

**Comparative antidiabetic effects and
mechanisms of actions of five Chinese and
South African indigenous teas**



**UNIVERSITY OF TM
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**INYUVESI
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By

Xin Xiao

Student number: 217082112

December 2020

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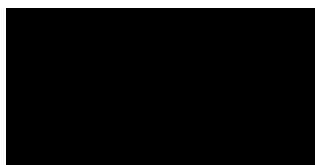
**Submitted in fulfilment of the academic requirements for the degree of
Doctor of Philosophy in Biochemistry, School of Life Sciences, University of
KwaZulu-Natal (Westville campus), Durban 4000, South Africa**

Supervisor: Professor MS Islam, PhD

PREFACE

The information presented in this thesis represents original work by the candidate. It was carried out in the Department of Biochemistry, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, Durban, South Africa from February 2018 to December 2020 under the supervision of Prof. MS Islam and has not otherwise been submitted in any form for any degree or diploma to any other University. Where use has been made of the work of others, it has been duly acknowledged in the text.

Candidate: Xin Xiao



Supervisor: Prof. MS Islam

DECLARATION 1 - PLAGIARISM

I, **XIN XIAO**-----, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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5. This thesis does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the thesis and in the references section.

Signature:..........

Date:.....10/12/2020.....

DECLARATION 2 - PUBLICATIONS AND PRESENTATIONS

Details of contribution to publications that make up part of the research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication).

In all the publications included in this thesis, I (with guidance from my supervisor) designed the work, performed all the experiments and wrote all the publications. The co-authors contributed by conducting an editorial work, checking the scientific content of the work and my correct interpretation of the findings.

Published/accepted

1. **Xin Xiao**, Ochuko L. Erukainure, Olakunle Sanni, Neil A. Koorbanally, Md. Shahidul Islam (2020) Phytochemical properties of Black tea (*Camellia sinensis*) and Rooibos tea (*Aspalathus linearis*); and their modulatory effects on key hyperglycaemic processes and oxidative stress. **Journal of Food Sciences and Technology**. 57, 4345-4354(2020), <https://doi.org/10.1007/s13197-020-04471-w>
2. **Xin Xiao**, Ochuko L. Erukainure, Brian Beseni, Neil A. Koorbanally, Md. Shahidul Islam (2020) Sequential Extracts of Red Honeybush (*Cyclopia genistoides*) Tea: Chemical Characterization, Antioxidant Potentials and Anti-hyperglycemic Activities. **Journal of Food Biochemistry**, <https://doi.org/10.1111/jfbc.13478>.

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3. **Xin Xiao**, Ochuko L. Erukainure, Nontokozi Zimbili Msomi, Neil A. Koorbanally, Md. Shahidul Islam (2020) Chemical characterization of sequential extracts of Green Rooibos (*Aspalathus linearis*) Tea, their antioxidative and antihyperglycemic potentials. **(In preparation)**
4. **Xin Xiao**, Ochuko L. Erukainure, Brian Beseni, Neil A. Koorbanally, Md. Shahidul Islam (2020) Chemical characterization of sequential extracts of Jasmine green tea (*Camellia sinensis*), their antioxidant activities and modulatory effects on Fe²⁺ induced hepatic injury ex vivo. **(In preparation)**
5. **Xin Xiao**, Ochuko L. Erukainure, Nontokozi Zimbili Msomi, Neil A. Koorbanally, Md. Shahidul Islam (2020) Phytochemicals content, antioxidative and antidiabetic activities of Jasmine green tea (*Camellia sinensis*) and Green rooibos tea (*Aspalathus linearis*): A

comparative study. **(In preparation)**

6. **Xin Xiao**, Ochuko L. Erukainure, Md. Shahidul Islam (2020) The *in vivo* antioxidant and antidiabetic activity of Jasmine green tea (*Camellia sinensis*) in fructose-streptozotocin induced type 2 diabetic model of Sprague-Dawley rats. **(In preparation)**
7. **Xin Xiao**, Ochuko L. Erukainure, Md. Shahidul Islam (2020) The antidiabetic effect of concentrated hot water-infusion of Green rooibos tea (*Aspalathus linearis*) in fructose-streptozotocin induced type 2 diabetic rats. **(In preparation)**
8. **Xin Xiao**, Ochuko L. Erukainure, Md. Shahidul Islam (2020) The *in vivo* antioxidant and antidiabetic activity of Red Honeybush (*Cyclopia genistoides*) Tea in fructose-streptozotocin induced type 2 diabetic model of rats. **(In preparation)**

PRESENTATIONS AT CONFERENCE

1. **Xin Xiao**, Ochuko L. Erukainure, Olakunle Sanni, Neil A. Koorbanally, Md. Shahidul Islam, Antioxidative and Antidiabetic Potentials of Black (*Camellia sinensis*) and Rooibos (*Aspalathus linearis*) tea: A Comparative Study, **College of Agriculture, Engineering, and Postgraduate Research and Innovation Symposium**, 17th October 2019, University of KwaZulu-Natal, Durban, South Africa.
2. **Xin Xiao**, Ochuko L. Erukainure, Olakunle Sanni, Neil A. Koorbanally, Md. Shahidul Islam, Antioxidative and Antidiabetic Potentials of Black (*Camellia sinensis*) and Rooibos (*Aspalathus linearis*) tea: A Comparative Study, **School of Life Sciences Research Day and Exhibition**, 29th April 2019, University of KwaZulu-Natal, Durban, South Africa.

DEDICATION

To My Family

To My Country

To the World

CONTENTS

ACKNOWLEDGEMENTS	i
ABSTRACT	ii
LIST OF TABLES	iii
LIST OF FIGURES	iv
1.0 CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW	1
1.1 Introduction	1
1.2 Prevalence of diabetes mellitus	1
1.3 Types of diabetes	4
1.4 Oxidative stress	12
1.5 Oxidative stress and type 2 diabetes (T2D)	15
1.6 Complications of diabetes	19
1.7 Management of T2D	23
1.8 Chinese teas and South African teas in the treatment and management of T2D	27
1.9 Rationale of the study	35
1.10 Scope and limitations of the study	35
1.11 Aim and objectives of the study	35
2.0 CHAPTER 2: MATERIALS AND METHODS	37
2.1 Experimental design	37
2.2 Collection of teas	37
2.2.1 Preparation of crude extracts	38
2.2.2 Preparation of hot infusions	38
2.2.3. Preparation of stock solutions	38
2.2.4. Total phenolic content	39
2.3 <i>In vitro</i> anti-oxidative activities assay	39
2.3.1 Free radical scavenging activity (DPPH) assay	39
2.3.2 Ferric cyanide (Fe ³⁺) reducing/antioxidant power (FRAP) assay	40
2.3.3 Nitric oxide (NO) radical scavenging assay	40
2.4 <i>In vitro</i> antidiabetic and antiobesogenic enzymes inhibitory assays	41
2.4.1 Determination of α -glucosidase inhibitory activity of tea extracts or infusions	41
2.4.2 Determination of α -amylase inhibitory activity of the tea extracts or infusions	42

2.4.3 Estimation of pancreatic lipase inhibitory activity.....	42
2.5 <i>Ex vivo</i> antioxidant assays	43
2.5.1 Animal.....	43
2.5.2 Preparation of tissue homogenates	43
2.5.3 Induction of <i>ex vivo</i> oxidative stress and treatment	43
2.5.4 Reduced glutathione (GSH) assay.....	44
2.5.5 Superoxide dismutase (SOD) enzyme activity.....	44
2.5.6 Catalase activity	45
2.5.7 Determination of lipid peroxidation.....	46
2.5.8 Determination of glutathione reductase (GR) activity	46
2.6 <i>Ex vivo</i> antidiabetic activity assay	47
2.6.1 Determination of glucose absorption in isolated rat small intestine	47
2.6.2 Determination of glucose uptake in harvested rat psoas muscle.....	48
2.7 Identification of phytochemicals in tea sequential extracts and infusions.....	49
2.7.1 Gas chromatography-mass spectrometric (GC-MS) analysis	49
2.7.2 Liquid Chromatography-Mass Spectrometric (LC-MS) analysis	50
2.8 <i>In vivo</i> antidiabetic activity of the tea hot water extract in a type 2 diabetes rats model ..	50
2.8.1 Animals	50
2.8.2 Induction of diabetes	52
2.8.3 Feeding and sampling.....	53
2.8.4 Determination of food and fluid intake, body weight (BW) and sampling.....	53
2.8.5 Oral glucose tolerance test (OGTT)	53
2.8.6 Estimation of final fasting blood glucose and insulin and calculation of HOMA scores	53
2.8.7 Serum lipid profiles.....	54
2.8.8 Hepatic glycogen content.....	54
2.8.9 Histopathological analysis of pancreatic tissue.....	54
2.8.10 <i>In vivo</i> antioxidant studies.....	55

2.9 Statistical analysis.....	55
3.0 CHAPTER 3: COMPARATIVE STUDY OF THE ANTIOXIDATIVE AND ANTIDIABETIC ACTIVITIES <i>IN VITRO</i>	56
3.1 Phytochemical properties of black tea (<i>Camellia sinensis</i>) and rooibos tea (<i>Aspalathus linearis</i>), and their modulatory effects on key hyperglycaemic processes and oxidative stress	56
3.1.1 Abstract	56
3.1.2 Introduction	57
3.1.3 Materials and methods	58
3.1.4 Results	58
3.1.5 Discussion	64
3.1.6 Conclusion.....	66
3.2 Antioxidative and antidiabetic activities of jasmine green tea (<i>Camellia sinensis</i>) and green rooibos tea (<i>Aspalathus linearis</i>): A comparative study	67
3.2.1 Abstract	67
3.2.2 Introduction	68
3.2.3 Materials and methods	69
3.2.4 Results	69
3.2.5 Discussion	78
3.2.6 Conclusion.....	81
4.0 CHAPTER 4: THE ANTIOXIDANT AND ANTIDIABETIC ACTIVITIES OF RED HONEYBUSH TEA <i>IN VITRO</i> AND <i>IN VIVO</i>	82
4.1 Background.....	82
4.1.1 Ethnomedical uses.....	82
4.1.2 Biological uses	83
4.1.3 Phytochemistry.....	83
4.2 Sequential extracts of red honeybush (<i>Cyclopia genistoides</i>) tea: chemical characterization, antioxidant potentials and anti-hyperglycaemia activities	84
4.2.1 Abstract	84
4.2.2 Introduction	85

4.2.3 Materials and methods	86
4.2.4 Results	86
4.2.5 Discussion	97
4.2.6 Conclusion.....	101
4.3 The <i>in vivo</i> antioxidant and antidiabetic effect of red honeybush (<i>Cyclopia genistoides</i>) tea hot water extract in fructose-streptozotocin induced type 2 diabetic model of rats	102
4.3.1 Abstract	102
4.3.2 Introduction	103
4.3.3 Materials and methods	105
4.3.4 Results	105
4.3.5 Discussion	118
4.3.6 Conclusion.....	124
5.0 CHAPTER 5: THE ANTIOXIDANT AND ANTIDIABETIC ACTIVITIES OF JASMINE GREEN TEA <i>IN VITRO</i> AND <i>IN VIVO</i>	125
5.1 Background	125
5.1.1 Biological uses	126
5.1.2 Phytochemistry.....	126
5.2 Chemical characterization of sequential extracts of jasmine green tea, their antioxidant activities and modulatory effects on Fe ²⁺ induced rat hepatic injury <i>ex vivo</i>	127
5.2.1 Abstract	127
5.2.2 Introduction	128
5.2.3 Materials and methods	129
5.2.4 Results	129
5.2.5 Discussion	136
5.2.6 Conclusions	139
5.3 The <i>in vivo</i> antioxidant and antidiabetic activity of jasmine green tea (<i>Camellia sinensis</i>) extract in fructose-streptozotocin induced type 2 diabetic rats.....	140
5.3.1 Abstract	140
5.3.2 Introduction	141

5.3.3 Materials and methods	142
5.3.4 Results	143
5.3.5 Discussion	155
5.3.6 Conclusion.....	160
6.0 CHAPTER 6: THE ANTIOXIDANT AND ANTIDIABETIC ACTIVITIES OF GREEN ROOIBOS TEA <i>IN VITRO</i> AND <i>IN VIVO</i>	161
6.1 Background.....	161
6.1.1 Ethnomedical uses.....	162
6.1.2 Biological activities.....	162
6.1.3 Phytochemistry.....	162
6.2 Chemical characterization of sequential extracts of green rooibos (<i>Aspalathus linearis</i>) tea, their antioxidative and antihyperglycemic potentials	163
6.2.1 Abstract	163
6.2.2 Introduction	164
6.2.3 Materials and methods	165
6.2.4 Results	165
6.2.5 Discussion	173
6.2.6 Conclusion.....	174
6.3 The <i>in vivo</i> antioxidant and ameliorative ability of green rooibos (<i>Aspalathus linearis</i>) tea hot water extract in fructose-streptozotocin induced type 2 diabetic rats.....	176
6.3.1 Abstract	176
6.3.2 Introduction	177
6.3.3 Materials and methods	179
6.3.4 Results	179
6.3.5 Discussion	192
6.3.6 Conclusion.....	196
7.0 CHAPTER 7: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS	197
7.1 General discussion	197

7.2 General conclusion.....	200
7.3 Recommendation	202
REFERENCES.....	203
APPENDIX A ANIMAL ETHICS APPROVAL LETTER.....	255
APPENDIX B PUBLICATIONS FROM THIS THESIS.....	256

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ABSTRACT

The present thesis assessed the *in vitro*, *ex vivo* and *in vivo* anti-oxidative and antidiabetic activities of five teas which are widely consumed in China or South Africa. Three of the selected five teas are from South Africa, namely red rooibos (*Aspalathus linearis*), green rooibos (*Aspalathus linearis*) and red honeybush (*Cyclopia genistoides*) tea. The remaining two from China are jasmine green (*Camellia sinensis*) and zhengshanxiaozhong (ZSXZ) black tea (*Camellia sinensis*). The different sequential solvent extracts following increasing polarity index (dichloromethane, ethyl acetate, ethanol, and water) and hot water extract of different teas were evaluated at *in vitro* and *ex vivo* conditions for their antioxidant properties, inhibitory potentials on α -glucosidase, α -amylase and pancreatic lipase, effects on ameliorating Fe²⁺-induced oxidative pancreatic or hepatic injury, as well as the glucose absorption inhibition in small intestine and the glucose uptake stimulation in isolated psoas muscle of rats. Possible bioactive components responsible for the activities of the extracts were identified by using Gas Chromatography-Mass Spectrometry (GC-MS) analysis or liquid chromatograph-mass spectrometry (LC-MS) analysis. *In vitro* and *ex vivo* tests presented promising antioxidant and antidiabetic activities of these five teas. The red honeybush, jasmine green and green rooibos teas, were further subjected to an *in vivo* intervention trial in a fructose-streptozotocin (STZ) induced T2D model of Sprague-Dawley rats. Assays were carried out to reveal the effects of these teas on lowering blood glucose level, improving oral glucose tolerance ability, stimulating insulin secretion and hepatic glycogen synthesis and ameliorating some diabetes related parameters such as serum lipid profile, hepatic and renal function tests and calculated insulin resistance (HOMA-IR), β -cell function (HOMA- β) from the blood glucose and serum insulin data. Furthermore, *in vivo* oxidative stress markers such as reduced glutathione, superoxide dismutase and catalase activity and lipid peroxidation were analysed in harvested organs (liver, kidney, heart and pancreas). The results of *in vivo* tests demonstrated that high dose of jasmine green tea showing the best activity followed by the high dose of red honeybush tea, low dose of jasmine green tea, high dose of green rooibos tea, low dose of red honeybush tea, when lowest activity was observed for the low dose green rooibos tea. The results of this study indicated promising anti-T2D properties of the above-mentioned teas. However, further clinical trials are needed to ascertain the results of these *in vitro*, *ex vivo* and *in vivo* studies.

LIST OF TABLES

Table 1.1:	Top 10 countries for number of diabetics aged 20-79 years in 2019, 2030 and 2045	4
Table 1.2:	Target tissues and mechanisms of some tea bioactive compounds on T2D	29
Table 1.3:	Summary of some major phytochemicals of <i>Camellia sinensis</i> tea, rooibos and honeybush herbal teas	31
Table 1.4:	Summary of some studies indicates the potential antioxidant and antidiabetic activity of rooibos and/or rooibos polyphenols in <i>ex vivo</i> , <i>in vivo</i> experimental model	31
Table 1.5:	Summary of some studies indicates the potential antioxidant and antidiabetic activity of honeybush and/or honeybush polyphenols in <i>ex vivo</i> , <i>in vivo</i> experimental model.	32
Table 3.1.1:	IC ₅₀ values of <i>C. sinensis</i> and <i>A. linearis</i> activities	59
Table 3.1.2:	LC-MS identified compounds in (A) <i>C. sinensis</i> and (B) <i>A. linearis</i>	64
Table 3.2.1:	IC ₅₀ , GA ₅₀ and GU ₅₀ values of jasmine green tea and green rooibos tea (<i>Aspalathus linearis</i>) activities	73
Table 3.2.2:	LC-MS identified compounds in (A) jasmine green tea (JGT) and (B) green rooibos tea (GRT)	78
Table 4.2.1:	IC ₅₀ values of different extracts of red honeybush (<i>Cyclopia genistoides</i>) tea	89
Table 4.2.2:	Compounds identified in different extracts of red honeybush (<i>Cyclopia genistoides</i>) tea by GC-MS	95
Table 4.3.1:	Liver weight, relative liver weight and liver glycogen levels in the different animal groups at the end of the intervention period	112
Table 4.3.2:	Serum biochemical parameters in different animal groups at the end of the experimental period	113
Table 4.3.3:	Serum insulin and fructosamine concentrations as well as computed HOMA-IR and HOMA-β scores in different animal groups at the end of the experimental period	114
Table 5.2.1:	IC ₅₀ values of different extracts of jasmine green tea activities	133
Table 5.2.2:	Compounds identified in DCM, ethyl acetate, ethanol extract of jasmine green tea by GC-MS	135

Table 5.3.1:	Liver weight, relative liver weight and liver glycogen level in the different animal groups at the end of the intervention period	150
Table 5.3.2:	Serum biochemical parameters in different animal groups at the end of the experimental period	150
Table 5.3.3:	Serum insulin and fructosamine concentrations as well as computed HOMA-IR and HOMA- β scores in different animal groups at the end of the experimental period	151
Table 6.2.1:	IC ₅₀ values of green rooibos tea (<i>Aspalathus linearis</i>) activities	166
Table 6.2.2:	Compounds identified in dichloromethane, ethyl acetate, ethanol extract of green rooibos tea (<i>Aspalathus linearis</i>) tea by GC-MS	170
Table 6.3.1:	The effects of different doses of GRT on liver weight, relative liver weight and liver glycogen levels in the different animal groups at the end of the intervention period	186
Table 6.3.2:	Serum biochemical parameters in different animal groups at the end of the experimental period	186
Table 6.3.3:	Serum insulin and fructosamine concentrations as well as computed HOMA-IR and HOMA- β scores in different animal groups at the end of the experimental period	187
Table 7.1:	Scoring value of jasmine green tea, green rooibos tea, and red honeybush tea for different diabetes-related parameters during the intervention period	217

LIST OF FIGURES

Figure 1.1:	Summary of estimated of people with diabetes worldwide and per region in 2019 and 2045 (20-79 years)	3
Figure 1.2:	Global diabetes distribution of estimated number of adults (20-79 years) with diabetes in 2019	3
Figure 1.3:	Possible pathogenesis of T1D	6
Figure 1.4:	Multiple risk factors are involved in the pathogenesis of T2D	7
Figure 1.5:	Pathogenesis of T2D	11
Figure 1.6:	Risk factors and pathogenic basis of gestational diabetes mellitus	12
Figure 1.7:	The sources and cellular response to ROS	15
Figure 1.8:	The polyol pathway	16
Figure 1.9:	The activation and synthesis of protein kinase C (PKC) isoforms	18
Figure 1.10:	Activation of hexosamine pathways	19
Figure 1.11:	Antidiabetic agents treatment of hyperglycaemia according to site of action	24
Figure 2.1:	Flowchart demonstrating the entire experimental approach	37
Figure 2.2:	Flowchart of the sequential extraction of tea samples	38
Figure 2.3:	Reaction mechanism of 1,1-diphenyl-1-picrylhydrazyl (DPPH) with antioxidant	39
Figure 2.4:	The oxidation of GSH to GSSG by DTNB	44
Figure 2.5:	The formation of MDA-TBA ₂	46
Figure 2.6:	Flow diagram describing the procedure of isolated rat small intestine glucose absorption and harvested rat psoas muscle glucose uptake	49
Figure 2.7:	Flow diagram of comprehensive <i>in vivo</i> animal treatment procedure	52
Figure 3.1.1:	Total phenolic contents of <i>C. sinensis</i> and <i>A. linearis</i>	59
Figure 3.1.2:	(A) DPPH scavenging activities and (B) Ferric reducing antioxidant power (FRAP) activities of <i>C. sinensis</i> and <i>A. linearis</i>	60
Figure 3.1.3:	Inhibitory effect of <i>C. sinensis</i> and <i>A. linearis</i> on α -amylase activity	61
Figure 3.1.4:	Effect of <i>C. sinensis</i> and <i>A. linearis</i> on (A) GSH level, (B) SOD activity, (C) catalase activity, and (D) MDA level in oxidative hepatic injury	62

Figure 3.1.5:	Effects of <i>C. sinensis</i> and <i>A. linearis</i> on glucose absorption in isolated rat jejunum (A) and glucose uptake (B) in isolated psoas muscle	63
Figure 3.2.1:	Total phenolic contents of jasmine green tea (JGT) and green rooibos tea (GRT)	70
Figure 3.2.2:	Ferric reducing antioxidant power (FRAP) activities of jasmine green tea (JGT) and green rooibos tea (GRT)	70
Figure 3.2.3:	(A) α -amylase inhibitory effect, (B) α -glucosidase inhibitory and (C) pancreatic lipase inhibitory effect of jasmine green tea (JGT) and green rooibos tea (GRT)	72
Figure 3.2.4:	Effect of jasmine green tea (JGT) and green rooibos tea (GRT) on GSH level in oxidative hepatic injury	74
Figure 3.2.5:	Effect of jasmine green tea (JGT) and green rooibos tea (GRT) on SOD activity in oxidative hepatic injury	74
Figure 3.2.6:	Effect of jasmine green tea (JGT) and green rooibos tea (GRT) on catalase activity in oxidative hepatic injury	75
Figure 3.2.7:	Effect of jasmine green tea (JGT) and green rooibos tea (GRT) on (D) MDA level in oxidative hepatic injury	76
Figure 3.2.8:	Effects of jasmine green tea (JGT) and green rooibos tea (GRT) on glucose absorption in isolated rat jejunum	77
Figure 3.2.9:	Effects of jasmine green tea (JGT) and green rooibos tea (GRT) on glucose uptake in isolated psoas muscle	77
Figure 4.1:	<i>Cyclopia genistoides</i> (Fabaceae)	82
Figure 4.2.1:	Total phenol content of different extracts of red honeybush (<i>Cyclopia genistoides</i>) tea	87
Figure 4.2.2:	DPPH of different extracts of red honeybush (<i>Cyclopia genistoides</i>) tea	88
Figure 4.2.3:	Ferric reducing antioxidant power (FRAP) of different extracts of red honeybush (<i>Cyclopia genistoides</i>) tea	88
Figure 4.2.4:	Nitric oxide scavenging activity (NO) of different extracts of red honeybush (<i>Cyclopia genistoides</i>) tea	89
Figure 4.2.5:	Inhibitory effect of different extracts of red honeybush (<i>Cyclopia genistoides</i>) tea on α -glucosidase activity	90

Figure 4.2.6:	Inhibitory effect of different extracts of red honeybush (<i>Cyclopia genistoides</i>) tea on pancreatic lipase activity	91
Figure 4.2.7:	Effect of red honeybush (<i>Cyclopia genistoides</i>) tea on GSH level in oxidative pancreatic injury	91
Figure 4.2.8:	Effect of red honeybush (<i>Cyclopia genistoides</i>) tea on SOD activity in oxidative pancreatic injury	92
Figure 4.2.9:	Effect of red honeybush (<i>Cyclopia genistoides</i>) tea on catalase activity in oxidative pancreatic injury	92
Figure 4.2.10:	Effect of red honeybush (<i>Cyclopia genistoides</i>) tea on MDA level in oxidative pancreatic injury	93
Figure 4.2.11:	Chemical structures of compounds identified in the DCM, ethyl acetate, ethanol, and aqueous extract of red honeybush tea (<i>Cyclopia genistoides</i>) by GC-MS	94
Figure 4.3.1:	Feed and fluid intake in different animal groups	106
Figure 4.3.2:	Mean body weight in different animal groups	107
Figure 4.3.3:	Weekly blood glucose concentrations of different animal groups	108
Figure 4.3.4:	Oral glucose tolerance test (OGTT)	109
Figure 4.3.5:	Histopathological examinations of the pancreas in different animal groups	110
Figure 4.3.6:	Serum lipid profile of different animal groups	111
Figure 4.3.7:	Thiobarbituric acid reactive substances (MDA equivalent) in the pancreas, liver, kidneys, and heart of all animal groups	115
Figure 4.3.8:	Reduced glutathione (GSH) levels in the pancreas, liver, kidneys, and heart of all animal groups	116
Figure 4.3.9:	Glutathione reductase (GR) activity in the pancreas, liver, kidneys, and heart of all animal groups	117
Figure 4.3.10:	SOD activity in the pancreas, liver, kidneys, and heart of all animal groups	117
Figure 4.3.11:	Catalase activity in the pancreas, liver, kidneys, and heart of all animal groups	118
Figure 5.1:	JGT (<i>Camellia sinensis</i>)	125
Figure 5.2.1:	Total phenolic content of different extracts of jasmine green tea	130

Figure 5.2.2:	DPPH radical scavenging activities of different extracts of jasmine green tea	130
Figure 5.2.3:	Ferric reducing antioxidant power (FRAP) activities of different extracts of jasmine green tea	131
Figure 5.2.4:	Inhibitory effect of different extracts of jasmine green tea on α -glucosidase activity	132
Figure 5.2.5:	Inhibitory effect of different extracts of jasmine green tea on pancreatic lipase activity	132
Figure 5.2.6:	Effect of jasmine green tea on (A) GSH level, (B) SOD activity, (C) catalase activity, and (D) MDA level in oxidative hepatic injury	134
Figure 5.2.7:	Compounds identified in the different extracts of jasmine green tea by GC-MS	136
Figure 5.3.1:	Feed and fluid intake in different animal groups during the intervention period	143
Figure 5.3.2:	Mean body weight in different animal groups during the experimental period	144
Figure 5.3.3:	Weight and relative weight (relative to body weight) of pancreas in different animal groups	145
Figure 5.3.4:	Weekly blood glucose concentrations	146
Figure 5.3.5:	Oral glucose tolerance test (OGTT)	147
Figure 5.3.6:	Histopathological examinations of the pancreas in different animal groups at the end of the experimental period	148
Figure 5.3.7:	Serum lipid profile of different animal groups	149
Figure 5.3.8:	Thiobarbituric acid reactive substances (MDA equivalent) in the pancreas, liver, kidneys and heart of all animal groups at the end of the experimental period	152
Figure 5.3.9:	SOD activity in the pancreas, liver, kidneys and heart of all animal groups at the end of the experimental period	153
Figure 5.3.10:	Catalase activity in the pancreas, liver, kidneys and heart of all animal groups	154
Figure 5.3.11:	Reduced glutathione (GSH) levels in the pancreas, liver, kidneys and heart of all animal groups	154

Figure 5.3.12:	Glutathione reductase (GR) activity in the pancreas, liver, kidneys and heart of all animal groups at the end of the experimental period	155
Figure 6.1:	<i>Aspalathus linearis</i> (Fabaceae)	161
Figure 6.2.1:	Total phenolic content of different extracts of green rooibos (<i>Aspalathus linearis</i>) tea	166
Figure 6.2.2:	DPPH of different extracts of green rooibos (<i>Aspalathus linearis</i>) tea	167
Figure 6.2.3:	Ferric reducing antioxidant power (FRAP) activities of different extracts of green rooibos (<i>Aspalathus linearis</i>) tea	168
Figure 6.2.4:	Inhibitory effect of different extracts of green rooibos (<i>Aspalathus linearis</i>) tea on α -amylase	169
Figure 6.2.5:	Inhibitory effect of different extracts of green rooibos (<i>Aspalathus linearis</i>) tea on α -glucosidase	169
Figure 6.2.6:	Compounds identified in the different extracts of green rooibos tea (<i>Aspalathus linearis</i>) by GC-MS	171
Figure 6.2.7:	GC-MS chromatograms of green rooibos tea (<i>Aspalathus linearis</i>) tea extracts: A) DCM, B), Ethyl acetate, and C) Ethanol	172
Figure 6.3.1:	Mean feed and water intake in different animal groups during the experimental period	180
Figure 6.3.2:	Mean body weight in different animal groups	181
Figure 6.3.3:	Weight and relative weight of pancreas in different animal groups	181
Figure 6.3.4:	Weekly blood glucose concentrations of different animal groups	182
Figure 6.3.5:	Oral glucose tolerance test (OGTT)	183
Figure 6.3.6:	Histopathological examinations of the pancreatic tissue from different animal groups at the end of the experimental period	184
Figure 6.3.7:	Serum lipid profile of different animal groups at the end of the experimental period	185
Figure 6.3.8:	Thiobarbituric acid reactive substances (MDA equivalent) in the pancreas, liver, kidneys, and heart of all animal groups at the end of the experimental period	188
Figure 6.3.9:	Reduced glutathione (GSH) levels in the pancreas, liver, kidneys and heart of all animal groups at the end of the experimental period	189

Figure 6.3.10:	Glutathione reductase (GR) activity in the pancreas, liver, kidneys and heart of all animal groups at the end of the experimental period	189
Figure 6.3.11:	SOD activity in the pancreas, liver, kidneys, and heart of all animal groups at the end of the experimental period	190
Figure 6.3.12:	Catalase activity in the pancreas, liver, kidneys and heart of all animal groups at the end of the experimental period	191

CHAPTER 1

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Diabetes was first discovered by ancient Egyptians around 1500 BC, and they considered it a rare condition in which the patient had excessive urination and lost weight. The term “*diabetes*” was first used by the Greek physician Aertaeus, meaning “to pass through”. However, it was not until 1798 that the British Surgeon-General, John Rollo used the Latin term “*Mellitus*” to characterize the honey-sweet taste of urine in such patients (Lakhtakia 2013).

Traditional Chinese Medicine (TCM), a medical system of diagnosis and healing approaches. Around 2000 years ago, the traditional term defined diabetes as “wasting thirst (xiao-ke)”, appearing in the first medical text in TCM history, *Huang Di Nei Jing*, or *The Yellow Emperor’s Classic of Internal Medicine*. This book records the primary symptoms of diabetes as “three increases and one decrease”, namely polydipsia, polyphagia, polyuria and weight loss. However, the contemporary term for diabetes in TCM, Tangniao-bing, means “sugar urine illness.” (Zhang et al. 2010).

Diabetes mellitus (DM) is a chronic disease linking with disorders of sugar, fat and protein metabolism. It is characterized by the presence of hyperglycaemia and insufficient production or defective action of insulin produced by the pancreas (Punthakee et al. 2018). It is determined by detecting the elevation of blood glucose levels. Insulin is an endocrine peptide hormone biosynthesized in pancreatic islet β -cells in response to the use of glucose providing energy to organ systems like muscle, liver, adipose tissue and brain (Guo 2014). The lack or inefficiency of insulin in diabetic patients will cause the high levels of glucose in the bloodstream. As the accumulation of blood glucose or high concentrations of blood glucose will damage tissues and may eventually develop into disability or life-threatening complications such as diabetic nephropathy, retinopathy, neuropathy, cardiomyopathy and diabetic foot disease.

1.2 Prevalence of diabetes mellitus

DM is one of the global epidemics in the 21st century. It is also an important cause of worldwide premature mortality and disability, which makes the number of diabetics two to four times than the general population (Papatheodorou et al. 2016, WHO 2016). Type 2 diabetes (T2D) is the most common type of diabetes, since its early symptoms are not obvious, or the symptoms associated with it are not completely confirmed to be diabetes, which delays the

diagnosis of patients, causing the failure of implementing timely and effective prevention and management programs (Chatterjee et al. 2017). Thus, leading to the incidence of global diabetes remain high. At present, diabetes is among the highest prevalent disease in the world, DM and its complications killed approximately 4.2 million people in 2019 and it was the 7th leading cause of death in 2016 (WHO 2016), and the 4th leading cause of disability in 2017 in the world (WHO 2018). Especially in recent years, the prevalence of diabetes has shown a rapid growth trend.

According to the International Diabetes Federation (IDF), there are approximately 463 million adults (aged 20-79 years) with diabetes worldwide in 2019, with a prevalence of 9.3%. It is estimated that by 2045, the number of patients worldwide may be beyond 700 million, the prevalence is reaching around 10.9% (**Figure 1.1**). The report also states that 50.1% of people with diabetes remain undiagnosed, with an estimated 232 million around the world. Among those, the largest increases will take place where economies are moving from low-to-middle-income status (IDF 2019).

According to IDF (2019), global diabetes is mainly divided into seven regions, which are ranked according to the decreasing number of patients: Western Pacific > South-East Asia > Europe > Middle East & North Africa > North America & Caribbean > South & Central America > Africa. Among them, the total number of patients in the Western Pacific region, including China, is at top of the rank, which is predicted to reach 212 million by 2045, while the number of patients in Africa, including South Africa, is expected to reach 47 million by 2045.

The incidence of diabetes in China and South Africa has been increasing constantly year by year. China is now among the top-ten diabetes prevalent country in the world, while South Africa is at the top in Africa. **Figure 1.2** depicts the situation of the diabetes population in different countries. Among them, countries with a patient population of more than 20 million are dark red, mainly including China, the United States and India, which means that the number of patients with diabetes in these three countries ranks top in the world.

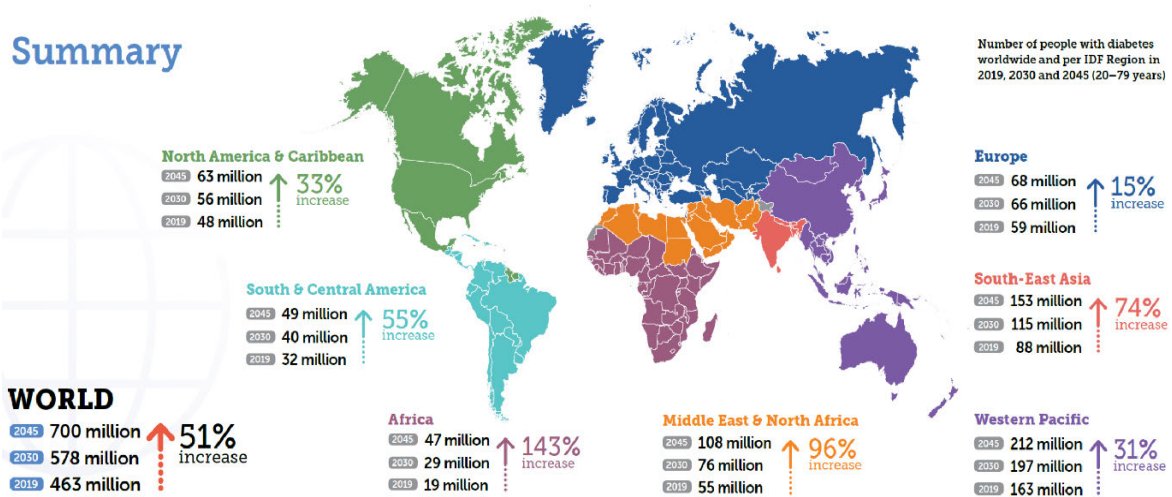


Figure 1.1: Summary of estimated of people with diabetes worldwide and per region in 2019 and 2045 (20-79 years), copied without permission from IDF (2019) diabetes atlas.

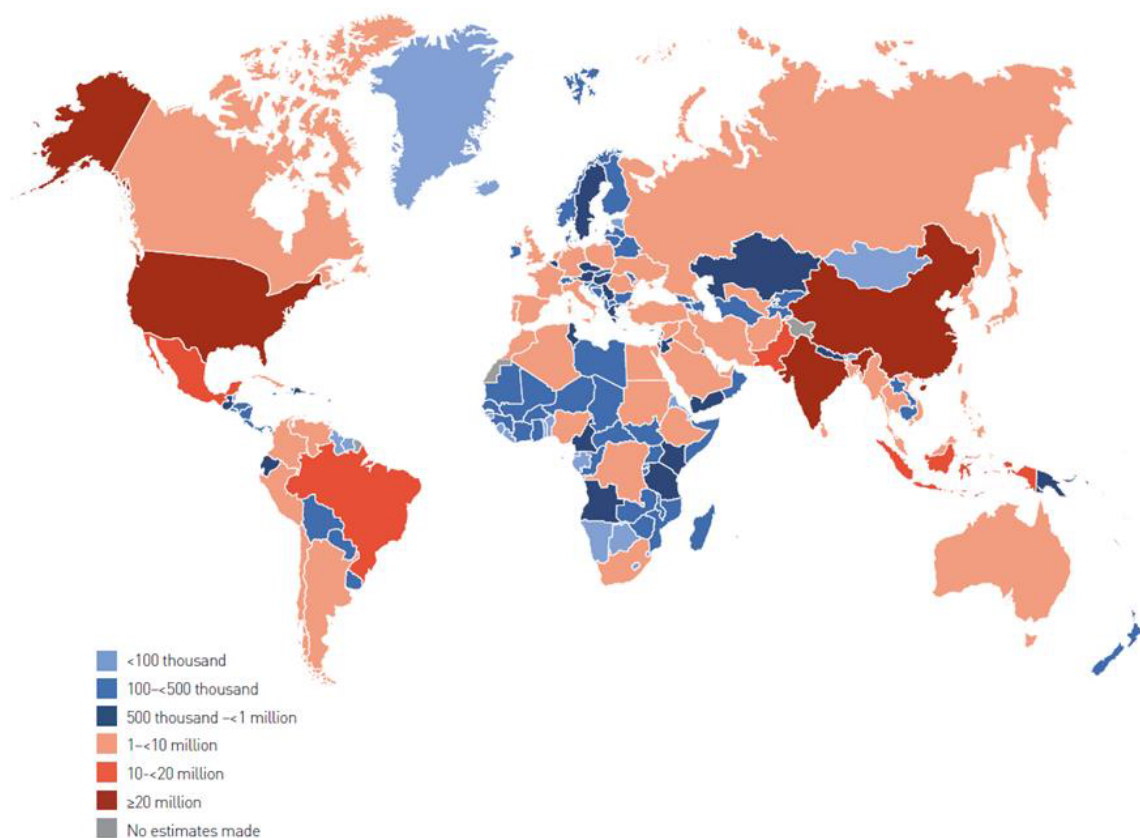


Figure 1.2: Global diabetes distribution of estimated number of adults (20-79 years) with diabetes in 2019, copied without permission from IDF (2019) diabetes atlas edition 9th.

IDF (2019) lists the top 10 countries/regions with the highest number of people with diabetes around the world (**Table 1.1**), namely China, India, the United States, Pakistan, Brazil, Mexico,

Indonesia, Germany, Egypt and Bangladesh. Among them, the prevalence of diabetes in China has reached 10.9%, when the number of patients is about 116 million, accounting for 1/4 of the total number of global patients. On the other hand, the prevalence of diabetes in South Africa reaches 12.8%, the number of patients about 4.58 million, which is around 24% of the total number of patients in Africa. It can be drawn that China is a large country with diabetes, the global focus of diabetes, and South Africa is the focus of diabetes in Africa.

Table 1.1: Top 10 countries for number of diabetics aged 20-79 years in 2019, 2030 and 2045, copied without permission from IDF (2019)

2019			2030			2045		
Rank	Country or territory	Number of people with diabetes (millions)	Rank	Country or territory	Number of people with diabetes (millions)	Rank	Country or territory	Number of people with diabetes (millions)
1	China	116.4 (108.6–145.7) ⁱ	1	China	140.5 (130.3–172.3)	1	China	147.2 (134.7–176.2)
2	India	77.0 (62.4–96.4)	2	India	101.0 (81.6–125.6)	2	India	134.2 (108.5–165.7)
3	United States of America	31.0 (26.7–35.8)	3	United States of America	34.4 (29.7–39.8)	3	Pakistan	37.1 (15.8–58.5)
4	Pakistan	19.4 (7.9–30.4)	4	Pakistan	26.2 (10.9–41.4)	4	United States of America	36.0 (31.0–41.6)
5	Brazil	16.8 (15.0–18.7)	5	Brazil	21.5 (19.3–24.0)	5	Brazil	26.0 (23.2–28.7)
6	Mexico	12.8 (7.2–15.4)	6	Mexico	17.2 (9.7–20.6)	6	Mexico	22.3 (12.7–26.8)
7	Indonesia	10.7 (9.2–11.5)	7	Indonesia	13.7 (11.9–14.9)	7	Egypt	16.9 (9.0–19.4)
8	Germany	9.5 (7.8–10.6)	8	Egypt	11.9 (6.4–13.5)	8	Indonesia	16.6 (14.6–18.2)
9	Egypt	8.9 (4.8–10.1)	9	Bangladesh	11.4 (9.4–14.4)	9	Bangladesh	15.0 (12.4–18.9)
10	Bangladesh	8.4 (7.0–10.7)	10	Germany	10.1 (8.4–11.3)	10	Turkey	10.4 (7.4–13.3)

i 95% confidence intervals are reported in brackets.

In summary, a more fast and effective way of preventing and curing diabetes needs to be developed regarding this serious global and national issue in many countries.

1.3 Types of diabetes

Patients with diabetes have multiple pathogenesis and types of complications, so it is relatively difficult to classify them (NDDG 1979, Susman and Helseth 1997, Mayfield 1998). The World Health Organization (WHO) classifies and revises diabetes many times (WHO 1980, WHO 1985). Eventually, diabetes is divided into four categories based on the etiology and clinical

presentation of diabetes shared by the WHO and the IDF. Type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes mellitus (GDM), and other specific types of diabetes (Gavin III et al. 1997, Alberti and Zimmet 1998, Unwin et al. 1998). However, T1D and T2D are two most common types of diabetes.

1.3.1 Type 1 diabetes (T1D)

T1D is also well-known as insulin-dependent diabetes mellitus (IDDM). It is a chronic autoimmune disease characterized by increased blood glucose levels (hyperglycaemia), which is because of the absolute insulin deficiency that occurs due to the permanent destruction of pancreatic islet β -cells (Atkinson et al. 2014).

The etiology of T1D has not yet been well elucidated, but autoimmune abnormalities are the most important pathogenic factors, for instance, T cell-mediated autoimmunity (Bach and Chatenoud 2011, Pugliese 2016). T1D induces due to autoimmune response in pancreatic islet's β -cell with islet inflammation, and damages in the β cells of islets to lose the function of synthesizing and secreting insulin, resulting in the disorders of glucose metabolism. Besides, other factors causing T1D may relate to genetics and environmental factors (microorganisms, chemicals, food components, etc.) (Pirot et al. 2008, Csorba et al. 2010, Steck and Rewers 2011, Wong 2019) (**Figure 1.3**).

T1D accounts for 5-10% of the total recorded cases of diabetes in the world (Gordon et al. 2020). It could take place at any age, however, it is most commonly occurring in childhood (Katsarou et al. 2017). T1D associated with the occurrence of autoantibodies many months or years before the onset of symptoms. These autoantibodies are not supposed to be pathogenetic but act as biomarkers during the progression of autoimmunity. Characteristic autoantibodies linked with T1D are those that target insulin, 65 kDa glutamic acid decarboxylase (GAD65; glutamate decarboxylase 2), zinc transporter 8 (ZNT8) or insulinoma-associated protein 2 (IA-2) (Howson et al. 2012, Katsarou et al. 2017, Eugster et al. 2019).

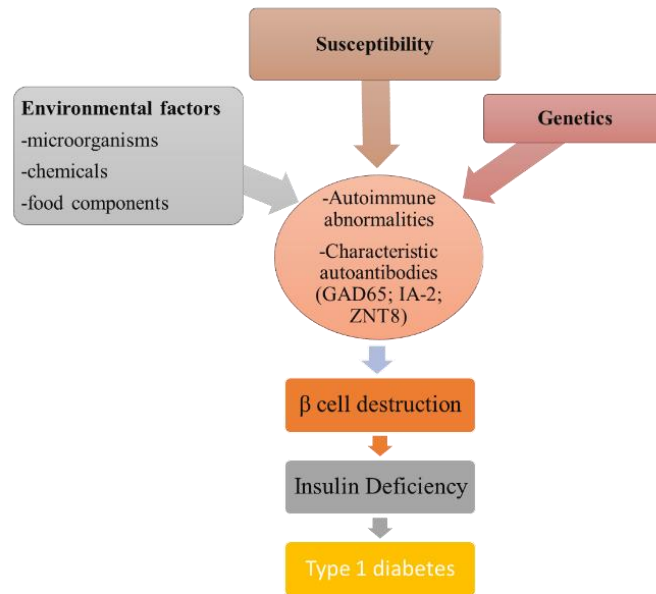


Figure 1.3: Possible pathogenesis of T1D, copied without permission from Ozougwu, Obimba et al. (2013).

1.3.2 Type 2 diabetes (T2D)

T2D also known as non-insulin-dependent diabetes mellitus (NIDDM), which is a type of diabetes caused by insufficient insulin secretion and/or insulin resistance as the main cause (ADA 2014). T2D attributes to about 90% of all diabetics (IDF 2019). The insufficiency of insulin secretion is a result of the carbohydrates in the diet exceeding the ability of the highest-level secretion of insulin could regulate, causing the blood glucose to constantly remain high, and in severe cases, urine glucose could appear.

Insulin resistance (IR) occurs when the pancreatic β -cell can produce enough insulin, but they cannot function due to the lack of insulin receptor to bind and maintain the blood glucose level. It refers to a general term for the reduction of insulin efficiency caused by the loss of effector cell sensitivity (such as muscle cells, fat cells, liver cells, etc.). In the past, T2D was recognized as the disease of the adult, children and adolescents have also however become a high-risk group of T2D in recent years (Kao and Sabin 2016, Yau et al. 2018).

Unlike T1D, T2D is mainly caused by IR as well as partial pancreatic β -cell dysfunction which leads to lower insulin secretion, rather than autoimmune destruction of pancreatic β cells.

1.3.2.1 Pathogenesis of T2D

Multiple risk factors (both genetic and environmental factors) interrelate in the pathogenesis of T2D, which includes obesity, family history of diabetes, aging, impaired glucose metabolism, physical inactivity causing pancreatic β -cell dysfunction as well as IR (Tuomilehto et al. 2001, Knowler et al. 2002). Furthermore, IR accompanies with β -cell dysfunction resulting in hyperglycaemia, which is associated with abnormalities leading to glucolipotoxicity, thus contributing to a vicious cycle (Wang et al. 1998, Patti et al. 1999) (**Figure 1.4**). The factors involved in the development of T2D are discussed below.

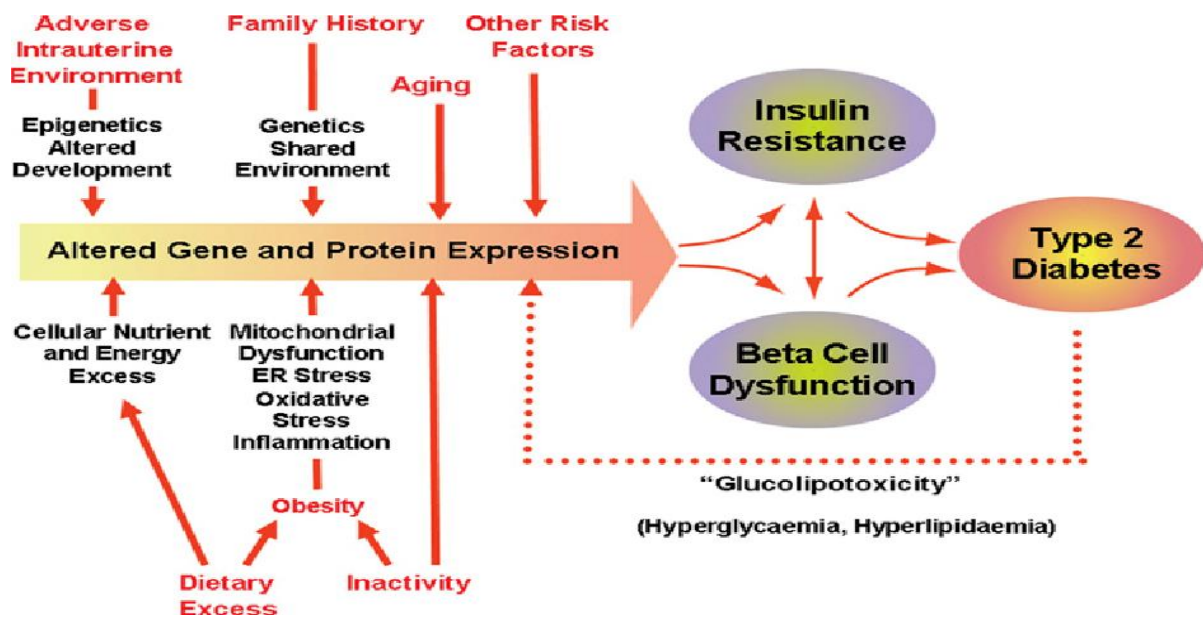


Figure 1.4: Multiple risk factors are involved in the pathogenesis of T2D, copied without permission from Jin and Patti (2009).

