ Topo 1 (10X); Lane 8-10, SC DNA+ HE 20, 5 and 1.25 µg/µL + Topo 1 (10X)+ Topo 1 (10X); Lane 11-13, SC DNA+ P25 extract 20, 5 and 1.25 µg/µL + Topo 1 (10X)+ Topo 1 (10X); Lane14-16, SC DNA+ P50 extract 20, 5 and 1.25 µg/µL + Topo 1 (10X); Lane17-19, Supercoiled DNA+ P80 extract 20, 5 and 1.25 µg/µL + Topo 1 (10X). B). Graphical representation of the cleavage data presented in 4.3 A. Column labels are the lane numbers of the gel shown in 4.2 A. Relaxed DNA is represented as Rel while cleavage is measured as band intensity.
Figure 4.4. A) Electrophoresis gel showing poisoning result by topoisomerase-I (Topo I)-induced cleavage by purified protease Inhibitor. Lane 1, SC DNA; Lane 2, Rx DNA; Lane 3 SC DNA +Topo 1 (10X); Lane 4, Rx DNA+10 µM CT+ Topo 1 (10X), Lane 5-7, Rx DNA+ Purified Protein fraction extract (P80p) with 20, 5 and 1.25 µg/µL; Lane 8, Marker. B), Graphical representation of the cleavage data presented in 4.4 A. Column labels are the lane numbers of the gel shown in 4.2 A. Relaxed DNA is represented as Rel while cleavage is measured as band intensity.
Figures 4.5 and 4.6 show topoisomerase suppression activity where morin was used as a standard sample due to its non-intercalating and non-poisoning property. Suppression activity was observed in all ME, AE and EE extracts at all concentrations. There was no suppression activity for WE, CE, BE and HE. Protein fraction 25 (P_{25}) extract, had suppression activity at the 1.25 μg/μL concentration only (lane 14, Figure 4.5) but with partial relaxation activity. Protein fraction 50 (P_{50}) extract had suppression activity at the 20 μg/μL only (lane 15, Figure) while protein fraction 80 (PI_{80}) extract had suppression activity at the 1.25 μg/μL only (lane 21, Figure…). Similarly, for the purified protease inhibitor PI_{80p}, suppression activity was observed at the lowest concentration only (5 & 1.25 μg/μL: lane 2 & 4 in Figure 4.7). Suppression activities for all CMEs are summarized in Figure 5.8.
Figure 4.5. Electrophoresis gel showing topoisomerase suppression activity. Lane 1, Marker; Lane 2, SC DNA; Lane 3 SC DNA+150 μM morin + Topo 1 (2X); Lane 4, Rx DNA; Lane 5, Supercoiled DNA+150 μM morin + Topo 1 (2X); Lane 6-8, Supercoiled DNA+ ME 20, 5 and 1.25 μg/μL + Topo 1 (10X)+ Topo 1 (2X); Lane 9-11, SC DNA + WE 20, 5 and 1.25 μg/μL + Topo 1 (10X) + Topo 1 (2X), Lane 12-14, SC DNA+ CE 20, 5 and 1.25 μg/μL + Topo 1 (10X)+ Topo 1 (2X); Lane15-17, SC DNA+ AE20, 5 and 1.25 μg/μL + Topo 1 (2X); Lane18-20, SC + BE 20, 5 and 1.25 μg/μL + Topo 1 (2X).
Figure 4.6. Electrophoresis gel showing topoisomerase suppression activity. Lane 1, Marker; Lane 2, SC DNA; Lane 3, Rx DNA; Lane 4 SC DNA + Topo 1 (10X); Lane 5, Rx DNA+10 µM CT+ Topo 1 (10X); Lane 6-8, Rx DNA+EE 20, 5 and 1.25 µg/µL + Topo 1 (10X); Lane 9-11, Rx DNA+HE 20, 5 and 1.25 µg/µL + Topo 1 (10X); Lane 12-14, SC DNA + P25 extract 20, 5 and 1.25 µg/µL + Topo 1 (10X); Lane 15, 16 and 17, SC DNA+ P50 extract 20, 5 and 1.25 µg/µL + Topo 1 (10X); Lane 18-20, DNA+ P80 extract 120, 5 and 1.25 µg/µL + Topo 1 (10X); Lane 21, Empty well; Lane 22, Marker.
Figure 4.7. Electrophoresis gel showing topoisomerase suppression activity. Lane 1, Rx DNA; Lane 2 x DNA+P$_{80p}$ extract 20 µg/µL + Topo 1 (10X); Lane 3, Rx DNA + Purified P$_{80p}$ extract 5 µg/µL + Topo 1 (10X); Lane 4, DNA+ P$_{80p}$ extract 1.25 µg/µL + Topo 1 (10X); Lane 5; Marker.
Figure 4.8. Percentage of topoisomerase-I suppression activity
4.4.2. Antidiabetic assay

IC$_{50}$ values for DPP-IV inhibition of the protease inhibitors (PIs) from canola meal and Diprotin A (standard sample) are shown in Figure 4.9. Diprotin A (19.4 μg/mL) showed the highest DPP-IV inhibition activity followed by PI from genotype-1 (61.8 μg/mL) and then genotype-2 (69.8 μg/mL) ($p < 0.05$).

Figure 4.10 also shows the percent DPP-IV inhibition for the seven CMES and Diprotin A. All extracts were used at a concentration of 2000 μg/mL whereas Diprotin A was used at 2 μg/mL due to its higher inhibition activity. All extracts from both genotypes showed some inhibition activity against DPP-IV ($p < 0.05$), the extent however was dependent on the genotype and the solvent used to prepare the extract. The order of inhibition was: BE > AE > WE > EE > ME > HE > CE for genotype-1 and BE > WE > AE > ME > HE > EE > CE for genotype-2. Overall, Diprotin A (standard sample) exhibited significantly higher inhibition activity when compared to the CMEs ($p < 0.05$).
Figure 4.9. DPP-IV inhibition activity of protease inhibitors (PIs) from two different genotypes) from canola meal represented as IC$_{50}$ value. Different letters on the top of each bar on graphs represent mean values (n=3) which are significantly different ($p<0.05$).
Figure 4.10. Percentage (%) of DPP-IV inhibition activity of canola extracts from two different genotypes. Diprotin A was used as standard control. Different letters on the top of each bar on graphs represent mean values (n=3) which are significantly different ($p<0.05$).
4.4.3 Angiotensin-converting enzyme (ACE) inhibitory activity

Figure 4.11 shows the IC<sub>50</sub> values for ACE inhibition of the PIs from the canola meal. The PI from genotype-1 possessed significantly higher ACE-inhibition activity than PI from genotype-2 (p < 0.05).

The ACE inhibition activities (%) of CMEs (at 2 mg/mL) are shown in Figure 4.12. The AE showed the highest ACE-inhibition potency followed by the ME with CE recording the least ACE-inhibition activity (p < 0.05). Similar ACE-inhibition activities were found for the WE, EE and BE (p > 0.05).
Figure 4.11. The ACE inhibition activity (IC\textsubscript{50}) of protease inhibitors obtained from canola meals of genotype-1 and genotype-2.
Figure 4.12. ACE inhibition activity (%) of canola meal extracts.
4.4.4 Pancreatic lipase inhibition assay (In-vitro)

The IC$_{50}$ values for pancreatic lipase inhibition of the seven CMEs are shown in Figure 4.13. All extracts showed the capacity to inhibit the PL with the acetone extract showing the highest inhibition activity (1.60±0.06 mg/mL) and the chloroform extract the least (5.42±0.07 mg/mL) for the CMEs ($p < 0.05$). The order for lipase inhibition was AE > BE > ME > WE > EE > HE > CE. Comparatively, Orlistate (standard sample) had greater potency against PL (IC$_{50}$ value of 0.74 mg/mL) than the CMEs ($p < 0.05$).
Figure 4.13. Inhibitory effect of different solvents (IC$_{50}$) extracts from canola meal on pancreatic lipase inhibition.
4.5 Discussion

4.5.1 In vitro topoisomerase-1 inhibition

Higher than normal levels of Topoisomerase-1 (topo-1) are often found in human cancer cells, hence, they are an attractive target for anticancer therapy (Tabassum et al., 2012). Natural compounds extracted from plants can be used in screening for useful compounds, which may act as antitumor pharmaceuticals. Gel electrophoresis was used for the characterisation and quantification of topo-I inhibition activity in canola meal extracts (CMEs). Plasmid PBR322 DNA (Supercoiled) was used as an indicator molecule which was readily relaxed by topo-I and was visualized on an agarose gel (Figure 4.1).