1.3.2.1.1 Family history

T2D has a clear genetic predisposition but does not conform to the classic Mendelian genetic laws and the genetic laws of mitochondrial matrilineal genesis, but rather a highly heterogeneous genetic disease controlled by multiple genes (Kwak and Park 2016, Anthanont et al. 2017). There are over 250 genes linked with the pathogenesis of diabetes, and most of these genes are related to glucose metabolism, fat metabolism, energy metabolism, and insulin signalling (Murea et al. 2012). Human Leukocyte Antigen (HLA) is one of the important genetic control factors for diabetes (Diamantopoulos et al. 2002, Ide et al. 2004), which has a total length of 3600 kbp and is located on the short arm of human chromosome six. HLA plays an important role in the human immune system (Mungall et al. 2003). Krook et al. (1998) found

that the expression of mitochondrial uncoupling protein 3 (UCP3) gene in skeletal muscle of T2D patients is 41% lower than that of normal people, however, UCP3 has a link with the insulin-mediated glucose metabolism, which can regulate systemic IR. Moreover, the family of insulin receptor substance (IRS) protein (IRS1-4) has implicated in the heritability of diabetes, especially IRS1 which locates on chromosome 2q36. IRS1 is an intracellular substrate for insulin receptors. There are two mutations in IRS1, Aal513Pro, and Gly972Arg, which have been confirmed the close relation with the pathogenesis of late-type T2D (Almind et al. 1993, Van et al. 2004, Rung et al. 2009).

1.3.2.1.2 Environmental factors

In addition to genetic factors, environmental factors including obesity, diet, and inactivity also has been implicated with the progression of diabetes.

1.3.2.1.3 Obesity

Obesity is a manifestation of abnormal metabolism (Parmar 2018). Obesity caused by overeating, especially the excessive accumulation of visceral fat is an important factor in triggering type 2 diabetes. Obesity can induce the body to resist insulin, which holds back the binding of insulin to its related receptors, ending with hyperglycaemia (Enzi et al. 1986). Kahn et al. (2001) reported that the higher the body-mass index (BMI), the higher the risk of diabetes. The type of fat distribution in obese people also has an important relationship with the occurrence of diabetes. The abdominal obesity is significantly higher than that of limb obesity (Gesta et al. 2007, Lakey et al. 2019).

1.3.2.1.4 Physical inactivity

With the development of society and changes in lifestyles, work pattern changes from heavy labour to sedentary occupations, physical activities are becoming less and less which also contributes to T2D risk (Levine et al. 2005, Imamura et al. 2019). However, physical activity can help with the reduction of weight, increasing muscle oxidative function and systemic insulin sensitivity, improving glucose tolerance, etc., thereby reducing the risk factors of diabetes (Wu et al. 2002, Zong et al. 2002, Russell et al. 2005).

1.3.2.1.5 Diets

With the increase of urbanisation and economy, many countries have experienced dietary changes favouring the proportion of high-fat, high-sugar, high-calorie foods such as animal

meat, fats, and fine grains in the diet more and more (Lakey et al. 2019). However, the supply of trace elements and fruits and vegetables, high-fibre foods are relatively inadequate. Thus, an unhealthy diet with dense caloric content has been regarded as an increased risk factor for developing T2D (Marshall et al. 1994, Toeller and Mann 2016).

1.3.2.1.6 Aging

With the improvement of medical conditions, the life expectancy of diabetic patients is significantly extended. The prevalence of diabetes shows a significant upward trend with age (Davidson 1979, Huang et al. 2008, Huang et al. 2014). The increase in blood glucose is closely related to age, which may be due to the decreased secretion and delayed release of insulin in the elderly, resulting in the reduction of glucose tolerance (Nathan et al. 1986, Gunasekaran and Gannon 2011, Thambisetty et al. 2013, Lipska et al. 2016).

1.3.2.1.7 Pancreatic β -cell dysfunction

At present, high glucose and high fat are considered to be the main factors that cause islet β cell dysfunction (Poitout et al. 2010). Persistent high blood glucose may cause glucotoxicity, which augments the generation of reactive oxygen species (ROS). However, ROS can down-regulate the expression of the transcription factor pancreas-duodenum homeobox-1 (PDX-1) and musculoaponeurotic fibrosarcoma oncogene A (MafA) or inhibit their binding with insulin neurons, reducing the synthesis and secretion of insulin (Gleason et al. 2000, Harmon et al. 2005, Raum et al. 2006, Wang et al. 2007).

Long-term excessive free fatty acid (FFA) in the bloodstream will cause lipotoxicity (DeFronzo 2004). Increased triglyceride (TG) synthesis caused by overexpression of diacylglycerol acyltransferase (DGAT) can inhibit glucose-stimulated insulin secretion (GSIS) and damage islet β cell function (Lee et al. 1994, Listenberger et al. 2003). High concentrations of FFA such as palmitic acid can inhibit the expression of insulin genes, and its lipotoxicity is even more pronounced when blood glucose continues to be at high levels (Briaud et al. 2001, Pardo et al. 2015, Palomer et al. 2018). High concentrations of FFA can also be oxidized by ceramide, peroxisome proliferator-activated receptor γ (PPAR γ), insulin receptor substrate (IRS), and endoplasmic reticulum (ER) stress causing β -cell apoptosis (Donath et al. 2005, Wilding 2007, Han et al. 2008, Muoio and Newgard 2008).

Type 2 diabetes is a natural immune and chronic subclinical inflammatory disease (Donath and Shoelson 2011, Donath 2013). Inflammatory factors participate in the progression of diabetes

by the NF- κ B inhibitor kinase pathway, c-Jun amino-terminal kinase (JNK) and other pathways (Cai et al. 2005, Shoelson et al. 2006, Tuncman, et al. 2006). Moreover, the inflammatory response process of diabetes further produces a large amount of inflammation factors, forming an inflammatory cascade. Therefore, cytokines can directly or indirectly affect the pancreas and damage islet β cells through different pathways (Rabinovitch and Suarez-Pinzon 1998, Donath et al. 2008, Eizirik et al. 2009).

Pancreatic β cell dedifferentiation is another mechanism behind T2D β cell dysfunction (Talchai et al. 2012). During the progressive deterioration of islet cell function, the specific marker molecules accompanied by the maintenance of cell differentiation phenotype continue to decline, such as insulin, PDX-1 And MafA (Kitamura et al. 2002, Zhou et al. 2008, Hang and Stein 2011).

When pancreatic islet β -cell function is deficient and the secreted insulin cannot compensate for demands, blood glucose will increase, and then factors such as glucotoxicity, lipotoxicity, inflammation, and increased reactive oxygen species will aggravate islet β -cell damage, ultimately T2D develop malignancy (Marchetti et al. 2010, Montane et al. 2014) (**Figure 1.5**).

1.3.2.1.8 Insulin resistance (IR)

IR refers to the impaired insulin sensitivity of tissues, mainly skeletal muscle, liver, and adipose tissues. The pathogenesis of IR is very complicated, and it may be a combination of genetic and environmental factors (Brown and Walker 2016). IR is one character of T2D, which could cause many complications such as hyperglycaemia, hyperlipidaemia, obesity, and hypertension (Petersen and Shulman 2018). It results in defects in the inhibition of hepatic glucose output and defects in insulin stimulating glucose uptake in peripheral tissues (muscles and fats), increasing in blood glucose (Tomás et al. 2002, Jellinger 2007). The liver is critical for maintaining glucose homeostasis through synthesis and decomposition of glycogen. It takes up about half of the insulin secreted from pancreatic β cells through the portal vein (Ekberg et al. 1999). While, hepatic IR leads to the impaired suppression of gluconeogenesis and glycogenolysis, causing an increase in blood glucose (Taylor 2008, Perry et al. 2014). Meanwhile, IR in skeletal muscle results in the reduction of muscle glycogen synthesis, causing the increase of blood glucose as well (Petersen and Shulman 2002, DeFronzo and Tripathy 2009). Furthermore, IR in adipose tissue decreases the insulin inhibitory effects on lipolysis causing the plasma FFA elevation (Taylor 2008). However, the increased plasma concentration of FFA can simultaneously promote the excessive synthesis of glycogen and inhibit skeletal

muscle insulin-mediated glucose transport and muscle glycogen synthesis (Baldeweg et al. 2000, Brehm et al. 2004). Moreover, the long-term excessive high levels of FFA can reduce the amount of glucose transporter 4 (GLUT4) on the cell membrane of skeletal muscle and adipose tissue by reducing the expression and activity of GLUT4, thereby inhibiting the oxidation and metabolism of glucose, which in turn aggravate IR (Baldeweg et al. 2000, Roden et al. 2017) (**Figure 1.5**).

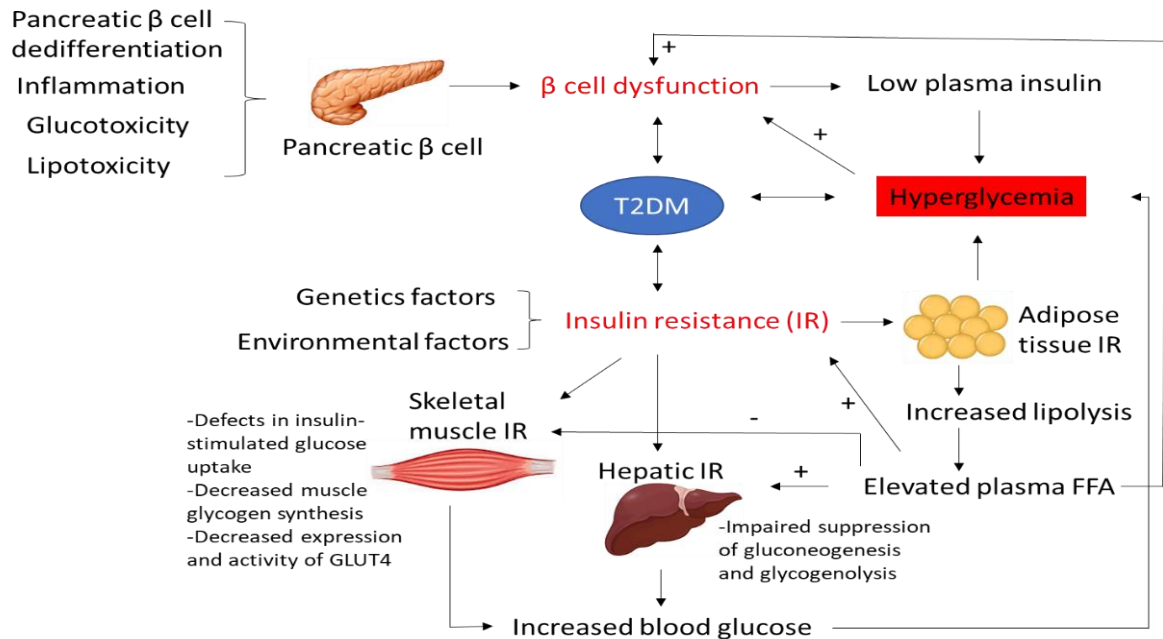


Figure 1.5: Pathogenesis of T2D, adapted without permission from (Stumvoll et al. 2005).

1.3.3 Gestational diabetes mellitus (GDM)

GDM refers to the fact of impaired glucose tolerance or elevated blood glucose in pregnant women due to the insufficient insulin secretion cannot compensate for physiological changes to respond the increase of antagonistic insulin substances during pregnancy (Liang et al. 2010, Yao et al. 2017). After childbirth, glucose tolerance will be able to back to normal in most patients. However, 30% of women with a history of GDM remaining the possibility to recur GDM during future pregnancy or in the later part of their lives (Kim et al. 2007).

GDM is a multifactorial disease that includes a history of previous GDM or impaired glucose tolerance, race, heredity, older maternal history, history of giant paediatrics, history of stillbirth or congenital malformations, metabolic syndrome or polycystic ovary syndrome, smoking during pregnancy, obesity, etc (Petry 2010) (**Figure 1.6**).

During pregnancy, pregnant women gain weight and decrease physical activities, which interact with external and personal reasons, contributing to the occurrence of peripheral IR and glucose intolerance. Furthermore, it will undermine the pancreatic β -cell function and may increase the incidence of GDM risk. The risk factors of GDM may associate with the environment, genetic allergy, epigenetic changes, aging, etc (Chiefari et al. 2017).

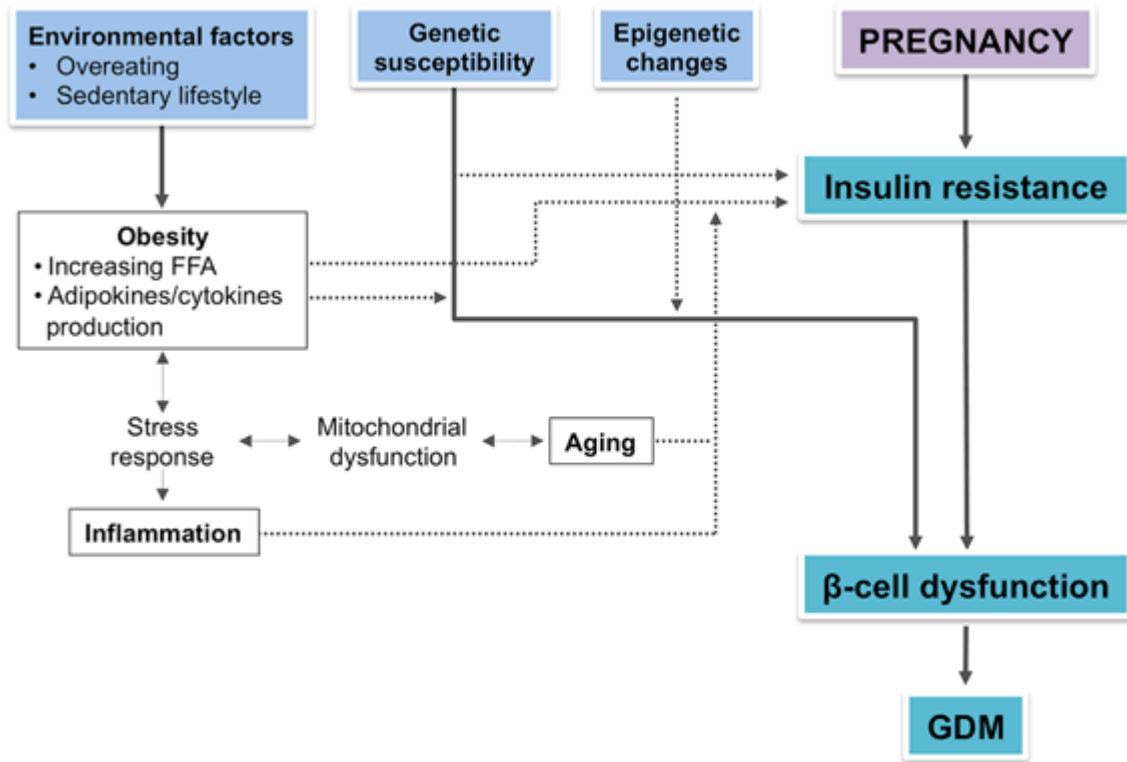


Figure 1.6: Risk factors and pathogenic basis of gestational diabetes mellitus, copied without permission from Chiefari et al. (2017).

1.3.4 Other specific types of diabetes

Other specific types of diabetes are uncommon, including a series of secondary or well-defined diabetes, particular: genetic defects of the insulin receptor, genetic defects of beta-cell function, pancreatic diseases, endocrine diseases, drugs or chemicals, infection, insulin autoimmune syndrome, and other genetic diseases with diabetes (NDDG 1995, Eastman and Vinicor 1997, ADA 2014).

1.4 Oxidative stress

1.4.1 Definition and sources of ROS

In 1956, Denham Harman proposed the free radical theory of aging, which believed that endogenous free radicals attacked cellular components and caused progressive damage related

to aging (Harraan 1955). In 1969, McCord and Fridovich found superoxide dismutase, which acts alone to remove superoxide anion free radicals, thus supporting Harman's hypothesis (McCord and Fridovich 1969).

Free radicals refer to groups containing one or more unpaired electrons. Free radicals can be divided into endogenous and exogenous according to the sources. They are produced either by endogenous oxidative metabolism activities or induced by exogenous environmental factors (Rahman 2007, Poljšak and Dahmane 2012). Endogenous free radicals are mostly derived from mitochondria, and superoxide anions of mitochondria are mainly produced by NADH dehydrogenase and coenzyme Q cytochrome C reductase on the electron transport chain. Furthermore, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system also produces superoxide anions. Besides, peroxisomes, lipoxygenases, cytochrome P450, etc. can produce free radicals (Inoue et al. 2003, Turrens 2003, Lambeth 2004, Finkel 2011, Nathan and Cunningham-Bussel 2013). The causes of exogenous free radicals are ultraviolet radiation, ionizing radiation, chemotherapy, inflammatory factors, and environmental toxins, etc (Cadenas 1989, Finkel and Holbrook 2000, Winterbourn 2008, Halliwell and Gutteridge 2015). The most common free radicals in living organisms are oxygen free radicals, which are often the cause of the production of other free radicals (Gerschman 1954, Gerschman et al. 1954). It contains oxygen and have unpaired valence electrons located on oxygen atoms (Riley 1994). Anion free radicals mainly include superoxide anion free radicals ($O_2^{\cdot-}$), hydroxyl radical (OH^{\cdot}), and dioxygen (O_2), singlet oxygen (1O_2), hydrogen peroxide (H_2O_2), and lipid peroxides (R, RO^{\cdot} , ROO^{\cdot} , $ROOH$) (Halliwell 2006, Liou and Storz 2010, Nathan and Ding 2010) (**Figure 1.7**). Among them, the superoxide anion radical ($O_2^{\cdot-}$) is produced as the earliest, the hydroxyl radical (OH^{\cdot}) is the most toxic and aggressive, and the chain reaction of $ROOH$ is the most durable (Bergamini et al. 2004, Li and Wang 2004).

1.4.2 Oxidative stress and endogenous antioxidant system

It has been established that ROS is a double-edge sword, which could have a harmful and beneficial effects involving in signalling in different process of biological systems (Lopaczynski and Zeisel 2001, Glade 2003). However, this signalling is controlled by a balance between ROS production and subsequent scavenging process. For instance, ROS produced by phagocytic cells is an important defence mechanism against infection (Mittler 2017). In contrast, excessive concentrations of ROS can result in oxidative stress in the body (Poli et al. 2004). Oxidative stress refers to the imbalance between the production of reactive oxygen

species (ROS) and the elimination of the antioxidant defence system in the organism. It can result in excessive production of ROS, leading to the lipid peroxidation of unsaturated fatty acids to generate lipid peroxides, such as malondialdehyde (MDA) and oxidized glutathione (GSSG). Furthermore, MDA and GSSG could damage biomembrane, break DNA, denature proteins and inactivate enzymes, and even cause disintegration and death of cells (Storz and Imlay 1999, Finaud et al. 2006, Rahman 2007, Mayor Oxilia 2010) (**Figure 1.7**).

1.4.2.1 Endogenous antioxidant system

As mentioned above, the body has an antioxidant defence system, which can remove oxygen free radicals promptly to maintain the balanced status of an organism (Halliwell 1996). Antioxidant defence system consists of two kinds of substances, which are enzymes and non-enzyme small molecules (McCord and Fridovich 1969, McCord et al. 1971). The antioxidant enzymes mainly include superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px). SOD can convert superoxide anions into hydrogen peroxide, while catalase and GSH-Px can convert hydrogen peroxide into H₂O (Matés et al. 1999, Altuntas et al. 2004, Imlay 2008). Meanwhile, GSH-Px has a response to lipid peroxidation products and eliminates active electrophilic mutants such as lipid peroxyaldehyde products (Imlay and Fridovich 1991, Devi et al. 2000, Imlay 2008). Non-enzyme small molecule includes reduced glutathione (GSH), ascorbate, pyruvate, and flavonoids (Thomas 1995, Finkel and Holbrook 2000, Lesser 2006).

In conclusion, in a normal state, the intracellular ROS level and the antioxidant defence system is in a dynamic equilibrium. However, the increase in intracellular ROS levels has a dual effect. On the one hand, ROS is the active substance of the body, which activates specific signalling pathways and exerts beneficial effects on the body; On the other hand, excessive ROS causes oxidative stress, trigger a series of pathological reactions (**Figure 1.7**).

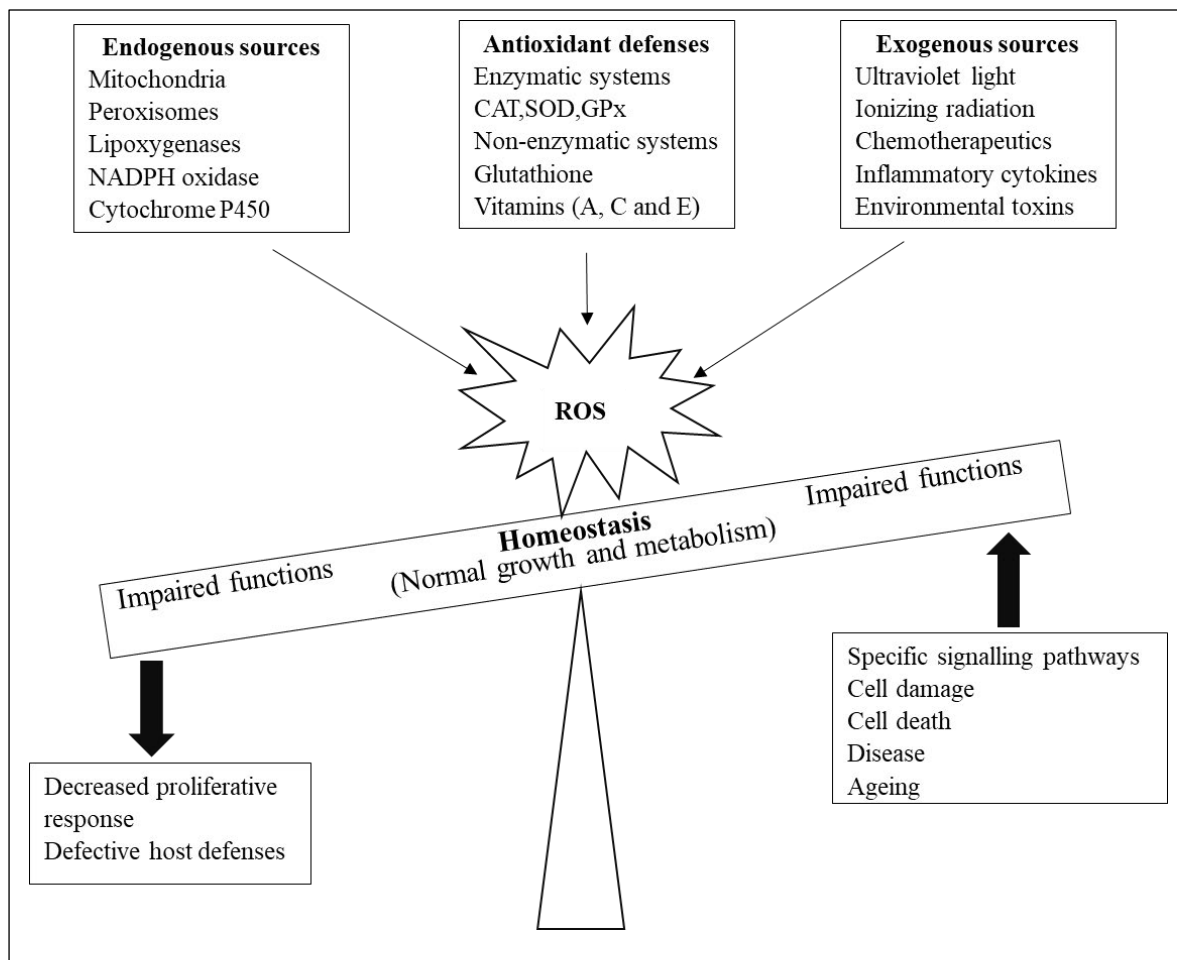


Figure 1.7: The sources and cellular response to ROS, copied without permission from Wang et al. (2013).

1.5 Oxidative stress and type 2 diabetes (T2D)

Oxidative stress has been closely related to the occurrence and progression of type 2 diabetes. Hyperglycaemia is mainly responsible for oxidative stress. It will increase the amounts of ROS and RNS in the living organism through the mitochondrial electron transport chain, glucose autoxidation, and polyol pathway. It is also the basis of other pathways to generate ROS. In the tricarboxylic acid cycle, the rise of blood glucose concentration will raise the generation of electron donors. Under the action of the proton pump, extravasation of mitochondrial membrane protons will arise, causing promotion in mitochondrial membrane electric potential. When the elevated membrane electric potential exceeds the mitochondrial threshold membrane electric potential, the enzyme complexes complex I and complex II in the mitochondrial electron transport chain will constantly produce anion free radicals, including superoxide anion (O_2^-), hydrogen peroxide (peroxide, H_2O_2) and hydroxyl radicals (OH^\cdot). These anion free

radicals in the mitochondria will further activate the polyol pathway, glucose autoxidation, and PKC pathway, etc., thereby generating more free radicals and creating a vicious cycle.

1.5.1 Activation of polyol pathway

In the polyol pathway, glucose is deoxidized to sorbitol by aldose reductase (AR) and NADPH, and then sorbitol is oxidized to fructose by sorbitol dehydrogenase, with nicotinamide adenine dinucleotide (NAD⁺) as a cofactor (Dunlop 2000) (**Figure 1.8**).

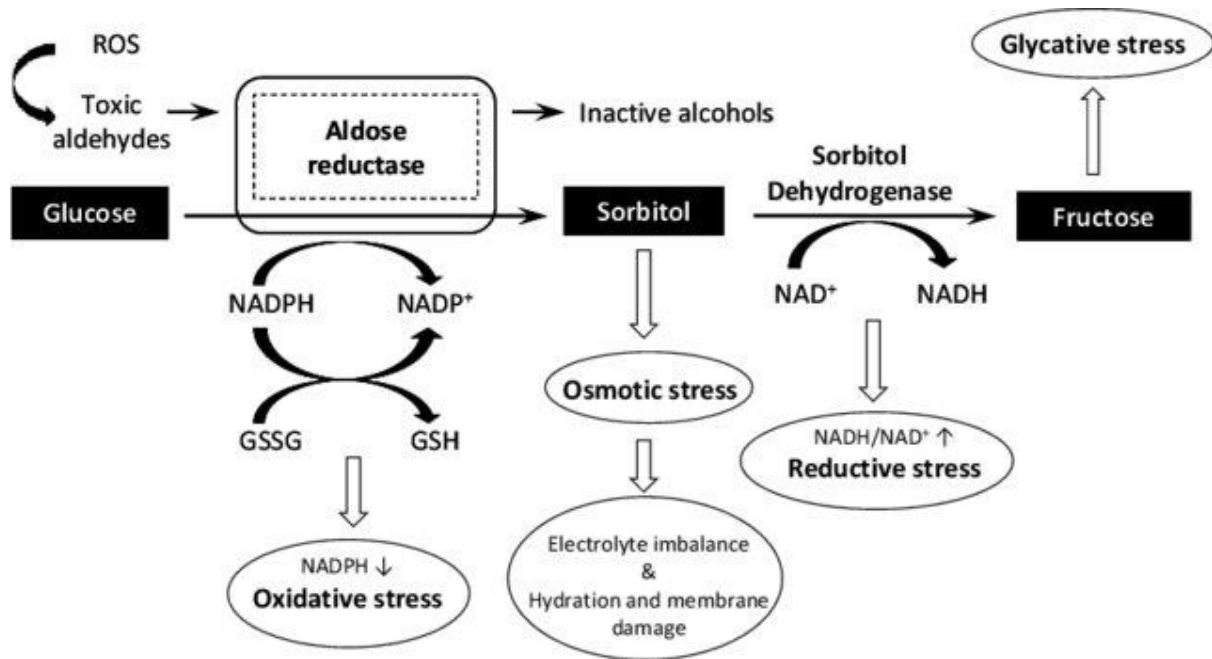


Figure 1.8: The polyol pathway, copied without permission from Singh Grewal, Bhardwaj et al. (2016).

AR is the key enzyme activate the polyol pathway in the hyperglycemic state, causing around 30% of glucose getting into this pathway to generate a large amount of sorbitol (Srivastava et al. 2005). The accumulation of sorbitol in cells may cause osmotic damage, and reduction of inositol and K⁺, Na⁺-K⁺-ATPase enzyme activity, which may result in the impact of the structural integrity and function of the cell, and eventually triggers a series of pathological changes in the body (Cohen et al. 1986, Reddy et al. 2012).

Several mechanisms have been brought up to elucidate how ROS is produced in the polyol pathway. The enzyme AR is competitively using the NADPH with glutathione reductase. Both the activation of AR and the reduction of GSSG to reduced glutathione (GSH) are conducted by using NADPH. However, in the hyperglycaemia state, a large amount of NADPH is consumed by AR, which reduces the production of GSH, and GSH is an important scavenger

of free radical (Vikramadithyan et al. 2005). Furthermore, oxidative stress also can be triggered as a result of sorbitol converting to fructose. During this process, NAD^+ is depleted, creating NADH, which is an important substrate for the production of ROS (Morre et al. 2000);

1.5.2 Increased formation of advanced glycation end-product (AGE)

Advanced glycation end products (AGEs) refer to carbonyl group of reducing sugars such as fructose and glucose with free amino groups of macromolecular substances like proteins, amino acids, nucleic acids, lipids, etc. with non-enzymatic conditions and prolonged exposure to hyperglycaemia, then producing a class of stable and irreversible end products by condensation, rearrangement, decomposition, and oxidation reactions (A Ajith and Vinodkumar 2016).

AGEs are mainly removed by the kidney, but slow and easy to accumulate tissues of diabetic patients for a long period, due to its stable and irreversible structure (Nowotny et al. 2015). Moreover, AGEs correlate with diabetic complications including diabetic atherosclerosis, nephropathy, retinopathy, cardiovascular disease, cataract and, other physiological diseases (Monnie et al. 2005, Pertynska-Marczewska and Merhi 2015, Yamagishi et al. 2015). AGEs compounds that enter the body via foods (such as foods grilled, fried, dehydrated, and ionized) are important sources of exogenous AGEs (Uribarri et al. 2010).

1.5.3 Activation of protein kinase C (PKC) isoforms

Protein kinase C is a family of protein kinases that phosphorylate serine/threonine residues and is broadly present in a wide variety of biological systems (Newton 2003). So far, there are multiple isoforms of PKC have been found, which are divided into three types according to their structure and activators. The conventional PKC (cPKC) isoforms, including α , β_i , β_{ii} , and γ , which are DAG-sensitive and calcium-dependent, whereas novel PKCs (nPKCs) such as δ , ϵ , η , θ , and μ , are DAG-sensitive and calcium-independent. The atypical PKCs (aPKCs), including ζ and λ , are not activated by DAG or calcium but can be activated by phosphatidylserine (Steinberg 2008). Protein kinase C (PKC) is an important second messenger. It transmits extracellular information to cells, thereby regulating cell growth and apoptosis, contraction, secretion, conduction, membrane permeability, extracellular matrix synthesis and gene expression, etc. In the state of diabetes, these functions are abnormal (Gerald and King 2010).

The DAG-PKC pathway is one of the most studied pathways in cellular signaling induced by diabetes (Nishizuka 1992). PKC can be activated by DAG and calcium in a variety of immune

receptor-mediated signal transduction. Hyperglycaemia can increase DAG in tissue cells and activate protein kinase C. Meanwhile, hyperglycaemia can also synergistically activate PKC with angiotensin II.

The activation of PKC is relevant to vascular endothelial dysfunction. In a diabetes state, the expression of endothelial nitric oxide synthase (eNOS) mRNA in retinal vascular endothelial cells is decreased, resulting in less production of nitric oxide (NO), while the inhibitor of PKC can raise NO production (Buckstein et al. 1999). Moreover, PKC α , PKC β and PKC ϵ play a key role in the occurrence and progression of cardiomyopathy. The activation of PKC ϵ and PKC δ are the primary factors for the pathogenesis of cardiomyopathy (Gisselbrecht 2002) (**Figure 1.9**).

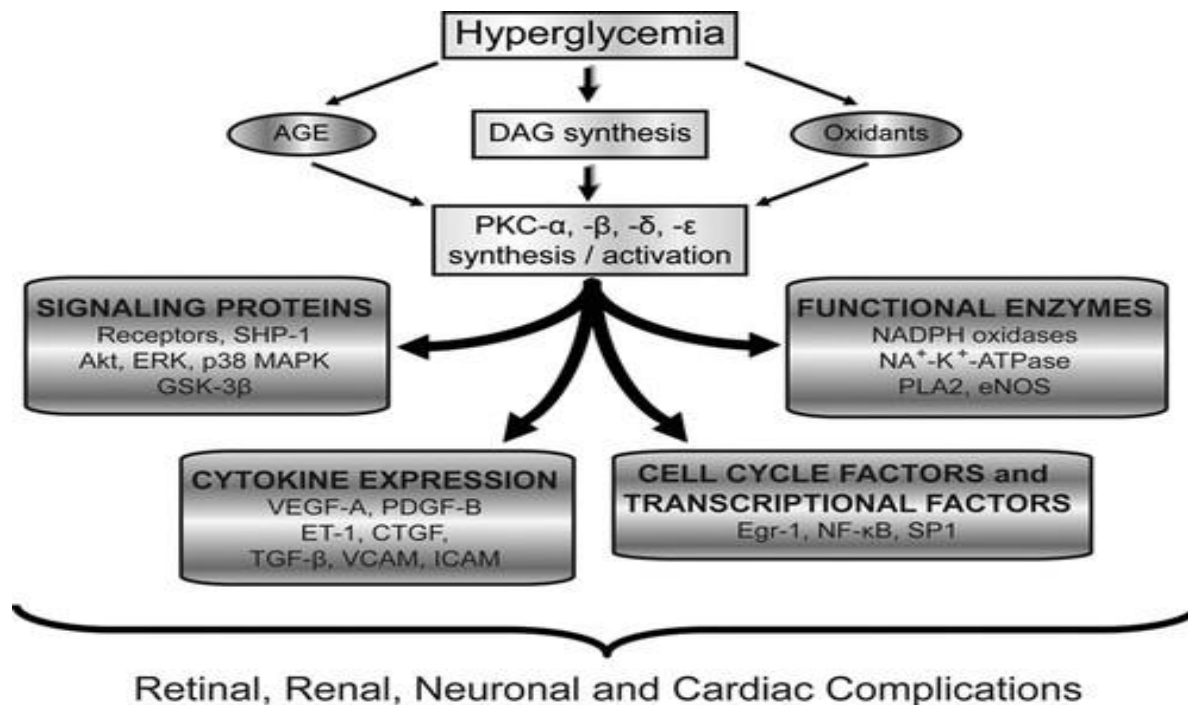


Figure 1.9: The activation and synthesis of protein kinase C (PKC) isoforms, copied without permission from Gerald and King (2010).

1.5.5 Activation of hexosamine pathway

After glucose enters the cell, most of it is metabolized by glycolysis, glycogen synthesis, and pentose phosphate shunt, and just about 1% to 3% of the glucose gets into the hexosamine pathway (**Figure 1.10**). It comprises the conversion of fructose-6 phosphate to glucosamine-6-phosphate by glutamine: fructose-6-P amidotransferase (GFAT), which acts as the rate-limiting enzyme to catalyze the first reaction of the hexosamine pathway. Uracil diphosphate-N-acetylglucosamine (UDP-GlcNAc) is the end product of the hexosamine pathway and then serves as

a donor for intracellular lipid and protein glycosylation (Giacco and Brownlee 2010). UDP-GlcNAc is also a substrate for protein Posttranslational modifications (Luo et al. 2016). Therefore, the activity of GFAT and the content of UDP-GlcNAc in cells or tissues can represent the activity of the hexosamine pathway.

However, the excess oxidation of fatty acids induced by hyperglycaemia can significantly upregulate the flux of fructose-6-phosphate into the hexosamine pathway, cause excessive activation of the hexosamine pathway, and induce IR in muscle, fat and other tissues (Marshall et al. 1991). Several studies have been reported that the main way of hyperglycaemia -induced aminohexose pathway is by increasing the transcription of the genes tumor necrosis factor- α (TNF- α) and TNF- β 1. Excessive activation activates pro-inflammatory factors through a large number of ROS, which in turn contributes to the complications of diabetes (Yang et al. 1970, Brownlee 2001, Brownlee 2005).

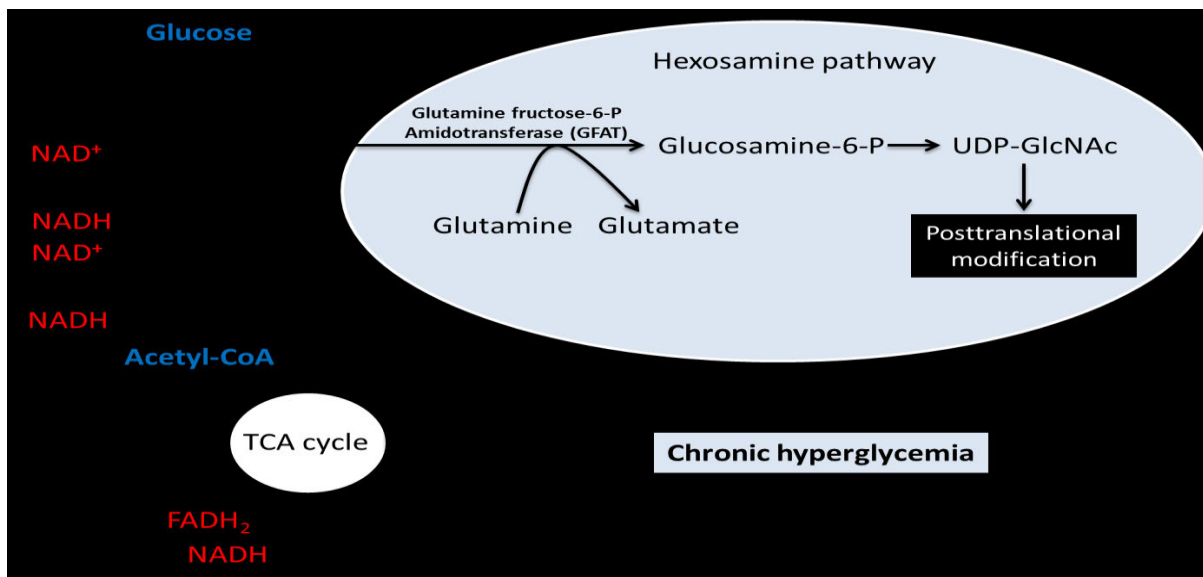


Figure 1.10: Activation of hexosamine pathways, copied without permission from Luo et al. (2016).

These pathways described above are considered as the potential unified mechanisms of hyperglycaemia-mediated oxidative cell dysfunction. This contributes to the understanding of the pathogenesis of T2D complications.

1.6 Complications of diabetes

Oxidative stress damage plays a key role in complications of diabetes. Diabetic with persistent abnormal glucose metabolism cause the disorders of lipid and protein metabolism, resulting in

excessive generating of free radicals. This in turn leads to the damage of organs and tissues, furthermore, brings out various diabetes complications (Brownlee 2005). Diabetic complications can be divided into diabetic acute complications and diabetic chronic complications (Brownlee 2001).

1.6.1 Acute complications of diabetes

There are three main types of acute complications of diabetes: diabetic ketoacidosis (DKA), hyperosmolar hyperglycemic state (HHS), and lactic acidosis (LA).

1.6.1.1 Diabetic ketoacidosis (DKA)

DKA refers to a syndrome characterized by hyperglycaemia, ketosis, and acidosis. It resulted from a relative or absolute insulin deficiency and an excess of insulin counter-regulatory hormones, and then symptoms of hyperventilation, vomiting, abdominal pain (Magee and Bhatt 2001, Charfen and Fernández-Frackelton 2005).

1.6.1.2 Hyperosmolar hyperglycaemic state (HHS)

HHS refers to a complication of diabetes in which hyperglycaemia causing a marked increase in osmolarity without significant ketoacidosis. HHS may lead to profound dehydration, electrolyte losses and other complications. In severe cases, the patient may experience a disturbance of consciousness or even a coma (Stoner 2005, Pasquel and Umpierrez 2014).

1.6.1.3 Lactic acidosis (LA)

LA refers to the rising of cellular anaerobic respiration under the conditions of alcohol abuse, hypoxia, and excessive adrenaline secretion, resulting in the excessive conversion of acetyl pyruvate to lactic acid after glycolysis. This leads to the excessive accumulation of lactic acid, which brings out a type of diabetic complications of acidosis (English and Williams 2004). LA is usually related to factors that may link with diabetes, myocardial infarction, cardiogenic shock, drugs, toxins and inborn errors of metabolism (Kreisberg 1984).

1.6.2 Chronic complications of diabetes

Patients with chronic diabetes complications, mainly microvascular and macrovascular disease.

1.6.2.1 Microvascular complications of T2D

Microvascular complications are those long-term diabetes complications that affect small blood vessels, which are the most distinguished complications of T2D, including retinopathy, neuropathy and nephropathy (Faselis et al. 2020).

1.6.2.1.1 Diabetic retinopathy (DR)

Diabetic retinopathy (DR) has been well-known as microvascular diseases, it is the most prevalent complication of diabetes mellitus (DM) (Flaxman et al. 2017), and is the leading inducement of visual loss amid adults aged from 20 to 74 years (Hirai et al. 2011, Wang and Lo 2018). The pathogenesis DR can occur in various ways, which may involve AR, glycoproteins, vascular endothelial growth factor (VEGF), growth hormone, hyperglycaemia and oxidative stress (Fong et al. 2004, Demiot et al. 2006, Keenan et al. 2007). Currently, there are three effective and rather invasive therapies in diminishing vision loss, including vitreous surgery, laser photocoagulation and intravitreal pharmacologic agents (Wang and Lo 2018).

1.6.2.1.2 Diabetic nephropathy

Diabetic nephropathy is the second most common and predominant complication of DM, which comprises beyond 50% of all the instances of kidney failure (Gheith et al. 2016). Diabetic nephropathy is characterized by the presence of persistent clinical albuminuria (albumin excretion rate > 300 mg/24 h) and a decline in the glomerular filtration rate over 5 years in defect of urinary tract infections, cardiac insufficiency, or other kidney diseases (Sifuentes-Franco et al. 2018). Hyperglycaemia, arterial hypertension, obesity, and an unhealthy lifestyle have been closely related with the occurrence and development of diabetic nephropathy (Rossing 2006). The kidney is the main organ liable for cleaning up body metabolic waste and also for sustaining the blood water level and blood salt level (NDDG 1998). The best administration for DM patients to manage the transformation of kidney impairment is the suitable regulation of glycemia (Sifuentes-Franco et al. 2018). In T2D, diabetic nephropathy predominantly occurs in patients with systemic arterial hypertension and previous kidney disease (Persson and Rossing 2018).

1.6.2.1.3 Diabetic neuropathy

Glucose metabolism plays an important role in the nervous system, glucose supply neurons constant and substantial energy to maintain brain main function. Abnormal cellular metabolism that occurs in diabetes state will inevitably affect the nervous system. Therefore, diabetic neuropathy is one of the common diabetes complications (Pellerin 2010). Diabetic neuropathy

is characterized by progressive distal-to-proximal degeneration of peripheral nerves (De Gregorio et al. 2018). Diabetic neuropathy has manifold forms, starting from discomfort to death, with chronic sensorimotor distal symmetric polyneuropathy as its most prevalent form, it may substantially induce pain, reduce motility, and even result in amputation (Dewanjee et al. 2018). Unfortunately, there is no specific treatment for diabetic neuropathy, even nowadays with various available drugs to choose from. Therefore, diabetic neuropathy demands urgent awareness of glycaemic control might through dealing with convertible risk factors, which include obesity, smoking, alcohol abuse (Feldman et al. 2019). However, diabetic neuropathy can be predicted from the duration of diabetes and the haemoglobin A1c (HbA1c) levels (Tesfaye et al. 2005).

1.6.2.2 Macrovascular complications

Macrovascular complications are diabetes complications that affect large blood vessels, which mainly include cerebrovascular disease, coronary heart disease, peripheral artery disease, etc. Among those macrovascular complications, cardiovascular disease (CVD) is the primary reason leading to the death of diabetic patients (Huang et al. 2017, Viigimaa et al. 2020).

1.6.2.2.1 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease which is a serious excuse for stroke and coronary heart disease emerging in humans (Malekmohammad et al. 2019). Intimal plaques and cholesterol accumulation in the arterial walls are the characteristics of atherosclerosis (Hennekens and Gaziano 1993).

The progression of atherosclerosis is the central pathological mechanism in macrovascular disease, which is believed to deriving from chronic inflammation and injury to the peripheral arterial wall or coronary vascular system. Vis-à-vis this, oxidized lipids from low- density lipoproteins (LDL) particles amass in the arteries endothelial wall. Thereafter, oxidized lipids accumulated through macrophages, which is differentiated from monocytes to form foam cells. Foam cells stimulate the proliferation of macrophage and the attraction of T-lymphocytes, which further cause smooth muscle proliferation in the arterial walls and collagen accumulation. All these processes provoke the emergence of a lipid-rich atherosclerotic region triggering atherosclerosis in T2D (Fowler 2011).

1.6.2.2.2 Cardiovascular disease (CVD)

CVD is a complex inflammatory process may lead to myocardial infarction, stroke, and peripheral artery disease (Petrie et al. 2018). CVD takes the main responsibility for the death of type 1 and type 2 diabetes, causing over 50% mortality seen in the diabetic population. It results in the greatest component concerning the health care expenditures for diabetic (Fowler 2011). CVD risk factors including obesity, hypertension, and dyslipidaemia are common in diabetic, positioning them at higher risk for cardiac events (Leon and Maddox 2015). In response to this, the elevation of CVD risk should start with prediabetes period along with IR and impaired glucose tolerance. As well as being the diagnostic hallmark of T2D, hyperglycaemia plays a vital role in the pathogenesis of CVD (Petrie et al. 2018).

1.7 Management of T2D

T2D is a comprehensive disease, and it almost needs a life-long treatment. Nowadays, there are four main methods for the management of T2D, diet management, physical exercises, psychological adjustment therapy, and oral hypoglycaemic drugs. A reasonable diet structure can reduce the intake of sugars, release the burden of islet B cells, and reduce the metabolic load, which closely related to glucose metabolism, thereby controlling the condition of T2D. The moderate amount of physical exercise can not only help to promote the energy substances catabolism in the tissues and organs of T2D patients but also increase the insulin sensitivity of the target cells, especially muscle cells. Meanwhile, mental and psychological counselling can also play a significant role in the management of T2D. Furthermore, the main medical treatment of T2D is taking oral hypoglycaemic drugs.

The management of T2D and its complications of oral hypoglycaemic drugs have been well studied. These hypoglycaemic drugs can be divided into six major classes namely: insulin secretion-promoting drugs, biguanide drugs, α -glucosidase inhibitors, thiazolidinediones, sodium-glucose co-transporters (SGLT2) and dipeptidyl peptidase (DPP) IV inhibitors. The different mechanisms of actions of these antidiabetic agents on hyperglycaemia treatment are shown in **Figure 1.11**.

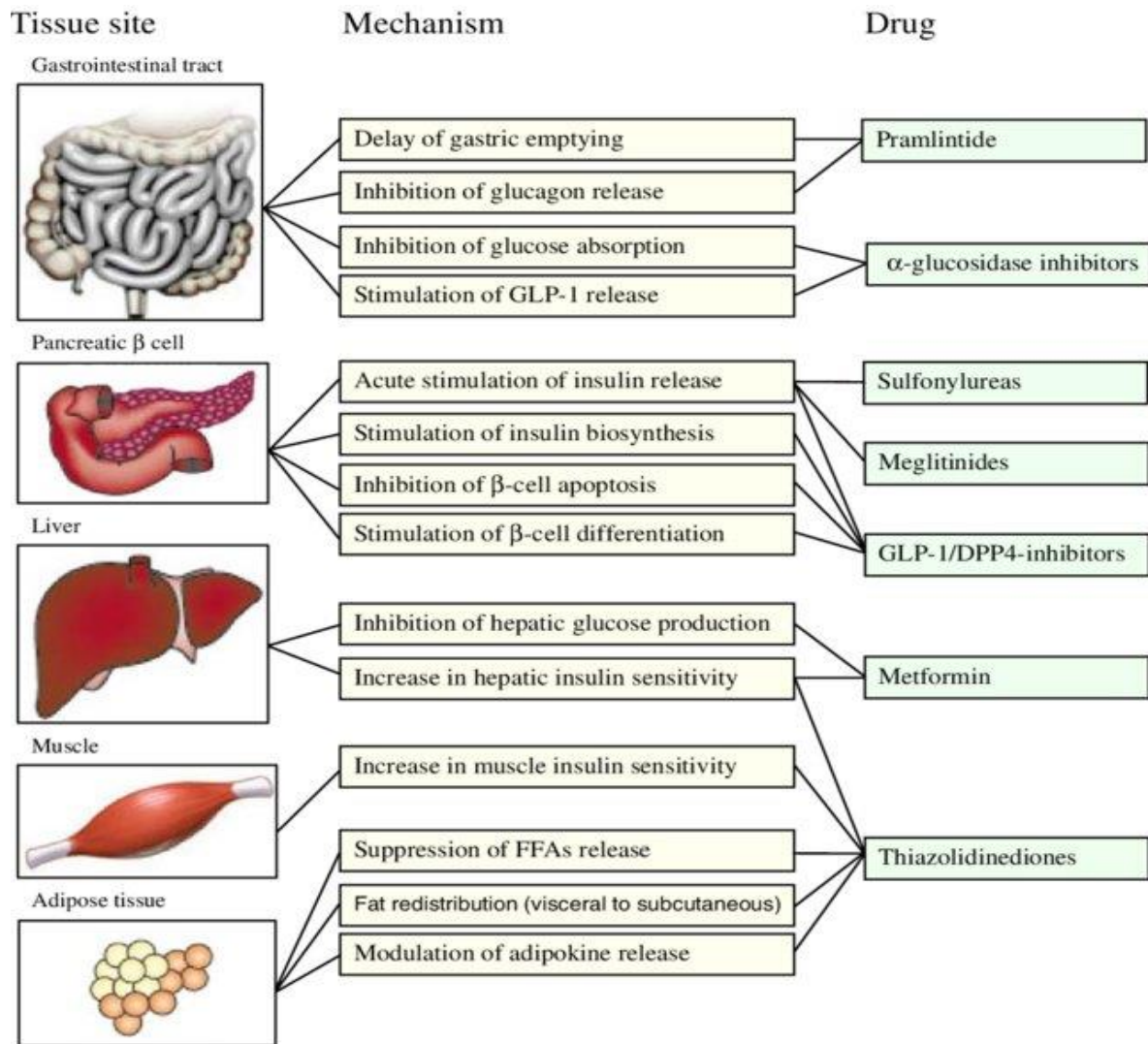


Figure 1.11: Antidiabetic agents treatment of hyperglycaemia according to site of action, copied without permission from Sena et al. (2013).

1.7.1 Sulfonylureas

Sulfonylureas were discovered in 1942 and became accessible by the 1960s. Sulfonylureas can be categorized into two generations. The application of the first-generation sulfonylureas (such as acetohexamide, chlorpropamide, tolbutamide, and tolazamide) rapidly eliminated. Meanwhile, the second-generation sulfonylureas (such as glipizide, gliclazide, glibenclamide, glimepiride) are commonly used around the world (Sola et al. 2015).

The major difference in sulfonylureas focuses on the potency and period of action. Compared to the first-generation sulfonylureas, the second-generation sulfonylureas are more effective when administered in lower doses. This because of their long half-lives, so they can be given

once daily. However, they can negatively affect the overnight hepatic glucose output, thereby escalates the risk of hypoglycaemia (Lorenzati et al. 2010).

Sulfonylureas decrease blood glucose by increasing pancreatic β -cells insulin secretion (Aguilar-Bryan et al. 1995) and may have a function on slightly ameliorate IR in peripheral target tissues such as muscle, fat (Bressler and Johnson 1997). The binding of sulfonylureas and its receptor leads to the inhibition of ATP-dependent potassium (K^+) channel, which leads to the changes in the resting potential of the cell, causes calcium influx and stimulates insulin secretion. This in turn results in more insulin being released from pancreatic secretory granules at different blood glucose concentrations (Lorenzati et al. 2010). Despite the effectiveness and tolerance of sulfonylureas, possible side effects include hypoglycaemia and weight gain (Kalra et al. 2018).

1.7.2 Biguanides

Biguanides are a class of drugs and chemicals formed upon the biguanide molecule, which originated from the *Galega officinalis* plant (Bailey and Turner 1996). Biguanides reduce hyperglycaemic by increasing insulin sensitivity and decreasing glucose absorption. Biguanides related compounds inclusive of guanidine, metformin, phenformin, biuret, L-arginine and some others. Among these compounds, metformin is the only marketed biguanide to treat non-insulin-dependent diabetes now (Perla and Jayanty, 2013). In therapeutic doses, metformin lower blood glucose levels through multiple mechanisms. It inhibits gluconeogenesis via insulin and glucagon-stimulated gluconeogenesis (Bailey and Turner 1996). Metformin can also positively modify the phosphorylation of insulin receptor and the activity of tyrosine kinase. It can likewise improve the translocation of GLUT-1 and GLUT-4 transporters along with suppressing the progression of IR in hepatocytes and adipocytes (Bailey 1992).

Adverse effects related to the therapeutic use of metformin can lead to nausea, vomiting and abdominal pain, Vitamin B₁₂ deficiency associated megaloblastic anaemia or neuropathies and diarrhoea associated hypomagnesaemia, hypocalcaemia, hypokalaemia (Wang and Hoyte 2019).

1.7.3 α -glucosidase inhibitors

Since α -glucosidase is so closely related to carbohydrate metabolism that makes it an attractive pharmacological target to ameliorate diabetes, obesity and other related complications. Among

all the accessible antidiabetic drugs, α -glucosidase inhibitors might be the most effective in decreasing postprandial hyperglycaemia through inhibiting the activity of α -glucosidase enzyme located in the brush border of enterocytes, thus delaying the digestion of complex carbohydrates and intestinal glucose absorption (Derosa and Maffioli 2012). Nowadays, commonly used α -glucosidase inhibitors are miglitol, voglibose, acarbose, and emigliate. Among them, acarbose introduced into the market in the 1990s as the first α -glucosidase inhibitor, which is the most used drug of this family. Nevertheless, some serious side-effects such as flatulence, abdominal pain, and diarrhoea impede the more comprehensive usage of these inhibitors (Santos et al. 2018).

1.7.4 Thiazolidinediones (TZDs)

TZDs also known as “insulin sensitizer”, the main mechanism of action is to increase the insulin sensitivity to effector cells, such as adipocytes, hepatic cells, muscle cells, etc., thereby accelerating the transformation of blood glucose to glycogen and other non-sugar substances. Meanwhile, it can also stimulate glucose metabolism to provide energy (Diamant and Heine 2003, McGuire and Inzucchi 2008). These drugs primarily regulate glucose and lipid metabolism through activating peroxisome proliferator-activated receptor gamma (PPAR γ). It can promote the glucose uptake of adipocytes, hepatic cells, and muscle cells, hence reducing the concentration of glucose in the blood, therefore reducing blood glucose (McGuire and Inzucchi 2008). In turn, it can lower the conversion of glycogen and non-sugar substances to glucose. Currently, widely used TZDs are mainly pioglitazone and rosiglitazone. Their side effects include body weight gain, increased risk of heart failure and fractures (Lipscombe et al. 2018). Therefore, diabetic patients with myocardial disease need to be treated with caution.

1.7.5 Dipeptidyl peptidase (DPP) IV inhibitors (DPP-IV)

Glucagon-like peptide (GLP)-1 promotes insulin biosynthesis by activating the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) signaling pathway via binding to the GLP-1 receptor of β -cells, thereby stimulating the gene expression of the insulin precursor, resulting in a reduction of β -cell apoptosis and increasing of β -cell mass. However, the half-life of GLP-1 present in the physiological state is relatively short, and it is easily degraded by the enzyme dipeptidyl peptidase-IV (DPP-IV) *in vivo* (Deacon 2004). However, DPP-IV inhibitor can inhibit the activity of DPP-IV and prolong the half-life of incretin GLP-1, thereby achieving the effect of enhancing insulin secretion and lowering plasma glucose (Dalla Man et al. 2009).

Examples of DPP-IV are alogliptin, linagliptin, saxagliptin, sitagliptin, and vildagliptin. Plenty of studies have reported these drugs are associated with fewer adverse side effects which are generally well tolerated (Deacon 2018).

1.7.6 SGLT2 inhibitors

Sodium-glucose cotransporter-2 (SGLT2) inhibitors were originally authorized as a new class of hypoglycaemic agents that bring insulin-independent blood glucose lowering in type 2 diabetes patients by increasing urinary glucose excretion through inhibiting SGLT2 in the proximal convoluted tubule, where glucose is reabsorbed. Owing to this kind mechanism, at the moment that pancreatic β -cell stocks are eternally absent in T2D, these agents may still be effective (Chaudhury et al. 2017, Ingelfinger and Rosen 2019). SGLT2 inhibitors are classes of glucosuria agents: canagliflozin, dapagliflozin, and empagliflozin. Potential adverse effects of these agents may include body weight loss, diuresis, polyuria, blood pressure-lowering, ketoacidosis and increased genital infections (Reusch and Manson 2017).

1.8 Chinese teas and South African teas in the treatment and management of T2D

Tea is the most worldwide popular consumed non-alcoholic beverage just next to water. Teas can be categorized as traditional tea made from *Camellia sinensis* or herbal tea made from non-*Camellia sinensis* teas or tisanes. **1.8.1 The effects of Chinese teas on the treatment and management of T2D.**

Tea (*Camellia sinensis*) is a healthy beverage that originated in China and has more than 5000 years of cultivation and application history since time immemorial. Tea and its brewing have been reported to possess bioactive compounds, such as polyphenols, polysaccharides, alkaloids, amino acids and pigments.

1.8.1.1 Tea polyphenols

Tea polyphenols account for 10% to 25% of the dry weight of tea. It mainly contains catechins, and their derivatives, including epicatechin (EC), gallic catechin (GC), epigallocatechin (EGC), catechin gallate (CG), epicatechin gallate (ECG), gallic catechin gallate (GCG) and epigallocatechin gallate (EGCG), which account for 60% to 80% of the total polyphenol content (Tang et al. 2019, Zhao et al. 2019). Among them, EGCG is the major catechin, which may account for 50 to 80% of the total catechin in tea. Moreover, other polyphenols like flavonoids and flavonoid glycosides, anthocyanins and phenolic acids are also present in tea (Khan and Mukhtar 2007, Zhao et al. 2019). Tea polyphenols are the primary bioactive

compounds responsible for various tea health beneficials, such as antioxidant, radiation protection, anti-aging, antibacterial, hypolipidemic, hypoglycaemic and enzyme inhibitory activities, which have been reported *in vitro* and *in vivo* (Oh et al. 2015, Zielinski et al. 2015, Satoh et al. 2016).

1.8.1.2 Tea polysaccharides

Tea polysaccharides is a group of heteropolysaccharides bond with proteins and mineral elements. The protein in polysaccharide is mainly composed of about 20 common amino acids, while the sugar unit is normally consisted of arabinose, xylose, rhamnose, glucose and galactose and the mineral elements composition of tea polysaccharide mainly include calcium, magnesium, iron, manganese, selenium and even trace elements (Wang et al. 2001, Du et al. 2016). Abundant previous studies on tea polysaccharide have suggested that polysaccharides may contribute to anti-oxidation, immune regulation, anti-cancer, anti-diabetic, anti-coagulation, anti-thrombosis and other biological properties (Ren et al. 2015, Yuan et al. 2015, Park et al. 2017).

1.8.1.3 Tea alkaloids

The alkaloids in tea are usually purine alkaloids, including caffeine, theobromine and theophylline, with caffeine accounting for the highest amount of tea alkaloids (Bi et al. 2016). Alkaloids is one of the most important phytochemicals in tea. Some previous studies have reported the antioxidant, anti-diabetic and anti-obesity effects of tea alkaloids (Xu et al. 2015, Luca et al. 2016).

1.8.1.4 Tea amino acids

Amino acids accounts for 1% to 4% of dry weight of tea leaves, in which arginine, alanine, theanine, glutamic acid, aspartic acid, and tyrosine cover over half the amount of free amino acids. L-theanine is the predominant amino acid and almost solely in tea (Horanni and Engelhardt 2013). It has been suggested that L-theanine contribute to lower blood pressure, improve learning ability, anticancer, antiobesity and produce relaxation effect in human beings (Juneja et al. 1999, Türközü and Şanlıer 2017).

1.8.1.5 Tea pigments

The pigments in tea include fat-soluble pigments and water-soluble pigments. Fat-soluble pigments include chlorophyll, xanthophyll and carotene. Water-soluble pigments include

flavonoids, anthocyanins and tea catechins oxidation products, which include theaflavins, thearubigins and theabrownins (Koch et al. 2017, Lv et al. 2017). Theabrownins is the most common pigments accounting for more than 85% of the tea pigments. Tea pigments have been reported for their health functions of such as antioxidant, anticancer, anti-inflammatory, hepato-protective effects and hypercholesterolemia (Huang et al. 2019, Xiao et al. 2020).

As a natural hypoglycaemic material, tea has been investigated worldwide, such as the inhibitory activity of tea polysaccharides on α -glucosidase (Shimizu et al. 1988, Wang et al. 2010, Wang et al. 2012), the inhibitory activity of theaflavins on α -amylase (Hara and Honda 1990), the inhibitory activity of polyphenols on sucrase (Matsumoto et al. 1993) and glucose-absorbing carrier protein in intestinal cells, such as SGLT1, GLUT2, and GLUT5 (Kobayashi et al. 2000, Shimizu et al. 2000) to manage T1D. The effect of teas on the treatment and management of type 2 diabetes is mainly manifested in increasing insulin secretion, enhancing insulin activity and sensitivity, enhancing intestinal insulin promoting activity, enhancing glycolysis and inhibiting gluconeogenesis and antioxidant as summarized as below (**Table 1.2**):

Table 1.2: Target tissues and mechanisms of some Chinese tea bioactive compounds on T2D

Category	Target tissue	Mechanism	Active compound	Reference
Insulin	Pancreas	Enhance insulin secretion	EGCG, unknown compounds in black tea, Green tea polyphenols	Wolfram et al. 2006 Islam and Choi 2007
Insulin	Liver muscle adipose	Increase insulin activity	EGCG, theaflavin, unknown compounds in black tea	Anderson and Polansky 2002, Tang et al. 2013
Insulin	Liver muscle adipose	Increase insulin sensitivity	EGCG, theaflavin, thearubigins	Wu et al. 2004 Nishiumi et al. 2010
Glycometabolism	Muscle	Enhance glycolysis	EGCG	Zhang et al. 2010 Jung et al. 2008
Glycometabolism	Adipose	Inhibit gluconeogenesis	EGCG	Waltner-Law et al. 2002 Collins et al. 2007
Others	Mirco and macro-	Antioxidant	Green tea Catechins,	Yan et al. 2012 Zhou et al. 2007

vascular tissues	Green tea polysaccharide, Puer polysaccharide	Xu et al. 2012
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1.8.2 The effects of South African teas in the treatment and management of T2D

Herbal teas can be made from different parts (such as seeds, fruits, flowers, leaves, stems and roots) of different plants and herbs through different ways such as infusion, decoction or maceration. They gain popularity worldwide in recent years because of their fragrance, aroma, and various health-promoting functions (Enioutina et al. 2017). In the African continent, over 30000 plant species accounting for 25% of the total number of higher plants in the world are found in sub-Saharan Africa (Light et al. 2005, Van 2008). While, in South Africa, an estimated 3000 plant species are used as medicines, and around 500 of them are traded in large quantities in informal markets (Van and Gericke 2000, Van 2008). Rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* species) are becoming increasingly popular worldwide, due to their multiple pharmaceutical and therapeutic properties-(Marnewick 2009).

1.8.2.1 Rooibos tea

Rooibos tea contains sodium, potassium, magnesium, calcium, and trace elements such as zinc.(Ku et al. 2015). Several phenolic compounds are presented in red rooibos tea and green rooibos tea, but their contents may have significant difference due to enzymatic and chemical modifications during the tea fermentation and manufacturing (Bramati et al. 2002, Bramati et al. 2003). Compounds comprising aspalathin, isoorientin, orientin, nothofagin, isovitexin and vitexin are degraded during the fermentation (Standley et al. 2001). Other predominant flavonoids identified in both teas are rutin, isoquercetin and hyperoside, quercetin, luteolin and chrysoeriol. Moreover, phenolic acids including ferulic acid, caffeic acid, vanillic acid, phydroxybenzoic acid, *p*-coumaric acid and protocatechuic acid have been reported presenting in red rooibos tea (McKay and Blumberg 2007). Specifically, rooibos tea is a rare source of the dietary dihydrochalcones, aspalathin and nothofagin.

1.8.2.1.1 Aspalathin

Aspalathin is a C-linked dihydrochalcone glycoside which has thus far uniquely found in *Aspalathus linearis*, which account for 4 % to 12 % of the dry plant material (Kreuz et al. 2008, Van et al. 2015). Studies have been focused on the biological activity of aspalathin linking with

multiple health beneficial effects including antioxidant, antidiabetic, cardioprotective, antihypertensive and antimutagenic effects (Han et al. 2014, Erlwanger and Ibrahim 2017).

1.8.2.1.2 Nothofagin

Nothofagin is also a dihydrochalcone which is similar in structure to aspalathin. This contributes to its similar antioxidant capabilities with aspalathin, such as antioxidant, antidiabetic, anti-inflammatory and antithrombotic (Erickson 2003, Snijman et al. 2009, Ku et al. 2015, Liu et al. 2015). Nothofagin is a C-linked phloretin glucoside found in rooibos (Koeppen and Roux 1966). It has only been identified in one other natural source besides rooibos, the heartwood of the red beech tree (*Nothofagus fusca* (Hook F.) Oerst, Nothofagaceae) (Joubert 1996). Green rooibos tea contains higher amount of nothofagin than red rooibos tea due to the oxidation of nothofagin to other substances during the fermentation (Erickson 2003).

Rooibos tea is abundant in unique antioxidants, which may relate closely to the management and treatment of T2D. Some studies indicate the potential anti-T2D activity of rooibos and/or rooibos polyphenols are summarized as following (**Table 1.3**).

Table 1.3: Summary of some studies indicates the potential antioxidant and antidiabetic activity of rooibos and/or rooibos polyphenols in *ex vivo*, *in vivo* experimental model

Material studied	Experimental model	Durati on	Mechanisms	Reference(s)
Aqueous or alkaline extract of fermented rooibos tea	STZ-induced diabetic rats	8 weeks	Reduced levels of lipid peroxidation, malondialdehyde (MDA) and AGEs in the plasma, lens, liver and kidney and decreased creatine and total cholesterol	Ulicna et al. 2006
Fermented rooibos extract	STZ-induced diabetic rats	7 weeks	Inhibited oxidative stress through suppression of lipid peroxidation and enhanced SOD and glutathione peroxidase activity	Ayeleso et al. 2015
Unfermented rooibos tea extract	T2D KK- <i>Aγ</i> mice	5 weeks	Decreased fasting blood glucose	Kamakura et al. 2015
Unfermented rooibos extracts	High fat fed diabetic Vervet monkeys	4 weeks	Improved glucose tolerance; prevent LDL oxidation; preserved endogenous coenzyme Q10 levels and inhibited oxidative stress	Orlando et al. 2017

Fermented rooibos tea	Normolipidemic individuals	6 hours	Modulated postprandial glycemia, oxidative stress and lipemia in subjects	Francisco 2010
Aspalathin - enriched unfermented rooibos extract or fermented rooibos hot water extract	Palmitate-induced insulin resistant C2C12 cells	3 hours	Increased glucose uptake; enhanced mitochondrial activity and ATP production	Mazibuko et al. 2013
Aspalathin and nothofagin	Human umbilical vein endothelial cells and male C57BL/6 mice	6 hours	Inhibited high-glucose induced vascular hyperpermeability and monocytes adhesion to human umbilical vein endothelial cells; suppressed ROS formation and NF- κ B activation	Ku et al. 2015
Z-2-(β -D-glucopyranosyloxy)-3-phenylpropenoic acid	Obese insulin-resistant rats	3 weeks	Increased glucose uptake, glucose tolerance and mRNA expression of liver GLUT1, 2, glucokinase, PPAR; decreased fasting blood glucose	Muller et al. 2013

1.8.2.2 Honeybush tea

Honeybush tea (*Cyclopia spp.*) is native to South Africa and have a growing worldwide market. It enjoys the reputation as a caffeine-free tea with a pleasant aroma and fruity honey-like flavour, which attributes to its aromatic volatiles, monoterpenes, phenylethyl alcohol and 5-methylfurfural (Wang et al. 2005). Honeybush is abundant in various phenolic compounds such as mangiferin, isomangiferin, luteolin 7-rutinoside, diosmin, hesperidin, luteolin, and hesperitin. The principal polyphenols in honeybush (*Cyclopia genistoides*) include the xanthone mangiferin and the flavonones hesperitin.

1.8.2.2.1 Mangiferin

Honeybush tea (*Cyclopia spp.*) is the greatest dietary source of mangiferin (Matkowski et al. 2013). Mangiferin (1,3,6,7-tetrahydroxyxanthone-C2- β -D glucoside) is a polyphenol compound found in honeybush tea, which makes up to about 4% of tea leaves by dry weight (Gold-Smith et al. 2016). Several *in vivo* studies have been reported its antitumor, antidiabetic, antioxidative, and hepato-protective effects (Guha et al. 1996, Sánchez et al. 2000, Yoshikawa et al. 2002).

1.8.2.2 Hesperidin

Hesperidin is another abundant polyphenol in honeybush tea. Some previous studies have been reported for its biologically beneficial activities, such as antioxidant, cholesterol lowering and anticarcinogenic effects (Garg et al. 2001, Muruganandan et al. 2005).

The antidiabetic effects of honeybush tea and some of its major polyphenols has been well documented *in vitro*, *ex vivo*, *in vivo* animal and human studies, some of which are shown in **Table 1.4**.

Table 1.4: Summary of some studies indicates the potential antioxidant and antidiabetic activity of honeybush and/or honeybush polyphenols in *ex vivo*, *in vivo* experimental model

Material studied	Experimental model	Duration	Mechanisms	Reference(s)
<i>Cyclopia maculata</i> (fermented and unfermented) and <i>Cyclopia subternata</i> (unfermented) extract	3T3-L1 mouse adipocytes	8 days	Inhibited intracellular triglyceride and fat accumulation, and decreased the expression of PPAR2	Dudhia et al. 2013
Aqueous <i>Cyclopia maculata</i>	Differentiated 3T3-L1 adipocytes	24 hours	Fermented <i>Cyclopia maculata</i> extract at 80 g/mL induce maximal lipolysis, along with increased protein expression of hormone sensitive lipase and perilipin	Pheiffer et al. 2013
Mangiferin	Partially pancreatectomized mice	14 days	Improve glycemia and glucose tolerance; increased serum insulin levels; enhanced β -cell hyperplasia; elevated β -cell proliferation and reduce β -cell apoptosis	Ajuwon et al. 2018
Mangiferin	STZ-diabetic rats	12 weeks	Decreased albuminuria; restored the nephrin expression and inhibited glomerular extracellular matrix expansion	Wang et al. 2018
Hesperidin	STZ-induced diabetic rats	4 weeks	Reduce serum urea and creatinine levels; decreased levels of MDA, TGF-1 β and 8-OHdG in the kidney; Increased renal glutathione concentration	Kandemir et al. 2018
Fermented honeybush (green ethanol soluble extracts); mangiferin; hesperidin	SHK-1 mice	10 days	Fermented honeybush tea decreased oedema, epidermal hyperplasia cyclooxygenase-2, ornithine decarboxylase, GADD45 and OGG1/2 expression; fermented honeybush tea extract reduced lipid peroxidation via increasing SOD	Petrova et al. 2011

			and catalase activity; hesperidin and magiferin were less than effective.	
Aqueous unfermented <i>Cyclopia maculata</i>	STZ-induced diabetes Wistar rats	15 days	Improved glucose tolerance; decreased fasting plasma glucose levels; reduced total triglyceride levels and decreased the glucose-to-insulin ratio.	Chellan et al. 2014
<i>Cyclopia intermedia</i> extract	Obese, insulin-resistant Wistar rats	3 months	Lowered fasting blood glucose concentrations; lowered total plasma cholesterol concentrations and decreased α -cell size	Muller et al. 2011

A review of the literature shows that rooibos tea and honeybush tea have significantly different phytochemical constituents from those found in *Camellia sinensis* (See **Table 1.5**).

Table 1.5: Summary of some major phytochemicals of *Camellia sinensis* tea, rooibos and honeybush herbal teas

<i>Camellia sinensis</i>	References	Rooibos (<i>Aspalathus linearis</i>)	References	Honeybush (<i>Cyclopia</i> species)	References
Epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), gallocatechin (GC), theanine, caffeine, theaflavin, thearubigin, vitamins (B1, C, B6)	Harbowy et al. 1997, Liang et al. 2001, Punyasiri et al. 2004, Mamati et al. 2006, Yousaf et al. 2014	Aspalathin; aspalalinin Z-2-(β -D-glucopyranosyloxy)-3-phenylpropenoic acid Nothofagin Flavones: luteolin, luteolin-7-O-glucoside, vitexin and iso-vitexin, orientin, iso-orietin and chrysoeriol	Koeppe and Roux 1966, Shimamura et al. 2006 Muller et al. 2013 Joubert et al. 2009 Koeppe and Roux 1965, Snyckers and Salemi 1974, Bramati et al. 2002,	Mangiferin, hesperidin, isosakuranetin isomangiferin, hesperidin Flavanones: hesperetin, enocitrin, narirutin, naringenin Flavanols: kaempferol glucosides, coumestans	De Nysschen, et al. 1996, Joubert et al. 2008 Joubert et al. 2008 Ferreira et al. 1998, Kamara et al. 2003, Joubert et al. 2009

Flavanones:	Kazuno et al.	Flavones:
Hemiphlorin, dihydro-	2005,	luteolin, diosmetin
orientin, dihydro-	Krafczyk	
isoorietin	and Glomb	
	2008,	
Flavanols:	Marnewick	Isoflavones:
rutin, quercetin, iso-	2009	formononetin,
quercitrin, quercetin-3-		calycosin, wistin
roninobioside		

1.9 Rationale of the study

A review of the literature shows the effectiveness of Chinese teas and South African teas in the management and treatments of T2D. With the need for the discovery of alternate therapies due to the deficiencies found in synthetic drugs, there is a need to do more research with teas with the hope of the better management of diabetes since these teas are widely consumed in China and South Africa.

1.10 Scope and limitations of the study

The focus of the present study was to compare some widely used Chinese and South African teas and to identify the antidiabetic potentials of these teas.

This study covered the antioxidant and antidiabetic screening of Chinese tea (zhengshanxiaozhong black tea, jasmine green tea) and South African teas (red rooibos tea, green rooibos tea, red honeybush tea) using several *in vitro*, *ex vivo* and ultimately *in vivo* experiments. Some bioactive components were also identified from the above-mentioned teas and different tea solvent extracts and hot water extracts using GC-MS and LC-MS analysis.

This study did not investigate the *in vivo* antidiabetic potentials of zhengshanxiaozhong black tea and red rooibos tea due to the unavailability of teas and the limited research time and number of animals.

1.11 Aim and objectives of the study

The present study aimed to examine not only the antioxidant and antidiabetic effects in multi-mode of selected Chinese teas (zhengshanxiaozhong black tea, jasmine green tea) and South African teas (red rooibos tea, green rooibos tea, red honeybush tea) but also to identify their possible bioactive antidiabetic phytochemicals and possible mechanism underlying those actions.

The specific objectives are as follow:

1. To evaluate the antioxidant activities of dichloromethane (DCM), ethyl acetate, ethanol and aqueous sequential extracts of Chinese and South African teas using *in vitro* and *ex vivo* models.
2. To assess the *in vitro* antidiabetic effects of the studied teas via inhibition of key enzymes linked to diabetes.
3. To evaluate the *in vitro* and *ex vivo* antioxidant and antidiabetic activities of the sequential extracts and concentrated hot water extract of Chinese and South African teas.
4. To assess the antioxidant and antidiabetic activities of the most effective infusion extract of Chinese and South African teas in T2D model of rats.
5. To identify the possible bioactive antidiabetic compounds in all studied teas employing Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS).

CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 Experimental design

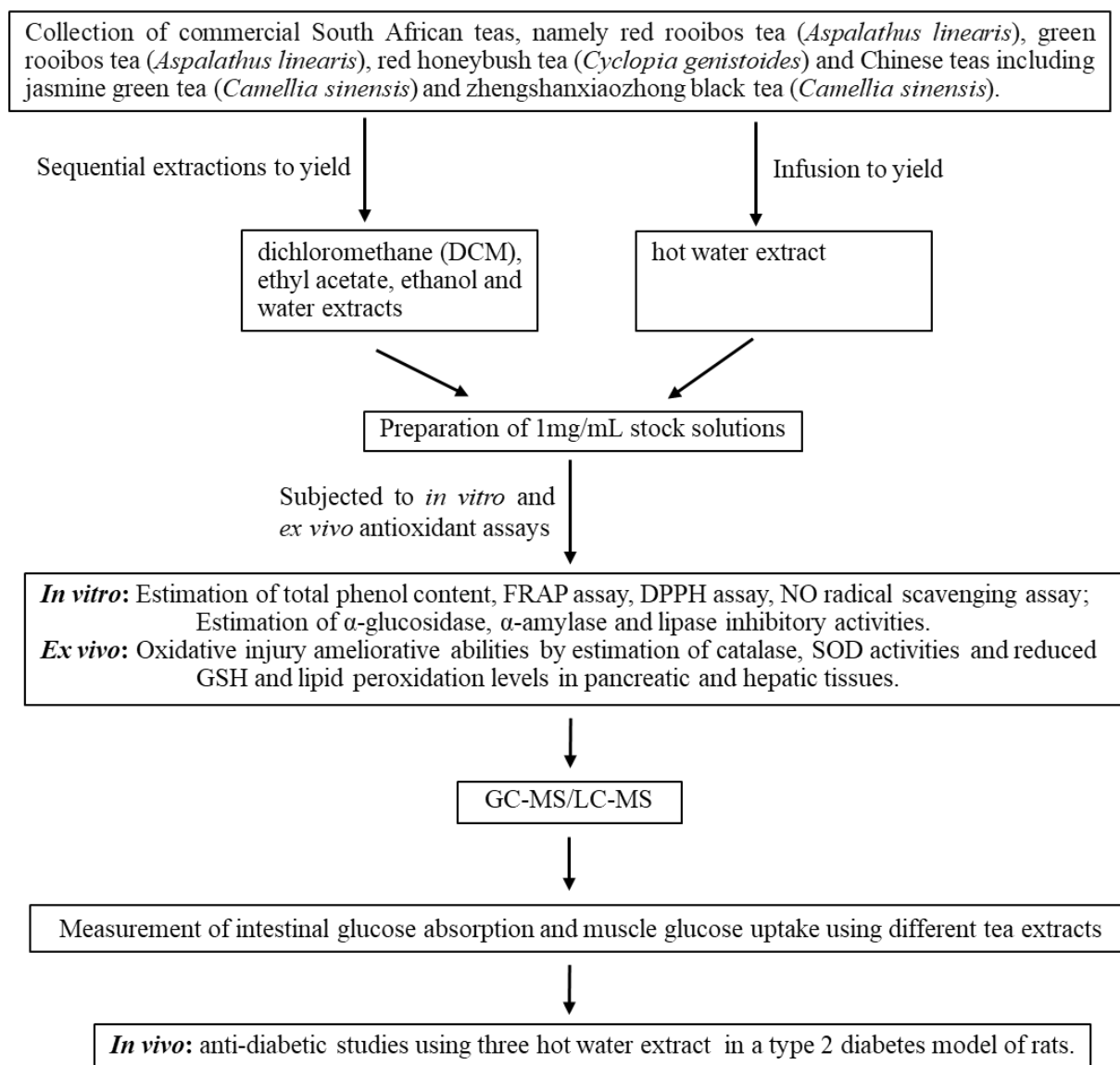


Figure 2.1: Flowchart demonstrating the entire experimental approach.

2.2 Collection of teas

Commercial Chinese teas including jasmine green (*Camellia sinensis*) and ZSXZ black tea (*Camellia sinensis*) in loose leaf were purchased from local tea shops in Fuzhou, China and South African tea, namely red rooibos (*Aspalathus linearis*), green rooibos (*Aspalathus linearis*), red honeybush (*Cyclopia genistoides*) in tea bags were purchased from local food malls in Durban, South Africa.

2.2.1 Preparation of crude extracts

Individually, 100 grams of each tea were sequentially extracted with different solvents following increasing polarity at 1:20 tea and solvents ratio and allowed to stand for 48 hours at room temperature respectively to yield dichloromethane (DCM), ethyl acetate, ethanol and aqueous extracts. All extracts except aqueous were then filtered using Whatman filter paper (No.1) and the filtrates were concentrated *in vacuo* using a rotatory evaporator (Buchi RII; Flawil, Switzerland) followed by drying under a fume hood at room temperature. The aqueous extracts were concentrated using a water bath at 50°C overnight (**Figure 2.2**). The weights of the different dry extracts were recorded, then stored in glass vials at 4°C until when needed.

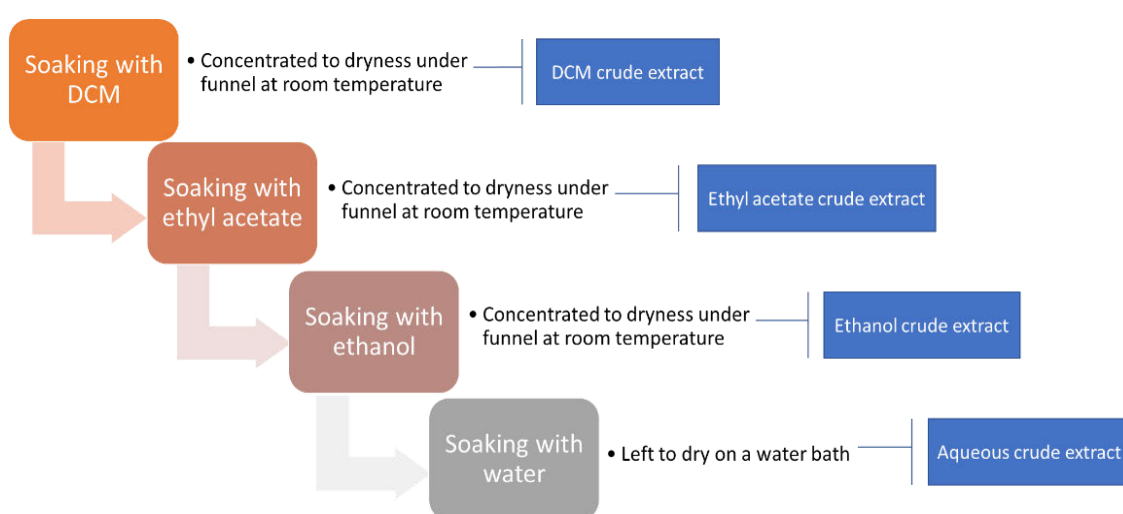


Figure 2.2: Flowchart of the sequential extraction of tea samples.

2.2.2 Preparation of hot infusions

Five tea bags (2 g each) or 10 g of each loose-leaf teas were infused in a boiling water bath at 1:10 tea and distilled water ratio and allowed to stand for 2 hours. The infusions were filtered with prewashed cotton and concentrated using a water bath at 50°C, then collected the hot water extracts and stored in airtight vials at 4°C until further analysis.

2.2.3. Preparation of stock solutions

A stock solution (1 mg/mL) was prepared from each tea sequential extracts or hot water infusions using distilled water, from which different working concentrations at 15, 30, 60, 120 and 240 µg/mL were prepared for subsequent *in vitro* and *ex vivo* studies.

2.2.4. Total phenolic content

The principle of this assay is that phenolic hydroxyl groups can react with specific redox reagents (Folin Ciocalteu reagent) under alkaline conditions to form a blue chromophore. The absorption is positively related to the content of phenolic compounds and the maximum absorption occurs at a wavelength of about 765 nm (McDonald, Prenzler et al. 2001). Briefly, 200 μL of the tea extracts or tea infusions (240 $\mu\text{g}/\text{mL}$) was incubated with 1 mL of 10 times diluted Folin Ciocalteu reagent and 800 μL of 0.7 M Na_2CO_3 at room temperature for 30 minutes. Then the absorbance was determined at 765 nm on a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). All measurements were done in triplicate and indicated in milligrams of gallic acid equivalent (GAE) per gram dry weight.

2.3 *In vitro* anti-oxidative activities assay

2.3.1 Free radical scavenging activity (DPPH) assay

The principle of this assay is that 1,1-diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical due to the delocalisation of the spare electron over nitrogen and its alcoholic solution is a deep violet colour, which is measured at about 517 nm. When the DPPH solution mixes with an antioxidant substance that donates a hydrogen atom, then its colour changes to a residual pale-yellow colour from the picryl group.

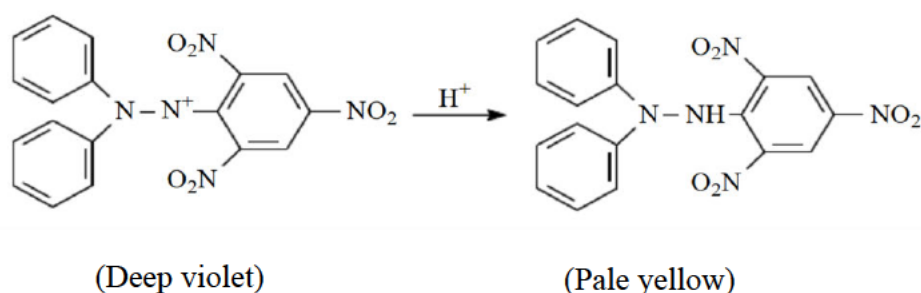


Figure 2.3: Reaction mechanism of 1,1-diphenyl-1-picrylhydrazyl (DPPH) with antioxidant (Oscar, Ngoung et al. 2018).

Spectrophotometer was used to detect the change in the absorbance of DPPH after reaction with the sample solution, which estimates the ability of the sample to donate hydrogen atoms and scavenge free radicals and resist oxidation (Ak and Gülçin 2008). Briefly, 50 μL at a 0.3 mM solution of DPPH (in methanol) was added to a 96 well plate contained 100 μL of tea sequential extract or infusion of various concentrations (15-240 $\mu\text{g}/\text{mL}$). Then, these solutions

were incubated in the dark condition at room temperature for 30 minutes. The absorbance was measured at 517 nm for the blank without tea sequential extract or infusion. Ascorbic acid, gallic acid and Trolox were used as standards. DPPH inhibition was calculated using the following formula:

$$\% \text{ Scavenging activity} = \frac{\text{Abs (control)} - \text{Abs (test sample)}}{\text{Abs (control)}} \times 100$$

Abs: absorbance

2.3.2 Ferric cyanide (Fe³⁺) reducing/antioxidant power (FRAP) assay

The principle of this assay is based on the ability of antioxidants to reduce Fe³⁺ to blue-violet Fe²⁺ at low pH leading to formation of a bluish violet ferrous-probe complex which is detected at a wavelength around 700 nm (Oyaizu 1986). Briefly, 100 µL of tea sequential extract or infusion (15-240 µg/mL) mixed with 1 mL sodium phosphate buffer (0.2 M, pH 6.6) and 100 µL 1% potassium ferricyanide were incubated at 50°C for 30 minutes. After incubation for 30 minutes, the reaction mixtures were acidified with 100 µL of 10% trichloroacetic acid. Then, 100 µL of distilled water and 200 µL of 0.1% FeCl₃ were sequentially added. The absorbance was measured at 700 nm. Ascorbic acid was utilized as a control in this assay. The results were expressed as a percentage of the absorbance of the sample to the absorbance of gallic acid and calculated using the following formula:

$$\% \text{ Ferric reducing antioxidant power} = \frac{\text{Abs (sample)}}{\text{Abs (gallic acid)}} \times 100$$

Abs: absorbance

2.3.3 Nitric oxide (NO) radical scavenging assay

This assay is based on sodium nitroprusside solution (in aqueous) at physiological pH spontaneously generating NO. NO interacts with oxygen to create nitrite ions which can be evaluated by using the Griess reagent. Thus, NO scavengers compete with oxygen reducing the NO generation (Kurian et al. 2010). Briefly, 100 µL of 10 mM sodium nitroprusside in

phosphate buffer saline (pH 7.4) was added to 100 μ L of each tea sequential extract or infusion at various working concentrations (15-240 μ g/mL) and then incubated at 37°C for 2 hours. A volume of 100 μ L Griess reagent was then mixed with the reaction mixture. The absorbance of the chromophore was read at 546 nm which was formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthyl ethylenediamine. Inhibition of NO generated was measured by comparing with the absorbance value of a control (sample without tea sequential extract or infusion). All assays were carried out in triplicate. Quercetin was used as the standard drug. The NO radical scavenging activities were calculated using the following formula:

$$\% \text{ inhibitory activity} = \left(1 - \frac{Abs}{Abc}\right) \times 100$$

Abs: absorbance of the sample and Abc: absorbance of the control

2.4 *In vitro* antidiabetic and antiobesogenic enzymes inhibitory assays

2.4.1 Determination of α -glucosidase inhibitory activity of tea extracts or infusions

This assay is based on the hydrolysis of the colourless p-nitrophenyl- α -D-glucopyranoside (pNPG) by α -glucosidase to release p-nitrophenol (pNP), which is detected at a wavelength around 405 nm. The inhibitory activity of the sample was determined according to the absorbance of the solution. Lowest absorbance shows the lowest concentration of pNP and its indication of the highest inhibitory activity of α -glucosidase (Ademiluyi and Oboh 2013). Briefly, a volume of 250 μ L of each tea sequential extract or infusion or acarbose at different working concentrations (15-240 μ g/mL) was mixed with 500 μ L of 1.0 U/mL yeast α -glucosidase solution in phosphate buffer (100 mM, pH 6.8) and incubated at 37°C for 15 minutes. Then, a volume of 250 μ L 5 mM pNPG solution dissolved in the same phosphate buffer was added. The reaction mixture was further incubated at 37°C for 20 minutes. The absorbance of the released pNP was recorded at 405 nm and the inhibitory activity was expressed as percentage of the control without the inhibitors. All assays were carried out in triplicate. Acarbose was utilized as the standard drug. The inhibitory activities of α -glucosidase were calculated using the following formula:

$$\% \text{ inhibitory activity} = \left(1 - \frac{Abs}{Abc}\right) \times 100$$

Abs: absorbance of the sample and Abc: absorbance of the control

2.4.2 Determination of α -amylase inhibitory activity of the tea extracts or infusions

This assay is based on reducing sugars undergoing a redox reaction with DNS, and the product is brownish-red when boiled. The depth of the product colour is proportional to the amount of reducing sugar, and the content of reducing sugar was measured by colorimetry (Shai et al. 2010). Briefly, 250 μ L tea sequential extract or infusion or acarbose at different working concentrations (15-240 μ g/mL) was mixed with 500 μ L porcine pancreatic amylase (2 U/mL in 100 mM pH 6.8 phosphate buffer) and later incubated at 37°C for 20 minutes. Then, 250 μ L 1% starch dissolved in the same phosphate buffer was added to the reaction mixture following by 1 hour incubation at 37°C. 1 mL of DNS solution (0.25 g DNS in 5 ml of 2 N NaOH) was then added and allowed to boil for 10 minutes. The absorbance of the resulting mixture was determined at 540 nm and the inhibitory activity was compared to the control without the inhibitors. All assays were carried out in triplicate. Acarbose was utilized as the standard drug. The inhibitory activities of α -amylase were calculated using the following formula:

$$\% \text{ inhibitory activity} = \left(1 - \frac{Abs}{Abc}\right) \times 100$$

Abs: absorbance of the sample and Abc: absorbance of the control

2.4.3 Estimation of pancreatic lipase inhibitory activity

This assay is based on the hydrolysis of the substrate p-NPB (p-nitrophenyl butyrate) in dimethylformamide to p-nitrophenol (pNP) by lipase. The p-NPB hydrolysis was monitored by absorbance changes at 405 nm (Kim, Lee et al. 2010). Briefly, a solution of porcine pancreatic lipase (2.5mg/mL) was mixed with 10 mM MOPS (morpholinepropanesulphonic acid) and 1 mM pH 6.8 EDTA to prepare an enzyme-containing buffer. A volume of 100 μ L of tea sequential extract or infusion or Orlistat (100 μ M) at different working concentrations (15-240 μ g/mL) and 169 μ L of Tris buffer (100 mM Tris-HCl and 5 mM CaCl₂, pH 7.0), 20

μL of freshly prepared enzyme buffer were pipetted into separate wells of a 96-well plate. The plates were initially incubated at 37°C for 15 minutes. Thereafter, 5 μL of 10 mM p-NPB was added and further incubated at 37°C for 30 minutes. The absorbance was determined by the hydrolysis of p-NPB to pNP at 405 nm. All assays were carried out in triplicate. Orlistat was utilized as the standard drug. The inhibitory activity of lipase was calculated using the following formula:

$$\% \text{ inhibitory activity} = \left(1 - \frac{Abs}{Abc}\right) \times 100$$

Abs: absorbance of the sample and Abc: absorbance of the control.

2.5 *Ex vivo* antioxidant assays

2.5.1 Animal

Fifteen adult male Sprague-Dawley rats with a mean body weight of 211.16 ± 22.43 g were procured from the Biomedical Resource Unit (BRU), Westville Campus, University of KwaZulu-Natal, Durban, South Africa. Before sacrifice, the food was removed and water given *ad libitum* for 12 hours. The animals were euthanized by overdose of isoflurane and the whole gastrointestinal tract, partial psoas muscle, pancreas and liver were harvested and immediately tested for psoas muscle glucose uptake, small intestine glucose absorption and oxidative stress studies. All animals were maintained following the rules and regulations of the Animal Ethics Research Committee of the University of KwaZulu-Natal, South Africa (protocol approval number: AREC/020/019D).

2.5.2 Preparation of tissue homogenates

A 0.5 gram of freshly excised tissue was homogenized in 5 mL of homogenization buffer (50 mM sodium phosphate buffer with triton X-100, pH 7.5). Then, the homogenate was centrifuged using a refrigerated centrifuge at 15000 rpm for 15 minutes at 4°C. After centrifugation, the supernatant was collected in sample tubes and stored at -20°C until needed.

2.5.3 Induction of *ex vivo* oxidative stress and treatment

Oxidative stress was induced in isolated hepatic or pancreatic tissue according to a previously published method described by Adefegha and Oboh (2011). Briefly, a volume of 30 μL freshly

prepared FeSO₄, 15 mM was added to 100 μL tea sequential extract or infusion and the standard drug at different working concentrations (15-240 μg/mL) and incubated with 100 μL tissue homogenate in 5% CO₂ for 30 min at 37°C. A reaction mixture contained no tea sequential extract or infusion or standard drug served as the negative control i.e. untreated. Thereafter, the incubated samples were assessed for reduced glutathione (GSH) (Ellman 1959); superoxide dismutase (SOD) (Misra and Fridovich 1977); catalase (Aebi 1984) and lipid peroxidation (LPO) (Chowdhury and Soulsby 2002).

2.5.4 Reduced glutathione (GSH) assay

This assay is based on the rapid oxidation of GSH via 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) to generate GSSG and 5'-thio-2-nitrobenzoic acid (TNB) (yellow). The formed oxidized glutathione (GSSG) can be converted to GSH through glutathione reductase in a reaction containing the cofactor NADPH (Rahman, Kode et al. 2006).



Figure 2.4: The oxidation of GSH to GSSG by DTNB.

Briefly, 300 μL of the incubated sample was precipitated with 100 μL of 10% TCA and then centrifuged at 15000 rpm for 10 minutes. Then, 80 μL of the supernatant was pipetted into a 96-well plate, 40 μL of 0.5 mM DTNB and a 200 μL of sodium phosphate buffer (0.2 M, pH 7.8) were sequentially added. The reaction mixture was then incubated at room temperature for 15 min before the absorbance was determined at 412 nm. Different GSH concentrations were used to plot a standard curve and the GSH concentration of tests was then extrapolated from the standard curve.

2.5.5 Superoxide dismutase (SOD) enzyme activity

This assay is based on the inhibitory activity of SOD converting superoxide radicals into molecular oxygen (O₂) and hydrogen peroxide (H₂O₂). The H₂O₂ produced from the dismutation of the superoxide ion (O₂^{·-}) by SOD can oxidize 6-hydroxydopamine (6-HD) to form a coloured product which can be spectrophotometrically determined at 492 nm, while the diethylenetriaminepentaacetic acid (DETAPAC) was employed to suppress the aerobic autoxidation of 6-HD (Kakkar et al. 1984). Briefly, a volume of 170 μL of 0.1 mM DETAPAC and 15 μL of the incubated homogenate were pipetted into respective wells of a 96-well plate.

Then, 15 μL of freshly prepared 6-HD (1.6 mM) was immediately added into the reaction mixture and gently tapping the sides of the plate to mix the solution. The absorbance was measured at 492 nm for 3 minutes at 1 min intervals. The SOD enzyme activity of test was calculated using the following formula:

$$\text{Activity} = \frac{(A1 - Ab) \times Rv \times Df}{\epsilon 49 \times Sv}$$

$\epsilon 490$: the standard molar absorptivity at 490 nm wavelength given as 1.742/mM/cm

A1: sample reaction rate, Ab: blank reaction rate, Rv: the volume of the reaction, Df: the dilution factor, Sv: the volume of the sample

2.5.6 Catalase activity

This assay is based on the reaction of undecomposed H_2O_2 with ammonium molybdate to form a yellowish colour, which has a maximum absorbance at 374 nm. Meanwhile, catalase catalyses the decomposition of H_2O_2 into water and molecular oxygen, and plays the role of transferring electrons in the reaction (Hadwan and Abed 2016). Briefly, 100 μL of the incubated homogenate was mixed with 1 mL of H_2O_2 (65 μM) in sodium phosphate buffer (6.0 mM, pH 7.4) and allowed to stand for 3 minutes. Then, 4 mL of ammonium molybdate (32.4 mM) was added. The absorbance was measured at 347 nm to record the alterations yellow-coloured molybdate/ H_2O_2 complex to compare the control containing H_2O_2 only. The catalase activity was calculated using the following formula:

$$\text{Catalase activity of test } kU = \frac{2.303}{t} \times \left[\log \frac{S^\circ}{S - M} \right] \times \frac{Vt}{Vs}$$

t: time, S° : absorbance of standard tube (containing all reagents except tissue sample), S: absorbance of test samples, M: absorbance of control test sample (contains all reagents except H_2O_2), Vt: summation of volume of total reagents in test tube, Vs: test sample volume

2.5.7 Determination of lipid peroxidation

This assay is based on the reaction in acidic and heated conditions between malondialdehyde (MDA) and thiobarbituric acid (TBA) from the homogenate, forming a pink and fluorescent chromogen (MDA-TBA₂), which can be spectrophotometrically determined at 532 nm (Oboh et al. 2012).