There are two mechanisms for topoisomerase inhibition: (i) topoisomerase poisoning where compounds interact directly with DNA. Poisoning is detected as an increased amount of nicked DNA and (ii) topoisomerase suppression which is indicated by the prevention of supercoiled DNA from being converted to relaxed DNA. Compounds that reduce topoisomerase-1 levels either by “poisoning” and/or “suppressing” the enzyme may result in the premature death of these cells, forming an effective treatment for cancer (Bailly, January 2000).

In the present study, topoisomerase suppression and poisoning products from CMEs were clearly distinguished by agarose gel electrophoresis. In the literature the differences between topo-I poisoning, suppression and
inhibition activity have not been clearly stated although some researchers have commonly used the term “inhibition” for suppression activity. In the current study it was found that topo-I inhibition by CMEs is a mechanisms of either topo-I poisoning or suppression activities. Both of these assays are necessary in order to differentiate between poisoning and suppression activities (Palchaudhuri & Hergenrother, 2007).

Figures 4.2, and 4.3, show poisoning activity of the CMEs indicated by a higher intensity in the nicked DNA band. Boege et al., (1996) reported similar results for poisoning activity for flavonoids where relaxed DNA had 1.8 times higher intensity than supercoiled DNA. CMEs showing high poisoning activity may have compounds that trigger cancer cell death by high amount of covalent protein–DNA intermediate (Cheng et al., 2013).

Our result also indicate that the topoisomerase inhibition activity was concentration dependent. The ME showed poisoning activity at all concentrations whereas the BE and WE had poisoning activity only at the lowest concentration of (1.25 µg/µL). The absence of DNA at higher concentrations of the water extract may be explained by the fact that the crude water extract may contain other proteinaceous (some proteins and enzymes) and non-proteinaceous (phenolic acids, vitamins, alkaloids or steroids) compounds. Some of these compound especially some enzymes, may have degraded the DNA when the WE was used at the higher concentrations (5 and 25 µg/µL) but the contrary occurred at the lowest concentration (1.25 µg/µL), hence, presence of the nicked band with higher intensity. A strong poisoning activity was observed for CE at all concentrations but these bands had a single
band with partial relaxation activity with different topoisomers. Also, the CE and WE had greater topo-I poisoning activity when compared to camptothecin. Similar results have been reported of acetone, methanol and chloroform extracts from Reynoutria japonica extract as reported by Hwangbo et al., (2012). This could suggest a high poisoning activity for these compounds than camptothecin, hence the need to exploit natural compounds extracted from plant materials for their anticancer properties. Csapi et al.,(2010) have also reported that certain compounds in chloroforms extract of Centaure arenaria had high levels chemotherapeutic activity against cervix adenocarcinoma (HeLa), breast adenocarcinoma (MCF7), and skin epidermal carcinoma (A431) cells. Active compounds identified in the chloroform extract included flavonoids such as isokaempferid, moschamine, apigenin, eupatorin, arctiin, matairesinol and arctigenin, and cnicin (Csapi et al., 2010).

RP-HPLC profile of CMEs indicated the presence of the following compounds such as sinapine, caffeoyl dihexoside, ferroyl choline guiacyl, ferroyl choline guiacyl isomer, sinapoyl dihexoside, 4’-glucosylsinapic acid, cyclic spermidin derivative, sinapoyl hexoside, sinapoyl hexoside isomer, feruloyl choline (5-8’) guaiacyl, kaempherol-sinapoyl-trihexoside, feruloyl choline (4-O-8’) guaiacyl-di-sinapoyl, disinapoyl dihexoside, trisinapoyl dihexoside, disinapoyl hexoside, methyl sinapate, and disinapoyl hexoside isomer were also identified. Major gluconsinlates were also founds in CMEs such as progitrin, sinigrin, glucalyssin, gluconapoleiferin, gluconapin, 4-hydroxyglucobrassicin, and gluconasturtin. It was analyzed that AE was best solvent for recovery of phenolic and antioxidant compounds. Glucosinolates
and their derivatives are already known for their anticancer activity (Vig, Rampal, Thind, & Arora, 2009). Glucoraphanin (4-methylsulfinylbutyl glucosinolate), sinigrin, and progoitrin, glucoalyssin and glucobrassicin is well known for its potent anticancer activity (Avila et al., 2013; Park et al., 2013). The presence of glucoraphanin extract was found in AE while 4-hydroxyglucobrassicin was found in all extracts with higher concentration in ME in canola meal. But all these glucosinolates were present in lesser quantity in CMEs, However, progoitrin in WE and glucoalyssin in ME were present in better amounts than other glucosinolates in CMEs.

The topo-I inhibition activity of kaempherol (Boege et al., 1996) and the antimutagenic activity of sinapic acid (Niciforovic & Abramovic, 2014) have already been reported. Kampa et al., (2004) have reported sinapic acid for the anticancer activity in vitro with the time and dose-dependent manner for the inhibition of cancer cells. Inhibitory effects of sinapic acid on tumorigenic colon and breast cells have also been reported by (Hudson, Dinh, Kokubun, Simmonds, & Gescher, 2000).

Cartea et al., (2010) have reported the synergistic inhibition effects of quercetin and kaempferol against cell growth in human gastrointestinal cancer lines and ethanol and water extracts from Asarum have also been reported to have positive inhibitory effects against tumor cell line (Castelli et al., 2013). These results demonstrate that sinapic acid, and kaempherol present in CMEs have potential as compounds with topoisomerase-I inhibition property. These compounds could be studied for their
topoisomerase-II inhibition which may providing great evidence for anticancer drugs (Kou et al., 2012).

The results for the suppression activity suggested the presence of different compounds in the CMEs extracts that may have topoisomerase inhibition activity and are highly dose-dependent. This was evident by the presence of different transition forms of the closed DNA from fully relaxed to fully supercoiled DNA. The dose-dependency observed in the suppression activities for the CMEs were consistent with the findings of Minderman et al., (2000). This would be relevant as the different compounds may be used at different concentration levels for the design on pharmaceuticals with anti-cancer properties, hence warranting further studies. Protein fractions (PI25, PI50, and PI80) and protease inhibitors (PI80p) have also shown strong topo-I inhibition activity at various concentration. Sequence of PI80p constitute some homology with serine protease inhibitor, cysteine protease inhibitor, papain like cysteine protease inhibitor and kunitz-Type Kallikrein inhibitor. Different Kallikrein inhibitor from other plants are known for antithrombotic properties in venous and arterial thrombosis model (Brito et al., 2014), and prostate cancer cell death (Ferreira et al., 2013).

The potency of compounds from canola meal extracts for topo-I inhibition has been demonstrated indicating their potential anticancer properties. CMEs inhibited topo-I either through suppression or poisoning or a combination of both. The present in-vitro study for topo-I inhibition of methanol, acetone and ethanol and other protein extracts including purified protease inhibitor (PI80p)
from canola meal may provide a pathway for an *in-vivo* study to investigate cytotoxicity activity.

### 4.5.2 Antidiabetics assay

The results from Figure 4.9 suggest that PIs from canola meal have potential antidiabetic activity. The PI from genotype-1 resulted in greater inhibition for DPP-IV than PI from genotype-2. The inhibition activity observed for the PIs from both genotypes may be attributed to the fact that both PIs belong to the serine family and DPP-IV is a serine protease (Chapter-3). The IC$_{50}$ value for Diprotin A in the current study was 19.4 μg/mL, which was consistent with the IC$_{50}$ value of 19.71 μg/mL reported by Yogisha & Ravisha, (2010).

There is interest in having DPP-IV inhibitors which are long lasting inhibitors and active at low concentration (Singh et al., 2011). This research on the inhibitory effects of canola PIs on DPP-IV shows that PIs have strong inhibitory activity at low concentrations. However Diprotin A has an inhibitory activity at lower concentration than PIs.

Figure 4.10 shows that the DPP-IV inhibitory activity was high in BE for both genotypes. These results indicate the presence of compounds in these extract that have potential antidiabetic activity. The HPLC profile for BE may have compound not present in other extracts (see chapter 2). Hence, it may be suggested that the observed DPP-IV inhibition may be attributed to the presence of this or other compounds found in AE, ME, EE and WE.
AE showed a higher recovery of antioxidant and phenolic compounds (chapter-2). This extract contains a wide range of phenolic compounds. Sinapic acid is known to possess antidiabetic activity in streptozotocin-induced diabetic rats (Kanchana, Shyni, Rajadurai, & Periasamy, 2011). Kaempferol are flavonoids are thought to be involved in antidiabetic activity (Yang et al., 2015a). Kaempferol glycosides have also been shown to have antidiabetic activity in high-fat-diet mice (Zang, Zhang, Igarashi, & Yu, 2015).

Likewise, sinapic acid and its derivatives are present in CMEs including other compounds such as kaempferol derivatives. Feruloyl choline (5-8') guaiacyl and its isomers have been also been found. All these compounds have high antioxidant activity (Chapter-2). This study has shown that CMEs can inhibit DPP-IV and may have potential in the treatment and management of diabetes by enhancing glucose tolerance and insulin secretion. These finding show the potential for the development of drugs that may help in the management of type-II diabetes. However, further studies are required to assess newly identified compounds to demonstrate their \textit{in-vivo} functionality.
4.5.3 Angiotensin-converting enzyme (ACE) inhibitory activity

The variation in the ACE inhibitory efficacy of the PIs from the two genotypes can be attributed to differences in the sequence of amino acids they contain. It has been reported that the presence of basic amino acids such as arginine or lysine and aromatic amino acids such as tyrosine, phenylalanine, tryptophan and proline at the C-terminal play a key role in the ACE inhibitory properties of proteins (Connolly et al., 2014; Erdmann, Cheung, & Schroder, 2008). Sequencing of the PIs from both genotypes showed variations in their amino acid composition (see chapter 3). These differences in amino acid subunits and their proportions may have accounted for the greater potency of PI from genotype-1 compared to the PI from genotype-2.

The current study is in agreement with previous studies by (Ee, Agboola, Rehman, & Zhao, 2012) which showed that PI from wattle seeds also possess ACE-inhibitory activities. Compounds from several plants including canola (Alashi et al., 2014a) broccoli (Lee, Bae, Lee, & Yang., 2006), soybean (Ademiluyi & Oboh, 2013), and sunflower (Megias et al., 2004) have been shown to have ACE-inhibitory properties. These include compounds such as protein hydrolysates, flavonoids, polyphenols, terpenes, tannins, lipids, alkaloids, xanthones, iridoids, glycosides and phenylpropanoid (Balasuriya & Rupasinghe, 2011).

The higher ACE-inhibition activity observed for the AE may be attributed to the presence of flavonoids, which have previously been shown to have a high
antioxidant activity (see chapter 2). The HPLC profiles of AE, ME and EE extract confirmed the presence of compounds such as sinapic acid, Ferroyl choline (4-0-8’) guiaeryl and kamepherol derivatives, suggesting that these compounds may have also contributed to the ACE inhibition activity of these extracts.

Furthermore, ME, EE and aqueous leaf extract of some medicinal plants including *Artocarpus altilis, Catharanthus roseus, Pongamia pinnata* and *Azadirachta indica* and other plant materials (Lacaille-Dubois et al., 2001) have been reported to have ACE inhibition activity (Siddesha, D’Souza, & Vishwanath, 2010). This was attributed to the presence of several compounds in the extracts such as luteolin, and kaempferol-3-o-β-galactopyranoside derivatives (Lacaille-Dubois et al., 2001). It was argued these polar solvents were effective for the extraction of ACE inhibitory compounds from plant materials.

In this study, all extracts displayed ACE-inhibition activity to varying degrees with AE having higher levels of inhibition. Further research is however needed in order establish those compounds that influence the ACE-inhibition activities of the CMEs. This will help to understand whether compounds act individually or synergistically in the inhibition of the ACE enzymes.

The results show that that PIs and CMEs may be beneficial as therapeutic and functional agents in the management of high blood pressure by their capacity to inhibit the ACE enzyme. Antioxidant activity and structure of PIs could have contributed for the ACE inhibition in CMEs. However further studies
are required using animal models (in vivo) so as to understand their mechanism in blood pressure regulation and their potential application and utilisation in pharmaceutical design and formulation.

4.5.4 Lipase Assay

Yang et al., (2014) analysed the inhibitory effect of different solvent extracts from walnut shells and found lipase inhibition in the ME, EE, WE, BE, with CE extracts recording the least inhibition activity. In the current study, the CE extract also showed the least PL inhibition activity which is consistent with the results of Yang et al., (2014).

Phenolic compounds are known for their role in the prevention and treatment of obesity (Yuda et al., 2012). Vuorela et al. (2005) showed that phenolic extracts from rapeseed meal inhibited lipid oxidation in cooked pork. Phenols such as ellagic acid (You, Chen, Wang, Jiang, & Lin, 2012), erulic acid, naringenin, catechin, quercetin, kaempferol (Sergent et al., 2012), gallic acid and epicatechin related derivatives (Zhu et al., 2015) are known for their lipase inhibition properties. According to Zhu et al., (2015), the lipase inhibition activity of these compound may be related to their attached catechol group. Linoleic acid in different conjugated forms is known for lipase inhibition in 3T3-L1 adipocytes (Lin, Kreeft, Schuurbiers, & Draijer, 2001).
Most of these compounds are present in the CMEs described here as confirmed by HPLC analysis. Ferroyl choline guaiacyl, sinapic acid and kaempferol derivatives were found for potent antioxidant activity in CMEs, which could have contributed to the observed lipase inhibition (see chapter 2). It is possible that the compounds present in the CMEs may have multiple modes of action and may be useful in the treatment and management of multiple diseases.

This study demonstrated that canola meal extract such as acetone, butanol and methanol extracts have lipase inhibition properties. Sinapic acid, kaempferol, and ferroyl choline guaiacyl in extracts could have been involved in antilipase activity. The compounds in the extracts could be used as dietary food or medicine in the fight against obesity. Further study is required on cellular and whole animal assays to confirm the mechanisms of action of these extracts as antiobesity agents.

**4.6 Conclusion**

In this chapter the benefits of enzyme-based assays as a way of predicting bioactivity have been demonstrated. The extracts investigated in these studies have shown a range of potential bioactivities that may provide potential health benefits to those consuming the compounds. While the use of enzyme-based assays is a convenient and cost effective way to assess the potential activities of compounds, it does not replace the assessment of these compounds in complex systems that more closely mimic a whole animal model. It is for this reason that the next chapter uses a cellular based assay to examine the
potential bioactivity of canola meal extracts, to provide a more accurate assessment of how these extracts may impact cellular systems.
CHAPTER 5. *In vitro* inhibition of adipogenesis by canola meal extract

5.1 Introduction

Obesity, an epidemic is spreading at an alarming rate worldwide (Hurt, Kulisek, Buchanan, & McClave, 2010). Adipocytes are specialized cells that store fat (triacylglycerides), secrete hormones and are insulin sensitive. It involves cellular and molecular processes underlying fat metabolism, such as adipocyte number, size and functions. Obesity is associated with a number of diseases such as diabetes, hypertension, cardiovascular disease; the hallmark of obesity is accumulation of fat through a prolonged increase in energy intake compared to output. Increases in fat cell tissue or adipocytes can be attributed to adipocyte hypertrophy (increased adipocyte size) and hyperplasia (increased adipocyte numbers). A potential anti-obesogenic approach may be to inhibit the differentiation of pre-adipocytes to mature cells, hence reducing adipocyte numbers.