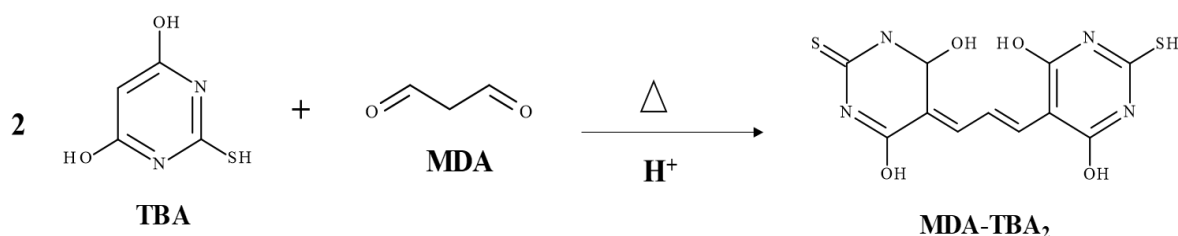
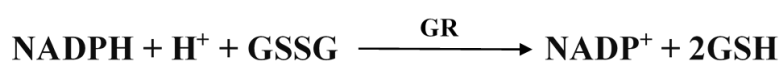


Figure 2.5: The formation of MDA-TBA₂.

Briefly, 200 μ L of the incubated homogenate was mixed with 200 μ L of 8.1% SDS solution, 750 μ L of 20% acetic acid, 2 ml of 0.25% TBA solution and 850 μ L of miliQ H₂O. Then the reaction mixture was boiled in a water bath for 1 hour. After cooling, the cooled incubated samples were pipetted into a 96-well plate. The absorbance was determined at 532 nm. The MDA concentration of the tests was extrapolated from the MDA standard curve.

2.5.8 Determination of glutathione reductase (GR) activity

This assay is based on measuring the glutathione reductase activity spectrophotometrically by recording a reduction in absorbance of the NADPH at 340 nm (Smith et al. 1988).



Briefly, 10 μ L of sample or glutathione reductase buffer (at a concentration of 50 mM Tris.HCl and 1 mM EDTA solution, pH 8.0) for blank was placed in a 96-well plate and 221 μ L of the buffer and 38 μ L of GSSG solution (8 mM) was added sequentially. Then, 10 μ L of NADPH was added into the reaction followed by immediate mixing by gently tapping the sides of the 96-well plate. The linear decrease in absorbance at 340 nm was quickly noted for 8 min at 2 min interval using a multi-well plate reader. The activity of the glutathione reductase enzyme was calculated using following formula:

$$\text{Activity} = 1000 \times \left(\frac{A1 - Ab}{\epsilon_{340}} \right) \times 0.5 \mu\text{mol}/\text{min}/\mu\text{g of protein}$$

ϵ_{340} : the molar absorptivity at 340 nm which is 6.22 mM/cm, A1: sample reaction rate and Ab: blank reaction rate

2.6 *Ex vivo* antidiabetic activity assay

2.6.1 Determination of glucose absorption in isolated rat small intestine

The inhibitory activity of the different working concentrations (30-240 $\mu\text{g/mL}$) of tea sequential extracts or infusions on small intestinal glucose absorption was determined according to a previously published method (Hassan et al. 2010) as modified by Chukwuma, Mopuri et al. (2018) which recorded the glucose concentrations in an incubation mixture consisting of the 5 cm pre-cut rat jejunum and the test samples (**Figure 2.6**). Briefly, the jejunal segments of the freshly harvested rat small intestine were cut into 5 cm segments and the inner jejunal lumen was removed and rinsed with Krebs buffer (118 mM NaCl, 5 mM KCl, 1.328 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 and 25 mM NaHCO_3) using a sterile syringe. Then, the villi of each divided segments were exposed and incubated with tea sequential extracts or infusions at different working concentrations (30-240 $\mu\text{g/mL}$) in an incubation tube consisting of 8 mL of Krebs buffer and 11.1 mM glucose in a Steri-Cult CO_2 incubator (Labotec, South Africa) with 5% CO_2 , 95% oxygen and 37°C settings for 2 hours. Rat small intestine incubated with glucose but without tea sequential extracts or infusions in Krebs buffer used as the normal control. Blank was Krebs buffer with glucose only. The glucose concentration in the samples were determined employing an Automated Chemistry Analyzer (Labmax Plenno, Labtest Inc., Costa Brava, Brazil). The small intestinal glucose absorption was extrapolated as the glucose quantity that absorbed by per centimetre of rat jejunum and calculated using the following formula:

$$\text{Abdominal glucose absorption} = \frac{GC1 - GC2}{\text{length of jejunum (cm)}}$$

GC1: glucose concentrations (mg/dL) before incubation

GC2: glucose concentrations (mg/dL) after incubation

2.6.2 Determination of glucose uptake in harvested rat psoas muscle

The effect of the different concentrations (30-240 µg/mL) of the tea sequential extracts or infusions on harvested rat psoas muscle glucose uptake was determined according to the procedure explained in a previous study (Hassan et al. 2010) as modified by (Chukwuma et al. 2018) (**Figure 2.6**). The harvested psoas muscle was briefly cleaned with Krebs buffer and diced into small chunks of the same weight (0.5 g). Then, different concentrations of the extracts were incubated with each chunk in 8 ml of Krebs buffer (pre-mixed with 11.1 mM glucose) in a Steri-Cult CO₂ incubator (Labotec, South Africa) with 5% CO₂, 95% oxygen and 37°C temperature settings for 2 hours. Psoas muscle incubated without tea sequential extracts or infusions in Krebs buffer used as the normal control. Blank was Krebs buffer with glucose only. Metformin at concentration of 2 mg/mL was employed as the standard drug i.e. positive control. A volume of 1 mL sample was collected before and after the incubation period from each incubation tube. The glucose concentration was measured employing a Chemistry Analyzer (Labmax Plenno, Labtest Inc., Costa Brava, Brazil). The muscle glucose uptake was extrapolated as the glucose quantity that used up by each gram of rat psoas muscle and calculated using the following formula:

$$\text{Rat psoas muscle glucose uptake} = \frac{GC1 - GC2}{\text{weight of muscle tissue (g)}}$$

GC1: glucose concentrations (mg/dL) before incubation

GC2: glucose concentrations (mg/dL) after incubation

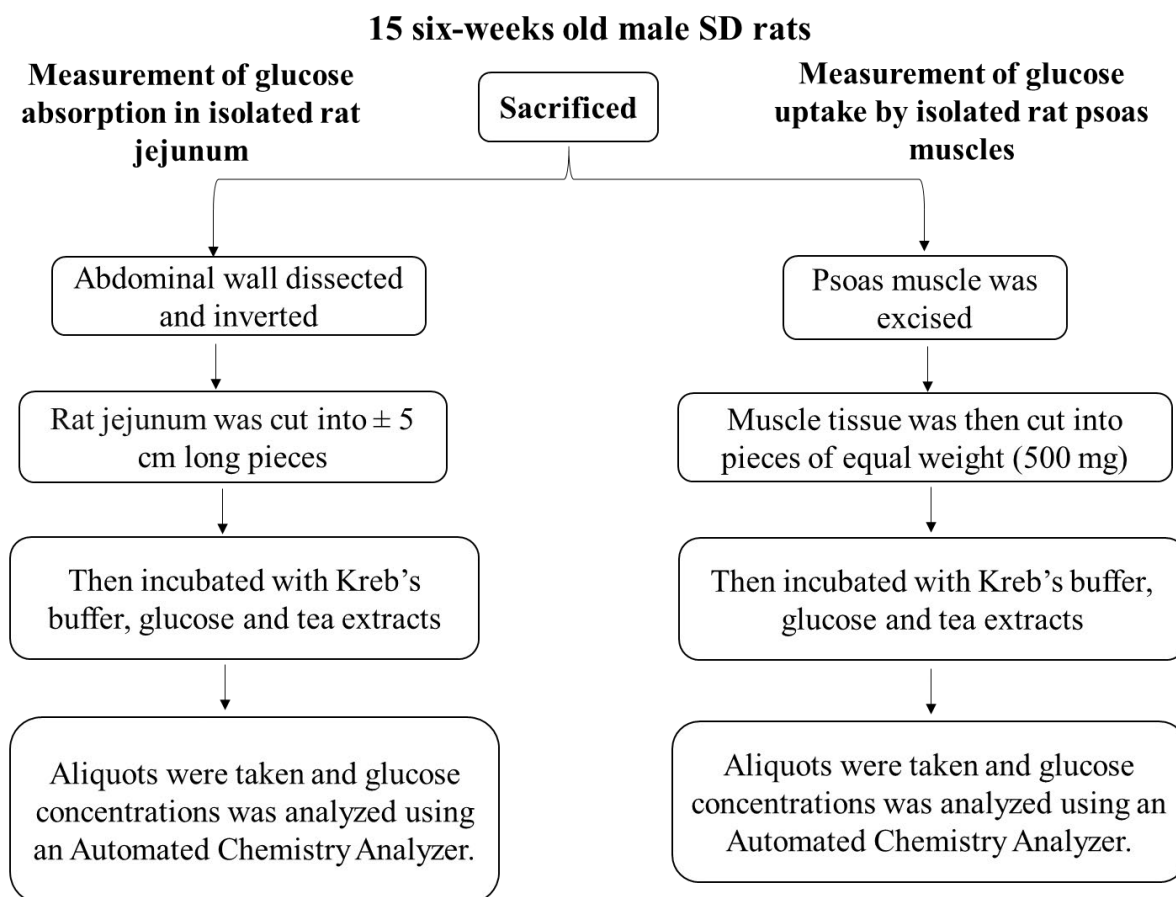


Figure 2.6: Flow diagram describing the procedure of isolated rat small intestine glucose absorption and harvested rat psoas muscle glucose uptake.

2.7 Identification of phytochemicals in tea sequential extracts and infusions

2.7.1 Gas chromatography-mass spectrometric (GC-MS) analysis

The tea sequential extracts were analysed on an Agilent Technologies 6890 Series GC coupled with (an Agilent) 5973 Mass Selective detector equipped with Agilent Chemstation software. An HP-5MS capillary column was used (30 m × 0.25 mm ID, 0.25 µm film thickness, 5% phenylmethylsiloxane). Ultra-pure helium was used as a carrier gas at a flow rate of 1.0 mL/min and a linear velocity of 37 cm/sec. The injector and oven temperatures were set at 250°C and 60°C, respectively. The oven was programmed to 280°C at the rate of 10°C/min with a hold time of 3 minutes. Injections of 1 µL were made in splitless mode with a split ratio of 20:1. The mass spectrometer was operated in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230°C, quadrupole temperature 150°C, solvent delay 4 minutes and scan

range 50-70 amu. Compounds were identified by direct comparison of their mass spectral data with those in the NIST library.

2.7.2 Liquid chromatography-mass spectrometric (LC-MS) analysis

The tea water extracts were subjected to LC-MS analysis using Shimadzu LC-MS-2020 Single Quadrupole Liquid Chromatograph Mass Spectrometer (LC-MS) by direct injection into the machine via a loop. The LC stop time was set at 4.00 min. The PDA sampling frequency was set at 1.5625 Hz and the operating mode was on low pressure gradient. Other operating parameters were as follows: Pump A: LC-2030 Pump, Flow: 0.2000 mL/min, B Conc.: 95.0%; C Conc.: 0.0%; D Conc.: 0.0%; Mobile Phase A: Water; Mobile Phase B: Methanol; Start Wavelength: 190 nm; End Wavelength: 800 nm; Cell Temp.: 40°C; Start Time: 0.00 min; End Time: 4.00 min; Acquisition Mode: Scan Polarity: Positive; Event Time: 1.00 sec; Detector Voltage: +1.00 kV; Threshold: 0; Start m/z: 50.00; End m/z: 1700.00; Scan Speed: 1667 u/sec. Compounds were identified by direct comparison of mass spectral data with those in the <http://foodb.ca/spectra/ms/search>.

2.8 *In vivo* antidiabetic activity of the tea hot water extract in a type 2 diabetes rats model

2.8.1 Animals

Six-week-old male Sprague-Dawley rats were procured from the Biomedical Resource Unit (BRU), Westville Campus, University of KwaZulu-Natal, Durban, South Africa. Animals were randomly grouped into thirteen (13) groups as follows, with five (5) rats each in the normal or non-diabetic groups and seven (7) rats each in the diabetic groups (**Figure 2.7**):

Normal control (NC): Normal rats supplied with normal diet and drinking water

Norma jasmine green tea (NJG): Normal rats administered with jasmine green tea infusion (300 mg per kg body weight).

Normal red honeybush tea (NRH): Normal rats administered with red honeybush tea infusion (300 mg per kg body weight).

Normal green rooibos tea (NGR): Normal rats administered with green rooibos green tea infusion (300 mg per kg body weight).

Diabetic control (DBC): Diabetic rats supplied with normal diet and drinking water

Diabetic jasmine green tea infusion low dose (DJGL): Diabetic rats administered with a low dose of jasmine green tea infusion (150 mg per kg body weight).

Diabetic jasmine green tea infusion high dose (DJGH): Diabetic rats administered with a high dose of jasmine green tea infusion (300 mg per kg body weight).

Diabetic red honeybush tea infusion low dose (DRHL): Diabetic rats administered with a low dose of red honeybush tea infusion (150 mg per kg body weight).

Diabetic red honeybush tea infusion high dose (DRHH): Diabetic rats administered with a high dose of red honeybush tea infusion (300 mg per kg body weight).

Diabetic green rooibos tea infusion low dose (DGRL): Diabetic rats administered with a low dose of green rooibos tea infusion (150 mg per kg body weight).

Diabetic green rooibos tea infusion high dose (DGRH): Diabetic rats administered with a high dose of green rooibos tea infusion (300 mg per kg body weight).

Diabetic glibenclamide (DBG): Diabetic rats administered with glibenclamide (5 mg per kg body weight).

Diabetic metformin (DBM): Diabetic rats administered with metformin (300 mg per kg body weight).

The animals were housed in a maximum of two-in-one medium-sized polycarbonate cage and kept in a temperature and humidity-controlled room in a natural photo period of 12-hour light-dark cycle. All animals were fed with a commercial rat pellet diet and given normal drinking water during the entire experimental period. Papers and tubes were put in cages for the rat's environmental enrichment. Reduction of body weight after the injection of streptozotocin is very common and it is also a part of the pathogenesis of the diabetic condition. However, most of the animals were started to regain their body weight within a short period after the injection of streptozotocin. All animals used were maintained under the rules and regulations of the Experimental Animal Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa (protocol approval number: AREC/020/019D).

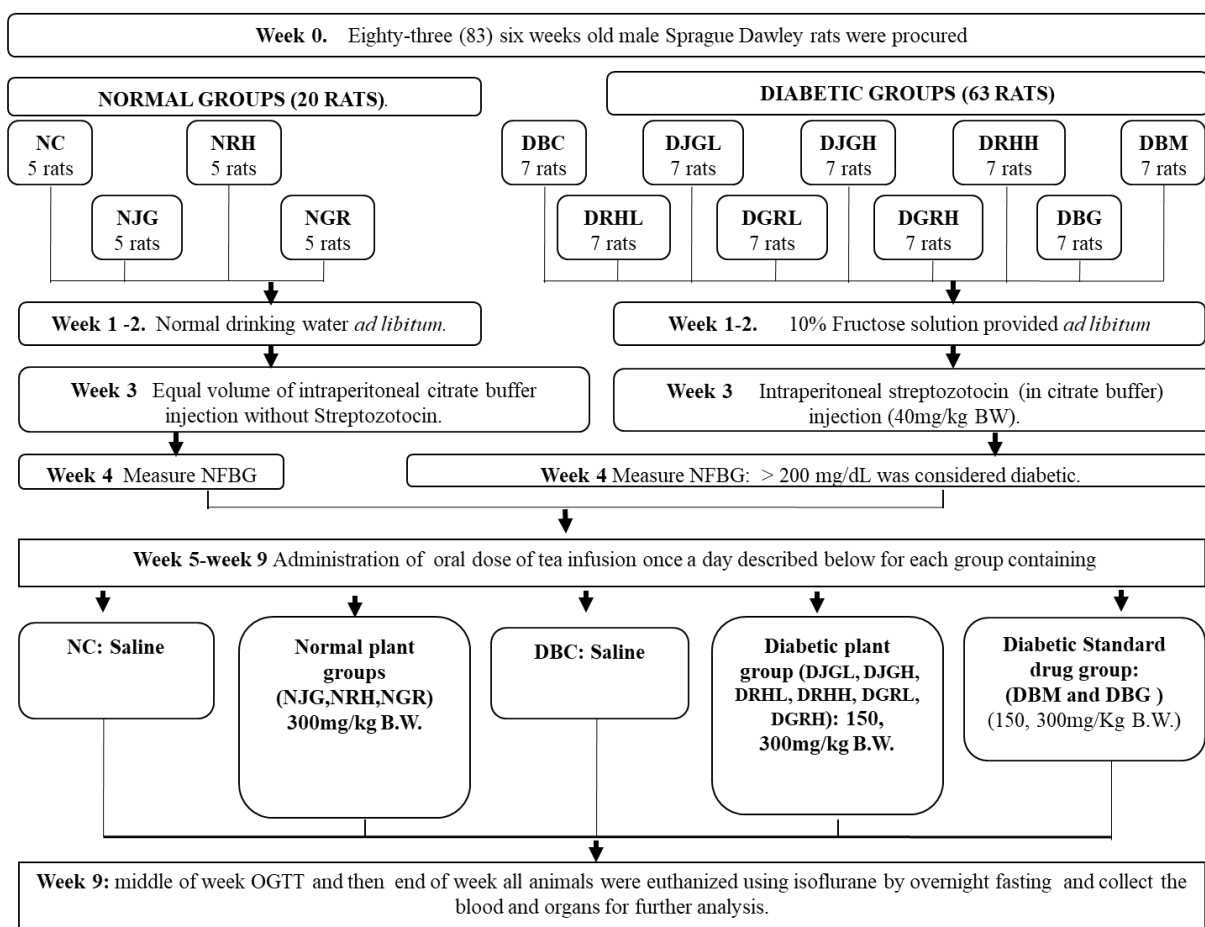


Figure 2.7: Flow diagram of comprehensive *in vivo* animal treatment procedure.

2.8.2 Induction of diabetes

The animals were allowed to acclimatize for one week. For next 2 weeks of the experiment, a 10% fructose solution was provided to the animals in the diabetic groups (DBC, DJGL, DJGH, DRHL, DRHH, DGRL, DGRH, DBM and DBG groups; 63 animals in total) to induce insulin resistance. Normal drinking water was provided to the animals in the normal or non-diabetic groups (NC, NJG, NRH, NGR groups; 20 animals in total). Thereafter, a single low dose of streptozotocin (40 mg/kg body weight) dissolved in a citrate buffer (pH 4.5) was intraperitoneally injected into the animals in the diabetic groups to induce partial pancreatic β -cell dysfunction. A similar volume of citrate buffer was injected to the animals in the normal or non-diabetic groups. One week after the streptozotocin injection, the non-fasting blood glucose (NFBG) levels of all rats were measured in the blood collected from the tail vein with a portable glucometer. Rats with blood glucose level \geq 200 mg/dl were considered as diabetic and included in the studies, while rats with blood glucose level $<$ 200 mg/dl were excluded.

2.8.3 Feeding and sampling

All animals were given normal drinking water *ad libitum* and pelletized chows after the diabetes induction. The animals in tea infusion groups (DJGL, DJGH, DRHL, DRHH, DGRL, DGRH, NJG, NRH and NGR) were orally administered with low (150 mg/kg bw) and high (300 mg/kg bw) dose of respective tea extract. While the animals in the DBM and DBG groups were orally administered with a single dose of metformin (300 mg/kg bw) and glibenclamide (5 mg/kg bw), respectively. The animals in the NC and DBC groups were provided with only an equivalent volume of normal drinking water via the same route. A 100 gram of each tea was infused in boiling water at 1:10 tea and distilled water ratio and allow to stand for 2 hours. The infusions were concentrated in a water bath at 50°C to yield concentrates, respectively.

2.8.4 Determination of food and fluid intake, body weight (BW) and sampling

The food and fluid intake, body weight and sampling were determined throughout the experimental period. The food and fluid intake of all animal groups were monitored daily. The body weights of rats were monitored weekly. At the end of the experiment, the overnight fasted animals were euthanized using an overdose of isoflurane and blood and organs (liver, kidney, heart, muscle, brain, and pancreas) were collected for further analysis. Blood was collected in sterile centrifuge tubes and preserved on ice for 2-3 hours. Then the serum was separated by centrifuging at 3000 rpm for 15 minutes and preserved at -30°C until needed.

2.8.5 Oral glucose tolerance test (OGTT)

The OGTT was conducted in the last week of the intervention period. After an overnight fast, a glucose solution was orally administered to each animal at a dose of 2 g/kg body weight and blood samples were collected from the tail vein at 0 (just before glucose ingestion), 30, 60, 90 and 120 minutes to measure the level of glucose. Glucose levels were measured by employing a portable glucometer (Glucoplus, Quebec, Canada).

2.8.6 Estimation of final fasting blood glucose and insulin and calculation of HOMA scores

The final fasting blood glucose was measured by using a commercially available assay kit. The serum insulin level was determined by using an ultrasensitive rat insulin ELISA kit from Mercodia, Uppsala, Sweden. The homeostatic model assessment (HOMA) scores for insulin resistance (HOMA-IR) and beta-cell functions (HOMA- β) were calculated from the final fasting blood glucose and fasting serum insulin data using following formulas:

$$HOMA - IR = \frac{\text{Fasting serum insulin (U/L)} \times \text{FBG (mg/dL)}}{22.5}$$

$$HOMA - \beta \text{ cell function} = \frac{20 \times \text{Fasting Serum insulin (U/L)}}{\text{FBG (mg/dL)} - 3.5}$$

Conversion factor: Insulin (1 U/L = 7.174 μ mol/L) and Blood glucose (1 mMol/L = 18 mg/dL)

2.8.7 Serum lipid profiles

The total cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol, fructosamine, urea, uric acid and creatinine, and liver function enzymes: aspartate and alanine transaminases (AST and ALT), and alkaline phosphatase (ALP) were determined with an Automated Chemistry Analyzer (Labmax Plenno, Labtest Co. Ltd., Lagoa Santa, Brazil) with commercial standard assay kits according to manufacturer's manual.

2.8.8 Hepatic glycogen content

Estimation of the levels of hepatic glycogen concentrations was done by using a phenol-sulfuric acid method (Lo, Russell et al. 1970). Briefly, 0.5 gram of the excised liver tissue was digested with 0.5 mL of 30% KOH saturated in Na₂SO₄ contained in a test tube immersed in ice. Then, it was immediately cooled on ice after boiled for 30 minutes. Thereafter, a volume of 670 μ L of 95% ethanol was added and followed by cold centrifugation twice at 840 g for 30 minutes to precipitate the glycogen content. Then, the supernatant was discarded, and the precipitate was re-suspended in 300 μ L of 95% ethanol and centrifuged for another 20 minutes at 840 g to precipitate the liver glycogen. The precipitate obtained was dissolved in 1 mL of distilled water and an aliquot of 20 μ L was in a test tube, later mixed with 180 μ L distilled water. Subsequently, 200 μ L of 5% phenol was added to the test tube followed by rapidly adding 1 mL concentrated H₂SO₄ and whereafter boiled for 20 minutes. After cooling, the absorbance was recorded at 490 nm by using Synergy HTX Multi-mode Reader. The glycogen content of the samples was extrapolated from a standard glycogen curve using log-log graph and expressed as μ g/mg glycogen.

2.8.9 Histopathological analysis of pancreatic tissue

The formalin-preserved pancreatic tissue was subjected to standard laboratory procedures for paraffin fixing/embedding. Tiny sections were cut to a size of approximately 4 μ m, followed by the deparaffination of the slides using *p*-xylene. This was consequently rehydrated using ethanol at different concentrations (100%, 80%, 70%, 50%) and cleansed with running water. Subsequently, hematoxylin was used to stain the slides for 5 minutes and then cleansed with running water. Next was counterstaining using eosin; finally, the stained slides were arranged

and mounted in Dibutylphthalate Polystyrene Xylene (DPX), with a sterile cover-slip and viewed with a microscope (Niko Eclipse E100, CFI optical system, Japan).

2.8.10 *In vivo* antioxidant studies

To fully understand the likely role of antioxidant activity in combination to the observed antidiabetic activity, the tea infusions were assayed for their *in vivo* antioxidant potentials in the serum and tissues of the animals utilizing oxidative stress biomarkers namely, reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARS) levels, catalase, superoxide dismutase (SOD), glutathione reductase (GR). Assays utilized for the determination of *in vivo* antioxidant activity were described above.

To conduct the *in vivo* antioxidant assays, centrifuged homogenates of the excised tissues (liver, kidney, heart, pancreas and brain) were initially prepared by homogenising 0.5 g of each organ tissue sample in 4000 μ L of homogenization buffer (50 mM sodium phosphate buffer with triton X-100, pH 7.5) with an Ultra Turrax Tube Drive Workstation Homogenizer (IKA-Works, Staufenim Breisgau, Germany). Soon after, the solution was transferred to a microtube and centrifuged for 15 minutes at 15000 rpm in a Hettich Mikro 200 Microcentrifuge (Hettich Lab Technology, Tuttlingen, Germany). The supernatant was then poured in another microtube and kept at -20°C for subsequent analysis.

2.9 Statistical analysis

The data were presented as mean \pm standard deviation (SD), and the significance difference established at $p < 0.05$ using a one-way analysis of variance (ANOVA) Tukey's-HSD multiple range post hoc test. Statistical analyses were conducted using IBM Statistical Package for the Social Sciences (SPSS) for Windows, version 25.0 (IBM Corp, Armonk, NY, USA).

CHAPTER 3

3.0 COMPARATIVE STUDY OF THE ANTIOXIDATIVE AND ANTIDIABETIC ACTIVITIES *IN VITRO*

3.1 Phytochemical properties of black tea (*Camellia sinensis*) and rooibos tea (*Aspalathus linearis*), and their modulatory effects on key hyperglycaemic processes and oxidative stress

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3.1.1 Abstract

The comparative phytochemicals, antioxidative and antidiabetic activities of *Camellia sinensis* (black tea) and *Aspalathus linearis* (rooibos tea) were studied *in vitro* and *ex vivo*. Concentrated infusions of the teas showed significant free radical scavenging activities *in vitro*. They significantly increased the glutathione level, superoxide dismutase and catalase activities in oxidative hepatic injury, while concomitantly depleting malondialdehyde level. The teas significantly inhibited intestinal glucose absorption and α -amylase activities, and elevated muscle glucose uptake. LC-MS phytochemical profiling revealed the presence of hydroxycaffeic acid, l-threonate, caffeine, vanillic acid, n-acetylvaline, and spinacetin 3-glucoside in *C. sinensis*. While quinolinic acid, coumestrol, phloroglucinol, 8-hydroxyquercetagenin, umbelliferone, and ajoene were identified in *A. linearis*. These results portray the antioxidant and antidiabetic potencies of both teas, with *A. linearis* showing the best activity. These teas may thus be used as functional foods in the management of the disease.

Keywords: Antioxidants; Antidiabetics; Black tea; Rooibos tea; and Type 2 diabetes

3.1.2 Introduction

Diabetes mellitus (DM) is the most endemic of all metabolic disease, as it was reported to affect over 425 million people in 2017 (IDF 2017). This depicts a 2.4% rise in prevalence from 415 million in 2015 (IDF 2015) and it has been predicted to rise by 48% to 629 million in 2045, with an upsurge of 156% expected for Africa (IDF 2017).

Diabetes mellitus is characterized by increased blood glucose level (hyperglycaemia) owing to disorder in the metabolism of carbohydrate, protein and lipids (Erukainure et al. 2013), which is caused by failure of the pancreatic β -cell to secrete insulin, and/or failure of the cells to use the secreted insulin (Erukainure et al. 2018). The former is referred to as type 1 diabetes (T1D), while the latter is often referred to as type 2 diabetes (T2D) and the most prevalent of all diabetes types as it is responsible for over 90% of morbidity and mortality due to DM (IDF 2016, IDF 2018). Hyperglycaemia leading to oxidative stress is the major trigger of T2D pathogenesis, that leads to micro- and macro- vascular complications such as retinopathy and neuropathy (Constantino et al. 2013, Tiwari et al. 2013, Chukwuma and Islam 2017, Erukainure et al. 2017). Oxidative stress occurs in T2D as a result of increased generation of reactive oxygen species (ROS) from increased glucose oxidation, which overwhelms the cell's endogenous antioxidative system (Maritim et al. 2003, Sanni et al. 2018). Increased activities of carbohydrate hydrolyzing enzymes particularly α -glucosidase and α -amylase have also been reported to contribute to hyperglycaemia owing to rapid breakdown of dietary carbohydrate leading to postprandial rise in blood glucose level (Oyebode et al. 2018).

Camellia sinensis is a well-known medicinal plant commonly referred to as tea and has been consumed as beverage from time immemorial. Its origin has been ascribed to Asia and it has been described as the most globally consumed beverage second to water (Macfarlane and Macfarlane 2004). *Camellia sinensis* is commercially available in most countries as black, green and white teas. Several studies have reported its antidiabetic and antioxidant properties (Dufresne and Farnworth 2001, Bhatt et al. 2010, Kumar and Rizvi 2015, Fu et al. 2017), which has been attributed to its phytochemical constituents particularly the catechins and alkaloids (Frei and Higdon 2003, Williamson et al. 2011, Han et al. 2016).

Aspalathus linearis is a medicinal plant native to South Africa, belonging to the Fabaceae family and the *Aspalathus* genus. Its leaves are utilized in the production of the herbal tea, rooibos or bush tea which is widely consumed globally (Joubert et al., 2008). Its medicinal properties have been widely studied and has been reported for antidiabetic and antioxidant

activities (Marnewick et al. 2003, Joubert et al. 2008, Patel et al. 2016). These medicinal properties have been attributed to its reported high ascorbic acid content as well as polyphenols such as the flavones and dihydrochalcones particularly aspalathin and nothofagin (Iswaldi et al. 2011, Lee and Bae 2015).

Camellia sinensis and *Aspalathus linearis* constitute the most common teas consumed in Southern Africa, and often used singly or combined in the management of various ailments including DM. However, there is a dearth in their comparative studies. This study thus aims at comparing the phytochemical, antidiabetic and antioxidative properties of *C. sinensis* (black tea) and *A. linearis* (rooibos tea) by investigating their ability to promote hypoglycaemic processes vis-à-vis muscle stimulation of glucose uptake, inhibiting intestinal glucose absorption and activities of major carbohydrate digestive enzymes, as well as improving antioxidant enzymes activities.

3.1.3 Materials and methods

Please refers to Chapter 2 for the detailed materials and methods for *C. sinensis* (black tea) and *A. linearis* (rooibos tea).

3.1.4 Results

Both tea samples had moderate phenolic contents as shown in **Figure 3.1.1**. The phenolic content of *A. linearis* was significantly ($p < 0.05$) higher than that of *C. sinensis*.

Both tea hot water extracts significantly ($p < 0.05$) scavenged DPPH, with *A. linearis* displaying the best scavenging activity as shown in **Figure 3.1.2A** and **Table 3.1.1**. Both teas showed a dose-dependent FRAP activity as shown in **Figure 3.1.2B**, with the highest activity observed at the highest concentration (240 $\mu\text{g/mL}$) of both tea extracts.

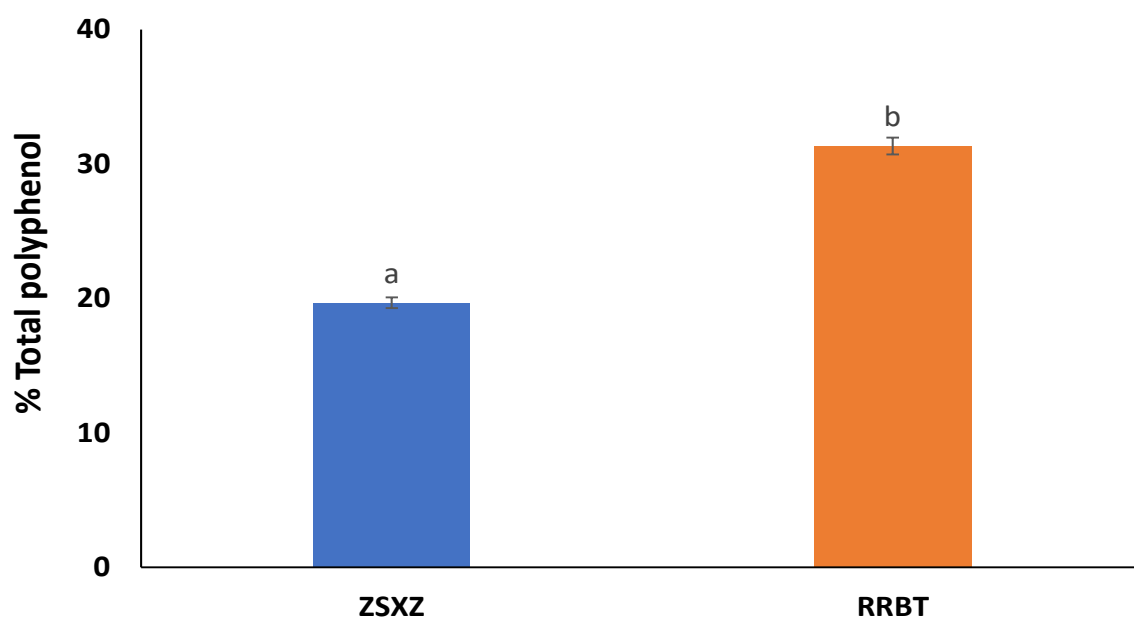


Figure 3.1.1: Total phenolic contents of *C. sinensis* and *A. linearis*. Data are presented as mean \pm SD; n = 3. ^{a-b}Values with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$). ZSXZ: zhengshanxiaozhong (*C. sinensis*); RRBT: red rooibos tea (*A. linearis*).

Table 3.1.1: IC₅₀ values of *C. sinensis* and *A. linearis* activities

Activities	<i>C. sinensis</i> ($\mu\text{g/mL}$)	<i>A. linearis</i> ($\mu\text{g/mL}$)	Ascorbic acid ($\mu\text{g/mL}$)	Acarbose ($\mu\text{g/mL}$)
DPPH	0.03	0.01	0.05	–
FRAP	>1000	>1000	>1000	–
α -amylase	>1000	>1000	>1000	>1000
GSH	112.02	84.10	90.34	–
SOD	>1000	87.27	797.56	–
Catalase	1.71	1.62	2.21	–
MDA	1.55	2.6	3.52	–
Glucose absorption	271.89	196.79	–	–
Glucose uptake	6.82	11.51	–	–

IC₅₀, concentration to inhibit 50% activity; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FRAP, ferric reducing antioxidant power; GSH: reduced glutathione, SOD: superoxide dismutase enzyme, MDA: malondialdehyde.

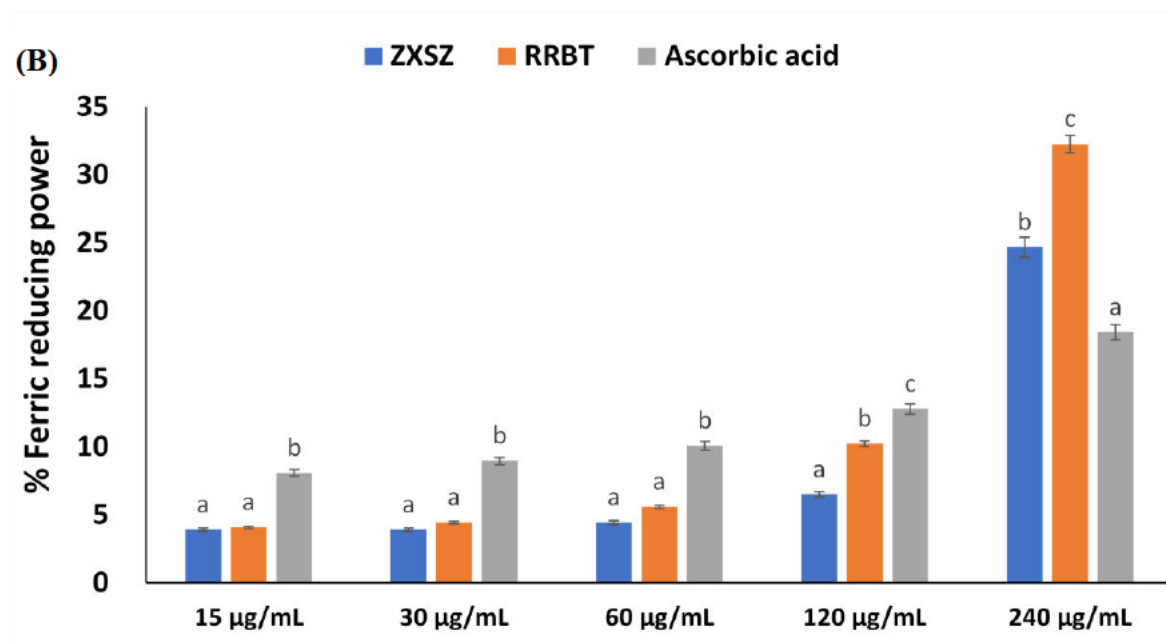
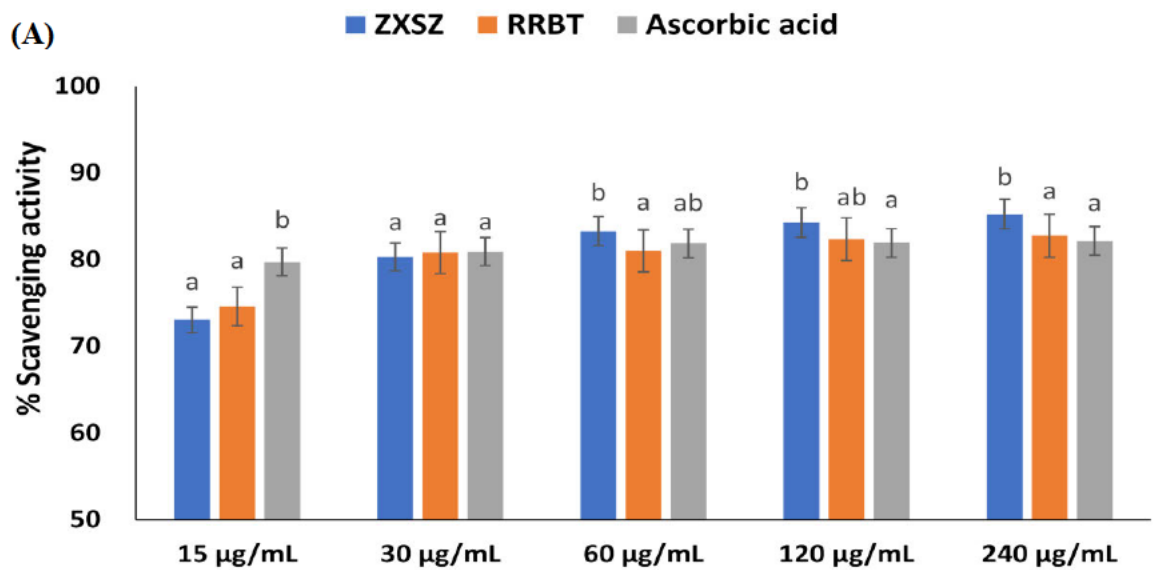


Figure 3.1.2: (A) DPPH scavenging activities and (B) Ferric reducing antioxidant power (FRAP) activities of *C. sinensis* and *A. linearis*. Data are presented as mean \pm SD. ^{a-c}Values with different letter above the bars for a given concentration are significantly ($p < 0.05$) different from each other. ZXSZ: zhengshanxiaozhong (*C. sinensis*); RRBT: red rooibos tea (*A. linearis*).

Both teas moderately inhibited the activities of α -amylase but were significantly ($p < 0.05$) lower when compared to the standard drug, acarbose as depicted in **Figure 3.1.3**.

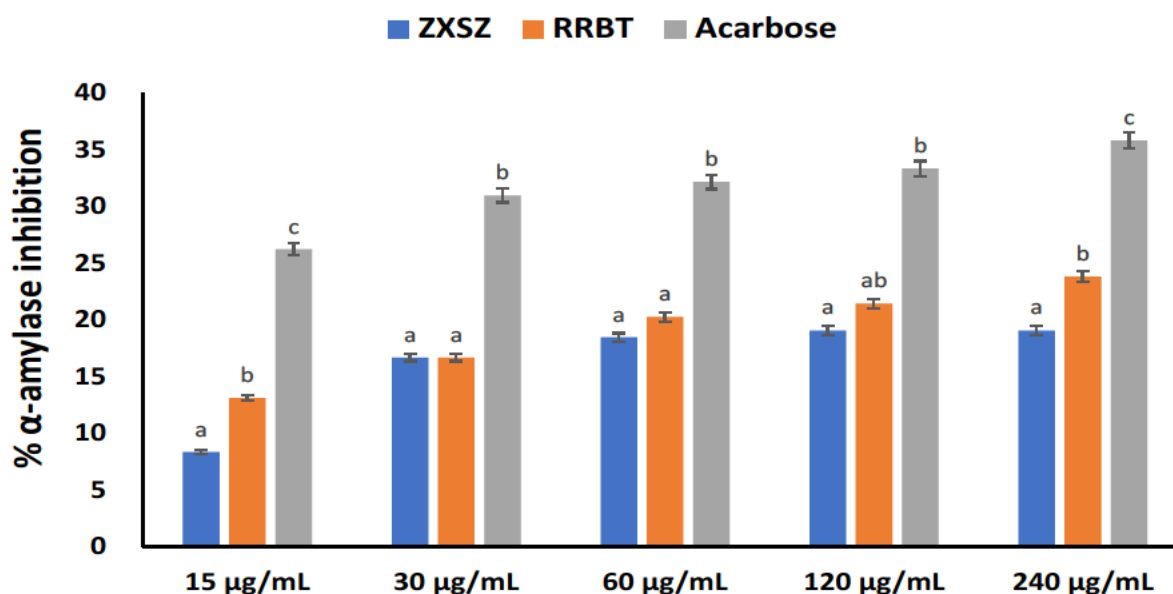


Figure 3.1.3: Inhibitory effect of *C. sinensis* and *A. linearis* on α -amylase activity. Data are presented as mean \pm SD. ^{a-c}Values with different letter above the bars for a given concentration are significantly ($p < 0.05$) different from each other. ZXSZ: Zhengshanxiaozhong (*C. sinensis*); RRBT: Red rooibos tea (*A. linearis*).

Incubation of hepatic tissue homogenates with FeSO_4 led to significant ($p < 0.05$) depletion of GSH level, SOD and catalase activities, while significantly ($p < 0.05$) increasing MDA level as depicted in **Figures 3.1.4A – D**. Incubation with the teas significantly ($p < 0.05$) increased the GSH level, SOD and catalase activities, and concomitantly depleted MDA level. The ability of both teas to increase the SOD activities were dose-dependent, with *A. linearis* showing the best activity (**Figure. 3.1.4B**). Based on the IC_{50} values (**Table 3.1.1**), *A. linearis* had the best activities except for MDA depletion.

Incubation of isolated rat jejunum with the teas significantly ($p < 0.05$) inhibited intestinal glucose absorption as depicted in **Figure 3.1.5A**, with *C. sinensis* showing a dose dependent activity. The low IC_{50} value of *A. linearis*, indicates a better activity compared to *C. sinensis*.

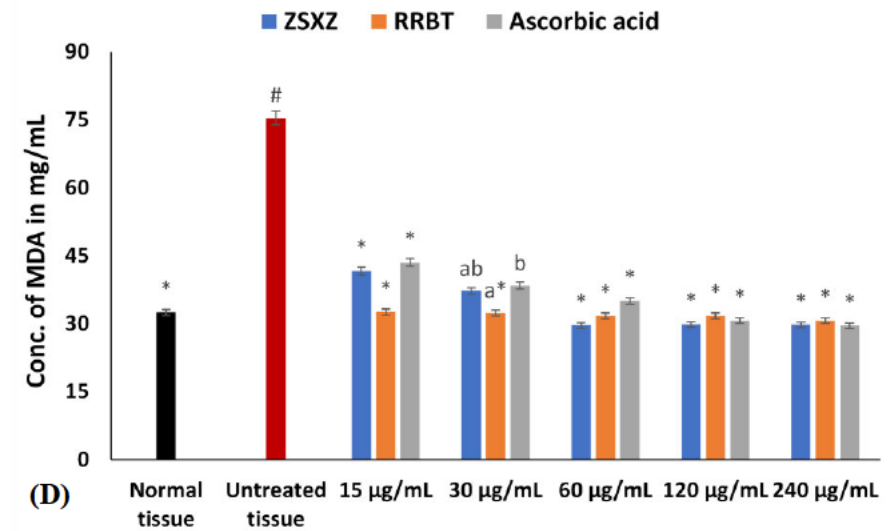
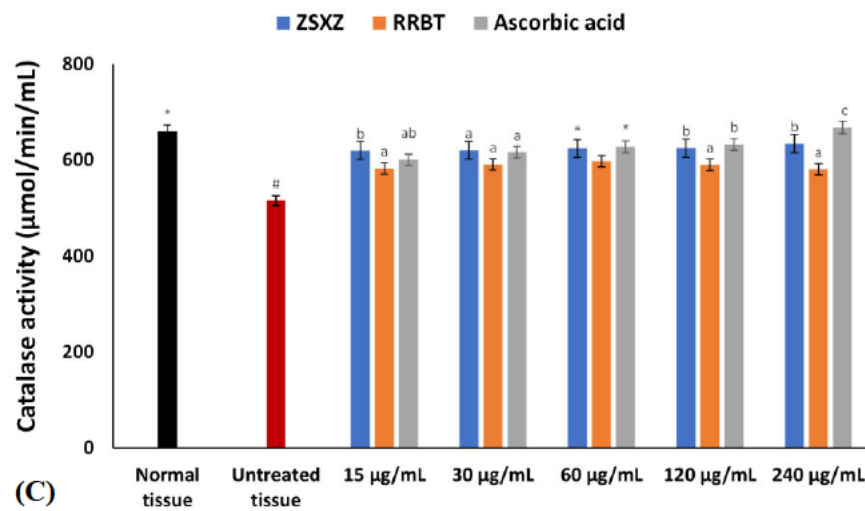
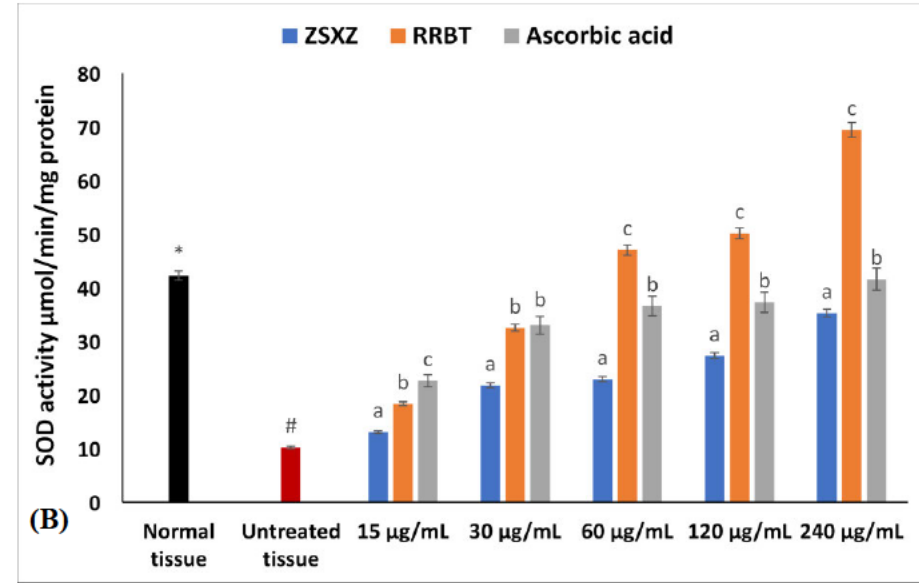
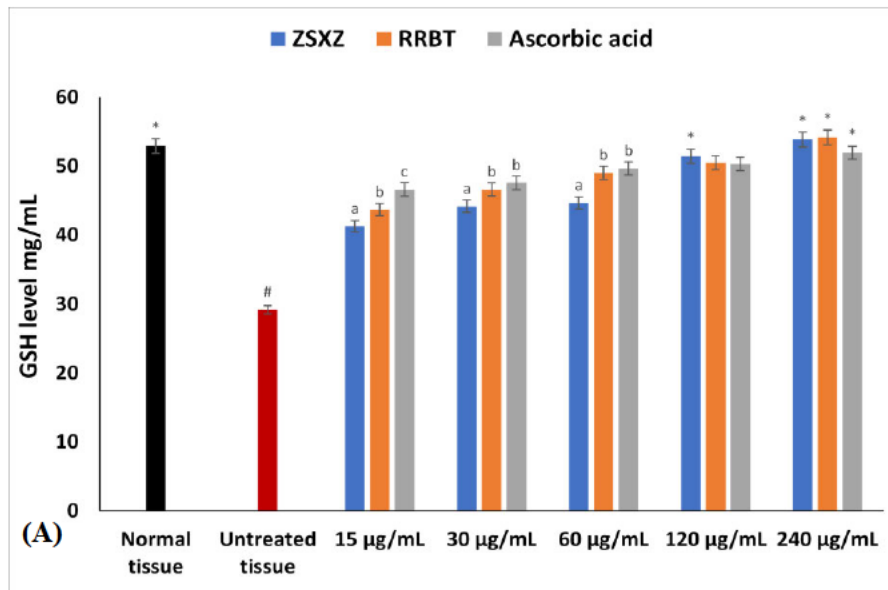


Figure 3.1.4: Effect of *C. sinensis* and *A. linearis* on (A) GSH level, (B) SOD activity, (C) catalase activity, and (D) MDA level in oxidative hepatic injury. Values = mean \pm SD; n = 3. ^{a-c}Values with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$). *Significantly different from untreated sample and #Significantly ($p < 0.05$) different from normal sample. ZSXZ: Zhengshanxiaozhong (*C. sinensis*); RRBT: Red rooibos tea (*A. linearis*).