In our previous study (chapter-4) canola meal extracts (CMEs) has showed properties associated with for lipase inhibition, and anti-diabetic activity. The aim of this study was to use an *in vitro* cell based assay to screen for anti-adipogenic activity in CMEs.

The study was carried out by oil Red O staining for accumulated fat droplets in differentiated C3H10T1/2 cells, and immunofluorescence staining of the PPARgamma transcription factor (PPARγ) in treated and untreated
C3H10T1/2 cells undergoing adipogenesis. Quantification of PPARγ was done for estimating the differences between the undifferentiated and differentiated CMEs treated cells using real-time polymerase chain reaction (RT-PCR).
5.2. Material and Methods

5.2.1 Material

Freeze dried extracts of canola meal (CMEs) were dissolved in a range of solvents and referred to as acetone extract (AE), methanol (ME), butanol (BE), ethanol (EE), hexane (HE) chloroform (CE) and water (WE) were used for the cellular assays.

The murine embryonic fibroblast mesenchymal stem cell line (C3H10T1/2) was purchased from the American Type Culture Collection (Rockville, MD, USA). Chemicals used were the CellTitre® aqueous non-radioactive cell proliferation assay kit, GoTaq Green 2X Master Mix purchased from Promega, Corporation (WI, USA). Aurum™ total RNA kit, and iScript™ advance cDNA synthesis kits from Bio-Rad Laboratories (Hercules, CA, USA); and RT-PCR grade water (Ambion, USA).

Cell culture media such as Dulbecco’s Modified Eagle’s medium (DMEM), Foetal Bovine Serum (FBS), 0.25% Trypsin-EDTA solution, Dexamethasone, Penicillin / Streptomycin, L-Glutamine, 3% Paraformaldehyde (PFA), and Phosphate Buffered Saline (PBS) were purchased from Sigma Chemicals (St. Louis, MO, USA).
5.2.2. Sample solubility, cell culture, viability and adipocyte differentiation

5.2.2.1 Sample solubility

The CM extracts AE, ME, BE, EE, HE, CE and WE were tested for solubility. All of the samples involved in solubility testing were suspended at a concentration of 20 mg/mL, using different diluents including DMEM, water, Phosphate buffer saline (PBS), ethanol and Dimethyl sulfoxide (DMSO). All samples were mixed using vortex and sonication in order to improve solubility of extracts. Solubility was observed visually to detect the presence of any precipitation or particulate.

5.2.2.2 Cell Culture

The C3H10T1/2 cells were cultured in T 75 tissue culture flasks (T75) in Dulbecco’s modified Eagle’s Medium (DMEM), containing 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin and 1% L-glutamine and incubated at 37°C in a humidified incubator with 5% CO2. The media in the flask was replaced after the third day. When the cells were 100% confluent, then cells were trypsinized (detached) using 0.25% Trypsin-EDTA solution for 2-5min. These cells were then resuspended in DMEM cell culture medium in a 1:10 ratio.
5.2.2.3 Cell Viability

Cell viability was assessed using the non-radioactive cell proliferation assay commonly known as the MTT assay, according to the manufacturer’s protocol, with C3H10T1/2 cells plated at 5000 cells/well in a 96 well plate treated with DMEM at the percentage of 1 to 3% and also with 0.1 to 0.3% DMSO with DMEM.

The cells were incubated in a humidified cell incubator at 37°C with 5% CO2. After completion of the reaction, microplates were taken for analysis of viability via spectrophotometry using a FLUOstar omega UV-VIS spectrophotometer (BMG Labtech, Offenburg, Germany) for measurement of absorbance at a wavelength of 570 nm.

5.2.2.4 Adipogenic differentiation

C3H10T1/2 cells were cultured in cell culture media until they approached 70% confluency for the induction of the adipogenic differentiation. Adipogenic differentiation media (ADM) comprised of DMEM including 0.5 μM rosiglitazone, 10 μM insulin, 0.25 μM indomethacin, 1 μM dexamethasone and 0.5 mM 3-Isobutyl -1-methyoxanthine (IBMX) was used. Cell culture media was substituted with the ADM to initiate adipocyte cell differentiation. ADM was replaced every 48 h, which allowed for three media replacements within the 7 day testing period before the cells were examined by oil Red O staining.
5.2.3 Oil-Red O staining and quantification of Intracellular Lipid Droplets

5.2.3.1 Oil-Red O staining
Oil Red O staining was performed according to the procedure mentioned by Singh et al., (2003). Briefly, C3H10T1/2 cells were treated with ADM containing 2 mg/mL of AE, ME, HE, CE and WE or 1.5 mg/mL of BE and EE extracts for 7 days as described earlier.

All cells were rinsed briefly with PBS, fixed with 3% PFA for 15 mins, rinsed again with PBS and air dried before staining with 0.3% oil Red O stain and incubating for 1 h at room temperature (RT). After incubation, the cells were rinsed carefully with PBS and viewed under an inverted microscope (Nikon Eclipse Ti – U inverted).

Quantification of intracellular lipid staining was performed by adding 100 μL of isopropanol to each well of 96-well plate and incubated for 10 min. After incubation, the solubilised material was removed and absorbance was measured using a BMG Labtech (Offenburg, Germany) UV microplate reader at 510 nm.

186
5.2.3.2 Immunofluorescence staining

Cells were treated with canola extract samples as detailed above (section 5.3.2.1), after which they were fixed with 3% PFA, rinsed with PBS, then treated with 0.1% Triton X-100 for 7 min at RT and rinsed again with PBS. The cells were then incubated for 30 min in blocking buffer prepared by mixing 5% goat serum (Gibco®, Eggenstein, Germany) in PBS. The blocking buffer was aspirated from the cells, and cells were then incubated with primary antibody for 1 h: PPARγ (81B8) rabbit monoclonal antibody (1:50; Cell signalling technology), cells were then washed gently with PBS three times for about 10 min. The cells were aspirated and incubated in dark with secondary antibody for 1 h: Anti-Rabbit IgG (Fab 2) – Alexa Fluor ® 488 (1:100; Cell signalling technology). The cells were again rinsed three times with PBS and incubated with 4’, 6-diamidino-2-phenylindole (DAPI) counter stain for 20 min. Cells were observed using a A1R+/A1+ confocal laser microscope system (Nikon, NY, USA).

5.2.4 RNA extraction, reverse transcription polymerase chain reaction (RT-PCR) and real time quantitative PCR (qPCR)

5.2.4.1 RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from treated and un-treated C3H10T1/2 cells using the Aurum™ total RNA kit (Bio-Rad), following the manufacturers protocol.
The concentration and quality of RNA was measured using the Nanodrop 2000’analyser (Thermo Scientific Ltd, Melbourne, Australia). The purity of the RNA samples was measured at an absorbance ratio at A260/A280 of ~1.84 was recorded.

cDNA was synthesized using the iScript Advance cDNA synthesis kit for RT-qPCR (Bio-Rad) according to the manufacturer’s protocol. The reverse transcription reaction was incubated in a thermo cycler using the amplification cycles at 25°C, 5 min; 42°C, 30 min; 85°C, 5 min, and 4°C, 5 min.

The amplification of the synthesized cDNA was performed by real time PCR at a final concentration of 300 ng/20 μL, using 12.5μL 2x GoTaq, with 0.5μL of forward and reverse primers, where 1 μM for gene specific primers of Peroxisome proliferator-activated receptor gamma (PPARγ), and a β-actin, house keeping gene was also used. All samples were prepared including positive and negative control.
The PCR primers for PPARγ1 were designed using the *Mus musculus* mRNA sequence (accession numbers: NM_011146.2) (Thyagarajan-Sahu, Lane, & Sliva, 2011). The gene specific primers were supplied by Sigma:

<table>
<thead>
<tr>
<th>Gene</th>
<th>primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARγ</td>
<td>Forward</td>
<td>TTTTCAAGGGTGCC AGTTTC</td>
<td>AATCCTTGCCCTCTGAGAT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>CACCCGCGAGTACAACCTTC</td>
<td>CCCATAACCACCACTCACACC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The PCR products sizes were 197 (PPARγ) and 207 (β-actin) base pair (bp) respectively. Amplified products were separated by electrophoresis on 2% agarose gel and visualized with ethidium bromide. The gel images were analysed and captured using the molecular imager gel doc XR+ system (Bio-Rad, NSW, Australia).

### 5.2.4.2 Real time quantitative PCR (qPCR)

For PCR amplification, a total volume of 20 μL reaction was prepared using 2X Soso fast mix (BioRad) in a transparent PCR tube as follows: 10 μL 2X Soso fast mix, 0.1 μL (at a final concentration of 0.2 μM) of Forward and Reverse primers, 1 μL of cDNA template (600 ng), with a total reaction volume of 20 μL with Dnase free water. All samples were prepared including positive and negative controls. The qPCR reactions were carried out using a ‘C1000 Thermal Cycler with Real-Time System CFX96’ (Bio-Rad). The amplification cycles for the cDNA products: were carried out with an initial 95°C, 3 min; followed by 95°C, 1 min; 59°C, 1 min; Go to step 2 of incubation and repeated 39 times.
5.2.5 Statistical analysis

Experiments were done in triplicates. Data are presented as the mean ± standard deviation (SD). All results were analyzed using Graph pad prism 5, Microsoft Excel 2013 and one way analysis of variance (ANOVA) using SAS® system for Window V8 (SAS institute, USA). Comparison between sample means were calculated using the Duncan multiple range test at a 5% probability level (p<0.05).
5.3 Result

5.3.1 Extract solubility, and measurement of cell viability

The results of the extent of solubility of lyophilized extracts of CME in AE, ME, BE, EE, HE, CE and WE are shown in Figure 5.1. The solubility of these seven extracts were tested. ME, EE, AE, BE and HE were soluble in DMSO without WE and CE. Unsolubilised material was present in suspension of WE and CE. All extracts were also soluble in DMEM, in addition to heating and more stirring excluding BE. None of the extracts were soluble in ethanol. Solubility test for all extracts are detailed in Table 5.1, which is shown by saturation concentration and homogeneity of a sample. Hence, it was found that the best way for re-suspension of the solvent extracts was in DMSO, with stirring only for all extracts excluding WE and CE which were soluble in DMEM.
Figure 5.1. Analysis of canola meal extracts (CME) solubility. This figure illustrates the solubility testing of canola meal extract such as Acetone extract using different solvents (1), DMEM (2), Water (3), DMEM Phosphate Buffer Saline (PBS) (4) Ethanol (5) DMSO.
Table 5.1. Analysis of canola meal extracts (CME) solubility.

<table>
<thead>
<tr>
<th>Canola Meal Extracts</th>
<th>Solvent solubility test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMEM</td>
</tr>
<tr>
<td>Methanol</td>
<td>**</td>
</tr>
<tr>
<td>Acetone</td>
<td>**</td>
</tr>
<tr>
<td>Ethanol</td>
<td>**</td>
</tr>
<tr>
<td>Butane</td>
<td>*</td>
</tr>
<tr>
<td>Chloroform&lt;sup&gt;a&lt;/sup&gt;</td>
<td>**</td>
</tr>
<tr>
<td>Water&lt;sup&gt;a&lt;/sup&gt;</td>
<td>**</td>
</tr>
<tr>
<td>Hexane</td>
<td>**</td>
</tr>
</tbody>
</table>

Note: Stars as “*” represent amount of solubility.

Yes, *(low, medium, high); No, –; Extracts with superscript are both soluble in water and DMEM.
The cell viability was determined by the MTT assay. The untreated cells functioned as the positive control, and were referred to as 100% viable, and used to determine the percentage viability of the cells treated with extracts or DMSO. Further it was revealed that 0.2% of DMSO in DMEM resulted in approximately for 70% viable cells (Figure 5.2).

Figure 5.3 indicates that Cells treated with all types of CMEs at a concentration of 2 mg/mL resulted in 70% viability, except for ethanol and butanol extracts, which resulted in viability at 1.5 mg/mL. Therefore 1.5 mg/mL of ethanol and butanol extracts were tested and 2 mg/mL of all other extracts was used for cell culture experiments. Results revealed that there was a marked reduction in the development of fat droplets in the CME treated C3H10T1/2 cells in a dose-dependent manner.
Figure 5.2. The effect of DMSO on C3H10T1/2 Cell Viability.

The effect of DMSO on cell viability containing 0.1, 0.15, 0.2 and 0.3% concentration. Results are expressed as mean ± standard deviation (n=3). Bars with different letters have mean values that are significantly different ($p<0.05$) at the different concentration.
Figure 5.3. The effect of canola meal extract on C3H10T1/2 cell (CME) for cell viability.
The effects of on cell viability at different concentrations (1, 1.5, 2, 2.5 and 3mg/L) of CMEs. Results are expressed as mean ± standard deviation (n=3). Bars with different letters have mean values that are significantly different (p<0.05) at the same concentration. A) 70% undifferentiated cell. B) Undifferentiated dead cell showing toxicity under 3 mg/ml of BE.
5.3.2 Oil-Red O staining staining and quantification of Intracellular Lipid Droplets

5.3.2.1 Effect of CME on intracellular lipid accumulation

To evaluate anti-adipogenic effect, the cells were treated with 1.5mg/ml of EE and BE, while 2 mg/mL was used for AE, ME, HE, CH, and WE in ADM for seven days. Finally, cells were stained with oil-red O stain and visualised by light microscopy.

Solubilised lipid was measured by absorbance of the oil-red O stain as shown in Figure 5.4 while the undifferentiated cells (Figure 5.5 i) acting as a negative control. The positive control which consisted of cells exposed to adipocyte differentiation media (Figure 5.5 ii).

The cells treated with AE showed the maximum inhibition as the accumulation of fat cells was totally diminished. Lower intracellular staining was observed in ME, BE, and HE treated cells, increasing levels of oil Red O red stain were seen in EE treated cells, whereas, higher levels of stain with larger fat droplets were observed in CE treated cells.
Figure 5.4. Quantitative analysis of Oil Red staining for the effect of adipogenic inhibition by CMEs. Differentiated mesenchymal stem (CH310T1/2) cells were quantified by Oil Red staining when measured at 510 nm. Result expressed are means ± standard deviation (n=3). Bars chart with different letters have mean significantly different ($p<0.05$) values.
Figure 5.5. Qualitative analysis for the effect of CME on adipogenic differentiation visualized at 100 µm. 

i) Undifferentiated cells-negative control; ii) Differentiated cells-positive control; Differentiated cells with CMEs iii) AE; iv) ME; v) BE; vi) EE; vii) HE; viii) CE; ix) WC
AE, showed significant inhibition ($p<0.05$) of adipocyte differentiation. The trend for inhibition of adipocyte differentiation was as follows; DMEM>AE>HE>ME>BE>WE>EE>CE. BE and HE also noticeably inhibited adipocyte differentiation. EE and CC however did not show adipogenic cell inhibition.