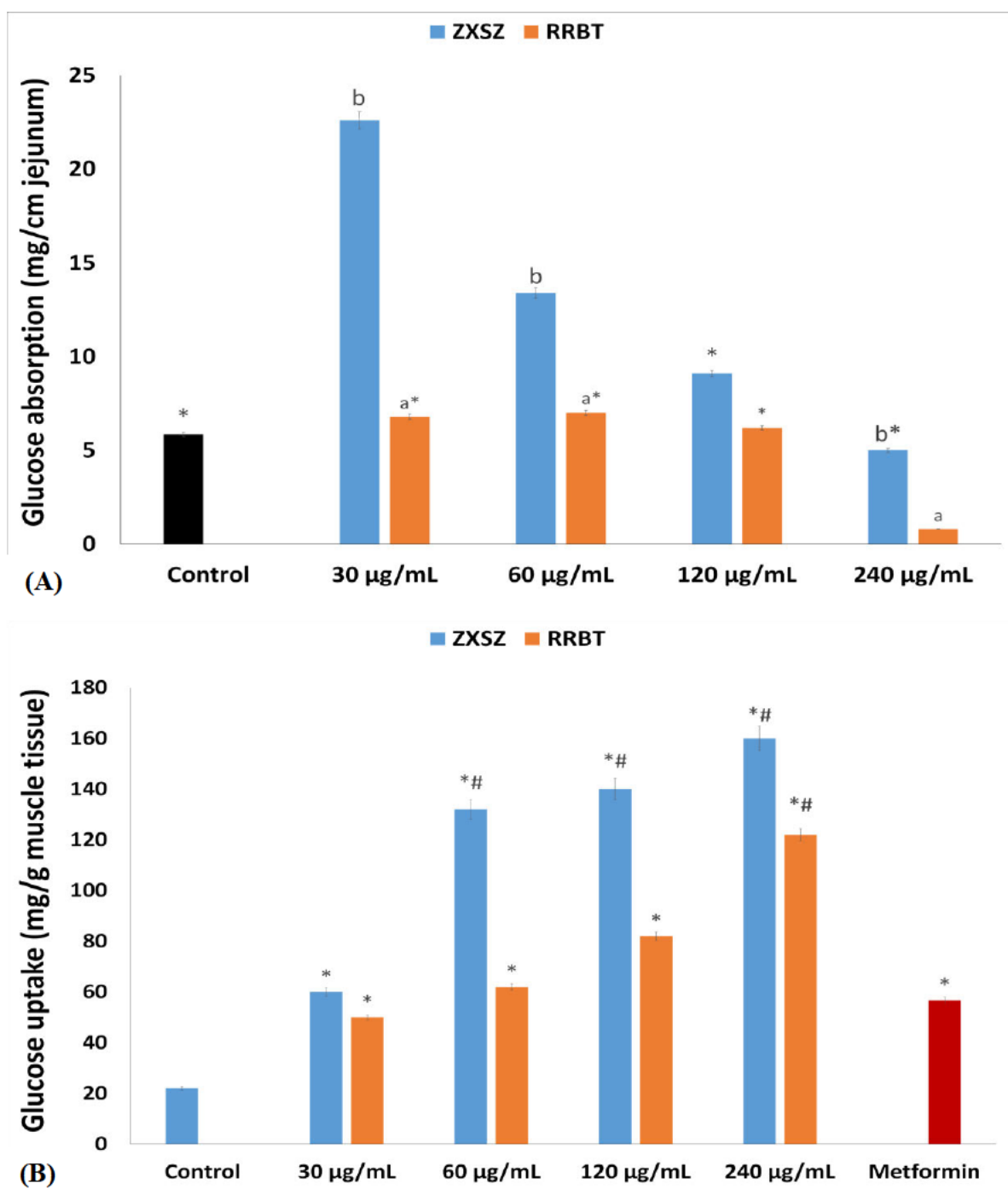


Figure 3.1.5: Effects of *C. sinensis* and *A. linearis* on glucose absorption in isolated rat jejunum (A) and glucose uptake (B) in isolated psoas muscle. ^{a-c}Values with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$). *Significantly different from untreated sample and #Significantly ($p < 0.05$) different from normal sample. ZXSZ: zhengshanxiaozhong (*C. sinensis*); RRBT: red rooibos tea (*A. linearis*).

Incubation of isolated psoas muscle with the teas led to significant ($p < 0.05$) increase in muscle glucose uptake as shown in **Figure 3.1.5B**. Both teas showed dose-dependent activities, with *C. sinensis* having the best activity.

Table 3.1.2: LC-MS identified compounds in (A) *C. sinensis* and (B) *A. linearis*.

Teas	Compounds					
<i>C. sinensis</i>	hydroxycaffeic acid	l-threonate	caffeine	vanillic acid	n-acetylvaline	spinacetin 3-glucoside
<i>A. linearis</i>	quinolinic acid	coumestrol	phloroglucinol	8-hydroxyquercetagenin	umbelliferone	ajoene

LC-MS analysis led to the identification of hydroxycaffeic acid, l-threonate, caffeine, vanillic acid, n-acetylvaline, and spinacetin 3-glucoside in *C. sinensis*, while quinolinic acid, coumestrol, phloroglucinol, 8-hydroxyquercetagenin, umbelliferone, and ajoene were identified in *A. linearis* as shown in **Table 3.1.2**.

3.1.5 Discussion

Tea drinking culture has been in practice from time immemorial, with Yunnan in western China said to be the birthplace (Kumakura 2002). Aside *C. sinensis*, there have been in increase in other types of tea notably herbal teas which also enjoy worldwide consumption (Joubert et al. 2008). Though often taken as recreational beverages and food, teas have been reported for their medicinal properties (Siddiqui et al. 2004, Sharma et al. 2007). This study reports the ability of *C. sinensis* and *A. linearis* teas to scavenge free radicals and to inhibit the activities of major carbohydrate catabolic enzymes linked to T2D as well as their phytoconstituents.

The total phenolic contents of both teas were rather very low (**Figure 3.1.1**) which corroborates previous studies by Anesini et al. (2008), Pal et al. (2012) and Bhebhe et al. (2015) which reported low total phenolic content for *C. sinensis* and *A. linearis*. This however contradicts previous reports that both teas were had rich contents of phenolics. Pereira et al. (2014) reported high phenolic contents for black, green and white *C. sinensis* and correlated them with the antioxidant properties of the studied teas. Damiani et al. (2019) also reported high phenolic properties for *A. linearis* and also correlated the antioxidant activity of the tea to the phenolic content. Although the present study reports low phenolic contents for both teas, they however contribute to the antioxidant properties of the teas as depicted by their ability to scavenge DPPH and reduce Fe^{3+} (**Figure 3.1.2**).

The influence of oxidative stress in the pathogenesis of type 2 diabetes and its complications due to hyperglycaemia induced increased production of free radicals are well documented (King and Loeken 2004, Erukainure et al. 2012, Tiwari et al. 2013). These free radicals have been shown to attack cellular proteins, DNAs, membrane lipids which may subsequently lead

to cell death (Maritim et al. 2003). Increased lipid peroxidation owing to suppressed GSH level, SOD and catalase activities is a major oxidative mechanism. The high DPPH scavenging and FRAP activities (**Figure 3.1.2**) of the teas indicates their free radical and reducing power properties. This corroborates previous reports on the potent antioxidant properties of *C. sinensis* and *A. linearis* (Pereira et al. 2014, Damiani et al. 2019). This is further depicted by the ability of both teas to elevate the levels of GSH, SOD and catalase activities, while suppressing lipid peroxidation in oxidative hepatic injury (**Figures 3.1.4A - D**). These potencies may be attributed to the LC-MS identified compounds of the teas (**Figure 3.1.6**), particularly the phenolics which are well known antioxidants (Zhao et al. 2014, Heleno et al. 2015).

Inhibition of major carbohydrate digestive enzymes has been reported in several studies to be effective in the treatment and management of T2D (Van et al. 2005, Van et al. 2006). The inhibition of α – amylase by the teas *C. sinensis* and *A. linearis* (**Figure 3.1.3**) corroborates previous studies (Muller et al. 2012, Ramírez et al. 2012, Gao et al. 2013, Mikami et al. 2015, Vinholes and Vizzotto 2017) and further portrays their antidiabetic properties. These studies attributed the enzyme inhibitory properties to the phytoconstituents of both teas (Muller et al. 2012, Wang et al. 2012, Gao et al. 2013, Dlundla et al. 2017), thereby implying that the total phenol content (**Figure 3.1.1**) and identified compounds (**Figures 3.1.6A & B**) may play a synergetic role in regard to this activity.

Inhibition of and/or delayed intestinal glucose absorption can also lead to decreased postprandial elevation of blood glucose level, thus can be employed in the treatment and management of T2D (Chukwuma and Islam 2015). Studies have reported the ability of plant extracts to suppress glucose absorption in the intestine mostly at the first quarter jejunal and duodenal regions (Erukainure et al. 2018, Oyebode et al. 2018, Erukainure et al. 2019). The inhibitory effects of the studied teas (**Figure 3.1.5A**) demonstrates their ability to delay the intestinal absorption of dietary glucose, thus preventing postprandial elevation of blood glucose level. This also corroborates with their ability to inhibit α -amylase activity (**Figure 3.1.3**).

The role of skeletal muscle in glucose and carbohydrate have been well documented (Sinacore and Gulve 1993, Oyebode et al. 2018). This can be attributed to their richness in the glucose transporter, GLUT-4 which facilitates the uptake of glucose (Satoh 2014, Oyebode et al. 2018). Some commercial antidiabetic drugs such as metformin exhibit their antidiabetic activity by triggering muscle glucose uptake (Natali and Ferrannini 2006). Thus, the ability of the studied

teas to stimulate muscle glucose uptake (**Figure 3.1.5B**) further insinuates their antidiabetic potentials. This may also portray an improved insulin sensitivity, as insulin resistance has been implicated in the defects in muscle glucose uptake (Sinacore and Gulve 1993, Satoh 2014).

Phytochemicals have been reported for their antioxidant and antidiabetic activities (Alasalvar and Bolling 2015, Chukwuma et al. 2019). The studied biological activities of *C. sinensis* and *A. linearis* maybe attributed to the identified phytochemicals (**Table 3.1.2**), thus depicting a synergistic effect. The presence of the phenolics, hydroxycaffeic acid, vanillic acid, and n-acetylvaline as well as the phenolic glycoside, spinacetin 3-glucoside in *C. sinensis* portrays a strong antioxidant potency as phenolics are well known for their antioxidant and antidiabetic properties (Erukainure et al. 2018, Chukwuma et al. 2019). The presence of caffeine may also contribute to the antidiabetic activity of *C. sinensis*, as the hypoglycaemic activity of caffeine has been reported in non-diabetics, pre-diabetics and diabetics (Lane 2011, Bhaktha et al. 2015, Lee et al. 2016). Similarly, the presence of phloroglucinol, 8-hydroxyquercetagenin, and umbelliferone in *A. linearis* may also contribute to its antioxidant and antidiabetic activities. Phytoestrogen, coumestrol and ajoene have also been shown to possess antioxidant and antidiabetic properties (Bhathena and Velasquez 2002, Hattori et al. 2005, Yuk et al. 2011), and may also contribute to the biological activities of *A. linearis*.

3.1.6 Conclusion

These results depict the antioxidative and antidiabetic potencies of *C. sinensis* and *A. linearis* as demonstrated by their ability to scavenge free radicals, suppress lipid peroxidation, inhibit α -amylase activity and intestinal glucose absorption, and concomitant increase in antioxidant enzymes activities and muscle glucose uptake. Thus, further affirming the utilization of these teas for managing T2D and its complications, with *A. linearis* being the most potent. Hence, they may be employed as functional foods in the management of the disease.

3.2 Antioxidative and antidiabetic activities of jasmine green tea (*Camellia sinensis*) and green rooibos tea (*Aspalathus linearis*): A comparative study

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3.2.1 Abstract

Jasmine green tea (JGT), a type of green tea (*Camellia sinensis*), is one of the most consumed teas in China, while green rooibos tea (*Aspalathus linearis*) (GRT) is endemic to South Africa. Both teas gained popularity worldwide and have been confirmed as sources of dietary antioxidants. There is, however, a shortage of studies on antioxidative, antiobesogenic and antidiabetic activities of JGT and its comparison to GRT for the management of diabetes mellitus (DM). In the present study, comparative studies on the phytochemicals content, antioxidative, antiobesogenic and antidiabetic activities of the concentrated infusions of JGT (*Camellia sinensis*) and GRT (*Aspalathus linearis*) were investigated *in vitro* and *ex vivo*. JGT exhibited higher total polyphenolic content, better *in vitro* and *ex vivo* antioxidative activity than GRT. The α -amylase and α -glucosidase inhibitory activities were higher for JGT, while anti-lipase activity was higher in GRT. The intestinal glucose absorption level was significantly lower, and muscle glucose uptake was significantly higher for JGT than GRT. LC-MS analysis identified trifoliol, catechin, L-theanine, vanillic acid, epigallocatechin, and caffeine in JGT, while vanillic acid, chrysoeriol, *p*-coumaric acid, dihydroferulic acid, luteolin and quercetin in GRT. These results highlight the antioxidant and antidiabetic potencies of both teas, while JGT exhibited better effects on type 2 diabetes (T2D) related parameters.

Keywords: Antioxidative; Antidiabetic; Jasmine green tea; Green rooibos tea; Type 2 diabetes

3.2.2 Introduction

Diabetes mellitus (DM) is a metabolic disease of multiple aetiologies characterized by chronic hyperglycaemia with disorders of fat, protein and carbohydrate metabolism, resulting from the absolute or relative insufficient insulin secretion, insulin action, or both (WHO 1999, ADA 2014, IDF 2019). According to the International Diabetes Federation (IDF), about 9.3% of the global population is affected by this disease. The prevalence of DM is increasing annually and is predicted to reach over 700 million by 2045 (IDF 2019). In 2019, China had the largest number of diabetics aged 20 - 79 years, ranking number one worldwide with an estimate of 116.4 million. While in South Africa, the number of diabetic adults (20 - 79 years) is about 4.6 million, which is the highest in the Africa region (IDF 2019).

There are two major categories of diabetes: type 1 diabetes and type 2 diabetes (T2D), with T2D being the most prevalent accounting for over 90% of diabetic cases (WHO 2016, IDF 2019). T2D occurs due to a combination of insulin resistance and pancreatic β -cell dysfunction, which results in chronic hyperglycaemia (WHO 1999, DeFronzo 2004).

Hyperglycaemia and hyperlipidaemia have been implicated in the excessive generation of reactive oxygen species (ROS) which overwhelms the endogenous antioxidant system, causing oxidative stress (Mohamed et al. 2016, Xiao et al. 2020). Oxidative stress is a primary trigger of T2D pathogenesis, that leads to micro and macro-vascular complications such as nephropathy, neuropathy, retinopathy, and cardiomyopathy (Chawla et al. 2016, Rani et al. 2016).

Increased activities of carbohydrate and lipid digesting enzymes, such as α -glucosidase, α -amylase, and pancreatic lipase, have been explained to contribute to hyperglycaemia and hyperlipidaemia, due to the rapid breakdown of dietary carbohydrate and lipids resulting in an increased level of postprandial blood glucose and fat (Erukainure et al. 2017, Xiao et al. 2020). Therefore, the inhibitory activities on these enzymes have attracted a growing interest to detect antidiabetic potentials of medicinal plants (Buchholz and Melzig 2016).

JGT is one of the most consumed teas in China. It is a type of green tea (*Camellia sinensis*), which is flower-scented and different from general green tea (Gao et al. 2009). It is produced through a specific step called “Ti-hua”, which is repeatedly mixing green tea (*Camellia sinensis*) with fresh jasmine (*Jasminum sambac*) flowers. This step transfers the fragrant compounds from the jasmine flower to the green tea, so that to form its unique aroma and health benefits of both tea and jasmine flower (Kuroda et al. 2005, Chen et al. 2017). The medicinal properties

of JGT that have been reported in several previous studies include anti-obesity, antioxidant and antidiabetic activities (Tao et al. 2011, Yao and Gui-nian 2011, Huang and Yang 2016, Huiying et al. 2016). These properties have been attributed by bioactive compounds of green tea and jasmine flower, such as phenolics, flavonoids, vitamins, tannins, caffeine, and aroma substances (Gao et al. 2009, Zayapor et al. 2019).

GRT (*Aspalathus linearis*), also known as unfermented rooibos tea, is consumed as a herbal tea and gaining popularity worldwide (Muller et al. 2012). It is an unoxidized form of traditional fermented rooibos tea made from a broom-like member of the *Fabaceae* family, which is endemic to South Africa (Joubert et al. 2008). GRT and its bioactive compounds, such as aspalathin, aspalinin, and nothofagin, have been reported in several studies correlating its pharmaceutical applications to its antidiabetic, antioxidative, anti-cancer, and hypoglycaemic properties (Kawano et al. 2009, Muller et al. 2012, Mazibuko et al. 2013, Kamakura et al. 2015).

The consumption of tea can offer a good source of dietary antioxidants. Both JGT and GRT have been confirmed as sources of dietary antioxidants in human (Villaño et al. 2010, Alappat et al. 2015, Kamakura et al. 2015, Huiying et al. 2016). There is, however, a shortage in their comparative studies on the management of DM. Thus, this study aims to compare the phytochemicals, antioxidative, antiobesogenic and antidiabetic properties of JGT and GRT by investigating their ameliorative effects against Fe²⁺-induced hepatic oxidative injury, and abilities to inhibit key enzymes linked to T2D, as well as the inhibition of intestinal glucose absorption and the stimulation of glucose uptake.

3.2.3 Materials and methods

Please refer to Chapter 2 for the detailed materials and methods for green rooibos tea (*Aspalathus linearis*) and jasmine green tea (*Camellia sinensis*)

3.2.4 Results

JGT displayed significantly ($p < 0.05$) higher phenolic content compared to GRT as presented in **Figure 3.2.1**.

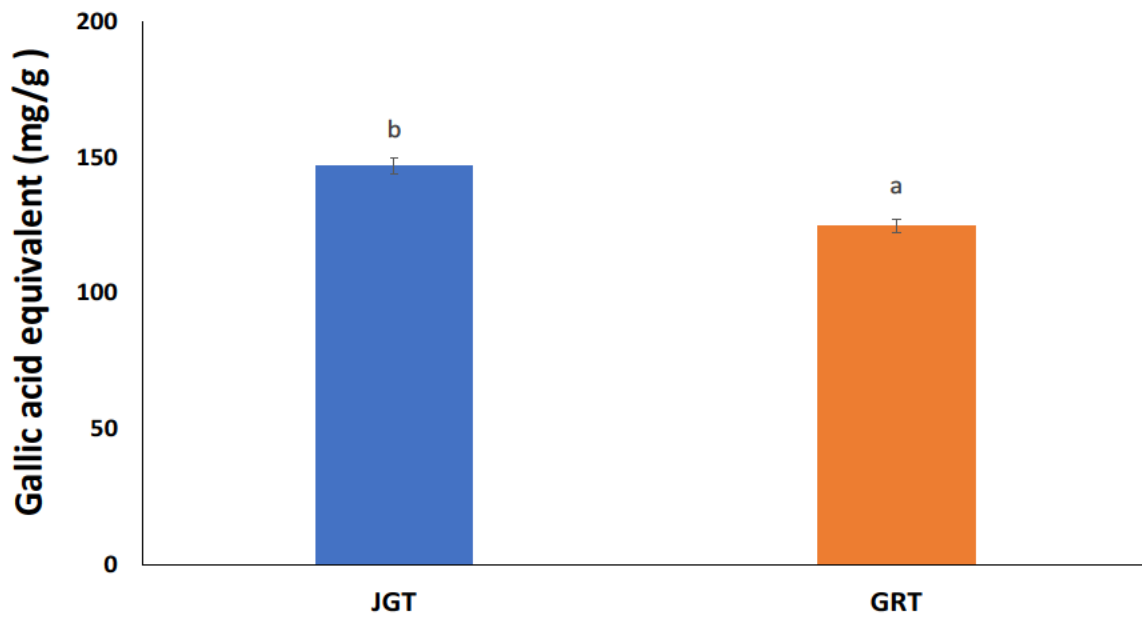


Figure 3.2.1: Total phenolic contents of jasmine green tea (JGT) and green rooibos tea (GRT). Data are presented as mean \pm SD; $n = 3$. ^{a,b}Values with different letters above the bars for a given tea are significantly different from each other ($p < 0.05$).

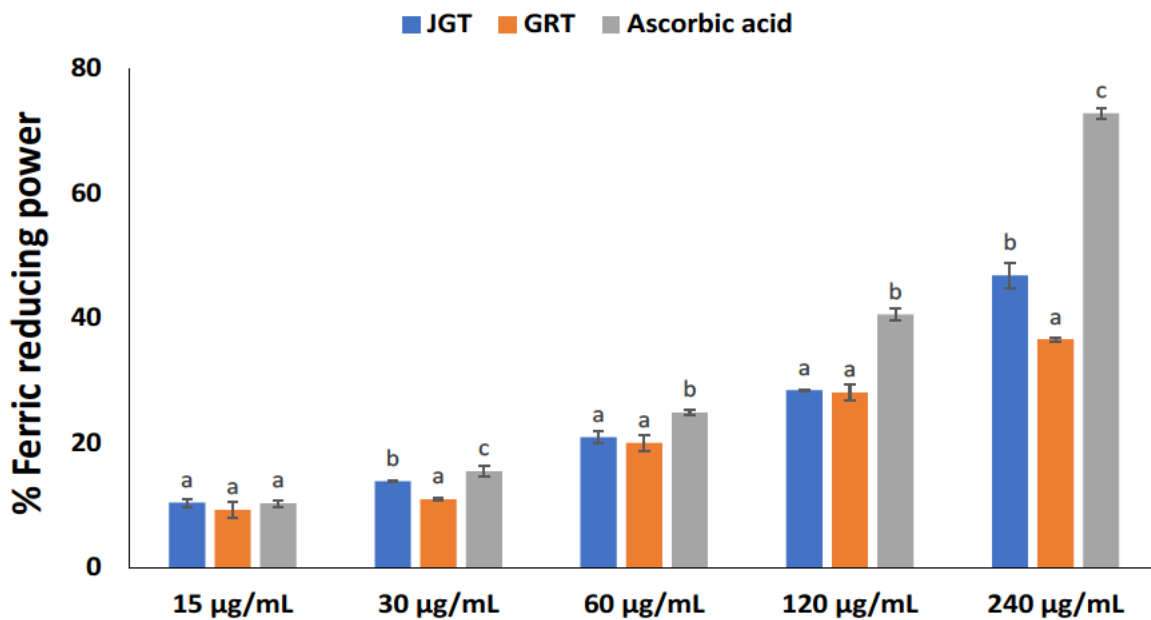


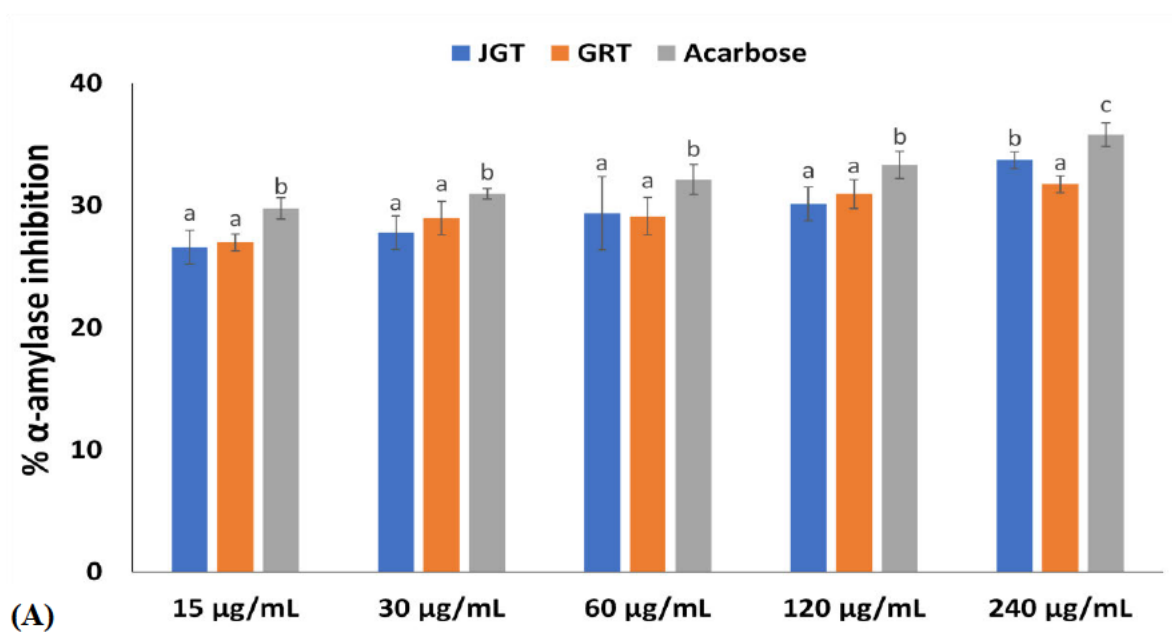
Figure 3.2.2: Ferric reducing antioxidant power (FRAP) activities of jasmine green tea (JGT) and green rooibos tea (GRT). Data are presented as mean \pm SD. ^{a-c}Values with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$).

Both teas exhibited a Fe^{3+} radical reducing power in a dose-dependent manner, and a significant ($p < 0.05$) difference among them demonstrated in the highest concentration of 240 $\mu\text{g/mL}$, as presented in **Figure 3.2.2**. Jasmine green tea ($\text{IC}_{50} = 469.35 \mu\text{g/mL}$) exhibited better activity than that of GRT ($\text{IC}_{50} = 990.21 \mu\text{g/mL}$) as depicted by its lower IC_{50} value (**Table 3.2.1**).

Both teas moderately inhibited α -amylase activity in a dose-dependent pattern **Figure 3.2.3A**. Both teas displayed significantly ($p < 0.05$) lower inhibitory activity when compared to the standard drug acarbose. However, the inhibition of JGT on α -amylase activity was significantly ($p < 0.05$) higher than GRT at the highest concentration (240 $\mu\text{g/mL}$).

Both teas inhibited α -glucosidase enzyme activity in a dose-dependent manner as presented in **Figure 3.2.3B**. JGT exhibited a significantly ($p < 0.05$) higher inhibitory activity compared with GRT as portrayed by its IC_{50} value (**Table 3.2.1**). Furthermore, there was not significant ($p > 0.05$) difference between the standard drug acarbose and JGT at the highest concentrations of 120 and 240 $\mu\text{g/mL}$.

Both teas inhibited pancreatic lipase activity in a dose-dependent manner as presented in **Figure 3.2.3C**. Both teas exhibited significantly ($p < 0.05$) lower activity than that of the standard drug, orlistat, while no consistent significant difference was exhibited between the studied teas. GRT ($\text{IC}_{50} = 0.52 \mu\text{g/mL}$) exhibited a better anti-lipase activity than that of JGT ($\text{IC}_{50} = 0.84 \mu\text{g/mL}$) as portrayed by its lower IC_{50} value (**Table 3.2.1**).



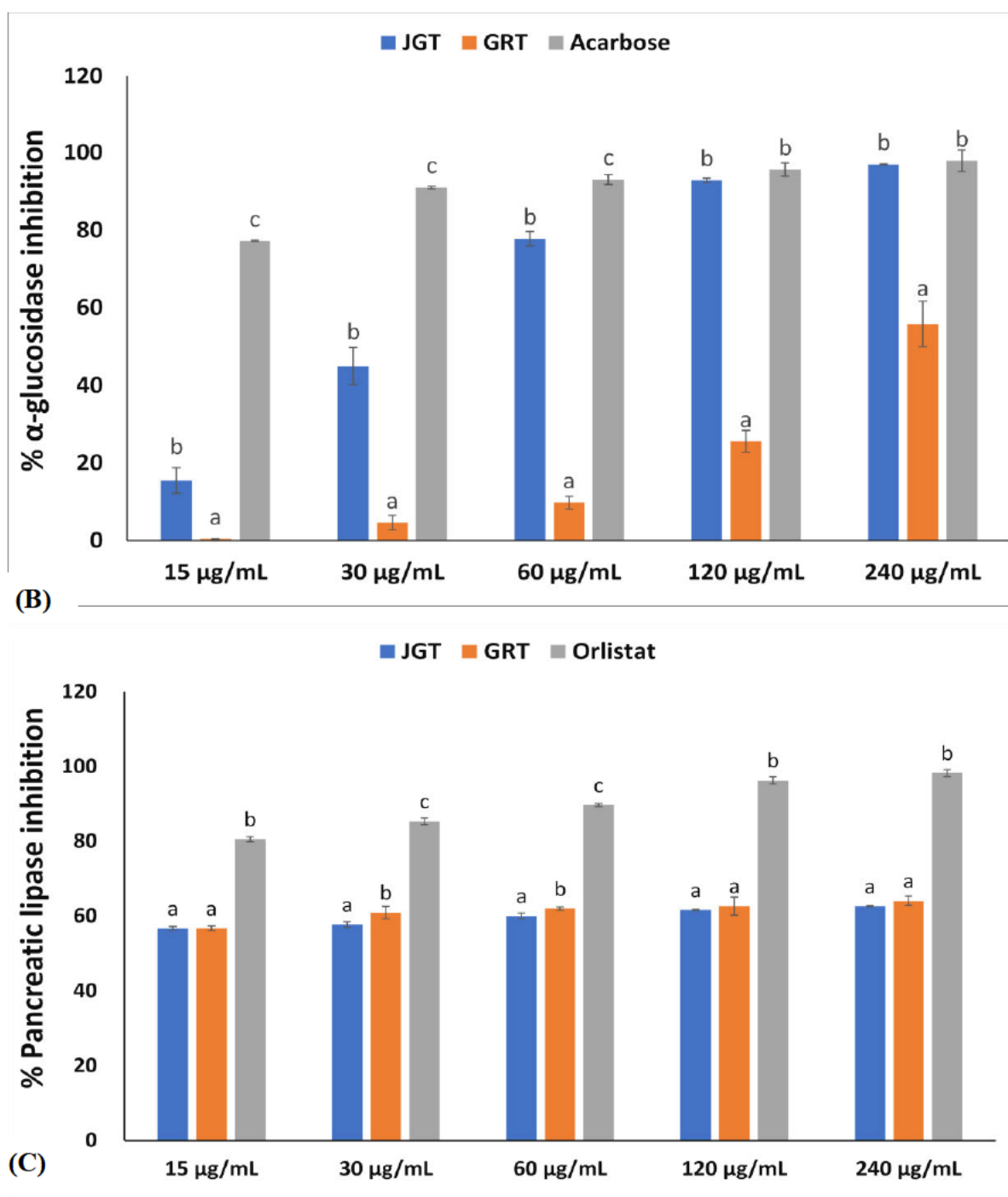


Figure 3.2.3: (A) α -amylase inhibitory effect, (B) α -glucosidase inhibitory and (C) pancreatic lipase inhibitory effect of jasmine green tea (JGT) and green rooibos tea (GRT). Data are presented as mean \pm SD of triplicate determination. ^{a-c}Values with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$).

Table 3.2.1: IC₅₀, GA₅₀ and GU₅₀ values of jasmine green tea and green rooibos tea (*Aspalathus linearis*) activities

Activities	JGT (µg/mL)	GRT (µg/mL)	Ascorbic acid (µg/mL)	Acarbose (µg/mL)	Orlistat (µg/mL)
DPPH	3.22±0.81	3.8±1.06	0.51±0.72	–	–
FRAP	469.35±0.79	990.21±0.85	132.75±0.72	–	–
α-amylase	>1000 ±1.56	>1000±1.09	–	>1000±0.92	–
α-glucosidase	35.93±2.45	303.31±2.11	–	0.12±1.26	–
Anti-lipase	0.84±0.92	0.52±0.18	–	–	0.15±0.78
GSH	46.05±2.21	20.90±1.75	13.02±0.75	–	–
SOD	1.72±2.11	2.38±3.21	0.38±2.43	–	–
Catalase	>1000±7.11	>1000±6.13	>1000±4.13	–	–
MDA	53.48±0.28	157.21±0.38	49.84±0.91	–	–
Glucose absorption (GA ₅₀)	89.53±0.13	123.07±0.20	–	–	–
Glucose uptake (GU ₅₀)	28.04±1.18	43.31±1.08	–	–	–

IC₅₀, concentration to inhibit 50% activity; GA₅₀, concentration to inhibit 50% glucose absorption; GU₅₀, concentration to stimulate 50% glucose uptake. JGT, jasmine green tea; GRT, green rooibos tea; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FRAP, ferric reducing antioxidant power; GSH, reduced glutathione; SOD, superoxide dismutase; MDA, malondialdehyde.

Incubation of hepatic tissue homogenate with FeSO₄ led to a significant ($p < 0.05$) depletion of GSH level in the untreated group, as presented in **Figure 3.2.4**. Incubation with both tea samples and ascorbic acid significantly ($p < 0.05$) restored the effect on GSH level, with GRT (IC₅₀ = 20.90 µg/mL) exhibiting better activity in increasing reduced GSH level than that of JGT (IC₅₀ = 46.05 µg/mL) as portrayed by its lower IC₅₀ value (**Table 3.2.1**).

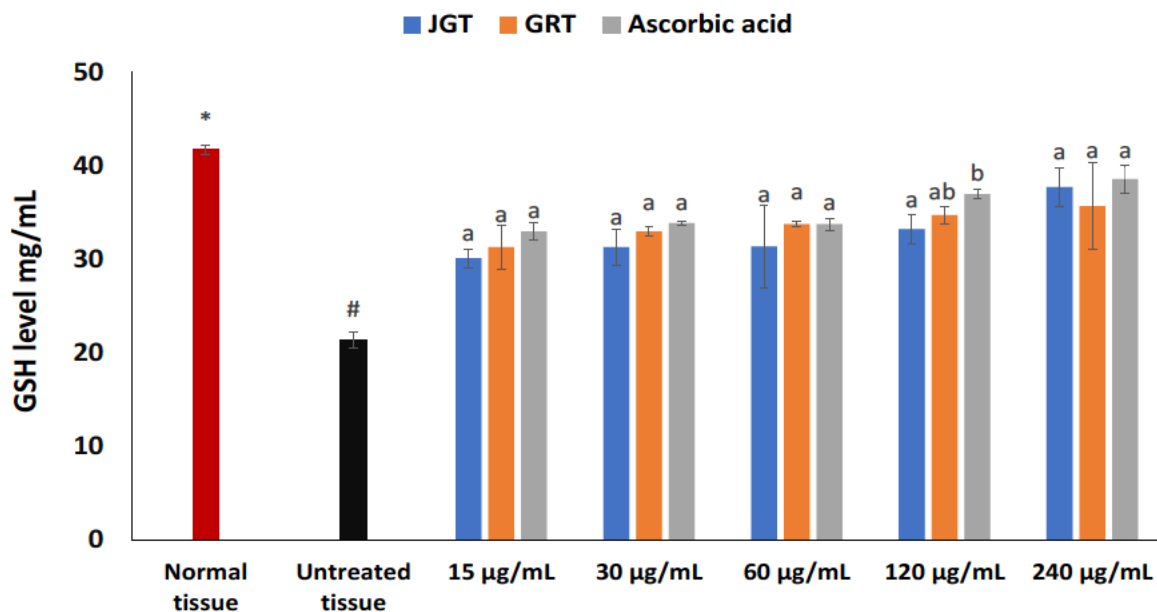


Figure 3.2.4: Effect of jasmine green tea (JGT) and green rooibos tea (GRT) on GSH level in oxidative hepatic injury. Values = mean \pm SD; n = 3. ^{a-c}Values with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$). *Significantly different from untreated tissue and #Significantly different from normal tissue ($p < 0.05$). GSH, reduced glutathione; SOD, superoxide dismutase; MDA, malondialdehyde.

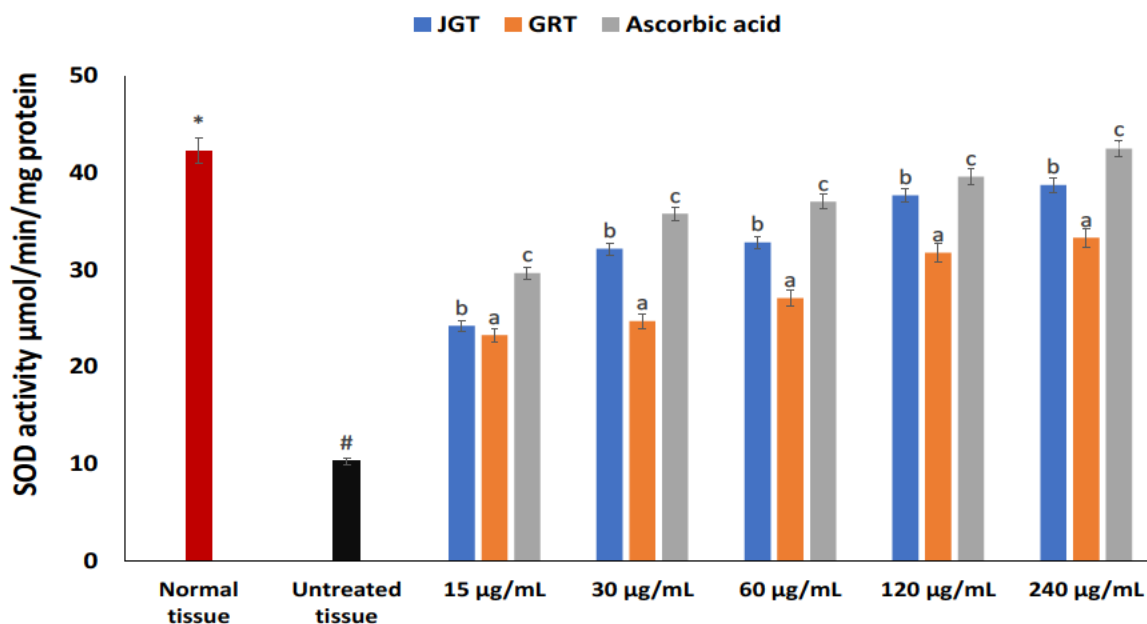


Figure 3.2.5: Effect of jasmine green tea (JGT) and green rooibos tea (GRT) on SOD activity in oxidative hepatic injury. Values = mean \pm SD; n = 3. ^{a-c}Values with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$). *Significantly different from untreated tissue and #Significantly different from normal sample ($p < 0.05$). GSH, reduced glutathione; SOD, superoxide dismutase; MDA, malondialdehyde.

The untreated hepatic oxidative injured tissue induced by FeSO₄ displayed significantly reduced SOD activity as presented in **Figure 3.2.5**. Both tea samples displayed significantly ($p < 0.05$) increased SOD activity in a dose-independent manner, when JGT (IC₅₀ = 1.72 µg/mL) exhibited better activity compared to GRT (IC₅₀ = 2.38 µg/mL) as portrayed by its lower IC₅₀, presented in **Figure 3.2.5** and **Table 3.2.1**.

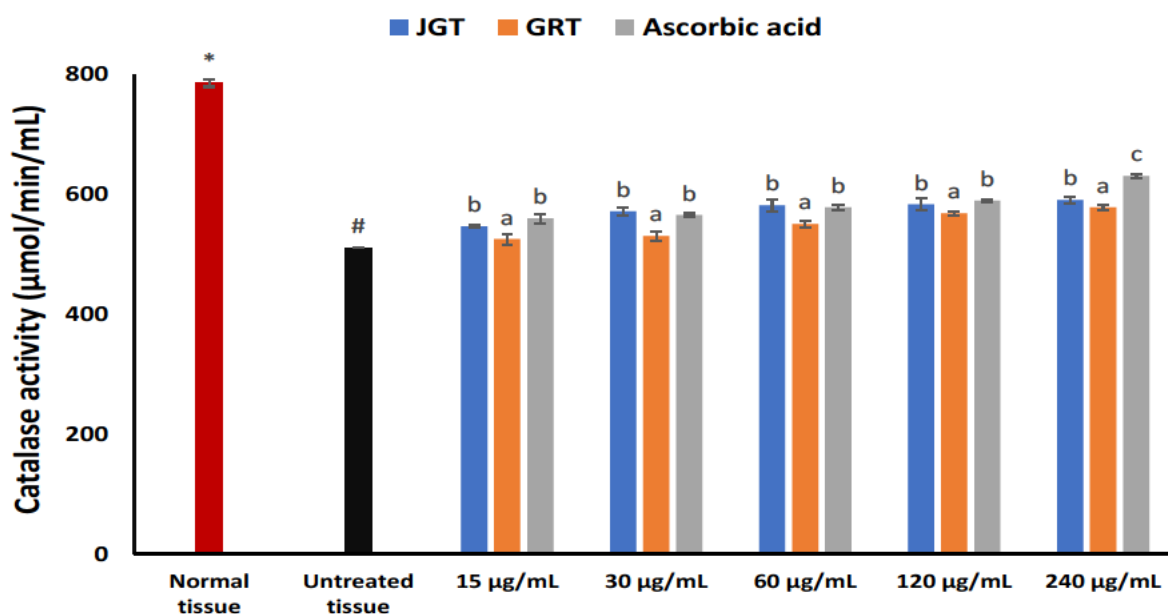


Figure 3.2.6: Effect of jasmine green tea (JGT) and green rooibos tea (GRT) on catalase activity in oxidative hepatic injury. Values = mean ± SD; n = 3. ^{a-c}Values with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$). *Significantly different from untreated tissue and #Significantly different from normal tissue ($p < 0.05$). GSH, reduced glutathione; SOD, superoxide dismutase; MDA, malondialdehyde.

There was a significant ($p < 0.05$) reduced catalase enzyme activity in hepatic oxidative injured tissue induced by FeSO₄ as presented in **Figure 3.2.6**. The treated groups displayed significantly ($p < 0.05$) increased catalase activity when compared to the untreated groups in a dose-dependent manner. JGT treated group displayed a significantly ($p < 0.05$) higher activity when compared with GRT.

The incubation of hepatic tissue homogenate with FeSO₄ led to a significant ($p < 0.05$) increase in the MDA level as presented in **Figure 3.2.7**. The MDA levels of treated groups displayed a significant ($p < 0.05$) decrease compared to that of the untreated group in a dose-dependent manner. The inhibitory potential of JGT on Fe²⁺-induced lipid peroxidation displayed better

activity than that of GRT, however, it was lower when compared to the standard drug ascorbic acid as portrayed by their IC₅₀ values (Table 3.2.1).

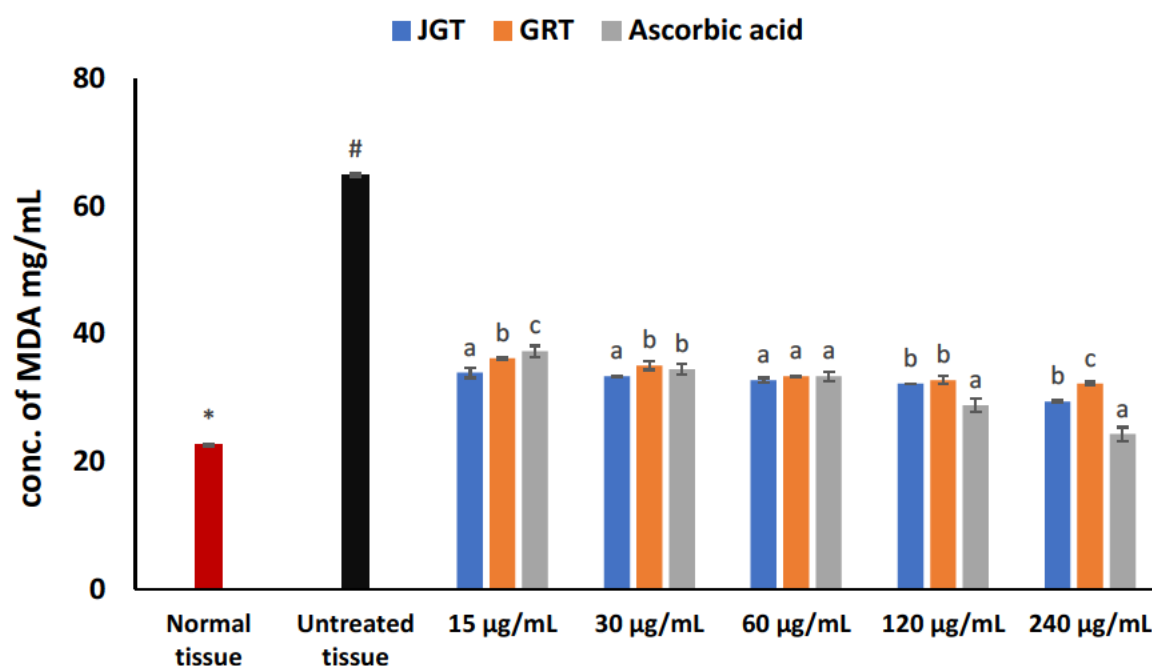


Figure 3.2.7: Effect of jasmine green tea (JGT) and green rooibos tea (GRT) on (D) MDA level in oxidative hepatic injury. Values = mean ± SD; n = 3. *^cValues with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$). *Significantly different from untreated tissue and #Significantly different from normal tissue ($p < 0.05$). GSH, reduced glutathione; SOD, superoxide dismutase; MDA, malondialdehyde

The incubation of isolated rat small intestine with both studied teas significantly ($p < 0.05$) inhibited glucose absorption in a dose-dependent manner as presented in Figure 3.2.8. The low GA₅₀ of JGT indicates its better activity compared to GRT (Table 3.2.1).

The incubation of isolated rat psoas muscle with both studied teas significantly ($p < 0.05$) increased muscle glucose uptake in a dose-dependent manner as presented in Figure 3.2.9. Both teas exhibited significantly higher activity than that of metformin at the highest concentration of 240 µg/mL. The low GU₅₀ of JGT indicates a better activity compared to GRT (Table 3.2.1).

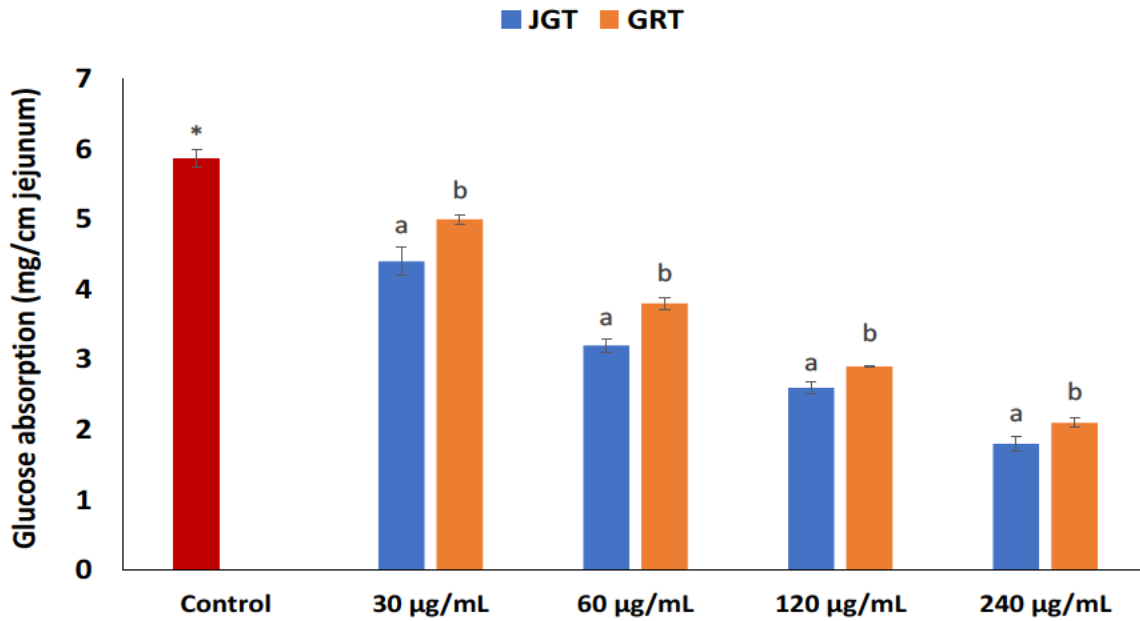


Figure 3.2.8: Effects of jasmine green tea (JGT) and green rooibos tea (GRT) on glucose absorption in isolated rat jejunum. Data are presented as mean \pm SD; $n = 3$. ^{a,c}Values with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$). *Significantly different from the treatment ($p < 0.05$).