5.3.3 Immunofluorescence staining of $PPAR_\gamma$ in C3H10T1/2 Cells

The effects of all CMEs on $PPAR_\gamma$ staining are shown in (Figure 5.6). There was no staining seen in the undifferentiated cells (Figure 5.6; UC). While the differentiated cells exhibited clear nuclear staining when observed by fluorescence microscopy using Fluorescein isothiocyanate (FITC) as filter shown in Figure 5.6; DC. There was no nuclear staining observed for AE, and BE treated cells, and a lower level of staining was observed in HE. The potency for nuclear staining in descending order relate to staining intensity and numbers of stained cells as AE>BE>ME>HE>WE>EE>CE, which demonstrate order of $PPAR_\gamma$ down regulation.
Figure 5.6. Immunofluorescence staining of PPARγ in mesenchymal stem cells (CH310T1/2) visualized at 10 µm. CH310T1/2 cells were treated with canola meal generated extracts (CME). UN) Undifferentiated cells- negative control; (DC) Differentiated cells positive control; differentiated cells treated with CMEs: (AE) acetone extract; (ME) methanol extract; (BE) butanol extract and (EE) ethanol extract; (HE) hexane extract ;(CE); chloroform extract; and (WE) water extract. Captured images with letters only are Fluorescein isothiocyanate (FITC). Letters with 1 are 4',6-diamidino-2-phenylindole (DAPI); letter with 2 are normal view of cells and letter with 3 are merged image of FITC, DAPI and normal image respectively.
5.3.4 Effect of CMEs on the gene expression of PPARγ in C3H10T1/2

5.3.4.1 Qualitative analysis

The present study also focussed on the molecular mechanism for expression of the PPARγ gene was observed for the inhibition of adipogenesis by CMEs. To evaluate the effect of CMEs in prevent the differentiation of mesenchymal stem C3H/10T1/2 cells, the expression of PPARγ was measured by RT-PCR. As shown in Figure 5.7, mRNA expression of PPARγ was decreased in the C3H/10T1/2 cells treated with CMEs in following order AE>BE>ME>HE>WE>EE>CE. AE treated C3H/10T1/2 cells exhibited down regulation in intensity of PPARγ on the gel when compared to DC and other treated cells.

5.3.4.2 Quantitative analysis

The relative quantity of PPARγ in the CMEs was determined using quantitative gene expression by PCR (Figure 5.8). Cells were treated with cell culture media only as negative control as (UD), ADM as (DC) positive control or with ADM treated with CMEs. There was variation in the expression level among all CMEs. The expression level of PPARγ in the treated C3H/10T1/2 cells exhibited a significant decrease in the expression of PPARγ, \((p<0.05)\) compared to DCs. BE treated cells showed the lowest expression of PPARγ, followed by HE, ME, WE, EE and CE treated cells, respectively.
AE and BE has also been documented to decrease the nuclear expression (of PPARγ and also its mRNA expression (Figure 1.8). This indicates that inhibition of adipocyte differentiation works mainly through the down regulation of PPAR γ. Similar changes were described for the expression of PPAR γ by Choi et al., (2014) and Ross et al., (2000) in 3T3-L1 adipocytes using extracts from other plants and compounds. Kong et al., (2012) observed remarkable adipogenic inhibition in mouse 3T3-L1 using extract of Salicornia herbacea having isorhamnetin glucopyranosides.
Figure 5.7. The effect of CMEs detected by qualitative PCR on PPARγ and β-actin expression during adipogenic differentiation. (A) PPARγ and (B) β-actin. GeneRuler low DNA Ladder (lane 1); UD (lane 2, negative control); DC (lane 3, positive control); AE, ME, BE, EE, HE, WE and CC (4-10) and control (lane 11) respectively.
Figure 5.8. Quantitative gene expression analysis of PPARγ in C3H10T1/2 cells treated with CMEs. Results are expressed as means ± standard deviation (n=3). Bars with different letters have mean values that are significantly different (p<0.05).
5.4 Discussion

As obesity is a multifactorial disease which includes diabetes mellitus, cancer, atherosclerosis, and hypertension (Rayalam, Della-Fera, & Baile, 2008), and, development of a therapeutic approach based on natural products may be a promising solution to this worldwide problem prevailing among one billion adults (Karmase, Jagtap, & Bhutani, 2013). At the cellular level obesity is associated with hypertrophy of adipocytes and production of new adipocytes from precursor cells (Choi et al., 2014). Medicinal and other plant extracts have been shown to inhibit adipogenesis (Rayalam et al., 2008). Therefore, \textit{in vitro} Inhibition of adipogenic differentiation is one approach to discover novel phytochemicals with anti-obesogenic properties (Gaya et al., 2013; Hwang et al., 2012).

Polyphenols like flavonoids, resveratrol, quercetin, epigallocatechin-3-gallate and curcumin, reduce elevated fat storage, blood pressure, blood glucose, lipid levels, hemoglobin-A1c and insulin resistance in mammals (Cherniack, 2011) Oxidative stress induces mitochondrial increase and arrest preadipocyte proliferation. Preconditioning preadipocytes with dietary polyphenols may totally or partially protect them against mitochondrial changes, obesity-associated diabetes and cardiovascular diseases (Barrett et al., 2013). Polyphenols present in green tea (Lee, Kim, Kim, & Kim., 2009), white tea (Sohle et al., 2009), grape seeds, orange and grapefruit combat adipogenesis at the molecular level and also induce lipolysis (Dallas et al., 2014).
In this study, the effect of canola meal extracts (CMEs) solubilized in seven different solvents/buffers named as AE, ME, BE, EE, HE, CE and WE were examined (Table 5.1) for their ability to inhibit adipocyte differentiation. AE and BE have been shown previously shown to exhibit properties for anti-diabetic and anti-lipase (Chapter-4). This research has also shown that AE and BE inhibit adipogenesis in C3H10T1/2 cells through assessment of oil Red O staining and immunofluorescence staining of PPARγ image analysis and and gene expression analysis expression of PPARγ protein and mRNA expression.

DMEM and water demonstrated the same solubility properties. Phosphate buffer saline (PBS) appeared to be a better solvent for all extracts but, DMSO solubilized all the extracts except CE and WE. Therefore it was concluded that DMSO in combination with DMEM was the superior solvent in all other extracts except CE and WE. CE and WE were mixed in DMEM only as DMEM is a recommended media for the growth of cells. Though PBS has good solubility for all extracts it was not used further for experiment as it contains salts, which may affect the cell growth, and usually DMEM and DMSO are mostly used medium for cell culture. DMEM and DMSO were also preferable to use as previously studies also used them for solubility of extracts (Kang, Okla, & Chung, 2014; Wilson, Liotta, & Petricoin, 2013).
All extracts showed 70% cell viability at 2 mg/mL except BE and EE which showed 70% viability only at 1.5 mg/ml. Results also revealed that at 70% cell viability, concentration of samples were not toxic. The concentration of DMSO used by Wilson et al., (2013) was 0.1%, which is similar to the concentration used in this study according to our results which shows that DMSO lower that 0.2% does not kill the cells. Hence, anti-adipogenic effects of samples at these concentration are not due to the effect of DMSO (Karmase et al., 2013).

The effect of AE and BE decreased the intracellular stain as indicated by suppression of red globules in cell images of oil Red O stain and with green colour in cell nuclei of immunofluorescence stain. Fat globules in fat cell appear as filled with numerous tiny red granules (Figure 5.5ii). After staining with oil Red O, a clear distinction can be seen in undifferentiated (Figure 5.5i) and differentiated cells (Figure 5.5ii).

Adipogenic differentiation involves different transcription factors [peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer binding proteins (C/EBPα, C/EBPβ, and C/EBPδ)] (Rosen & MacDougald, 2006, p. 889); however, research has shown that adipocyte development is firmly dependent on the activation of the transcription factor PPARγ (Siersbaek, Nielsen, & Mandrup, 2010). PPARγ belongs to the nuclear receptor super family and plays a role in the progression of adipogenesis (Tyagi, Gupta, Saini, Kaushal, & Sharma, 2011). Expression
level of adipocyte linked genes, including PPARγ1, is representative for the process of cell’s changes toward adipogenesis. In order to find out the effect of canola meal extracts (CMEs), on the process of adipogenic differentiation, it was important to assess the level of PPARγ expression between C3H10T1/2 cells.