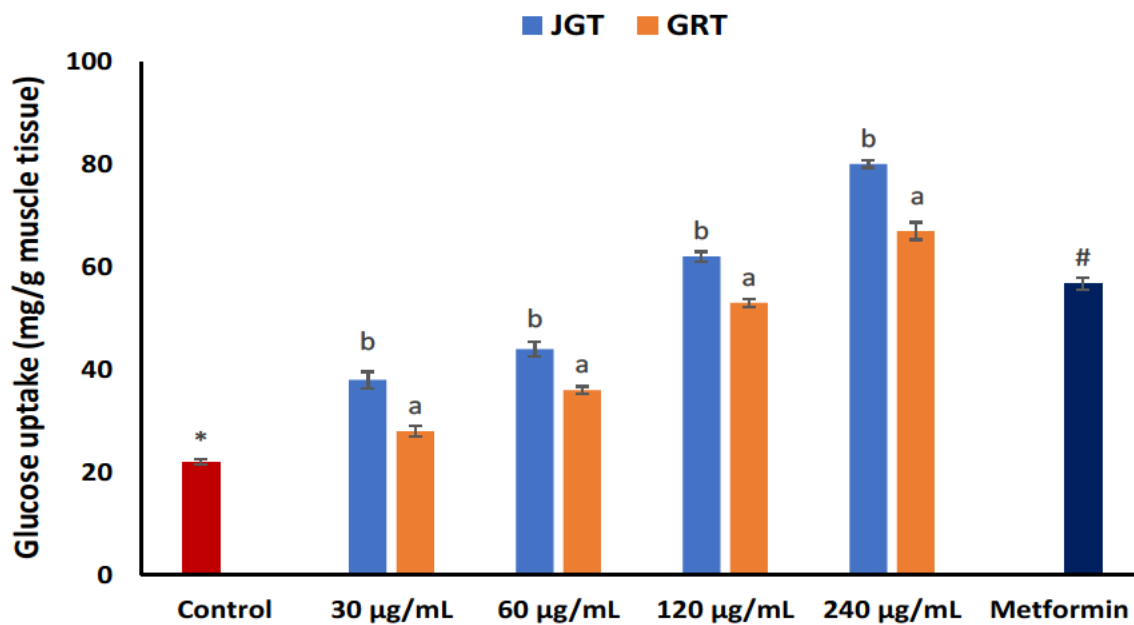


Figure 3.2.9: Effects of jasmine green tea (JGT) and green rooibos tea (GRT) on glucose uptake in isolated psoas muscle. Data are presented as mean \pm SD; $n = 3$. ^{a,b}Values with different letters above the bars for a given concentration are significantly different from each other ($p < 0.05$). *Significantly different from treatment and [#]Significantly different from control ($p < 0.05$).

Table 3.2.2: LC-MS identified compounds in (A) jasmine green tea (JGT) and (B) green rooibos tea (GRT).

Teas	Compounds					
JGT	trifoliol	epigallocatechin	caffeine	catechin	L-theanine	vanillic acid
GRT	vanillic acid	chrysoeriol	luteolin	<i>p</i> -coumaric acid	quercetin	dihydroferulic acid

LC-MS analysis of JGT led to the identification of trifoliol, epigallocatechin, caffeine, catechin, L-theanine, and vanillic acid, while vanillic acid, chrysoeriol, luteolin, *p*-coumaric acid, quercetin, and dihydroferulic acid were identified in GRT, as presented in **Table 3.2.2**.

3.2.5 Discussion

Tea is the most ancient drink that was first discovered in China almost 5000 years ago, which is the most worldwide popular beverage just next to water at present (Martin and Cooper 2011). Besides consumed as beverages, tea and its extracts have been applied in many other products, such as cereal, confectionery, dairy, edible oil, for shelf life, new flavour, or healthier benefits purposes (Sharma et al. 2010). The health benefits of drinking tea have been related to the regulation of lipid metabolism, anti-cancer, bacteriostatic and antioxidants activities (Yan et al. 2020). This study reports the effects of various concentrations of JGT and GRT infusions on free radicals scavenging and inhibition on principal carbohydrate and lipid catabolic enzymes linked to T2D, as well as their phytochemicals.

Plants rich in phenols have been well documented in their ability to inhibit the effects of oxidative stress associated diseases such as T2D (Ajuwon et al. 2018). The presence of phenols in both tea hot water extracts (**Figure 3.2.1**) give credence to their potential ability in the treatment and management of T2D. Some studies have reported the high phenolic content of JGT and correlated it with the antioxidant properties of the tea (Huiying et al. 2016, Xiao et al. 2020). Bramati et al. (2003) also reported the high phenolic content for GRT compared to the fermented rooibos tea and linked the antioxidant activity of the tea with the phenolic content. In the present study, the high phenolic content of both teas is correlated by their antioxidant properties in their ability to reduce Fe^{3+} (**Figures 3.2.2**). Phenolic compounds are known to have reducing power because of their electron donor capacity and can react with free radicals to terminate radical chain reactions by converting free radicals to more stable products (Lü et al. 2010, Nimse and Pal 2015).

The implication of oxidative stress in the pathogenesis of T2D and its complications is well

known (Folli et al. 2011). Oxidative stress results from the severe imbalance of free radicals and antioxidants in the body, which can lead to membrane lipids peroxidation, denaturation of cellular proteins and enzymes, DNA damage, even cell death (Bhattacharya 2015). Antioxidants can reverse oxidative damage contributing to a lower risk of diabetes and its complications (Matough et al. 2012).

Fe²⁺-induced oxidative stress in hepatic tissue, resulting in increased MDA level and decreased activities of SOD, catalase and GSH level, has been documented in previous studies as a major oxidative mechanism (Xiao et al. 2020). In this study, both teas elevated the enzyme activities of SOD and catalase, and the level of GSH, while decreasing the MDA level in oxidative hepatic injury (**Figures 3.2.4 – 3.2.7**). This further indicates their antioxidative potential. These potencies can be attributed to the LC-MS identified phytochemicals (**Figure 3.2.10**), such as L-theanine, epigallocatechin, quercetin, which have been reported for their potent antioxidant properties (Chan et al. 1999, Saeed et al. 2017, Morishita et al. 2019).

The inhibition of major carbohydrate enzymes activity has been reported in previous studies in the treatment and management of T2D (Ibrahim et al. 2016). These enzymes are involved in the hydrolysis of dietary carbohydrates to glucose (Giustarini et al. 2009). Thus, the inhibition of their activity can delay the process of carbohydrate digestion to decrease the glucose concentration in the bloodstream. The inhibitory activities on α -glucosidase and α -amylase by JGT and GRT (**Figures 3.2.3A & B**) therefore indicate an ability to reduce postprandial blood glucose level. Thus, insinuating an antidiabetic potential which is consistent with previous reports (Chukwuma and Islam 2015, Xiao et al. 2020).

Pancreatic lipase is the most active enzyme accountable for the breakdown of dietary fat in the intestine. Various reports have confirmed that productive anti-lipase activity could minimize fat accumulation in obese-T2D patients (Gulua et al. 2018). The inhibitory activity by the teas (**Figure 3.2.3C**) thus indicates an anti-obesity effect and is consistent with earlier published studies on the potent antiobesogenic properties of teas (Gulua et al. 2018). The inhibition of carbohydrate and lipid digesting enzymes activity displayed in this study may attribute to the synergetic effect of total phenolic content and phytochemicals of the studied teas (**Figures 3.2.1 & 3.2.10**).

Dietary glucose and fatty acid resulting from the breakdown of carbohydrate and fat are rapidly absorbed in the small intestine, leading to the elevation of blood glucose and fatty acid which could be detrimental to diabetic patients (Musso et al. 2011, Russell et al. 2016). Thus, the

inhibition and/or delaying these processes could be employed in the control and prevention of diabetes. The inhibitory effects of the studied teas (**Figure 3.2.8**) demonstrate their ability to slow down the dietary glucose absorbed by intestine, therefore preventing increase of postprandial blood glucose level. This is also in accordance with their effects on α -glucosidase activity inhibition (**Figure 3.2.3B**).

Antidiabetic drugs including metformin display their antidiabetic activity by improving muscle glucose uptake (Martineau et al. 2010). This occurs by targeting glucose transporter GLUT4, which promotes glucose uptake (Oyebode et al. 2018). The ability of the studied teas to improve glucose uptake (**Figure 3.2.9**) is in line with previous studies on *Camellia sinensis* and *Aspalathus linearis* (Muller et al. 2012, Yamashita et al. 2012, Ajuwon et al. 2018, Ueda-Wakagi et al. 2019, Xiao et al. 2020). Therefore, this further insinuates their antidiabetic potentials and potentials to modulate hyperglycaemia as well. This may also indicate improved insulin sensitivity as insulin resistance plays an important role in impaired glucose uptake (Chukwuma and Islam 2015).

The antioxidant and antidiabetic activities of phytochemicals have been well-documented (Muller et al. 2012, Saeed et al. 2017). Both teas were subjected to LC-MS to identify the possible bioactive phytochemicals. The presences of trifoliol, catechin, L-theanine, vanillic acid, epigallocatechin, and caffeine in JGT (**Table 3.2.2**) have been reported for their antioxidative, antidiabetic, antiaging, and anticancer activities (Cabrera et al. 2003, Hung et al. 2012, Alappat et al. 2015, Saeed et al. 2017). Moreover, Chan et al. (1999) pointed out that epigallocatechin isolated from JGT exhibited hypolipidemic activity, which may also correlate with its antioxidant and antidiabetic activity. Furthermore, caffeine has been reported to have hypoglycaemic activity in people with/without diabetes (Bhaktha et al. 2015). Similarly, the presences of vanillic acid, chrysoeriol, *p*-coumaric acid, dihydroferulic acid, luteolin, quercetin in GRT (**Table 3.2.2**) have been recognized as possessing activities such as antioxidant, antidiabetic, anti-inflammatory, antitumor, antimicrobial, and free radical scavenging activities (Muller et al. 2012, Van et al. 2015). Among them, quercetin was reported as the major *in vitro* active component found in rooibos tea, which may possess antiallergic, antioxidative, antidiabetic activities (Persson et al. 2010, Morishita et al. 2019). Thereby, the identified phytochemicals of JGT and GRT may play a synergetic role regarding their exhibited biological activities in the present study.

3.2.6 Conclusion

This study demonstrated the antioxidative, antiobesogenic and antidiabetic activities of jasmine green tea and green rooibos tea through their ability to suppress Fe²⁺-induced lipid peroxidation, inhibition of carbohydrate and lipid digesting enzyme activities, reducing intestinal glucose absorption and muscle glucose uptake. These activities may be attributed to the synergistic effect of phytochemical constituents. This further highlights their utilization in the management of T2D and its complications, with jasmine green tea having better activities compared to green rooibos tea. Hence, they could be applied as a functional beverage for the management of diabetes. Further *in vivo* antioxidant and antidiabetic studies in diabetic rats' models are however needed to confirm the antidiabetic activity of jasmine green tea until we make final recommendation.

CHAPTER 4

4.0 THE ANTIOXIDANT AND ANTIDIABETIC ACTIVITIES OF RED HONEYBUSH TEA *IN VITRO* AND *IN VIVO*

4.1 Background

Cyclopia genistoides belongs to *Cyclopia* species which is utilized for making herbal teas (Joubert et al. 2011). It is a small fynbos shrub in the Fabaceae family which can grow to about one metre with golden yellow stems, short needle-like leaves (**Figure 4.1**). *C. genistoides* is found mostly on high mountain peaks, marshy areas, shale bands and wet southern slopes, flats and sandy coastal areas in the Western Cape, from the Cederberg Mountains, southwards to the Cape Peninsula and eastwards to Port Elizabeth (Joubert et al. 2008).



Figure 4.1: The appearance of the plant *Cyclopia genistoides* (*Fabaceae*) used in the thesis, copied without permission from Agricultural Research Council (ARC) Stellenbosch. http://www.africanplants.senckenberg.de/root/index.php?page_id=78&id=8881#.

4.1.1 Ethnomedical uses

Cyclopia genistoides was the earliest *Cyclopia* species consumed as a tea at the Cape, which has a sweet, astringent taste. A decoction of *C. genistoides* was used as a restorative and as an expectorant in chronic catarrh and pulmonary tuberculosis (Van Wyk and Gorelik 2017). An

infusion of *C. genistoides* was valued as a stomachic which could aid weak digestion, increase the appetite, without any serious stimulating effects to the heart (Watt and Breyerbrandwijk 1932, Van et al. 1997). It also stimulates milk production in breast-feeding women and treats colic in babies (Rood 2008).

4.1.2 Biological uses

Honeybush tea originated making from *C. genistoides*, has proven health benefits, including skin photo-protection and skin cell viability, antioxidant, antimutagenic, anticarcinogenic, anticancer, antiobesity and antidiabetic properties (Joubert et al. 2008, Muller et al. 2011, Petrova et al. 2011, Van Wyk 2011, Dudhia et al. 2013, Im et al. 2014, Im et al. 2016, Magcwebaba et al. 2016, Schulze et al. 2016, Jack et al. 2017). Extracts of *C. genistoides* enriched in xanthenes and benzophenones, exhibit potentials to prevent postprandial hyperglycaemia while reducing the effective dose of acarbose (Miller et al. 2020). Some phytochemicals include glycosylated xanthenes, mangiferin and isomangiferin, and benzophenones (3- β -D-glucopyranosylriflophenone, I3G, and 3- β -D-glucopyranosyl-4-O- β -D-glucopyranosylriflophenone, IDG) found in *C. genistoides* have also been confirmed as active α -glucosidase inhibitors in mammal (Beelders et al. 2014, Bosman et al. 2017).

4.1.3 Phytochemistry

C. genistoides contains glycosylated xanthenes mangiferin and isomangiferin. Other phenolics include 4-hydroxycinnamic acid, flavones (hesperitin, hesperidin, naringenin, eriodictyol, isosakuranetin), benzophenones (3- β -D-glucopyranosylriflophenone, I3G, and 3- β -D-glucopyranosyl-4-O- β -D-glucopyranosylriflophenone, IDG), isoflavanones (afrormosin, formononetin, calycosin) and coumestans (medicago, flemichapparin) are present (Joubert et al. 2014, Van Wyk and Wink 2018).

4.2 Sequential extracts of red honeybush (*Cyclopia genistoides*) tea: chemical characterization, antioxidant potentials and anti-hyperglycaemia activities

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4.2.1 Abstract

Red honeybush tea (RHT) is enjoyed widely in South Africa and around the world due to its no caffeine and very low tannin content, as well as many health-giving attributes. There are however no scientific reports for its sequential extraction by different solvents on antidiabetic effects. In this study, the antioxidant, antidiabetic and antiobesogenic potentials of different extracts (dichloromethane, ethyl acetate, ethanol, and aqueous) of the red honeybush (*Cyclopia genistoides*) tea were investigated *in vitro* and *ex vivo*. The aqueous and ethyl acetate extracts were the most potent in all *in vitro* analyses, except for anti-lipase activity. All extracts increased catalase and SOD activities, and glutathione level in oxidative pancreatic injury. GC-MS analysis revealed the presence of fatty acids (n-hexadecanoic acid, *cis*-6-octadecenoic acid, 4-hydroxybenzoic acid, shikimic acid), fatty acid ester (propanoic acid, 2-methylpropyl ester, hexadecanoic acid methyl ester, 9,12-octadecadienoic acid, methyl ester, 9,12-octadecadienoic acid (Z,Z)-,2-hydroxy-1-(hydroxymethyl)ethyl ester, pentadecyl acrylate), phytols (n-nonadecanol-1, tyrosol, phytol, 3-n-butylthiolane), sterols (sitosterol, vitamin E), saccharide (3-O-methyl-D-glucose), ketones (4-(4-hydroxyphenyl)butan-2-one, 4-(4-hydroxy-3-methoxyphenyl)-2-butanone), and triterpenes (stigmastan-3,5-diene). These results imply that the sequential extracts of honeybush tea (particularly the aqueous and ethyl acetate extracts)

may not only exhibit antioxidant potentials but also mediate anti-hyperglycaemia activities by inhibiting lipid and carbohydrate digestion. These findings will promote its utilization as a potential nutraceutical in the management of diabetes and its complications.

Keywords: Antioxidants; Honeybush tea; Sequential extraction; and Type 2 diabetes.

4.2.2 Introduction

Diabetes mellitus (DM) is a group of metabolic diseases whose hallmark characteristic is chronic hyperglycaemia. It is caused by disorders of carbohydrate, protein and fat metabolism resulting from defects in insulin secretion and/or action (WHO 1999, ADA 2014). Diabetes is associated with long-term damage, dysfunction, and even failure of multiple organs in severe circumstances. Currently, around 90% of all patients with diabetes worldwide are type 2 diabetes (T2D), or non-insulin-dependent diabetes mellitus (NIDDM).

T2D may result due to the inability of insulin responsive cells to utilize insulin or relatively low insulin levels being secreted by the pancreatic β -cells of the islets of Langerhans. Due to the mild nature of T2D especially in the early stages the first line management strategies do not involve the injection of insulin (Chatterjee et al. 2017). Nowadays, the clinical management of T2D mainly involves the use of oral pharmacological agents that stimulate the secretion of insulin from pancreatic β -cell, increase sensitivity of insulin to effector cells and retard post-prandial assimilation into the blood. These strategies are focused on achieving good glycaemic control to ultimately reduce the risk of development or progression of diabetes complications (Stein et al. 2013, Marín-Peñalver et al. 2016). Although oral antidiabetic drugs have exhibited some success in the management of hyperglycaemia, their effects are not always sustained and may be associated with undesirable side effects such as hypoglycaemic shock risk, weight gain and other comorbidities (Khardori and Griffing 2011, Chaudhury et al. 2017). Therefore, looking for therapeutic agent with reduced side effects will be of great significance in the management and possible treatment of diabetes. Medicinal plants have been reported for their uses in the management of diabetes. They are postulated to have superior benefits over synthesized drugs as they are believed to have less potential undesirable side effects, more affordable and readily available (Hung et al. 2012, He et al. 2019).

Honeybush tea, which is indigenous to South Africa and grows exclusively in the Cederberg Mountains north of Citrusdal to the Cape Peninsula in the South and Port Elizabeth in the Eastern Cape region (Joubert et al. 2009, Kokotkiewicz and Luczkiewicz 2009, Marnewick 2009). About 23-24 *Cyclopia* species have been discovered but only three species are

commercially made into honeybush tea namely, *Cyclopia intermedia*, *Cyclopia subternata* and *Cyclopia genistoides* (Joubert et al. 2009, Kokotkiewicz and Luczkiewicz 2009). Honeybush tea is characterized by sweet honey-like aroma, which is made from the leaves, stem, and flowers of the *Cyclopia* species (Kokotkiewicz and Luczkiewicz 2009). The effects of honeybush tea on antimutagenic, antioxidant, cancer modulating, and cardiovascular has been shown *in vitro* and *in vivo* studies (Marnewick et al. 2011, Panti et al. 2011).

The methanolic extracts of 22 *Cyclopia* species were screened by De Nysschen et al. (1996) who reported that the xanthone, mangiferin, the flavanone, hesperidin, and isosakuranetin were the major flavonoids common to all the species. Along with a more recent study by Joubert et al. (2008) showed that mangiferin, isomangiferin, and hesperidin are also present in all *Cyclopia* species. Although the phytochemicals of methanolic extracts of honeybush tea have been reported, the antioxidative and antidiabetic potentials of different extracts of red honeybush (*Cyclopia genistoides*) tea are still poorly documented.

Therefore, this study was designed not only to analyze phytochemical constituents of different extracts of RHT through GC-MS analysis but also to investigate their effects on key enzymes linked to T2D vis-à-vis α -glucosidase and lipase, as well as their mitigative effect on oxidative pancreatic injury, *ex vivo*.

4.2.3 Materials and methods

Please refer to Chapter 2 for the detailed materials and methods for red honeybush (*Cyclopia genistoides*) tea

4.2.4 Results

The aqueous and ethyl acetate extracts showed the highest total phenol content compared to the ethanol and dichloromethane (DCM) extracts with significant difference as presented in **Figure 4.2.1**.

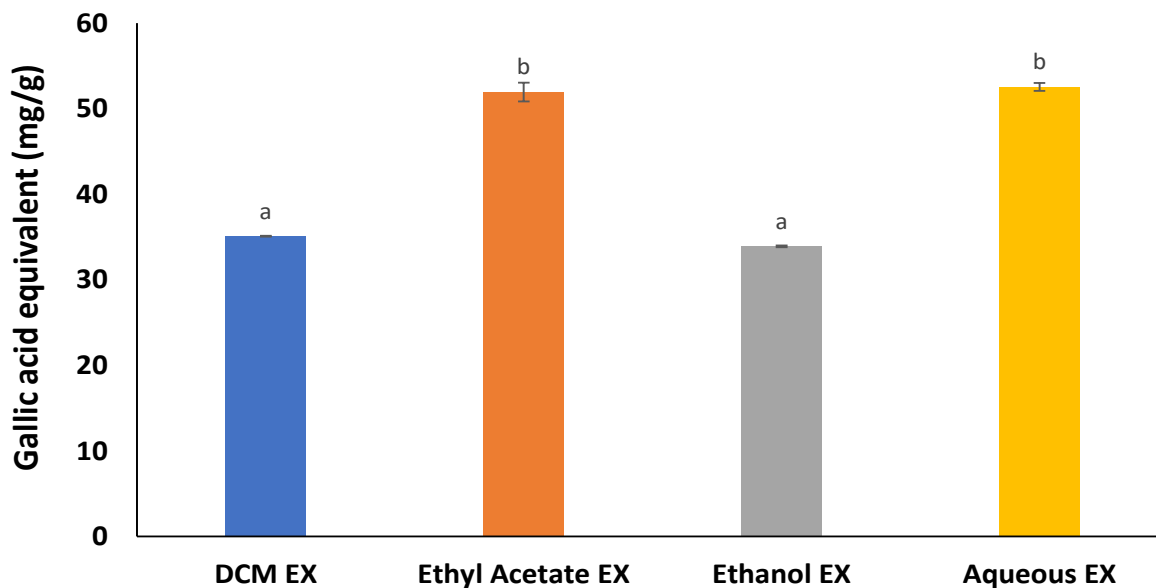


Figure 4.2.1: Total phenol content of different extracts of red honeybush (*Cyclopia genistoides*) tea. Values represent mean \pm standard deviation ($n = 3$). ^{ab} Different alphabets over the bars for a given extract represent significance of difference ($p < 0.05$). DCM = dichloromethane, EX = extract.

All the RHT extracts showed a dose-dependent DPPH free radical scavenging activity, where the aqueous showing the most potent activity ($IC_{50} = 0.12 \mu\text{g/mL}$). The activity of aqueous, ethyl acetate ($IC_{50} = 0.72 \mu\text{g/mL}$) and ethanol ($IC_{50} = 1.90 \mu\text{g/mL}$) extract were comparable to the positive control, ascorbic acid ($IC_{50} = 1.93 \mu\text{g/mL}$). The aqueous extract exhibited a significantly higher ($p < 0.05$) activity than other extracts except at concentration of $240 \mu\text{g/mL}$ (**Figure 4.2.2**).

The reducing power of the extracts are presented in **Figure 4.2.3**. All the extracts showed a dose-dependent activity, where the aqueous extract was exhibiting the most potent activity ($IC_{50} = 861.00 \mu\text{g/mL}$), while other extracts IC_{50} were over $1000 \mu\text{g/mL}$. However, the reducing power of all extracts were significantly higher ($p < 0.05$) than ascorbic acid standard with the aqueous extract showing significantly higher ($p < 0.05$) activity than other extracts at concentration of 120 and $240 \mu\text{g/mL}$ (**Figure 4.2.3**).

All the RHT extracts showed a dose-dependent nitric oxide radical scavenging activity and a significantly ($p < 0.05$) higher activity against NO-radicals than quercetin, with the aqueous extract showing the highest activity compared to other extracts except at the concentration of $240 \mu\text{g/mL}$ as depicted in **Figure 4.2.4**. The ethyl acetate extract showed the most efficient activity, as depicted by its IC_{50} value of $1.07 \mu\text{g/mL}$ compared to that of the positive control, quercetin ($116.65 \mu\text{g/mL}$) (**Table1**).

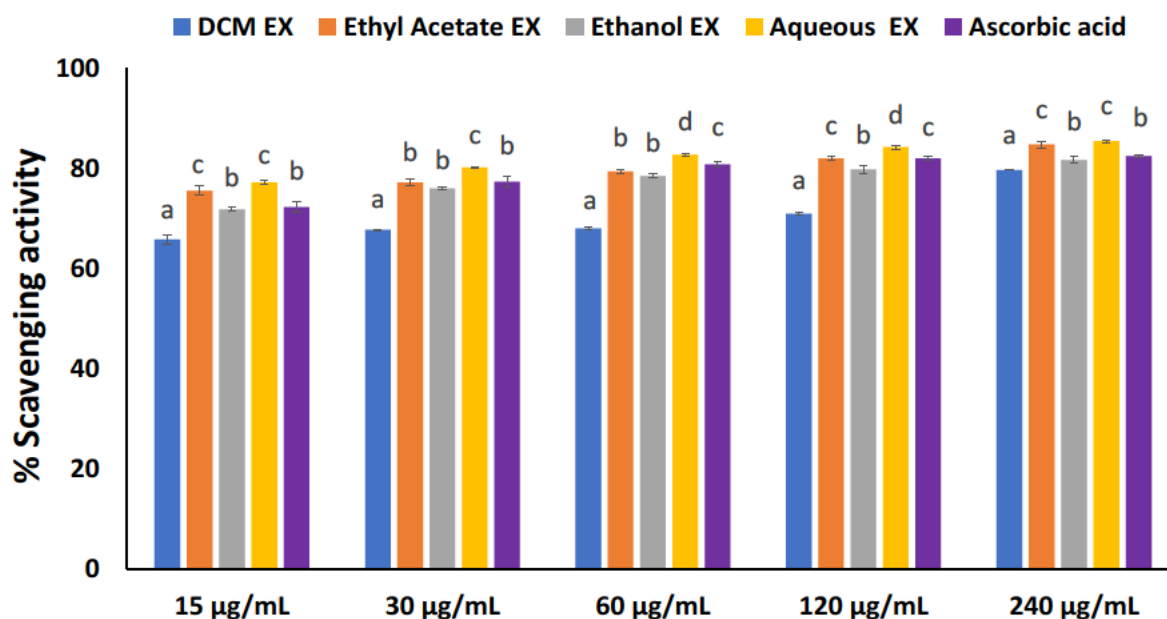


Figure 4.2.2: DPPH of different extracts of red honeybush (*Cyclopia genistoides*) tea. Values represent mean \pm standard deviation ($n = 3$). ^{a-d} Different alphabets over the bars for a given concentration for each extract represent significance of difference ($p < 0.05$). DCM = dichloromethane, EX = extract.

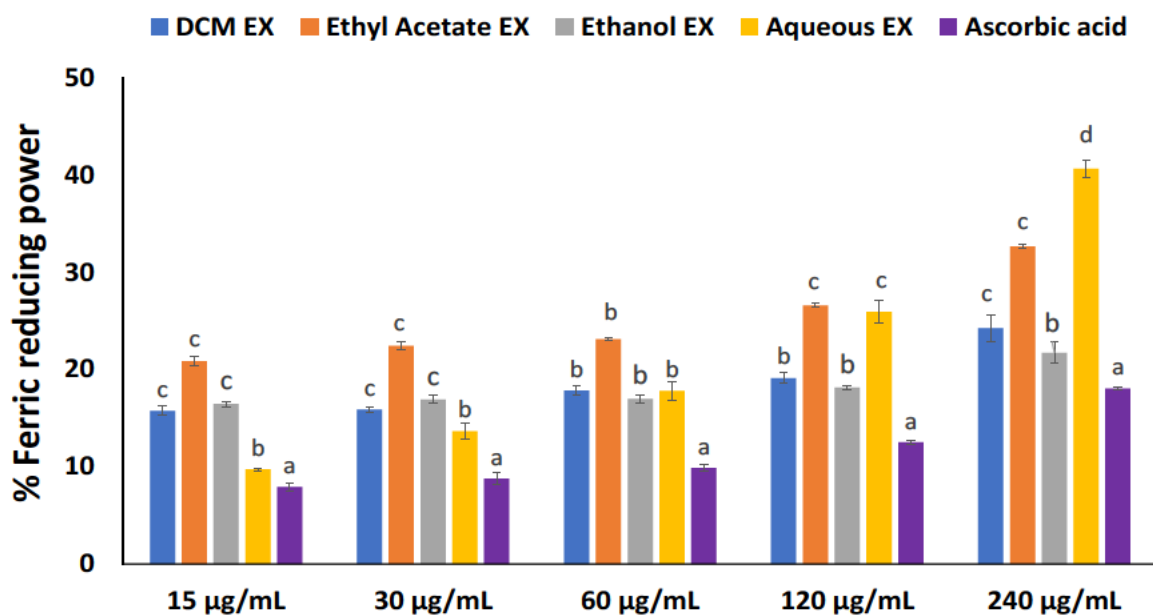


Figure 4.2.3: Ferric reducing antioxidant power (FRAP) of different extracts of red honeybush (*Cyclopia genistoides*) tea. Values represent mean \pm standard deviation ($n = 3$). ^{a-d} Different alphabets over the bars for a given concentration for each extract represent significance of difference ($p < 0.05$). DCM = dichloromethane, EX = extract.

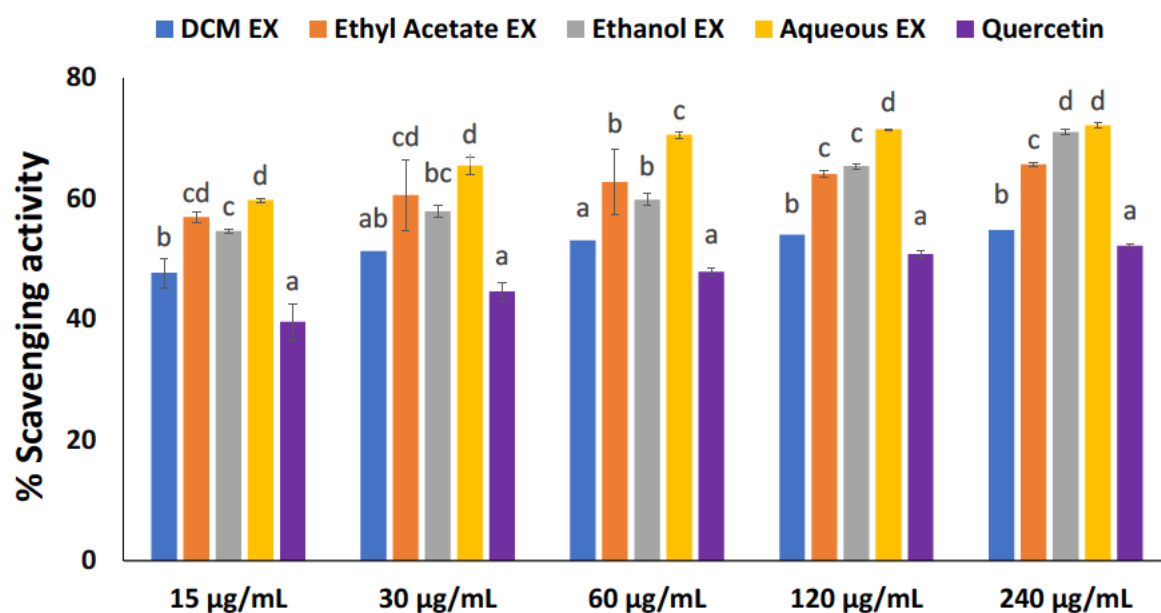


Figure 4.2.4: Nitric oxide scavenging activity (NO) of different extracts of red honeybush (*Cyclopia genistoides*) tea. Values represent mean \pm standard deviation (n = 3). ^{a-d} Different alphabets over the bars for a given concentration for each extract represent significance of difference ($p < 0.05$). DCM = dichloromethane, EX = extract.

Table 4.2.1: IC₅₀ values of different extracts of red honeybush (*Cyclopia genistoides*) tea

Activities	Dichloromethane Extract (µg/mL)	Ethyl Acetate Extract (µg/mL)	Ethanol Extract (µg/mL)	Aqueous Extract (µg/mL)	Ascorbic acid (µg/mL)	Quercetin (µg/mL)	Acarbose (µg/mL)	Orlistat (µg/mL)
DPPH	6.13	0.72	1.90	0.12	1.93	-	-	-
FRAP	>1000	>1000	>1000	861	>1000	-	-	-
NO	24.64	1.14	8.01	1.07	-	116.65	-	-
α -glucosidase	91.70	63.26	90.12	59.05	-	-	0.12	-
Anti-lipase	9.01	6.29	4.84	5.86	-	-	-	0.15
GSH	552.85	105.95	132.71	87.32	3.9	-	-	-
SOD	5.83	3.85	4.74	1.28	2.96	-	-	-
Catalase	>1000	>1000	>1000	>1000	>1000	-	-	-
LPO	12.67	10.36	12.42	9.23	7.87	-	-	-

IC₅₀, concentration to inhibit 50% activity. DPPH, 1,1-diphenyl-2-picrylhydrazyl; FRAP, ferric reducing antioxidant power; NO, nitric oxide; GSH, reduced glutathione; SOD, superoxide dismutase; LPO, lipid peroxidation.

All the RHT extracts significantly ($p < 0.05$) inhibited the activities of α -glucosidase in a dose-dependent manner, with the aqueous extract showing the highest activity except significantly lower than acarbose standard (Figure 4.2.5). The pancreatic lipase inhibitory activity of all extracts is shown in Figure 4.2.6. All extracts inhibited lipase activity in a dose-dependent manner. However, the activities were significantly lower ($p < 0.05$) than orlistat standard (Figure 4.2.6).

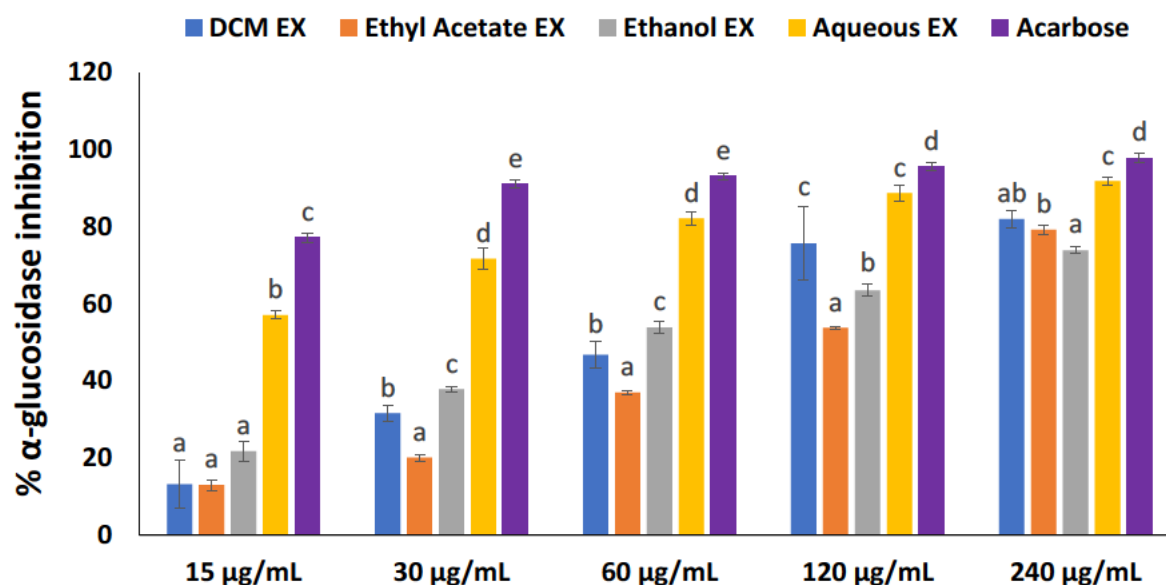


Figure 4.2.5: Inhibitory effect of different extracts of red honeybush (*Cyclopia genistoides*) tea on α -glucosidase activity. Data are presented as mean \pm SD. ^{a-e} Different alphabets over the bars for a given concentration are significantly ($p < 0.05$) different from each other. DCM = dichloromethane, EX = extract.

The induction of oxidative injury in the pancreatic tissue with FeSO_4 caused significant ($p < 0.05$) decrease in the levels of reduced GSH as depicted in Figure 4.2.7. Treatment with the aqueous and ethyl acetate extracts exhibited a significant increase in a dose-dependent effect, where the aqueous extract showing a better activity and lower IC_{50} value (Table 4.2.1).

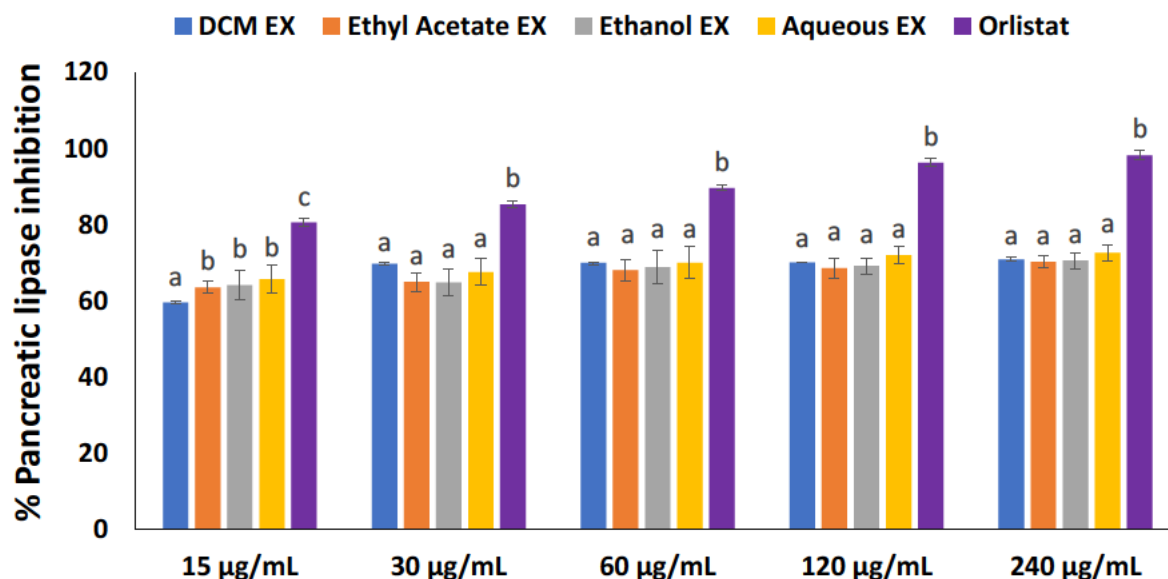


Figure 4.2.6: Inhibitory effect of different extracts of red honeybush (*Cyclopia genistoides*) tea on pancreatic lipase activity. Data are presented as mean \pm SD. ^{a-c} Different alphabets over the bars for a given concentration are significantly ($p < 0.05$) different from each other. DCM = Dichloromethane, EX = extract.

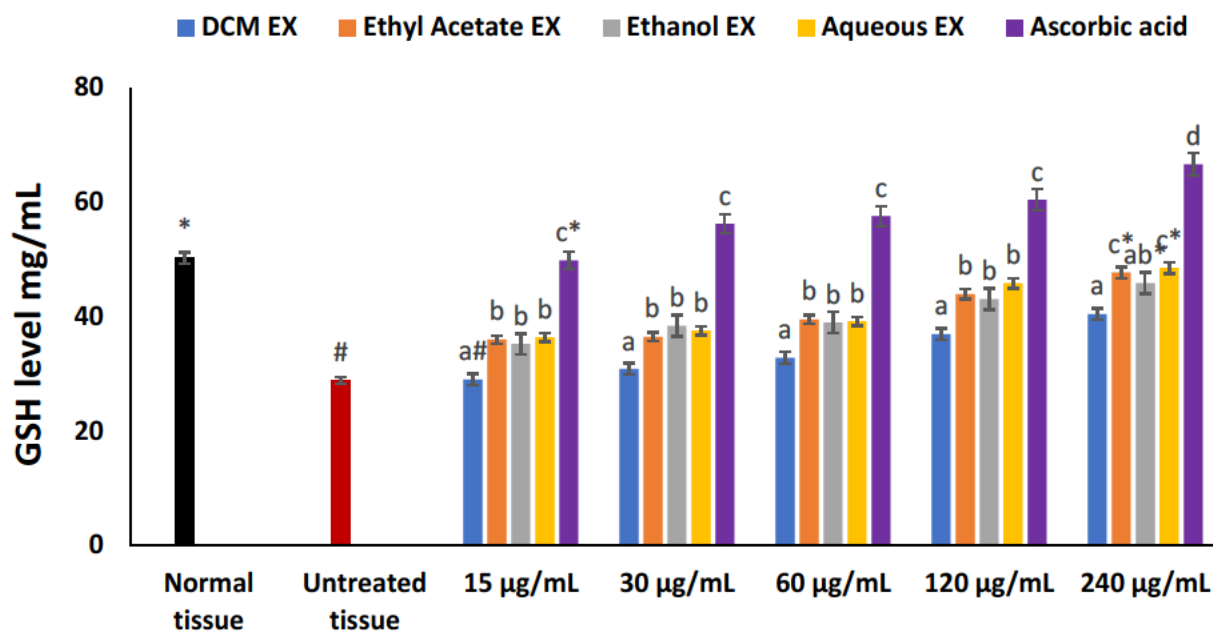


Figure 4.2.7: Effect of red honeybush (*Cyclopia genistoides*) tea on GSH level in oxidative pancreatic injury. Values = mean \pm SD; n = 3. *Significantly different from untreated sample and #Significantly ($p < 0.05$) different from normal sample. ^{a-d} Different alphabets over the bars for a given concentration are significantly ($p < 0.05$) different from each other. DCM = dichloromethane, EX = extract.

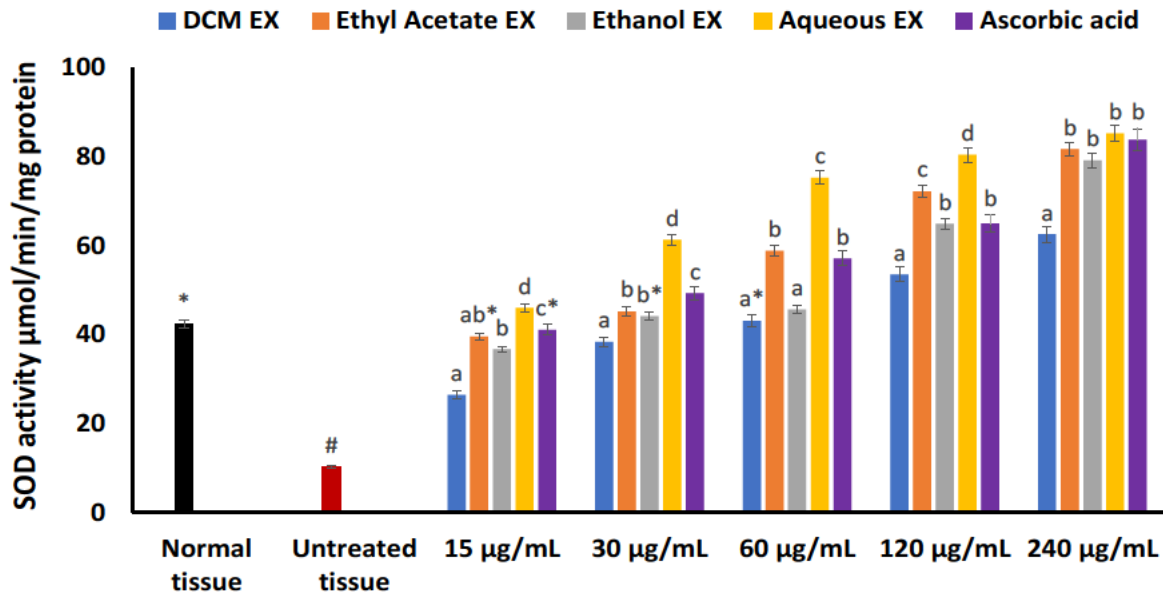


Figure 4.2.8: Effect of red honeybush (*Cyclopia genistoides*) tea on SOD activity in oxidative pancreatic injury. Values = mean \pm SD; n = 3. *Significantly different from untreated sample and #Significantly ($p < 0.05$) different from normal sample. ^{a-d}Different alphabets over the bars for a given concentration are significantly ($p < 0.05$) different from each other. DCM = dichloromethane, EX = extract.

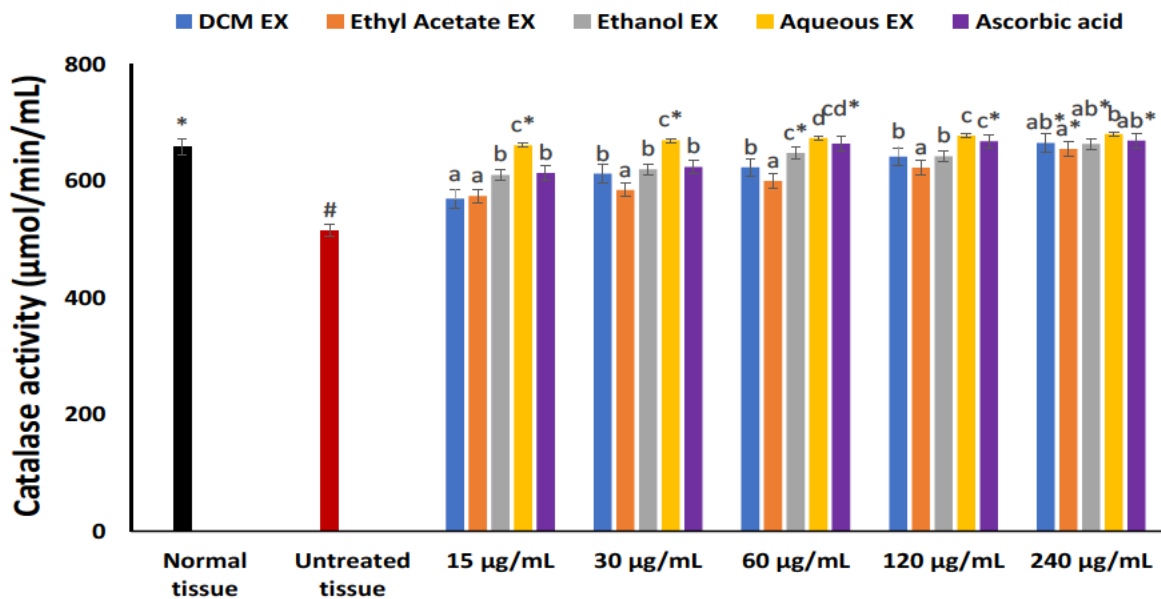


Figure 4.2.9: Effect of red honeybush (*Cyclopia genistoides*) tea on catalase activity in oxidative pancreatic injury. Values = mean \pm SD; n = 3. *Significantly different from untreated sample and #Significantly ($p < 0.05$) different from normal sample. ^{a-d} Different alphabets over the bars for a given concentration are significantly ($p < 0.05$) different from each other. DCM = dichloromethane, EX = extract.

There was a significant ($p < 0.05$) reduction in the SOD activity on induction of oxidative injury in the untreated pancreatic tissues. Treatment with the different extracts led to a dose-

dependent restorative effect, with the aqueous extract exhibiting the highest activity compared to other treatments (**Figure 4.2.8**) as depicted by its low IC₅₀ value in **Table 4.2.1**.

As shown in **Figure 4.2.9** induction of oxidative stress in the untreated pancreatic tissues led to decreased catalase activity. The activity of the honeybush tea extracts treated groups was significantly ($p < 0.05$) reversed.

The inhibitory effect of the honeybush tea extracts on Fe²⁺-induced lipid peroxidation in isolated pancreatic tissue is presented in **Figure 4.2.10**. There was an elevated level of MDA following the induction of oxidative injury. All extracts significantly reduced the MDA levels, with the aqueous extract being the most potent but exhibited less activity compared to that of ascorbic acid as depicted by their IC₅₀ values in **Table 4.2.1**.

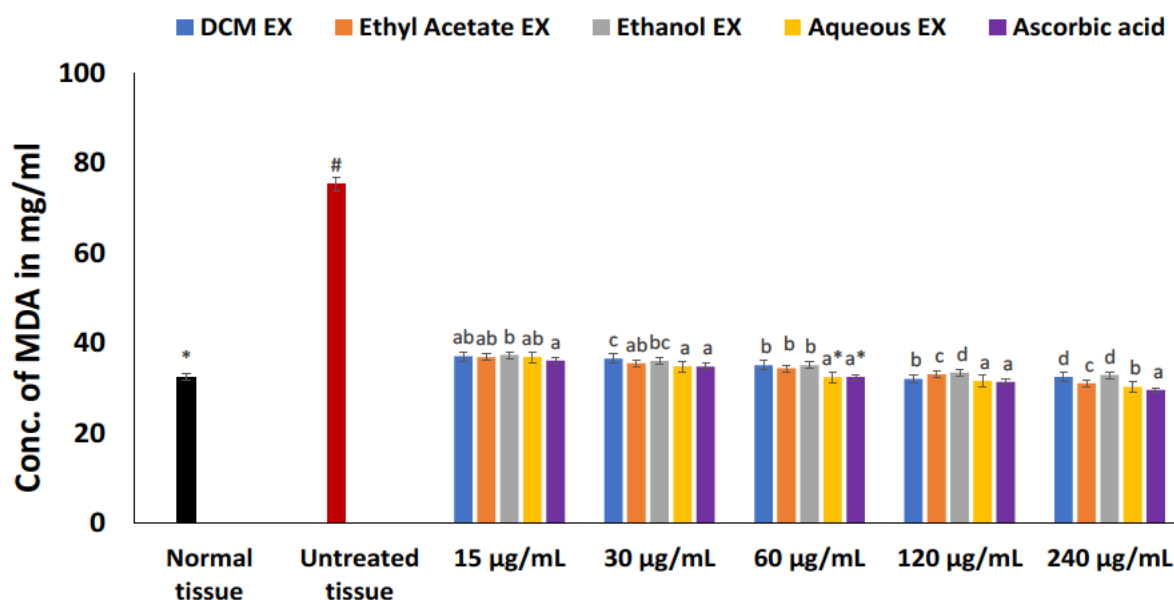


Figure 4.2.10: Effect of red honeybush (*Cyclopia genistoides*) tea on (D) MDA level in oxidative pancreatic injury. Values = mean ± SD; n = 3. *Significantly different from untreated sample and #Significantly ($p < 0.05$) different from normal sample. ^{a-d} Different alphabets over the bars for a given concentration are significantly ($p < 0.05$) different from each other. DCM = dichloromethane, EX = extract.

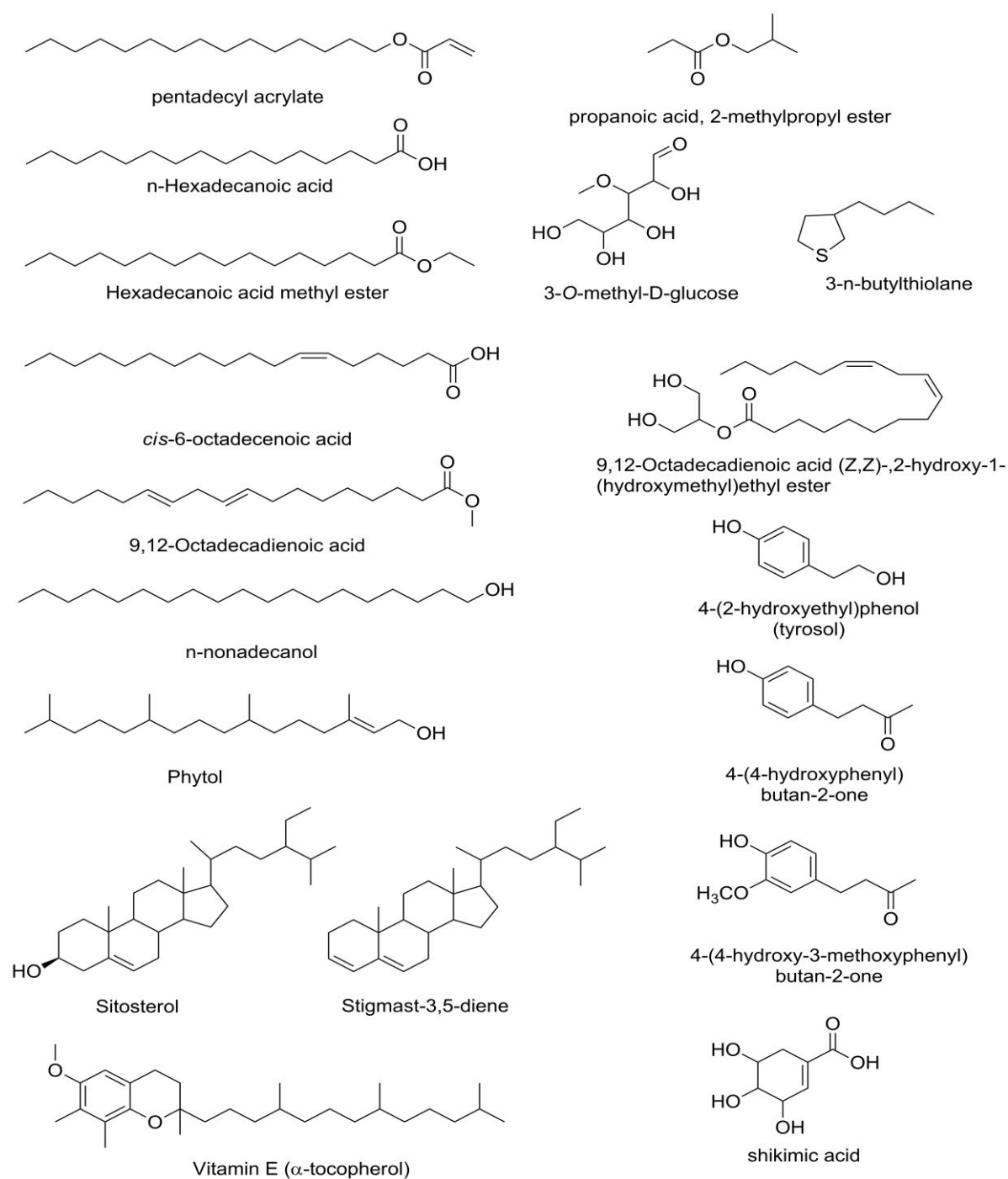


Figure 4.2.11: Chemical structures of compounds identified in the DCM, ethyl acetate, ethanol, and aqueous extract of red honeybush tea (*Cyclopia genistoides*) by GC-MS.

Table 4.2.2: Compounds identified in different extracts of red honeybush (*Cyclopia genistoides*) tea by GC-MS

Compounds	Molecular formula	RT (min)	Molecular mass	Relative abundance (%)
DCM extract				
Propanoic acid, 2-methylpropyl ester	C ₇ H ₁₄ O ₂	5.05	130	1.94
n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	18.82	256	8.80
Hexadecanoic acid methyl ester	C ₁₈ H ₃₆ O ₂	19.13	284	0.68
n-Nonadecanol-1	C ₁₉ H ₄₀ O	20.02	284	0.49
<i>cis</i> -6-Octadecenoic acid	C ₁₈ H ₃₄ O ₂	20.54	282	10.10
4-(4-hydroxyphenyl)butan-2-one	C ₁₀ H ₁₂ O ₂	14.32	164	0.33
4-(4-hydroxy-3-methoxyphenyl)-2-butanone	C ₁₁ H ₁₄ O ₃	15.46	194	0.73
Phytol	C ₂₀ H ₄₀ O	20.25	296	0.48
Vitamin E (α -tocopherol)	C ₂₉ H ₅₀ O ₂	28.31	430	0.85
sitosterol	C ₂₉ H ₅₀ O	30.21	414	0.87
Ethyl acetate extract				
n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	18.82	256	11.33
n-Nonadecanol-1	C ₁₉ H ₄₀ O	19.15	284	1.89
9,12-Octadecadienoic acid, methyl ester	C ₁₉ H ₃₄ O ₂	20.49	294	17.52
<i>cis</i> -6-Octadecenoic acid	C ₁₈ H ₃₄ O ₂	20.54	282	13.71
Pentadecyl acrylate	C ₁₇ H ₃₂ O ₂	21.93	268	1.09
tyrosol	C ₈ H ₁₀ O ₂	12.70	138	1.58
4-hydroxybenzoic acid	C ₇ H ₆ O ₃	13.64	138	0.45
4-(4-hydroxyphenyl)butan-2-one	C ₁₀ H ₁₂ O ₂	14.33	164	0.37
4-(4-hydroxy-3-methoxyphenyl)-2-butanone	C ₁₁ H ₁₄ O ₃	15.46	194	0.36
Phytol	C ₂₀ H ₄₀ O	20.25	296	0.46
stigmastan-3,5-diene	C ₂₉ H ₄₈	28.14	396	0.76
Vitamin E (α -tocopherol)	C ₂₉ H ₅₀ O ₂	28.32	430	0.64

Ethanol extract

Shikimic acid	C ₇ H ₁₀ O ₅	17.02	174	1.21
3- <i>O</i> -Methyl-D-glucose	C ₇ H ₁₄ O ₆	16.04	194	2.38
n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	18.81	256	12.12
6-Octadecenoic acid	C ₁₈ H ₃₄ O ₂	20.54	282	17.18
9,12-Octadecadienoic acid (Z,Z)-,2-hydroxy-1-(hydroxymethyl)ethyl ester	C ₂₁ H ₃₈ O ₄	25.11	354	1.19

Aqueous extract

3-n-Butylthiolane	C ₈ H ₁₆ S	15.85	144	2.14
3- <i>O</i> -Methyl-D-glucose	C ₇ H ₁₄ O ₆	15.925	194	2.19
n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	18.82	256	9.80
9,12-Octadecadienoic acid, methyl ester	C ₁₉ H ₃₄ O ₂	20.51	294	22.76
<i>cis</i> -6-Octadecenoic acid	C ₁₈ H ₃₄ O ₂	20.55	282	13.94
9,12-Octadecadienoic acid (Z,Z)-,2-hydroxy-1-(hydroxymethyl)ethyl ester	C ₂₁ H ₃₈ O ₄	25.11	354	3.40

The compounds presented in the table are those which matched similar compounds in the NIST library software.

The identification of some major bioactive compounds of different honeybush tea extracts were carried out using GC-MS analysis. The retention time (RT), molecular formula, molecular mass and relative abundance are shown in **Table 4.2.2** and **Figure 4.2.11**. The structures of the possible compounds presented in **Figure 4.2.11**, 19 phytochemicals were identified from the different extracts, including seven major classes of compounds: fatty acid (n-hexadecanoic acid, *cis*-6-Octadecenoic acid, 4-hydroxybenzoic acid, shikimic acid), fatty acid ester (propanoic acid, 2-methylpropyl ester, hexadecanoic acid methyl ester, 9,12-octadecadienoic acid, methyl ester, 9,12-octadecadienoic acid (Z,Z)-,2-hydroxy-1-(hydroxymethyl)ethyl ester, pentadecyl acrylate), phytols (n-nonadecanol-1, tyrosol, phytol, 3-n-butylthiolane), sterols (sitosterol, vitamin E (α -tocopherol)), saccharide (3-*O*-Methyl-D-glucose), ketones (4-(4-hydroxyphenyl)butan-2-one, 4-(4-hydroxy-3-methoxyphenyl)-2-butanone), and triterpenes (stigmastan-3,5-diene).

4.2.5 Discussion

The global prevalence of diabetes has reached a record high and continues to rise. This has prompted the search for cost-effective novel antidiabetic agents. Traditional medicinal plants which have been employed for therapeutic purposes since time immemorial have emerged as readily available options providing templates for development of antidiabetic pharmacological agents. The therapeutic nature of plants emanates from groups of secondary metabolites they contain such as terpenoids, saponins, alkaloids and polyphenols. Polyphenols are well-known for their beneficial health effects (Bazzano et al. 2002, Wang et al. 2008). They mainly act as antioxidants due to the presence of hydroxyl substituents and aromatic ring structures (Kefalas et al. 2003). The moderately high total phenol contents of the ethyl acetate and aqueous extracts the tea (**Figure 4.2.1**), depicts the presence of phenolics in the tea, which insinuates its beneficial health properties.

The ability of the different extracts of honeybush tea to scavenge free radicals was determined using the DPPH method. This colorimetric assay is based on the colour change that occurs when an unstable form of the DPPH (hydrazyl-purple) is converted to a more stable form (hydrazine-yellow). This conversion is facilitated by the coupling of the lone pair of electrons in this synthetic free radical to those from an antioxidant compound (Villaño et al. 2007). *In vitro* DPPH analysis of the different solvent extracts (**Figure 4.2.2 and Table 4.2.1**) showed dose dependent DPPH scavenging activity. The ethyl acetate and aqueous extracts exhibited good DPPH radical scavenging activity with IC₅₀ values of 0.72 µg/mL and 0.12 µg/mL respectively. These extracts marginally outperformed ascorbic acid (positive control) which had an IC₅₀ value of 1.93 µg/mL.

Furthermore, the antioxidant capacity of the extracts in terms of their electron donating potential was analysed by the ferric reducing antioxidant power assay (FRAP) (Benzie and Strain 1996). In this assay, extracts that are capable of electron donation result in the formation of a Prussian coloured ferric-ferrocyanide complex (Vijayalakshmi and Ruckmani 2016). The deepness of the resulting blue solution is directly proportional to the electron donating ability of the extract. The aqueous extract resulted in the best ferric ion reducing activity with an IC₅₀ of 861 µg/mL while the rest had IC₅₀ values greater than 1000 µg/mL including ascorbic acid (positive control) (**Figure 4.2.3**). Both the DPPH and FRAP are robust routine antioxidant assays that are used to determine the hydrogen and electron donating capabilities of plant derived extracts. Previous studies have shown a high level of correlation between the amount

of total phenolic content and antioxidant activity (Oyebode et al. 2018, Xiao et al. 2020). A similar trend was observed in the current study where the aqueous extract which had the highest total phenolic content had the best activity in both the DPPH and FRAP assays. Phenolic compounds are rich in delocalised electrons on their π ring structure that can be readily donated especially to unstable electron deficient radicals. The enhanced antioxidant activity of the aqueous extract observed in this study was also previously reported in fermented *C. genistoides* (Joubert et al. 2008). Exogenous antioxidants help supplement the endogenous antioxidant capacity within the body thereby aid to prevent the harmful effects of oxidative stress.

Under conditions of oxidative stress, there is overproduction of nitric oxide (NO) which is an important secondary messenger under homeostatic conditions. The excess nitric oxide (NO) may subsequently interact with superoxide anion to form a much more powerful oxidant peroxynitrite (ONOO⁻), which is implicated in the induction β cell apoptosis (Oyadomari et al. 2001, Pacher et al. 2007). Moieties that retard the production of excess nitric oxide may therefore play a protective function in the afore mentioned β cell destruction. Interestingly, all the extracts assayed in the current study showed remarkable NO production inhibition capabilities (**Figure 4.2.4 and Table 4.2.1**), especially the ethyl acetate and aqueous extract with IC₅₀ values of 1.143 μ g/mL and 1.067 μ g/mL.

Ingested dietary complex carbohydrates are successively degraded into simpler forms by an array of glycosidic bond cleaving enzymes. The α -glucosidase enzyme is one such enzyme that is located on the intestinal cell membrane where it facilitates the final hydrolysis of oligosaccharides, trisaccharides, and disaccharides to various mono-saccharides including glucose. Absorption of these simple sugars is responsible for the post-prandial blood glucose spikes observed after a carbohydrate rich meal. In the current study among all extracts assayed, the aqueous extract of honeybush tea (**Figure 4.2.5**), showed the best α -glucosidase inhibitory activity with an IC₅₀ value of 59.05 μ g/mL (**Table 4.2.1**). However, the extract did not surpass the inhibitory activity of acarbose (positive control) which had an IC₅₀ value of 0.12 μ g/mL. The significant difference in inhibitory activity between the aqueous extract of honeybush tea and acarbose may be attributed to the former being a crude mixture of compounds while the latter is a pure compound. Identification and isolation of the active compound in the honeybush tea may help enhance its activity. Compounds with α -glucosidase inhibitory activity have been recognised as potent antidiabetic agents, because they can effectively retard the digestion of complex carbohydrates and their subsequent absorption, thereby reducing the postprandial blood glucose spikes (Hara and Honda 1990). Such activity has been shown in previous studies

(Beelders et al. 2014). Thus, persuades us to suggest that the ethyl acetate and aqueous extracts of honeybush tea can be exploited as potent candidates for isolation of α -glucosidase inhibitors which might be effective as therapeutic agents against T2D.

The excessive intake of calories in the diet together with a sedentary lifestyle may cause obesity. Obesity is the accumulation of body fat causing one to be overly overweight and is associated with several negative health effects. Dietary triglycerides serve as a high-calorie energy source for the body but are often not utilised and are therefore usually stored as fats in the adipose tissue (Rial et al. 2016). Pancreatic lipase is an enzyme that catalyses the hydrolysis of triglycerides in the gastrointestinal tract. Therefore, inhibiting pancreatic lipase activity can prevent triglyceride absorption, thus contributes to the management of obesity and its related diseases. In the current study it was shown that the ethanol extract of honeybush tea had the highest pancreatic lipase inhibitory activity (**Table 4.2.1**), followed by the aqueous extract with IC₅₀ values of 4.84 μ g/mL and 5.86 μ g/mL respectively.

Oxidative stress occurs when the bodies antioxidant defence systems are overwhelmed by the amount of reactive species being produced during normal tissue metabolism. Diabetes is known to aggravate the extent to which oxidative stress occurs in tissues throughout the body. However due to the low expression of antioxidant enzymes such as catalase and superoxide dismutase in the pancreatic tissues, β cells are particularly prone to damage by oxidative stress (Limón-Pacheco and Gonsebatt 2009). In the current study FeSO₄ was employed as a pro-oxidant as it is known to generate substantial amounts of oxidized free radicals through the Haber-Weiss reaction (Rajpathak et al. 2009; Schulze and Hu 2005). This reaction was employed to mimic conditions of elevated oxidative stress that is particularly observed in diabetic patients. The incubation of the pancreatic tissue homogenate with FeSO₄ in the current study was shown to significantly decrease of GSH levels and SOD, catalase enzyme activities while concomitantly the increasing of lipid peroxidation with respect to the untreated group (**Figures 4.2.7 – 4.2.10**). This observation coincides with previous studies on the effects of oxidative stress on antioxidant enzyme activity and lipid peroxidation (Erukainure et al. 2017, Sanni et al. 2018).

Glutathione GSH is an important antioxidant in the body, it serves as a direct free-radical scavenger, as a substrate for glutathione peroxidase activity, and as a cofactor for many enzymes (Aslani and Ghobadi 2016). The increased level following treatments with the extracts (**Figure 4.2.7**) therefore depicts an improved antioxidant activity.

Superoxide dismutase (SOD) is among the endogenous antioxidant enzymes. SOD consists of active protein peptides with metal ion groups which can specifically catalyses the disproportionation reaction of superoxides generated in the metabolism to hydrogen peroxide (Mirończuk-Chodakowska et al. 2018). The products of this reaction are effectively decomposed to water and oxygen in a reaction catalysed by catalase (Raza et al. 2017). The marked increase in the activity of catalase and SOD enzymes after the treatment with different concentrations (**Figures 4.2.8 & 4.2.9**) demonstrated the antioxidant activity of honeybush tea solvent extracts.

Lipid peroxidation is a multistep chain of reactions which includes initiation, propagation and termination in which oxidants attack the polyunsaturated fatty acids. Various secondary products such as malondialdehyde (MDA) are formed in this reaction. Reaction of MDA and thiobarbituric acid is routinely used to spectrophotometrically to determine lipid peroxidation (Halliwell and Chirico 1993). In the current study, there is significant reduction of lipid peroxidation after the treatment with different concentrations of honeybush tea, shows the antioxidant potential of the aqueous and ethyl acetate extracts (**Figure 4.2.10**). As lipids are the basic biomolecules on which membrane structure is built. They also perform other functions such as chemical messengers and as prerequisites for the cholesterol and vitamin formation. Therefore, extracts such as those from RHT that retard their destruction through peroxidation have a therapeutic function in curbing the development of various degenerative complications that result from extreme oxidative stress conditions. These may furthermore assist the endogenous antioxidant enzymes that normally counter the harmful effects of reactive oxygen species.

The sequential extraction method was used to obtain differentiated extracts of RHT. Solvents were chosen based on their polarity gradient in order to extract the compounds soluble at different polarity levels. GC-MS analysis of the extracts was done in order to identify the presence of possible bioactive compounds that could be responsible for the observed antioxidant and antidiabetic activities. 19 phytochemicals were identified from this plant (**Figure 4.2.11 and Table 4.2.2**), which included seven major classes of compounds: fatty acids, fatty acid esters, ketones, sterols, phytols, saccharide and triterpenes, many of them have been reported to possess various medicinal properties including antioxidant and antidiabetic activity (Orhan et al. 2012, Sosa et al. 2016, Islam et al. 2018). Hexadecanoic acid, ethyl ester and vitamin E have been reported for their potent antioxidant activities (Garg and Bansal 2000,

Kumar et al. 2010). While 4-(4-hydroxy-3-methoxyphenyl)-2-butanone, sitosterol and shikimic acid have been reported for their antidiabetic activities (Gupta et al. 2011, Orhan et al. 2012, Ahmad et al. 2015). These identified compounds may thus be responsible for the studied antioxidative and antidiabetic activities of honeybush tea.

4.2.6 Conclusion

These results demonstrate the antioxidant and antidiabetic activities of the different extracts of honeybush tea, especially the ethyl acetate and aqueous. Their antioxidative and antidiabetic potentials are evident by free radical scavenging, lipid peroxidation elimination, decrease of α -glucosidase and lipid digesting enzymes activities, as well as the increase of antioxidant enzymes activities. These activities may be attributed to the total phenolic content and the synergistic effect of the identified phytochemicals.

4.3 The *in vivo* antioxidant and antidiabetic effect of red honeybush (*Cyclopia genistoides*) tea hot water extract in fructose-streptozotocin induced type 2 diabetic model of rats

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4.3.1 Abstract

This study was conducted to investigate the *in vivo* antioxidant and antidiabetic activity of red honeybush tea (RHT) (*Cyclopia genistoides*) concentrated hot water extract in type 2 diabetes (T2D) model of rats. T2D was induced by starting with feeding 10% fructose solution *ad libitum* for 2 weeks instead of drinking water, followed by a single intraperitoneal injection of streptozotocin (40 mg/kg body weight (BW)). After the confirmation of T2D, a low or high dose (150 and 300 mg/kg BW) of RHT in drinking water was orally administered to two groups of diabetic rats for 5 weeks (5 days/week) respectively. Metformin and glibenclamide served as standard drugs. The normal toxicology group was orally treated with RHT (300 mg/kg BW), while normal rats were given water served as normal control. Five weeks of treatment of RHT led to significant ($p < 0.05$) elevation in serum insulin, pancreatic β -cell function, HDL-c levels with concomitant decrease in AST, ALT, ALP, urea, CK-MB, fructosamine, total cholesterol, triglycerides, LDL-c, and insulin resistance in diabetic rats. RHT also significantly ($p < 0.05$) decreased MDA levels and enhanced level of GSH, activity of SOD, catalase, GR in most of organs (pancreas, liver, kidneys, and heart). Significantly ($p < 0.05$) improved morphological changes in the islets and β -cells were observed in rats treated with RHT. The data of this study suggest that RHT demonstrated outstanding antioxidant and antidiabetic effects in STZ-induced T2D model of rats.

Keywords: Red honeybush tea (*Cyclopia genistoides*), Antioxidant, Antidiabetic, STZ-induced T2D rats.

4.3.2 Introduction

Diabetes mellitus (DM) is a multifactorial chronic metabolic disorder rapidly increasing and becoming a major health related global pandemic. According to the 9th edition of *Diabetes Atlas* as published by International Diabetes Federation (IDF), the number of diabetics aged 20-79 years worldwide were around 463 million in 2019, which accounts for 9.3% of the world's population. And it is predicted to escalate to 578 million (10.2%) by 2030 and to 700 million (10.9%) by 2045 (IDF 2019). Moreover, DM and its complications killed approximately 4.2 million people in 2019 and was the 7th leading cause of death in 2016 (WHO), and the 4th leading cause of disability in 2017 in the world (WHO 2018). Globally, the prevalence of DM varies across different regions, income, age and gender groups. African has the lowest regional prevalence, but with the highest proportion of undiagnosed adult diabetes compared to other IDF regions. South Africa has the highest number of diabetics aged 20-79 years, the highest DM mortality, and the largest percentage of DM-related health expenditure in Africa in 2019 (IDF 2019).

DM has been primarily classified into type 1 diabetes mellitus and type 2 diabetes mellitus (T2D) (WHO 2019). T2D is the most common form of DM which account for over 90% of patients with diabetes. It is characterized by chronic hyperglycaemia and impaired lipids, carbohydrates and proteins metabolism caused by abnormal insulin secretion or insulin insensitivity (Chatterjee et al. 2017). Chronic hyperglycaemia leads to the accumulation of reactive oxygen species (ROS) and impairment of endogenous antioxidant defence system, which most likely trigger oxidative stress. Oxidative stress in turn combined with hyperglycaemia increasing insulin resistance and aggravating β -cell dysfunction, exacerbating T2D (Folli et al. 2011, Aouacheri et al. 2015). In addition, mounting evidence suggest that oxidative stress plays a causal role in the pathogenesis of T2D micro- and macro- vascular complications, that exist as comorbidities. The microvascular complications are principally diabetic nephropathy, retinopathy, neuropathy, and lower extremity amputations. while the macrovascular complications include cardiovascular diseases, myocardial infarction, stroke, coronary artery disease and peripheral arterial disease (Susan van et al. 2010, Halim and Halim 2019). Furthermore, a multitude of risk factors contribute to the widespread of T2D. This consists of unmodifiable risk factors including aging, genetics predispositions, race/ethnicity. While relatively more actions can be done to regulate modifiable risk factors such as an unhealthy diet change, sedentary lifestyle modification, body overweight control (Wu et al.

2014, Rawshani et al. 2018). Thus, it is crucial to modulate hyperglycaemia and oxidative stress to control or treat T2D and its complications.

Currently, most pharmaceutical drugs used in the treatment of T2D are usually expensive and most unlikely available to lots people, particularly, those who lives in low- and middle-income countries, and they often have unwanted side effects (Chaudhury et al. 2017, Bommer et al. 2018). Numerous evidence exhibits the multiple health effects such as antioxidant, antidiabetic, anticancer and antimicrobial nature of antioxidants including polyphenols contained in many nutraceuticals (Srinivasan 2005, Tapas et al. 2008, Chanda et al. 2019). Around 80% of the emerging world prefer to employ nutraceuticals including tea, vegetables, fruits, and herbals in the prevention, management and treatment of T2D, which might because of they are more accessible to self-care, safer and widely available alternative compared to that of existing pharmaceuticals (Ekor 2014, Windvogel 2019). This practise may involve the utilization of indigenous South African plant species, including RHT.

Honeybush (*Cyclopia spp.*), member of the fynbos biome, is a bushy shrub endemic to Cape Floristic Region, Western cape and Eastern Cape, South Africa (Windvogel 2019). Honeybush tea is characterized by its sweet honey-like aroma, which is made from different parts of *Cyclopia* species, especially their sweet-smelling flowers (Kokotkiewicz and Luczkiewicz 2009). The traditional fermented (brown-reddish) type of honeybush tea enjoys its commercial success in the market, which is also known as red honeybush tea (Ajuwon et al. 2018). Honeybush comprises 23-24 species, with 11 of them has been consumed as herbal tea, and six has been demonstrated for their commercial use or medicinal properties (Van Wyk and Gorelik 2017). These are *Cyclopia genistoides*, *Cyclopia intermedia*, *Cyclopia sessiflora*, *Cyclopia maculata*, *Cyclopia subternata* and *Cyclopia longifolia* (Joubert et al. 2011, Van Wyk 2011). The antioxidant, antidiabetic, anticancer and health-promoting properties of *Cyclopia species* have been well-documented, which mainly attributed to their phytochemicals such as mangiferin, isomangiferin, hesperidin and isokuranetin (McKay and Blumberg 2007, Marnewick 2009, Xing et al. 2014, Ajuwon et al. 2018, Windvogel 2019).

Cyclopia genistoides is the earliest *Cyclopia* species consumed as herb tea, and the only species with documented traditional medicinal use (Watt and Breyer-Brandwijk 1962). A recent review has mentioned *C. genistoides* used as in the form of decoction or expectorant in the treatment of tuberculosis, chronic catarrh, and consumption (Van Wyk and Gorelik 2017). Aadditionally, Beelders et al. (2014) reported the inhibition activity of *C. genistoides* isolated compounds on

mammalian α -glucosidase, which further highlighted by works that demonstrated the synergistic effects of *C. genistoides* with acarbose in the inhibition activity of mammalian intestinal α -glucosidase *in vitro* (Miller et al. 2020). One earlier paper published by Hubbe and Joubert (2000) exhibited the *in vitro* antioxidant of *C. genistoides*. Furthermore, Murakami et al. (2018) reported the immunostimulatory activity of 40% Et-OH water extracts of *C. genistoides* in primary murine splenocyte and lymphocyte cell cultures model. However, to date, there is no *in vivo* antidiabetic study has been published on red honeybush (*Cyclopia genistoides*) tea.

Therefore, the current study focuses on the hot water extract of RHT as it represents its normal way of daily preparation as herbal tea, to evaluate its *in vivo* antioxidant activity and antidiabetic effects in a fructose-fed streptozotocin (STZ)-induced T2D model of rats.

4.3.3 Materials and methods

Please refer to the Chapter 2 for details of the methods used for red honeybush (*Cyclopia genistoides*) tea

4.3.4 Results

All the diabetic groups had significantly ($p < 0.05$) higher mean daily feed and water intake than NC group and NRH group, with DBC group exhibiting the highest daily feed and water intake as shown in **Figure 4.3.1**. DRHH group displayed slightly lower feed intake and higher water intake when compared with DRHL group. Meanwhile, DBM group had significantly lower feed and water intake than that of RHT-treated groups. No statistically significant ($p > 0.05$) difference was observed between RHT-treated groups and DBG group in feed intake, when DBG group exhibited significantly ($P < 0.05$) lower water intake than DRHH group.

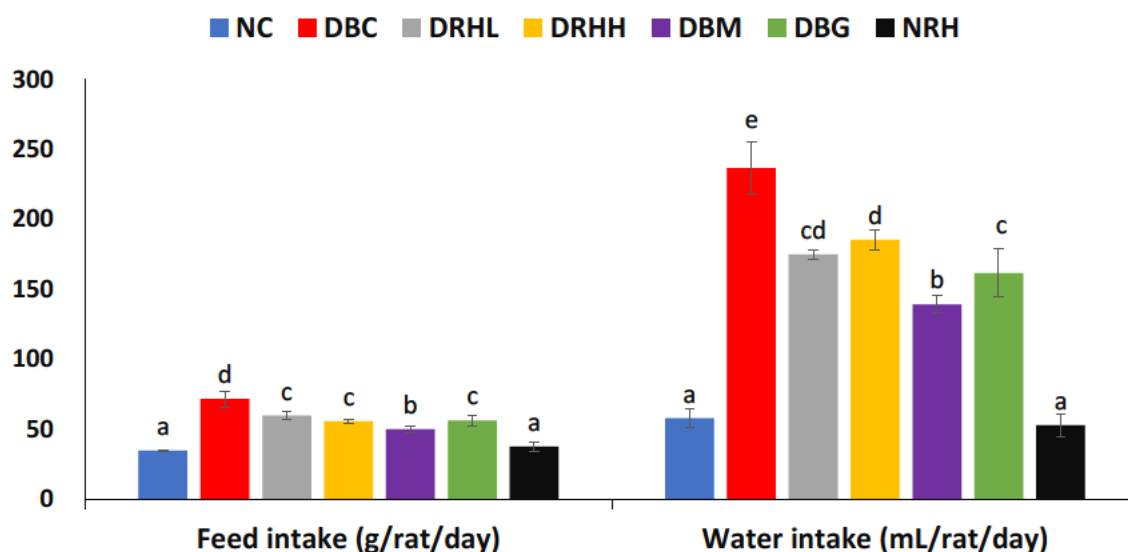


Figure 4.3.1: Feed and fluid intake in different animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given animal group represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DRHL: Diabetic red honeybush (*Cyclopia genistoides*) tea low dose; DRHH: Diabetic red honeybush (*Cyclopia genistoides*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NRH: Normal red honeybush (*Cyclopia genistoides*) tea high dose (toxicological control).

The weekly body weight (BW) changes in all animal groups was presented in **Figure 4.3.2**. No significant difference of initial BW (week 0) was seen in different groups; however, the BW gains of diabetic groups were significantly ($p < 0.05$) lower than that of NC group and NRH group after the STZ injection. As the remaining experimental period (week 2 to week 6) proceeded, there were only slightly, but not significantly ($p > 0.05$) elevated BW showing in RHT- and glibenclamide- treated groups compared with DBC group. DBM group exhibited a significantly ($p < 0.05$) higher BW gain than DBC group. There was no significant ($p > 0.05$) difference between DRHL and DRHH groups.

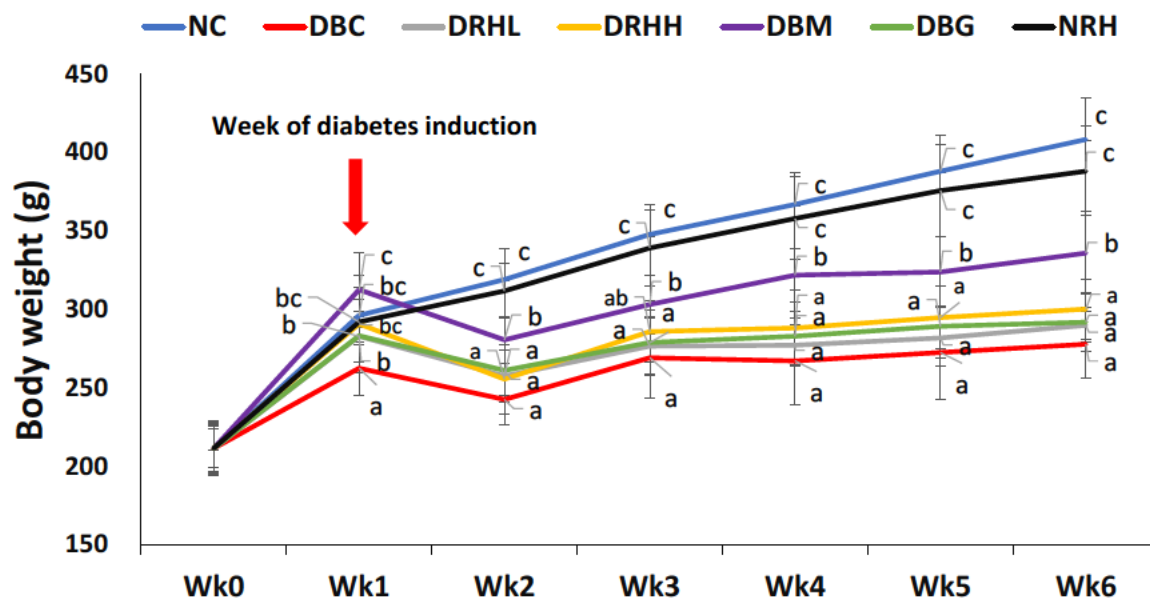


Figure 4.3.2: Mean body weight in different animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given animal group represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DRHL: Diabetic red honeybush (*Cyclopia genistoides*) tea low dose; DRHH: Diabetic red honeybush (*Cyclopia genistoides*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NRH: Normal red honeybush (*Cyclopia genistoides*) tea high dose (toxicological control).

There were significantly ($p < 0.05$) higher blood glucose concentrations in all diabetic groups when compared to normal groups upon induction of T2D as indicated in **Figure 4.3.3**. Besides, all diabetic treatment groups displayed a reduction in the blood glucose levels compared to DBC group, with a markedly lower blood glucose level showing during the last two weeks of the intervention period.

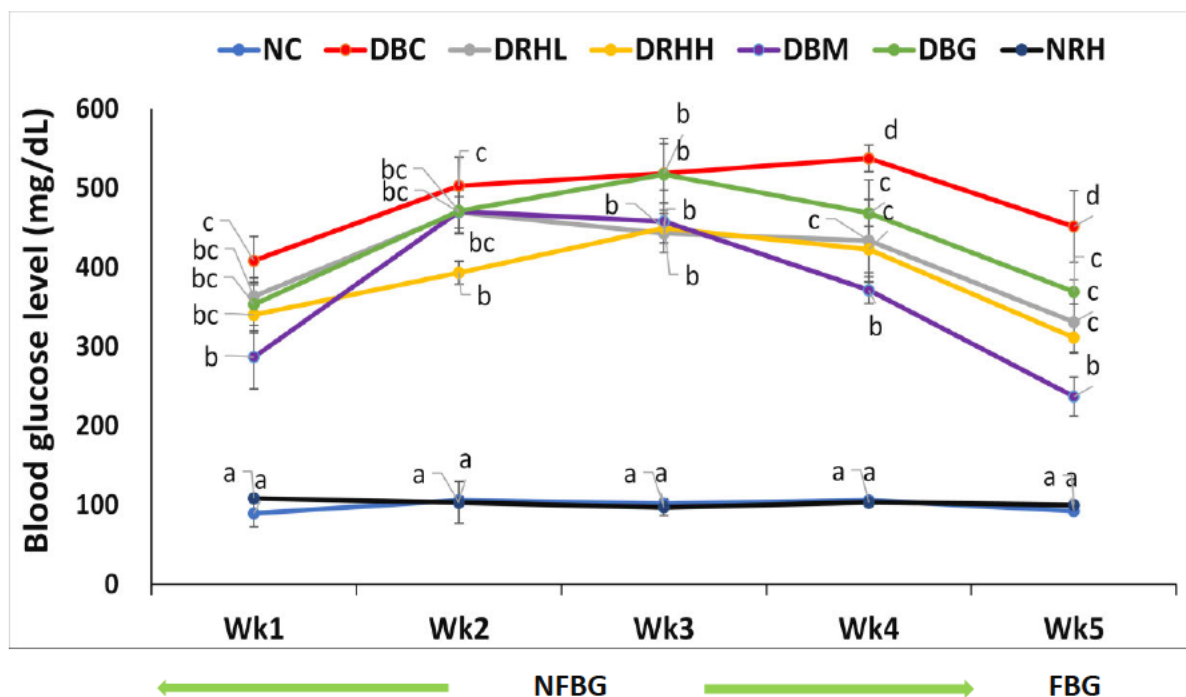


Figure 4.3.3: Weekly blood glucose concentrations of different animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given animal group represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DRHL: Diabetic red honeybush (*Cyclopia genistoides*) tea low dose; DRHH: Diabetic red honeybush (*Cyclopia genistoides*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NRH: Normal red honeybush (*Cyclopia genistoides*) tea high dose (toxicological control).

The results of OGTT and the corresponding area under curve (AUC) were presented in **Figure 4.3.4**. The glucose tolerance ability of normal groups (NC and NRH) was significantly ($p < 0.05$) better than that of all the diabetic groups. There was a significant ($p < 0.05$) evaluation in blood glucose level at 30 minutes for all groups after an oral administration of a dose of 2 g/kg BW glucose. Except for DBC group, the blood glucose level of all diabetic groups was peaked at this time and started declining to the end of the OGTT test. All diabetic groups with treatment exhibited significantly ($p < 0.05$) lower blood glucose levels than DBC group at 60, 90- and 120-minutes, except DBG at 120 minutes. The lowest levels of all animal groups were recorded at 120 minutes. RHT- treated diabetic group displayed notably lower blood glucose than that of other diabetic groups after the peak, with DRHH showing better result than DRHL. The AUC of normal groups and other diabetic treated groups were significantly ($p < 0.05$) lower than DBC, which again confirms their better glucose tolerance ability than DBC, with DRHH exhibiting better result than DRHL.

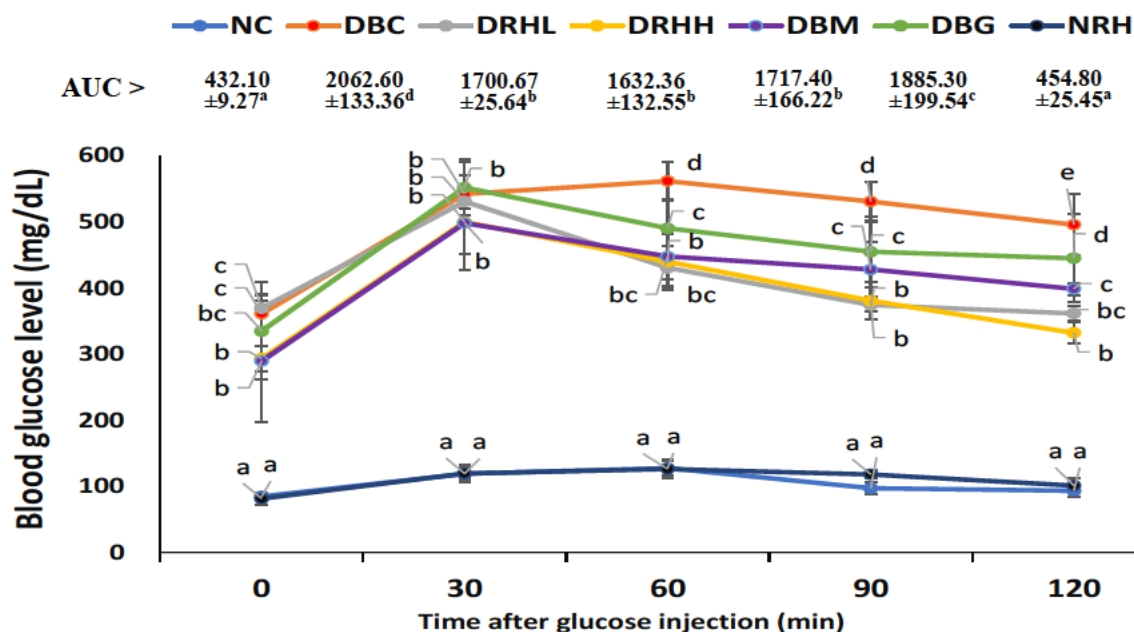


Figure 4.3.4: Oral glucose tolerance test (OGTT) with corresponding area under the curve (AUC) of different animal groups during the whole intervention period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given animal group represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DRHL: Diabetic red honeybush (*Cyclopiya genistoides*) tea low dose; DRHH: Diabetic red honeybush (*Cyclopiya genistoides*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NRH: Normal red honeybush (*Cyclopiya genistoides*) tea high dose (toxicological control).

The histopathological analysis of pancreatic tissues harvested from all animal groups was shown in **Figure 4.3.5**. Microscopic examination of the pancreas sections in NC (blue arrow) displayed the normal morphological characteristics of pancreatic islets, which showed light colour stained than the surrounding acini cells. The boundary between the endocrine and exocrine glands of pancreatic islets was clear. The morphology of pancreatic islets was regular. There were a number of and densely arranged islets in the islets of Langerhans with uniform nucleus and rich cytoplasm. While in the DBC (red arrow) group, the induction of T2D led to the destruction of pancreatic islets structure with some tissues severely lysed and nucleus disappeared. The number of islet cells in the pancreatic islets decreased remarkably compared to NC, with only few pancreatic β -cells islets left in STZ-injected rats. However, this condition was improved in RHT- and standard drugs treated diabetic groups, with DRHH (yellow arrow) treatment exhibiting better protection and regeneration abilities of pancreatic islets than DRHL (grey arrow) group. Whereas DBM (purple arrow) displayed better results than DBG (green arrow) group.

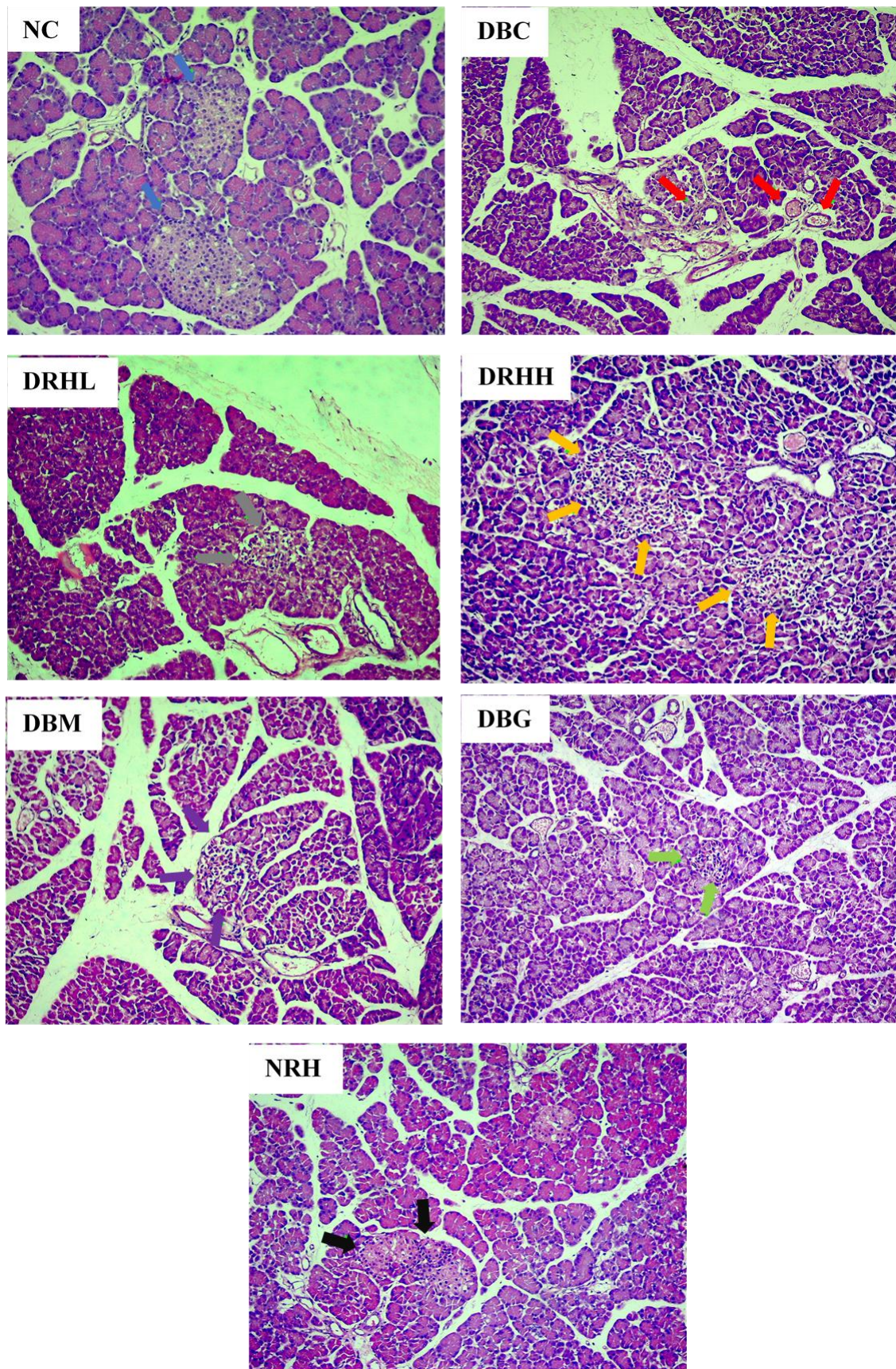


Figure 4.3.5: Histopathological examinations of the pancreas in different animal groups. Magnification: 10x. NC: Normal control; DBC: Diabetic control; DRHL: Diabetic red honeybush (*Cyclopia genistoides*) tea low dose; DRHH: Diabetic red honeybush (*Cyclopia genistoides*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NRH: Normal red honeybush (*Cyclopia genistoides*) tea high dose (toxicological control).

The concentrations of total cholesterol and LDL-cholesterol in DBC group was significantly ($p < 0.05$) higher than that of the normal groups as presented in **Figure 4.3.6**. Likewise, all the diabetic groups with treatment exhibited a significant ($p < 0.05$) lower levels of these cholesterols, with significantly lower level was observed in DRHH group than that of DRHL group. Furthermore, there was a concomitant significant reduction in HDL-cholesterol level in DBC group when compared to normal groups, this however was significantly reversed in the treatment groups, with DRHH group showing better result than DRHL group. The serum triglycerides in DBC group was significantly ($p < 0.05$) higher than other groups, while all diabetic treatment groups exhibited significantly ($p < 0.05$) higher levels than normal groups, with DRHL showing the lowest level among the treated diabetic groups.