DAPI counter-stain enabled visualization of nuclei in cells, which makes the nuclei appear blue in cells (Figure 5.6; second image of every sample). Since PPARγ is classified as a transcription factor, therefore immunofluorescence staining of PPARγ was expected to produce localized nuclear fluorescence of green colour as in Figure 5.6 first image of every sample. Fei et al., (2011) demonstrated PPARγ detection in the nuclei of adipogenic cells as green intense nuclei, confocal fluorescence microscope.

Our previous studies showed CMEs demonstrates major antioxidant peaks associated with sinapic acid and its derivatives including other compounds such as caffeoyl dihexoside, ferroyl choline guiacyl, ferroyl cholineguaiacyl isomer, sinapoyl dihexoside, 4'-glucosylsinapic acid, sinapoyl hexoside, feruloyl choline (5-8') guaiacyl, kaempherol-sinapoyl-trihexoside, cis-sinapic acid, kaempherol 3-dihexoside-7-sinapyl-hexoside, trans-sinapic acid, disinapoyl dihexoside, trisinapoyl dihexoside 1, disinapoyl hexoside, tetrasinapoyl dihexoside, and methyl sinapate which demonstrate that these compounds could potentially contribute to antiadipogenic properties. All these compounds were present in AE at a higher concentration (Chapter 2).
In this study, AE and BE has also been documented to decrease the nuclear expression (of PPARγ and also its mRNA expression (Figure 5.8). This indicates that inhibition of adipocyte differentiation works mainly through the down regulation of PPAR γ. Similar changes were described for the expression of PPAR γ by Choi et al.(2014) and Ross et al., (2000) in 3T3-L1 adipocytes. Kong et al., (2012) observed remarkable adipogenic inhibition in mouse 3T3-L1 using extract of Salicornia herbacea having isorhamnetin glucopyranosides.

Extracts of cranberries, quercetin-rich onion peel and salicornia herbacea inhibited adipogenic differentiation by down regulating the expression of PPARγ (Kong et al., 2012; Kowalska et al., 2014; Moon, Do, Kim, & Shin, 2013), similarly dramatic reduction for the expression of the PPARγ was achieved in using CMEs.

AE showed significant results for the inhibition of adipogenic cells, because it may be rich in phenolic compounds and their antioxidant activity as demonstrated earlier in chapter-2. EE and CE were not as effective for inhibition as compared to other extracts. CE cell image appeared quite big in size represent fat accumulation as in Figure 5.5H. This could be due to lack of phenolic compounds or presence of other phytochemicals in the extract promoting fat cell accumulation in mature cells only.

In this study BE also contributed to inhibition of adipogenic differentiation at 1.5mg/ml. Previous literature also demonstrated that BE from Walnut shell (Juglans regia L.) (Yang et al., 2014) and Salicornia herbacea (Kong et al.,
2012) inhibited anti-adipogenic activity. Choi et al., (2014) reported BE extract derived alkaloid from Coptis chinensis for antiadipenic inhibitory effects. Kim et al., (2010) used ethanol extract from Spirodea polyrhiza (weed) from the isolated butanol soluble fraction and found potent inhibition of adipogenesis in 3T3 cells at 200µg/ml.

Phytochemicals such as non methoxylated flavonoids with one or two OH groups in the side chain have also been reported to be associated with inhibition of adipogenesis (Bai et al., 2008). Singh & Kakkar (2014), documented antiadipogenic activity of flavonoids including kaempferol. Other flavonoid compounds such as quercetin, catechin have also been reported for inhibitory properties associated with for adipogenesis (Chien, Chen, Lu, & Sheu, 2005). Most of the compounds in CMEs with antioxidant properties are non methoxylated with one or two OH groups in side chain. The antiadipogenic activity in AE and BE could be due to the presence of flavonoid or antioxidant compounds in CMEs. All extracts were observed daily and there was not a single cell image in oil Red O and immunofluorescence staining showing mature adipocyte cells for AE or BE treatment.

However, WE was not associated with adipogenesis inhibition compared with AE, but appeared as an effective extract. This could be due to the presence of enzymes, phenolic compounds or glucosinolates, which do not have a role in the inhibition of fat cells. HPLC profile of WE has shown the presence of glucosinolates such as progitrin, sinigrin, glucalyssin, gluconapoleiferin, gluconapin, 4-hydroxyglucobrassicin, and gluconasturtin (Chapter 2).
Previously in chapter 4, we demonstrated lipase inhibition, and diabetic inhibition associated with CMEs, where AE, ME and BE had been documented to show properties with biological health beneficial molecules. From these results it is clear that the same compounds in these extracts are active in inhibition of lipid accumulation in adipocytes.

Hence, further preparative purification of individual compounds is required to study their individual or synergistic actions as regards adipogenic inhibition. This research will be needed to explore further the effects of canola meal extracts for in-vivo studies and potential clinical trials.

5.5 Conclusion

The results obtained in this present study demonstrate that AE has potent anti-adipogenic activity. BE also contributes to the inhibition of cell differentiation but is not as effective as AE. There was also marked reduction in mRNA expression of PPARγ as a result of treatment with AE and BE. Other extracts did not significantly inhibit adipogenesis, such as EE and CE. These result clearly demonstrate that AE and BE may contain unique potent therapeutic compounds. CE were worst of all extracts. These result clearly demonstrate that AE and BE has some potent therapeutic compounds which may not be present in other extracts.
CHAPTER 6. General Discussion

The processing of oil from canola (Brassica napus L.) seed has increased dramatically in volume in the past few decades. Increased canola production is leading to an excess of the meal by-product. Canola meal is a relatively low value product compared to oil. This meal is usually incorporated into animal diets but at low inclusion rates; its potential at high inclusion rates is limited by the presence of various anti-nutritional compounds. However, for human and animal use, there may be beneficial components present in the meal, which may have therapeutic aspects, and which could increase the value of the meal if they could be extracted economically and sold as food additives or pharmaceuticals.

The present study was undertaken to characterise the phenolic compounds (PC) and protease inhibitors (PIs) from canola meal extracts (CMEs) and assess their potential bioactivities. Both qualitative and quantitative analyses were employed for the determination of PI activities and total phenolic contents (TPC). Furthermore, the bioactive properties of the CMEs were studied, focusing principally on antioxidant and chemopreventive (topoisomerase inhibition) properties, as well as antihypertension, antidiabetic and antiplase activities. In addition, an in vitro system was used to assess antiadipogenic activity employing C3H10T1/2 stem cells.

The current study commenced by generating a range of canola meal extracts (CMEs) using different solvents for two canola genotypes. All the canola meal extracts (CMEs) were named according to the solvent used for extraction from the meal: water extract (WE), methanol extract (ME), ethanol
extract (EE), acetone extract (AE), butanol extract (BE), chloroform extract (CE) and hexane extract (HE). All CMEs were freeze dried after extraction and preserved for further testing. The yield of CMEs was in increasing order of their polarity WE>ME>EE>AE>BE>CE>HE. Our results demonstrated that mixing of meal in solvents by stirring using a rotary shaker and freeze drying of these extracts increased the amount of phytochemicals recovered. Furthermore, it also reduced the chance of having false positive results from solvent and extracts.

Characterisation and quantification of phenolic and antioxidant compounds was done for all extracts. The highest amount of phenolic and antioxidant activity was estimated in AE, followed by ME and EE, as this solvent was able to recover higher amount of compounds than any other solvents. When comparing genotype-1 with 2, genotype-2 had a higher antioxidant activity in AE. A major peak of sinapic acid was present in all extracts excluding WE and CE. There were other known and unknown compounds present in other extract such as CE with antioxidant activity which need further investigation.

This study also indicated the presence of seven major glucosinolates in CMEs where, WE was demonstrated to be the best extract for the recovery of glucosinolates. Glucosinolates did not have any antioxidant activity. Higher phenolic and glucosinolates amount were observed in genotype-2 rather than genotype-1.

Protease Inhibitors (PIs) are in high demand in the biotechnology and pharmaceutical industries. This study reports the purification and
characterisation of PIs from canola meal. The results confirm the presence of PI using chromatography and gel electrophoresis methods which revealed three different PIs in both genotypes, with different electrophoretic motilities. PAGE under reducing and non-reducing conditions revealed the presence of PI subunits with molecular weights of 13, 16 and 30 KDa for genotype-1, while genotype-2 exhibited subunits of 7, 15.5 and 19 KDa. Amicon ultra-15 filters appeared to be superior for the quick recovery of PIs. N-terminal amino acid sequencing of PIs from genotype-1 revealed they were not identical to previously reported PIs but they had some sequence homology to Kunitz-type serine and cysteine PIs. N-terminal sequencing showed that a protease inhibitor from genotype-1 was a previously unreported trypsin inhibitor. However it had some amino acid sequences homology with sequences from other species with antibacterial activities and the formyl peptide receptor-like 1 (FPRL1) inhibitory protein.

PIs from genotype-2 resemble previously identified PIs from the Brassicaceae family. It also appeared that all PIs from genotype-2 belong to serine protease inhibitors (Defence Proteins). Furthermore, some amino acid residues of this PI were similar in sequence to proteins from other species with antiviral properties as well as the ability to inhibit leukaemia development and plasminogen activator. These PIs should be further explored and used to assess their potential therapeutic applications.

Chronic diseases such as metabolomic syndrome, cancer, hypertension, diabetes, and obesity are becoming more widespread due to poor diet and lifestyle choices. Plant derived phytochemicals such as phenols with
Antioxidant properties have gained interest due to their potential role in the prevention of lifestyle related diseases. CMEs have shown remarkable antioxidant activities in the current study. Therefore, CMEs need to be further investigated using in vivo studies to determine their potential to treat these lifestyle related diseases.

This study indicates the presence of compounds in CMEs which act as topoisomerase inhibitors. Topoisomerase inhibitors are cytotoxic agents commonly known for their anticancer therapies. AE, EE were demonstrated to contain potent topoisomerase inhibitors having both poisoning and suppression activity. WE, at lower concentrations, also showed topoisomerase poisoning activity. Different protein fractions including PI also showed topoisomerase inhibition activity. The results suggest that canola meal may contain compounds that could potentially be used in cancer therapy. A thorough analysis is needed to identify the compounds responsible for this activity, so they can be assessed initially in cell culture based assays, followed by animal models to determine their anticancer potential.

Many studies have demonstrated that diabetes and hypertension are frequently linked, which leads to an additional risk of developing life-threatening cardiovascular diseases (Schutta, 2007). Inhibitors of the enzyme dipeptidyl peptidase-IV (DPP-IV) enzyme decreases blood glucose by the production of insulin and inhibition of glucagon. Angiotensin-converting enzyme (ACE) inhibitors are commonly used for lowering hypertension. The present study showed that PIs and BE contain promising therapeutic agents for the inhibition of DPP-IV enzyme, ACE inhibition AE, ME and PIs are
involved. Further work is needed to characterise the compounds responsible for these activities and assess their potential therapeutic applications.

Lipid metabolism disorders, linked to imbalances in fatty acid and cholesterol levels in the blood, are causes of obesity, diabetes, and cardiovascular diseases (Nadler & Attie, 2001). Pancreatic lipase is an enzyme which plays a key role in the breakdown of triglycerides into monoglyceride and fatty acids (Birari & Bhutani, 2007). Therefore, inhibition of fat digestion is a logical target for achieving a reduction of fat absorption. Lipase inhibition was observed in this study with AE, ME and BE. Furthermore, CMEs were examined for their effects on adipogenic differentiation in murine mesenchymal stem cells (C3H10T1/2) using various cellular functions. According to our knowledge, this is the first study to demonstrate that CMEs can inhibit adipogenic tissue differentiation. The present findings suggest that CMEs have significant \((p>0.05)\) inhibition of adipocyte differentiation without cell toxicity, demonstrating a remarkable reduction in the expression of the expression of PPAR\(\gamma\) gene with AE, ME and BE. However AE was most effective for the inhibition for adipogenesis.

Canola meal derived PIs and crude extracts could be a useful in the formulation of anticancer, antidiabetic, hypotensive and antiobesity drugs. This study confirms that CMEs contain compounds could be a source of potential new bioactive compounds that could be used for the treatment of life style disease.
Future studies require screening of identified compounds from canola meal extract that could contribute to future novel functional food products and medical-related research for the benefit of human health. Sinapine and sinapic acid appeared as major compound in CMEs. Sinapine is reported with antimicrobial, antioxidant, anticancer and anti-inflammatory properties. It is also known as acetylcholinesterase inhibitor (Niciforovic & Abramovic, 2014). These properties suggest that CMEs may have high value in pharmaceutical industry.

Unknown compounds need to be characterised using technique such as NMR, as some of these demonstrated high antioxidant activity suggesting they may have potentially useful bioactivities. This study focused on the identification of phenol, glucosinolates and protease inhibitors from canola meal. However, there could be some other bioactive compounds such as carotenoids, vitamins, anthocyanins, anthraquinone, carbohydrates, steroids, terpenoids, fatty acids, enzymes, proteins (not known yet) that are still to be identified. The full sequence of the protease inhibitor in genotype-1 purified in this study needs to be determined to understand its full potential as a functional compound, and to establish whether or not it is unique, and whether it has been identified in other plant species. Complete sequence of amino acid will allow possible structural genes to be identified. As protease inhibitors can be used for the treatment of antiviral disease such as hepatitis C virus (HCV) and human immunodeficiency virus (HIV) these protease inhibitors should be explored for their ability to treat a range of viral diseases. The next logical step in this research is to move to more complex models including cellular, mice and human systems which will ultimately demonstrate the value of the bioactive compounds discussed in this study.
REFERENCES


220


Cheng, B., Cao, S., Vasquez, V., Annamalai, T., Tamayo-Castro, G., Clardy, J., & Tse-Dinh, Y.-C. (2013). Identification of anziaic acid, a lichen depside


Gaya, M., Repetto, V., Toneatto, J., Anesini, C., Piwien-Pilipuk, G., & Moreno, S. (2013). Antiadipogenic effect of carnosic acid, a natural compound present in *Rosmarinus officinalis*, is exerted through the C/EBPs and PPARγ pathways at the onset of the differentiation program. *Biochimica et Biophysica Acta, 1830*(6), 3796-3806.


their introduction from Antiretroviral drugs to antifungal, antibacterial and antitumor agents based on aspartic protease inhibitors. *Current Medicinal Chemistry, 14*(26), 2734-2748. doi: 10.2174/092986707782360141


Wiesner, M., Schreiner, M., & Glatt, H. (2014). High mutagenic activity of juice from pak choi (Brassica rapa ssp. chinensis) sprouts due to its content of 1-methoxy-3-indolylmethyl glucosinolate, and its enhancement by
elicitation with methyl jasmonate. Food and Chemical Toxicology, 67, 10-16.


APPENDIX 1. Antioxidant properties by FRAP activity showing linear regression line for Trolox dilution with concentration (Con) in µM/ml
APPENDIX 2. Phenolic and antioxidant activity profile in AE and WE (A) RP-HPLC (absorbance at 280 nm), (a) PCD (absorbance at 414 nm).
APPENDIX 3. Canola meal extracts representing topoisomerase Inhibition activity

<table>
<thead>
<tr>
<th>Topoisomerase Inhibition</th>
<th>Concentration (µg/µL)</th>
<th>Canola meal extracts (CMEs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WE</td>
</tr>
<tr>
<td>Poisoning</td>
<td>1.25</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Suppression</td>
<td>1.25</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>-</td>
</tr>
</tbody>
</table>

Organic solvents represent different extract prepared from 80% solvents with CM
PI represents protein fractions prepared by ammonium sulphate precipitation (AS) of 25, 50 and 80 percentages from canola meal
Note: Yes, +; No, -