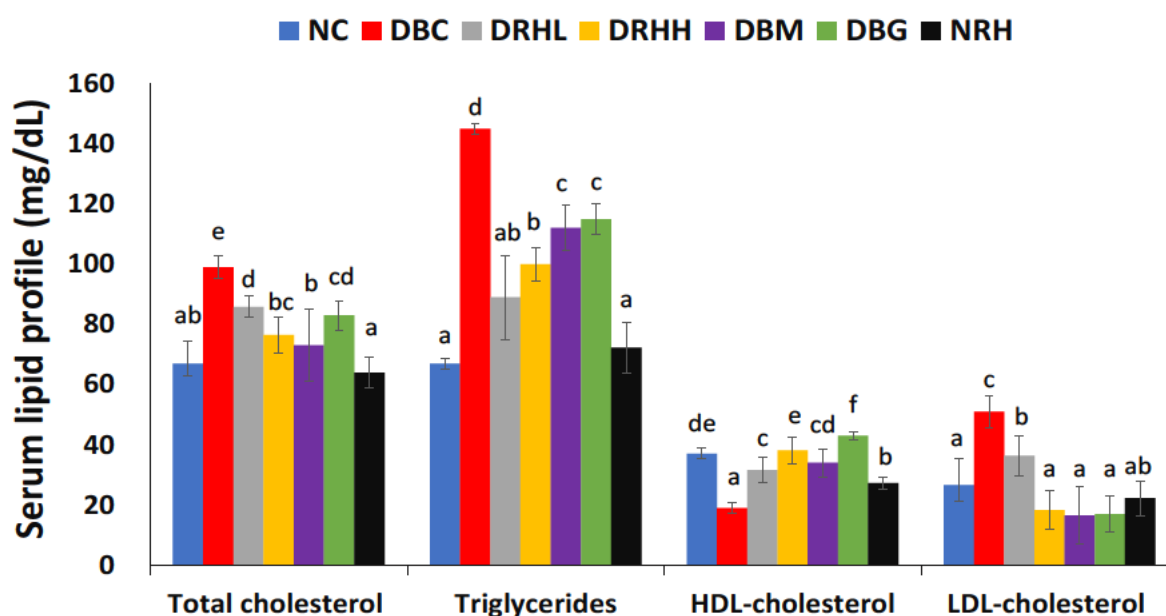


Figure 4.3.6: Serum lipid profile of different animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given animal group represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DRHL: Diabetic red honeybush (*Cyclopia genistoides*) tea low dose; DRHH: Diabetic red honeybush (*Cyclopia genistoides*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NRH: Normal red honeybush (*Cyclopia genistoides*) tea high dose (toxicological control). HDL: High density lipoprotein; LDL: Low density lipoprotein.

There was not significant ($p > 0.05$) difference in the liver weight of all groups as depicted in **Table 4.3.1**. The relative liver weight in DBC group was significantly ($p < 0.05$) higher when compared with the normal groups, while not statistically significant ($p > 0.05$) difference was observed among the diabetic groups. Liver glycogen was showing the lowest level in DBC group. However, this was significantly reversed in all other groups, with not significant

difference between DRHH and DBM groups. DRHH was showing significantly ($p < 0.05$) higher liver glycogen level than DRHL group.

Significant ($p < 0.05$) higher levels of the serum AST, ALT, ALP, urea, uric acid, creatinine and CK-MB were displayed in DBC group when compared with normal groups, with the exception of urea in NRH group as presented in **Table 4.3.2**. Animals in DBC presented a significant ($p < 0.05$) higher level of serum AST, ALP, CK-MB in all diabetic groups, however these levels were significantly ($p < 0.05$) reversed in all treated diabetic groups. Serum ALT level of treated diabetic rats was significantly lower than that of DBC group, except DBG group. The level of serum uric acid and creatinine in DBC group rats was significantly ($p < 0.05$) higher than that of other diabetic groups except for DRHL group. DRHL showed better results in parameters including ALT, ALP than that of DRHH group.

Table 4.3.1: Liver weight, relative liver weight and liver glycogen levels in the different animal groups at the end of the intervention period

Parameter	NC	DBC	DRHL	DRHH	DBM	DBG	NRH
Liver weights (g)	13.32±1.14	11.13±1.23	11.02±1.04	11.81±1.38	12.41±1.48	11.87±0.89	11.95±0.02
Rel. liver weights (%)	3.18±0.07 ^a	4.03±0.59 ^b	3.69±0.34 ^{ab}	3.97±0.68 ^b	3.59±0.24 ^{ab}	4.07±0.27 ^b	3.12±0.26 ^a
Liver glycogen (mg/g tissue)	6.26±0.23 ^c	2.25±0.27 ^a	3.33±0.14 ^b	5.42±0.83 ^{cd}	4.89±0.16 ^c	3.66±0.67 ^b	5.91±0.085 ^{de}

Results are expressed as mean ± SD (n = 5 to 7 animals) ^{a-c}Different superscript letters along a row indicate the significance of difference (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$). NC: Normal control; DBC: Diabetic control; DRHL: Diabetic red honeybush (*Cyclopia genistoides*) tea low dose; DRHH: Diabetic red honeybush (*Cyclopia genistoides*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NRH: Normal red honeybush (*Cyclopia genistoides*) tea high dose (toxicological control).

Table 4.3.2: Serum biochemical parameters in different animal groups at the end of the experimental period

Parameter	NC	DBC	DRHL	DRHH	DBM	DBG	NRH
AST (U/L)	84.20±1.33 ^a	156.20±14.96 ^c	128.00±1.41 ^d	105.00±4.24 ^c	91.17±5.19 ^{ab}	96.60±15.93 ^{bc}	89.00±3.61 ^{ab}
ALT (U/L)	84.20±2.17 ^a	93.40±3.65 ^c	66.67±8.91 ^a	84.50±1.87 ^b	85.50±2.26 ^b	88.67±4.73 ^{bc}	85.20±6.14 ^b
ALP (U/L)	72.40±2.30 ^a	364.60±15.98 ^e	169.33±10.84 ^b	209.00±10.62 ^c	173.33±11.48 ^b	351.00±6.32 ^d	75.40±7.77 ^a
Urea (mg/dL)	56.00±3.08 ^a	77.80±2.05 ^e	73.17±2.86 ^{cd}	70.33±3.33 ^{bc}	68.67±3.83 ^b	69.20±4.49 ^b	75.20±2.95 ^{de}
UA (mg/dL)	2.60±0.55 ^a	7.17±3.89 ^c	5.06±2.41 ^{bc}	2.87±1.38 ^{ab}	3.21±1.12 ^{ab}	4.17±3.38 ^{ab}	1.72±0.50 ^a
Creatinine (mg/dL)	1.81±0.39 ^a	3.37±0.90 ^c	2.88±0.58 ^{bc}	2.40±0.19 ^{ab}	2.19±0.32 ^a	2.38±0.55 ^{ab}	1.95±0.02 ^a
CK-MB (U/L)	4.90±1.46 ^a	44.74±2.99 ^d	21.53±2.33 ^c	13.53±3.11 ^b	7.20±2.84 ^a	11.62 ± 1.19 ^b	6.64±1.69 ^a

Results are expressed as mean ± SD (n = 5 or 7). NC: Normal control; DBC: Diabetic control; DRHL: Diabetic red honeybush (*Cyclopia genistoides*) tea low dose; DRHH: Diabetic red honeybush (*Cyclopia genistoides*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NRH: Normal red honeybush (*Cyclopia genistoides*) tea high dose (toxicological control); AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; UA: Uric acid; CK-MB: Creatinine Kinase-myocardial band.^{a-e}Different superscript letters along a row indicate the significance of difference (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$).

The serum insulin and fructosamine concentrations, calculated HOMA-IR and HOMA- β scores are depicted in **Table 4.3.3**. There was a significantly ($p < 0.05$) lower level of serum insulin in DBC group than that of normal groups, while a significantly reversed results were exhibited in treated diabetic groups. Significantly ($p < 0.05$) higher HOMA-IR score was observed in DBC when compared to other groups when it is significantly ($p < 0.05$) decreased in RHT- treated diabetic groups with DRHL exhibiting better result than DRHH group. Additionally, there was a significantly ($p < 0.05$) lower HOMA- β score displayed in DBC group compared with normal groups and diabetic groups treated with RHT exhibiting a significantly ($p < 0.05$) better HOMA- β score than that of DBC group. For serum fructosamine, significantly ($p < 0.05$) higher concentration was showed in DBC than that of normal groups, this was significantly reduced in other diabetic treated groups, with DRHH having significantly lower concentration than DRHL group.

Table 4.3.3: Serum insulin and fructosamine concentrations as well as computed HOMA-IR and HOMA- β scores in different animal groups at the end of the experimental period

	NC	DBC	DRHL	DRHH	DBM	DBG	NRH
Serum							
Insulin ($\mu\text{mol/L}$)	24.56 \pm 0.29 ^e	9.35 \pm 0.23 ^a	10.12 \pm 0.29 ^b	12.72 \pm 0.13 ^d	12.52 \pm 0.41 ^d	10.88 \pm 0.44 ^e	29.44 \pm 0.34 ^f
Fructosamine ($\mu\text{mol/L}$)	387.67 \pm 22.50 ^a	1210.33 \pm 60.00 ^d	829 \pm 28.28 ^c	587.00 \pm 27.58 ^b	574.67 \pm 58.45 ^b	546.33 \pm 33.13 ^b	336.67 \pm 47.38 ^a
HOMA-IR	0.80 \pm 0.01 ^a	1.73 \pm 0.04 ^e	1.15 \pm 0.03 ^c	1.36 \pm 0.01 ^d	1.02 \pm 0.03 ^b	1.38 \pm 0.06 ^d	1.01 \pm 0.01 ^b
HOMA- β	39.58 \pm 0.46 ^e	0.99 \pm 0.02 ^a	1.90 \pm 0.05 ^b	2.57 \pm 0.03 ^c	3.62 \pm 0.12 ^d	1.78 \pm 0.07 ^b	39.84 \pm 0.47 ^e

Results are expressed as mean \pm SD (n = 5 or 7). ^{a-f}Different superscript letters along a row indicate the significance of difference (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$). NC: Normal control; DBC: Diabetic control; DRHL: Diabetic red honeybush (*Cyclopia genistoides*) tea low dose; DRHH: Diabetic red honeybush (*Cyclopia genistoides*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NRH: Normal red honeybush (*Cyclopia genistoides*) tea high dose (toxicological control); HOMA-IR: Homeostatic model assessment for IR (insulin resistance); HOMA- β : Homeostatic model assessment for β (β -cell function).

Significantly ($p < 0.05$) higher level of MDA was exhibited in the pancreas, liver, kidneys, and heart of the animals as portrayed in DBC when compared to the normal groups as indicated in **Figure 4.3.7**. The MDA level was significantly depleted in diabetic treated groups, this was also replicated in the different studied organs, with DRHH exhibiting significantly ($p < 0.05$) lower MDA level when compared with DRHL.

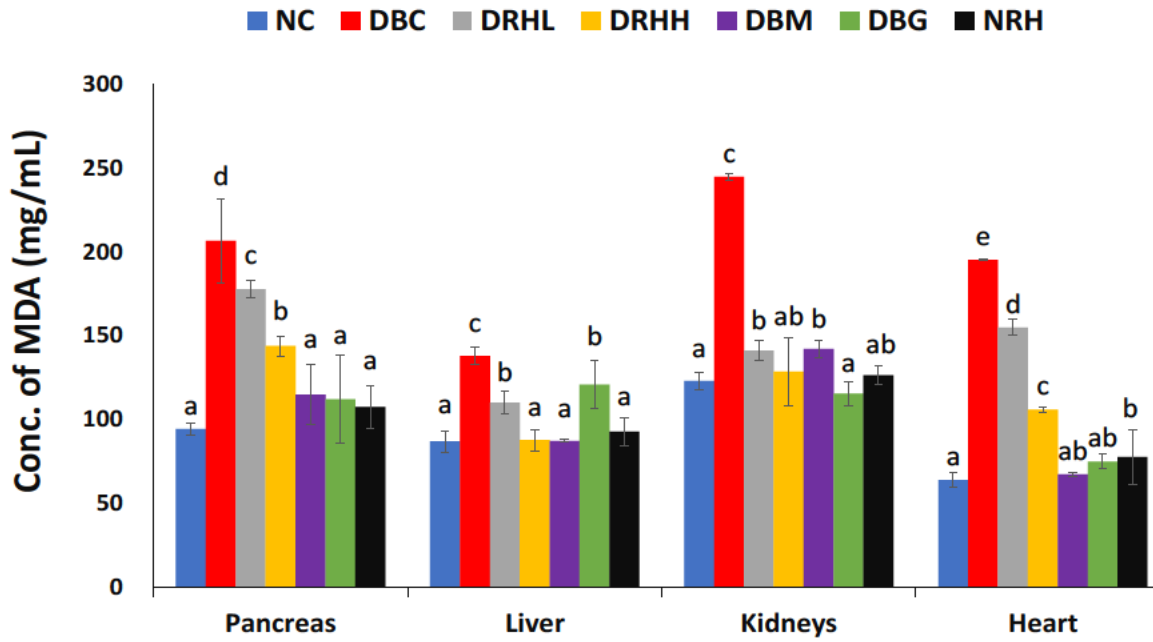


Figure 4.3.7: Thiobarbituric acid reactive substances (MDA equivalent) in the pancreas, liver, kidneys, and heart of all animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given animal group represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DRHL: Diabetic Red honeybush (*Cyclopia genistoides*) tea low dose; DRHH: Diabetic Red honeybush (*Cyclopia genistoides*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NRH: Normal Red honeybush (*Cyclopia genistoides*) tea high dose (toxicological control).

The GSH levels of organs in all animal groups were presented in **Figure 4.3.8**. Significantly ($p < 0.05$) lower GSH levels were seen in pancreas, liver, and kidneys in DBC compared to normal groups. GSH level of RHT- and standard drugs- treated groups compared to DBC group was significantly increased in pancreas, liver, and kidneys, with DRHH significantly higher than DRHL group in liver. While in the heart, there was no significant ($p > 0.05$) difference among all groups.

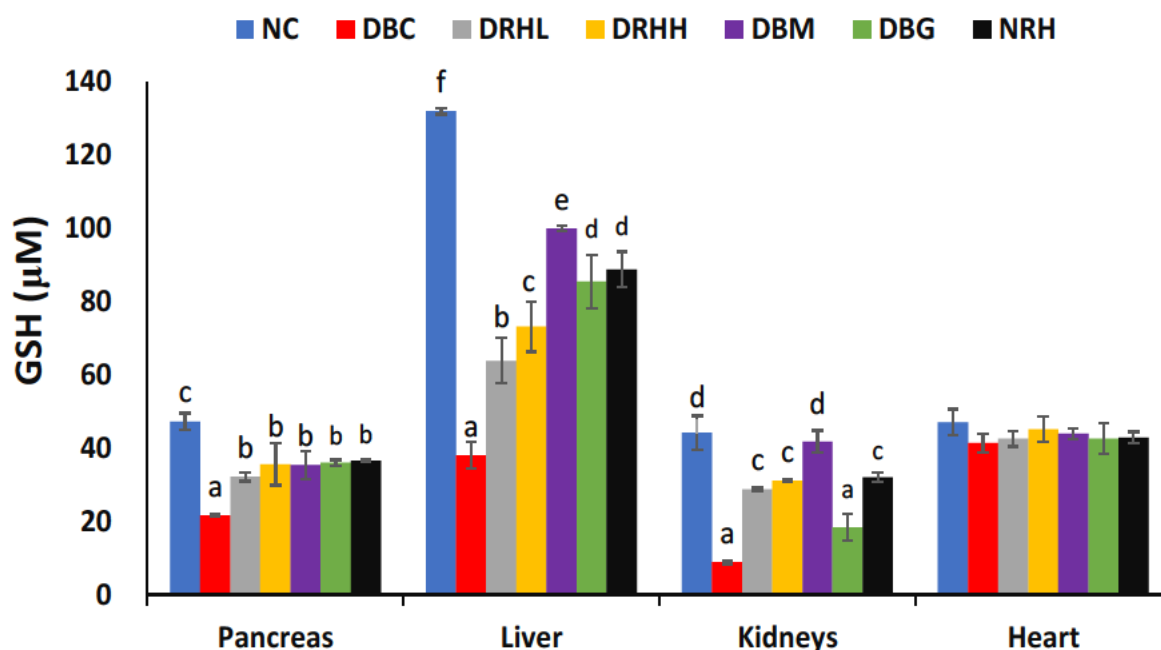


Figure 4.3.8: Reduced glutathione (GSH) levels in the pancreas, liver, kidneys, and heart of all animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given animal group represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DRHL: Diabetic red honeybush (*Cyclopia genistoides*) tea low dose; DRHH: Diabetic red honeybush (*Cyclopia genistoides*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NRH: Normal red honeybush (*Cyclopia genistoides*) tea high dose (toxicological control).

There was a significantly ($p < 0.05$) lower GR activity in organs of DBC than that of normal groups as displayed in **Figure 4.3.9**. RHT- and standard drug- treated groups significantly improved the GR activity in organs, with DRHH having higher GR activity than DRHL group in heart and pancreas. While in the liver and kidneys, DRHL improved the enzyme activity significantly higher than DRHH group.

The SOD activities in pancreas, liver, kidneys, and heart of all animal groups were presented in **Figure 4.3.10**. There was a significantly ($p < 0.05$) lower SOD activity in rats of DBC than that of normal groups (NC and NRH). However, this was significantly reversed in the pancreas, liver, kidneys, and heart of RHT- and standard drugs- treated group with the exception in the heart of DRHL group. DRHL group displaying significantly ($p < 0.05$) higher SOD activity than DRHH group in terms of liver, and not statistically significant ($p > 0.05$) difference in pancreas and kidneys. While in heart, DRHH group exhibited significantly higher SOD activity than DRHL group.

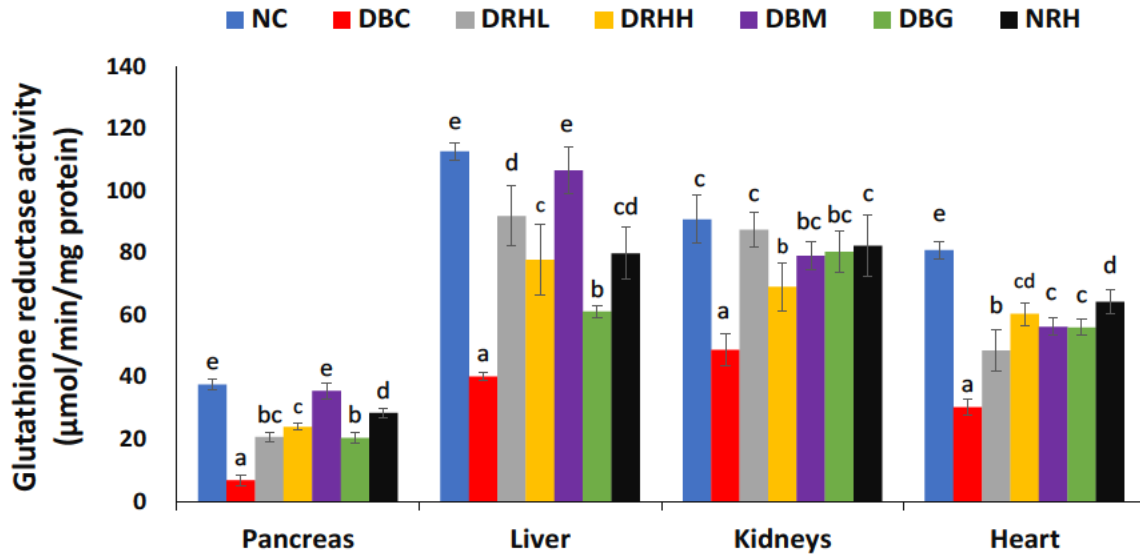


Figure 4.3.9: Glutathione reductase (GR) activity in the pancreas, liver, kidneys, and heart of all animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given animal group represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DRHL: Diabetic red honeybush (*Cyclopia genistoides*) tea low dose; DRHH: Diabetic red honeybush (*Cyclopia genistoides*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NRH: Normal red honeybush (*Cyclopia genistoides*) tea high dose (toxicological control).

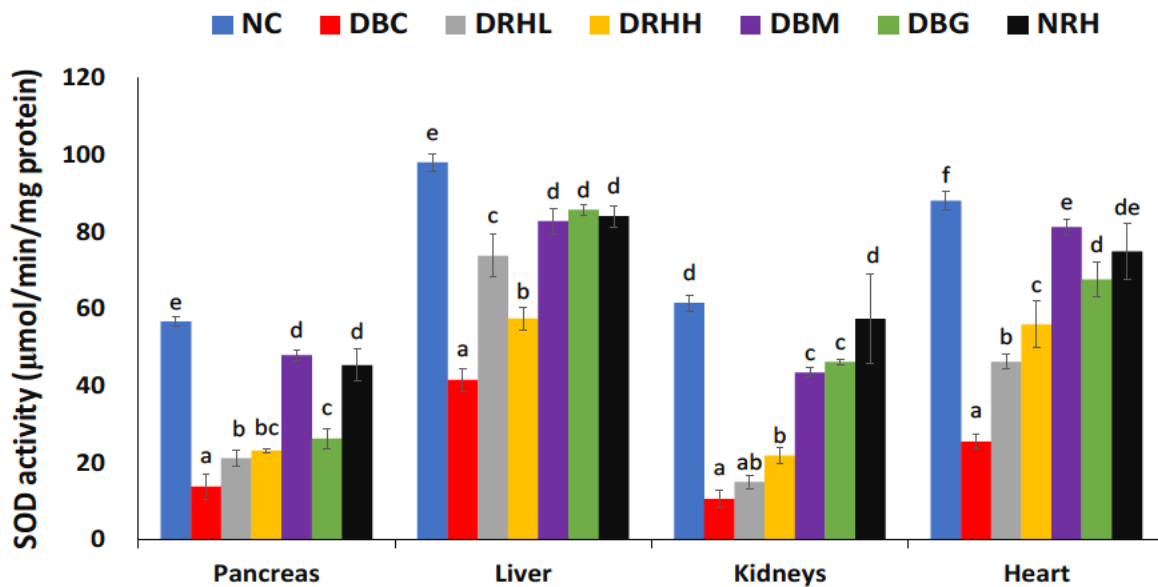


Figure 4.3.10: SOD activity in the pancreas, liver, kidneys, and heart of all animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given animal group represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DRHL: Diabetic red honeybush (*Cyclopia genistoides*) tea low dose; DRHH: Diabetic red honeybush (*Cyclopia genistoides*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NRH: Normal red honeybush (*Cyclopia genistoides*) tea high dose (toxicological control).

The activities of catalase in organs of all animal groups were displayed in **Figure 4.3.11**. The catalase activity in the pancreas, liver, kidneys, and heart of DBC group was significantly ($p < 0.05$) lower than that of the normal groups. RHT-treated diabetic groups in liver, kidneys and heart were displaying significantly higher catalase activity than DBC group, with DRHL group showing better result than DRHH group. While in pancreas, DRHL group was showing higher catalase activity than DRHH group, which is not statistically significant ($p > 0.05$) compared with DBC group.

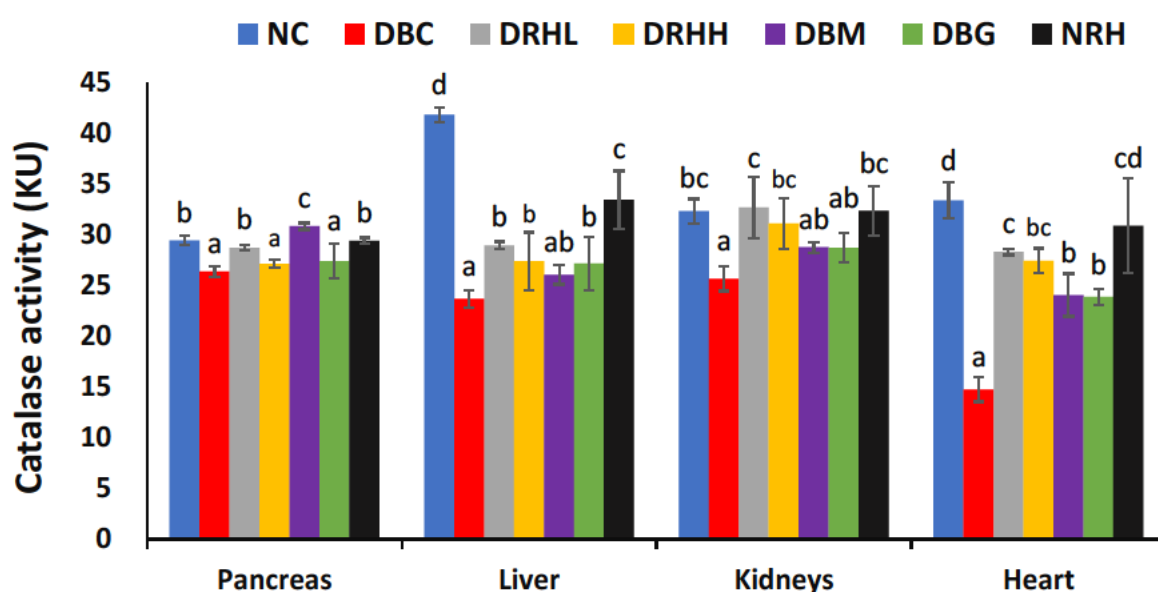


Figure 4.3.11: Catalase activity in the pancreas, liver, kidneys, and heart of all animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given animal group represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DRHL: Diabetic red honeybush (*Cyclopia genistoides*) tea low dose; DRHH: Diabetic red honeybush (*Cyclopia genistoides*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NRH: Normal red honeybush (*Cyclopia genistoides*) tea high dose (toxicological control).

4.3.5 Discussion

T2D is strongly associated with chronic hyperglycaemia and characterized by pancreatic β -cell dysfunction, and IR (Fonseca 2009). There are two major rodent models normally used in diabetes research namely: spontaneously induced genetic and experimentally induced non-genetic models (Wilson and Islam 2012). Non-genetic T2D rat model was employed in our experiment and was induced by feeding with fructose-containing diet (10% fructose solution) two weeks combined with a single intraperitoneal injection of streptozotocin (STZ; 40 mg/kg BW). Fructose solution was used to induce insulin resistance, while STZ was employed to

produce partial pancreatic β -cell destruction causing hyperglycaemia (Wilson and Islam 2012). Several studies reported the success of this model to mimic the natural onset progression of pathological changes in diabetics (Chukwuma et al. 2018, Erukainure et al. 2020). Male Wistar rat was employed due to its non-wild tranquil behaviour, smaller in size, easy to handle, and more sensitive than female to STZ-induced cytotoxicity (Furman 2015).

In the early clinical stage, typical diabetic symptoms and signs such as polyuria, polydipsia, polyphagia, and body weight loss are related with hyperglycaemia (Islam 2011, Ibrahim et al. 2016). Polyuria (data not shown) is attributed to the excess of renal threshold for glucose causing glucose excreting through the urine. Polydipsia results from hyperosmotic state due to water excessive loss in urine. Furthermore, polyphagia developed due to the inadequate utilization of glucose, causing the consumption of fat, muscle and tissue protein with a concomitant body weight loss (Okon et al. 2012). In our experiment, the significant ($p < 0.05$) increased water and feed intake, and significantly lower BW in DBC group compared to normal groups confirmed the T2D induction (**Figure 4.3.1 & 4.3.2**). In the rats treated with RHT although could not completely reverse these parameters, however the STZ-induced T2D polydipsia, and polyphagia were significantly ameliorated. Additionally, during the whole intervention period, better BW gain in the diabetic treated groups compared to the DBC group suggesting the gradual recovery of rats from diabetic condition. The reduced BW in DBC group may result from the inability of glucose utilization derived from feeds due to the ablated pancreatic β -cells and subsequent impaired insulin signalling. Thus, the elucidated parameters revealed a potential therapeutic effect of RHT against common T2D symptoms.

Blood glucose homeostasis is accomplished by actions of glucagon and insulin released from pancreas, which is essential for the evaluation and prevention of T2D as blood glucose is the main indicator for diagnosing the onset and development of diabetes (Röder et al. 2016). As the main pathological characteristic of T2D, chronic hyperglycaemia attributes to the reduced pancreatic β -cell mass and increased secretory demand of remaining β -cells, leading to β -cell dysfunction and IR, which may in turn induce β -cell apoptosis aggravating β -cell deficit (Meier and Bonadonna 2013). This corresponds with the heightened weekly blood glucose level (**Figure 4.3.3**), increased IR, depleted serum insulin level, and pancreatic β -cell function in DBC (**Table 4.3.3**). This also coincides with the reduction of β -cell cells as portrayed by the pancreatic histological analysis (**Figure 4.3.5**). Surprisingly, the lower blood glucose (NFBG and FBG) measured during the intervention period in the treatment (RHT and standard drug)

groups as well as increased serum insulin level at the end of the experiment confirmed the recovery of pancreatic β -cell function (**Table 4.3.3**), as well as the regeneration of pancreatic β -cells (**Figure 4.3.5**). These results support the anti-hyperglycemic as well antidiabetic activity and therapeutic effect of RHT on controlling and treating diabetes. This concurs with Chellan et al. (2014) who reported glucose metabolism improvement, pancreatic β -cells protection and preservation effects of aqueous extract of unfermented *cyclopia maculata* pretreated in STZ-induced diabetic rats and as well supported by Wang et al. (2014) and Lauricella et al. (2017) who showed mangiferin as one the major bioactive component of RHT in improving glycemia and glucose tolerance, while increasing serum insulin levels and reducing β -cell apoptosis in STZ-induced diabetic rats.

In addition, the significantly ($p < 0.05$) reduced level of fructosamine in RHT-treated rats compared to DBC also implies an antidiabetic effect of RHT (**Table 4.3.3**), which may support by report from Gondi and Rao (2015) who reported that mango exocarp extracts increases insulin level and decreases fructosamine level in STZ-induced diabetic rats. Mangiferin is not unique to honeybush, and presents in mangoes as well (*Mangifera indica*) (Augustyn et al. 2011). Fructosamine treated in some cases as a reliable biomarker of hyperglycaemia and glycemic control due to it relates to average serum glycaemic level in early stage of diabetes control (Cohen and Sacks 2012, Malmström et al. 2014).

Disorders of glucose and lipid metabolism, decreased insulin sensitivity and insulin signal conduction disorders leads to the onset of IR, resulting in increased FBG in fructose-feeding and STZ-induced T2D rats have been reported in several studies (Mohammed et al. 2016, Erukainure et al. 2019). The monitoring of FBG and glucose tolerance plays an important role in detection of IR in diabetes (Fonseca 2003, Alqahtani et al. 2013). OGTT has been regularly utilized to investigate the ability of blood glucose regulation in glucose load state and the function of pancreatic β -cells in experimental animals, it is also one criterion for clinical diagnosis of diabetes (Bartoli et al. 2011, Ibrahim and Islam 2014, Magnone et al. 2015). Under normal circumstances, the blood glucose concentration of the body will return to the level of FBG within two hours after giving glucose, while the glucose area under the curve (AUC) can be an index of glucose excursion which shows the overall the blood glucose change in this process (Sakaguchi et al. 2016). Although the blood glucose level of DBC group had a slight drop at 90- and 120 min, it still fluctuated within a high level after an oral administration of glucose, which indicated its worse and impaired glucose tolerance than that of normal groups (NC and NRH) (**Figure 4.3.4**). This implied the occurrence of pancreatic β -cell dysfunction

and insulin insensitivity, which supported by the significantly increased HOMA-IR and reduced HOMA- β scores in DBC group compared to normal groups (**Table 4.3.3**). However, the significantly ($p < 0.05$) declined level of blood glucose in RHT-treated groups compared to DBC group indicates the improved glucose tolerance of the treatment which further supports its anti-T2D effects. This is evidenced by previous studies that reported the effects of aqueous extract of *Cyclopia subternata* and *Cyclopia maculata* in improving glucose tolerance in STZ-induced diabetic rats (Chellan et al. 2014, Schulze et al. 2016).

As one of the main target organs of insulin action, the liver is essential for maintaining normal glucose homeostasis (Home and Pacini 2008). The synthesis of liver glycogen is an important way to decrease blood glucose level in human (König et al. 2012). In our experiment, the significantly ($p < 0.05$) depleted liver glycogen level observed in the rats of DBC (**Table 4.3.1**) could possibly be due to IR upon the induction of T2D, leading to the increased glycogen phosphorylation or reduced glycogen synthase activity (Islam 2011). While the significantly ($p < 0.05$) elevated liver glycogen level in the RHT- treated rats compared with DBC (**Table 4.3.1**) demonstrated the ability of RHT to improve hepatic glycogen synthesis. This collaborated with the decreased the blood glucose level (**Figure 4.3.3**) and increased the glucose tolerance (**Figure 4.3.4**) in RHT-treated diabetic rats further revealing its antidiabetic potentials which may attribute to its improvement of insulin sensitivity. These results may explained by Jung et al. (2004) who reported hesperidin, another major phenolic compound present in *Cyclopia*, decreased the blood glucose level, increased concentration of liver glycogen, and hepatic glucokinase activity.

Fructose-streptozotocin induced T2D rats with increased levels of serum TC, TG, LDL-c, and decreased level of serum HDL-c has been reported in several previous studies (Islam 2011, Moodley et al. 2015, Erukainure et al. 2019), which corresponds to the serum lipid profile of DBC rats in our study (**Figure 4.3.6**). The detection of these blood lipids was used to assess the fat metabolism of diabetics and is also a common index for clinical evaluation of the effects in the prevention and treatment of diabetes (Kelley and Kelley 2007, VinodMahato et al. 2011). The significantly ($p < 0.05$) decreased levels of serum TG, TC, LDL-c and significantly ($p < 0.05$) increased HDL-c level in the RHT-treated groups suggest the antidiabetic potentials of RHT, which may through improving dyslipidemia and insulin sensitivity. This in conformance with phytochemicals presented in *Cyclopia* by Xing et al. (2014) and Schulze, Beelders et al. (2015) who reported the effects of mangiferin on TG content reduction and benzophenone iriflophenone-3-C- β -D-glucoside on TG synthesis inhibition, respectively. One previous study

by Wang et al. (2011) reported the flavanone hesperidin decreased serum TG in C57BL/KsJ-db/db mice. In one previous paper by Jung et al. (2006) reported that hesperidin improves hyper-cholesterolemia and fatty liver through inhibition of cholesterol synthesis and absorption in high cholesterol diet fed Wistar rats.

The elevation of serum levels of ALT, AST, and ALP in STZ-reduced T2D rats has been reported in several studies as a biomarker suggesting the occurrence of hepatic injury (Hamzah et al. 2018, Oyebode et al. 2020), which are consistent with the significantly ($p < 0.05$) higher level of these parameters showed in DBC than the normal groups (**Table 4.3.2**) in our study. The significantly ($p < 0.05$) decreased levels of these enzymes in the RHT treated rats compared to DBC demonstrated the hepatoprotective properties of RHT. This coincide with the findings of Das et al. (2012) who reported that mangiferin plays a hepatoprotective role in galactosamine exposed hepatotoxic rats through induction of antioxidant defence including reducing activities of serum ALP, ALT and levels of TG, TC and lipid peroxidation.

Usually elevated serum urea and uric acid levels showed in T2D as a causal role in diabetic kidney failure have been reported by some previous studies (Johnson et al. 2013, Ryu et al. 2013). As expected, RHT exhibited its renal-protective potentials in STZ-induced rats through reducing the levels of above-mentioned biomarkers (**Table 4.3.2**). This accords with findings of Saha et al. (2019) who reported the renal-protective effect of mangiferin administration to *t*BHP induced renal injury mice on improving kidney dysfunction through ameliorate parameters including serum urea and uric acid.

Increased levels of CK (creatine) and CK-MB (creatine kinase isoenzymes) signals increased risk of heart injury have been reported before, due to their usage as biomarkers of T2D related coronary heart disease (Wallimann et al. 2011, Nogueira et al. 2019). In comparison to untreated diabetic rats, RHT demonstrated its cardiac-protective ability through reducing the levels of creatine and CK-MB (**Table 4.3.2**), which is interestingly in agreement with its effects on suppressing the levels of serum TG, TC, LDL-c and improving HDL-c level (**Figure 4.3.6**). This may support by the cardiac protection of some bioactive compounds of honeybush tea including mangiferin, iriflophenone-3-C-glucoside, hesperidin, hesperetin reported in several studies (Prabhu et al. 2006, Sellamuthu et al. 2013, Arozal et al. 2015, Núñez Selles et al. 2016, Yin et al. 2017, Li et al. 2018).

The disorder of glucose and lipid metabolism leads to an imbalance between peroxidation and antioxidant (Suryawanshi et al. 2006). Oxidative stress generated when endogenous

antioxidant system overwhelming by the overdue produced free radicals (Birben et al. 2012). The decreased level of GSH and increased level of MDA, as well as decreased activities of antioxidant enzymes such as SOD, catalase and GR have been well-documented in STZ-mediated oxidative stress (Erukainure et al. 2019, Mchunu et al. 2019, Sanni et al. 2019, Oyeboode et al. 2020). MDA is a biomarker of lipid peroxidation, GSH is responsible for protecting cells against oxidative stress. While GR reduces GSSG to GSH to maintain the content of GSH supporting the endogenous antioxidant defence system (Waggiallah and Alzohairy 2011, Gawlik et al. 2016). Kandemir et al. (2018) reported that hesperidin exhibited significantly ($p < 0.05$) decreasing MDA level and increasing level of GSH in STZ-induced diabetic nephropathy. Moreover, Pal et al. (2014) reported orally administered mangiferin (40 mg/kg BW) reduced ROS production and the decreased activities of antioxidant enzymes including catalase, SOD and GR in STZ-induced diabetic kidney. In our study, DBC exhibited significantly ($p < 0.05$) higher MDA level and lower GSH level and GR activity than normal groups (**Figure 4.3.7 – 4.3.9**). While the MDA level in the organs of diabetic rats was significantly decreased after the treatment with RHT, GSH level was significantly increased except no significant difference in heart and GR activity was significantly elevated in all studied organs, which may be attributed to the phenolics of RHT.

SOD and catalase are antioxidant enzymes mutually against oxidative stress in first line defence systems (Ighodaro and Akinloye 2018). Petrova et al. (2011) reported the fermented honeybush (*Cyclopia intermedia*) extract significantly ($p < 0.05$) decreased the MDA level and depletion of catalase and SOD activity in ultraviolet B (UVB)-induced skin damage SKH-1 mice, while less effective effects displayed in its phenolics mangiferin and hesperidin. While the increased catalase and SOD activities of fermented honeybush (*Cyclopia intermedia*) extract was further supported by its treatment on UVB-induced HaCaT human keratinocytes (Im, Yeon et al. 2016). In addition, Ku and Bae (2016) reported other flavonoids of *Cyclopia subternata* (scolymoside and vicenin-2) increased expression of SOD and catalase in human umbilical vein endothelial cells and C57BL/6 mice. In our study, diabetic groups treated with RHT significantly ($p < 0.05$) elevated SOD activity in all studied organs, while catalase activity in studied organs were significantly improved compared to DBC group except high dose of RHT in pancreas. This exhibited potent ability of RHT in enhancing the first line defence system in organs of STZ-induced T2D rats (**Figure 4.3.10 & 4.3.11**). This may evidence by its phenolics and the plants in the same family in different experimental models, which corroborates its *in vivo* antioxidant potentials.

4.3.6 Conclusion

All results taken together suggest that the *in vivo* antioxidant and antidiabetic property of the administration of RHT hot water extract in drinking water exhibited antihyperglycemic, antihyperlipidemic, antioxidant effects in STZ-induced T2D rats, therefore demonstrating its potential in ameliorating T2D and some of its associated complications.

CHAPTER 5

5.0 THE ANTIOXIDANT AND ANTIDIABETIC ACTIVITIES OF JASMINE GREEN TEA *IN VITRO* AND *IN VIVO*

5.1 Background

Jasmine green tea (JGT) is a type of green tea (*Camellia sinensis*), which is flower-scented and different from general green tea (Gao, Hu et al. 2009) (**Figure 5.1**). The production of JGT is featured by a specific process namely “Ti-hua” (Gao et al. 2009, Shen et al. 2017). “Ti-hua” is generally using green tea leaves as the base tea which is allowed to absorb the fragrance of the jasmine flower (*Jasminum sambac*) by amalgamation with 90% bloomed fresh flower buds at 38 - 44°C. Then, the flowers are separated and discarded from the tea leaves. While this process is repeated several times with new flower buds until sufficient fragrance has been absorbed by the tea leaves. This process forms the unique feature of JGT which is not only for the gastronomic role of the flower in flavouring the tea, but also for its synergistic effect with green tea to heighten their antioxidant capacities (Kuroda et al. 2005, Chen et al. 2017, Zayapor et al. 2019). JGT is the most welcomed scented tea in many countries including China, Thailand, and Japan (Kunhachan et al. 2012).



Figure 5.1: The appearance of commercial product of jasmine green tea (*Camellia sinensis*) used in the thesis.

5.1.1 Biological uses

The effects of JGT on antiobesity, antioxidative, and antidiabetic activities in animal models have been reported in several studies (Tao et al. 2011, Yao and Gui-nian 2011, Huang and Yang 2016, Huiying et al. 2016). JGT infusion at doses of 300 and 600 mg/kg displayed anti-hyperglycaemic effects on streptozotocin (STZ)-induced hyperglycaemia in SD rats (Huang and Yang 2016). While anti-obesity function, antioxidant activity of JGT has been reported *in vitro* and in breast cancer cells (MDA-MB-231) (Okuda et al. 2001, Alappat et al. 2015). In some other animal studies, JGT and its epicatechin isomers demonstrated hypocholesterolaemia and anti-tumour effects (Han and Xu 1990, Yang and Koo 1997). Hypolipidemic and free radical scavenging activities of this tea have also been found in some other studies (Zhang et al. 1997, Chan et al. 1999).

5.1.2 Phytochemistry

JGT is abundant in natural polyphenol antioxidants such as epigallocatechin gallate (EGCG), (-) epigallocatechin (EGC), (-) epicatechin gallate (ECG) and (-) epicatechin (EC) (Zhang et al. 1997). It has beneficial bioactive compounds from both the green tea and jasmine flower (Chen et al. 2017), including polyphenols, polysaccharides, vitamins, caffeine and aromatic substances, which are highly admired by consumers for health benefits and sensory quality (Ito et al. 2002, Gao et al. 2009, Zayapor et al. 2019).

5.2 Chemical characterization of sequential extracts of jasmine green tea, their antioxidant activities and modulatory effects on Fe²⁺ induced rat hepatic injury *ex vivo*

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5.2.1 Abstract

The antioxidant and antidiabetic activities of jasmine green tea (JGT) extracts of varying polarity solvents (dichloromethane (DCM), ethyl acetate, ethanol) were investigated *in vitro* and *ex vivo*. The ethanol extract displayed the best antioxidative activity in the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging and ferric reducing antioxidant power (FRAP) assays. The enzyme inhibitory effects of different extracts were examined on α -glucosidase and pancreatic lipase activities. The ethanol extract exhibited the best inhibitory potential on α -glucosidase activity, while the ethyl acetate extract exhibited the best inhibitory potential on pancreatic lipase activity. Fresh hepatic tissue homogenates were incubated with FeSO₄ to induce oxidative injury and thereafter the extracts' modulatory capabilities on oxidative stress biomarkers were investigated *ex vivo*. Catalase activity, superoxide dismutase (SOD) activity, and reduced glutathione (GSH) levels which had been reduced by oxidative injury were significantly ($p < 0.05$) increased by JGT extracts (especially the ethanol extract). The JGT extracts concomitantly resulted in significantly ($p < 0.05$) decreased levels of malondialdehyde (MDA). All the extracts were subjected to gas chromatography-mass spectroscopy (GC-MS) analysis to identify their possible bioactive compounds. Caffeine, styrene, n-hexadecanoic acid, octadecanoic acid, tetradecanoic acid, 1-benzoyl-4,5-dihydro-4,4,5,5-tetramethyl-3-phenyl-1h-pyrazole and 1,2-propanediol, 3-benzyloxy-1,2-diacetyl were identified. Results in this study implied the possible antioxidative, antidiabetic, and antiobesogenic potentials of different

extracts of JGT, particularly the ethanol extract. The results warrant further *in vivo* studies to determine the *modus operandi* of JGT extracts on the bioactivities postulated in the current study.

Keywords: Antioxidant, Sequential extraction, GC-MS, Jasmine green tea, Type 2 diabetes

5.2.2 Introduction

Diabetes mellitus (DM) is a metabolic disorder which is characterized by hyperglycaemia resulting from the absolute or relative insulin secretion deficiency, action or both (Ozougwu et al. 2013, Alam et al. 2014). According to the International Diabetes Federation (IDF), an estimated 463 million people are living with diabetes worldwide. Furthermore, it is predicted that over 700 million people will be affected by 2045 (IDF 2019). In China, there are about 116 million adults have diabetes, accounting for a quarter of the total number of people with diabetes globally. On the other hand, the number of diabetics in South Africa is about 4.58 million, which is around 24% of the total number of diabetics in Africa (IDF 2019).

There are two major etiologies of diabetes: insulin-dependent diabetes mellitus (IDDM), also known as type 1 diabetes, which is an autoimmune disease. It has an early onset in life the incidence group is mostly children or adolescents (Chiang et al. 2014). The second etiology is non-insulin-dependent diabetes mellitus (NIDDM), also known as type 2 diabetes (T2D). It is usually observed later on in life and accounts for 90% of the incidence of diabetes (Ozougwu et al. 2013). T2D may occur resulting from a combination of several factors including insulin resistance and pancreatic β -cell dysfunction which eventually lead to chronic hyperglycaemia (Erukainure et al. 2017). Hyperglycaemia is known to negatively changes the blood's biochemical milieu and leads to an increase in the generation of reactive species (RS) including reactive oxygen species (ROS). Furthermore, chronic hyperglycaemia reduces the ability of antioxidants in the endogenous defence system to mop up excess reactive species. This imbalance is termed oxidative stress and is closely associated with the damage of various functional cellular and biochemical elements including proteins, lipids, and DNA. Oxidative stress leads to islet β -cell dysfunction and peripheral insulin resistance, thus aggravates the progression of diabetes (Tangvarasittichai 2015). The impact of oxidative stress on the body may be studied through several intrinsic biomarkers which includes the enzyme activities of catalase, superoxide dismutase (SOD), and levels of reduced glutathione (GSH) and malondialdehyde (MDA) (Xiong et al. 2011). When these endogenous antioxidant systems are overwhelmed by reactive species, dietary exogenous antioxidants may be taken to bolster the

bodies antioxidant capacity. Some Chinese teas has been found to be a healthy source of these antioxidant compounds which can prevent and treat hyperglycaemia-induced oxidative stress through scavenging reactive species (Yashin et al. 2011, Xiao et al. 2020).

Jasmine green tea is made by green tea (*Camellia sinensis*) scenting with fresh jasmine flowers so that the tea leaves can absorb and integrate the floral fragrance of jasmine (*Jasminum sambac*) flowers (Gao et al. 2009). This process forms the unique feature of jasmine tea which is known as tea taste and jasmine fragrance. Thus, JGT has beneficial bioactive compounds from both the green tea and jasmine flower (Chen et al. 2017). Among these beneficial compounds are tea polyphenols, tea polysaccharides, vitamins, caffeine and aromatic substances, which are highly sought after by consumers to satisfy their need for the health care of tea and its sensory quality (Ito et al. 2002, Gao et al. 2009). Several studies have been reported the effects of JGT on antiobesity, antioxidative, and antidiabetic activities in the animal model (Tao et al. 2011, Yao and Gui-nian 2011, Huang and Yang 2016, Huiying et al. 2016). However, to the best of our knowledge, there is no scientific report on the antioxidant and antidiabetic effects of the sequential extracts of JGT.

Therefore, this study aims to investigate the antioxidative potentials of the DCM, ethyl acetate, and ethanol extracts of JGT on Fe²⁺-induced hepatic oxidative injury. Furthermore, the inhibitory activities of the extracts on key carbohydrate and lipid digesting enzymes linked to T2D were determined. The phytochemical constituents of different extracts of JGT were characterized as well.

5.2.3 Materials and methods

Please refer to Chapter 2 for the detailed materials and methods for jasmine green tea (*Camellia sinensis*)

5.2.4 Results

The TPC of the ethanol extract of JGT was significantly ($p < 0.05$) higher than that of other extracts as displayed in **Figure 5.2.1**.

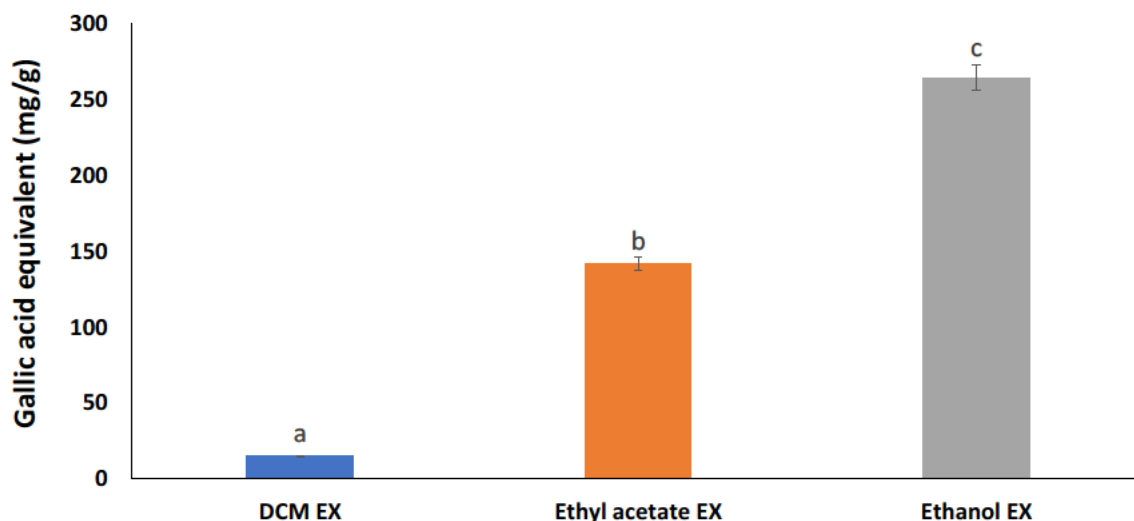
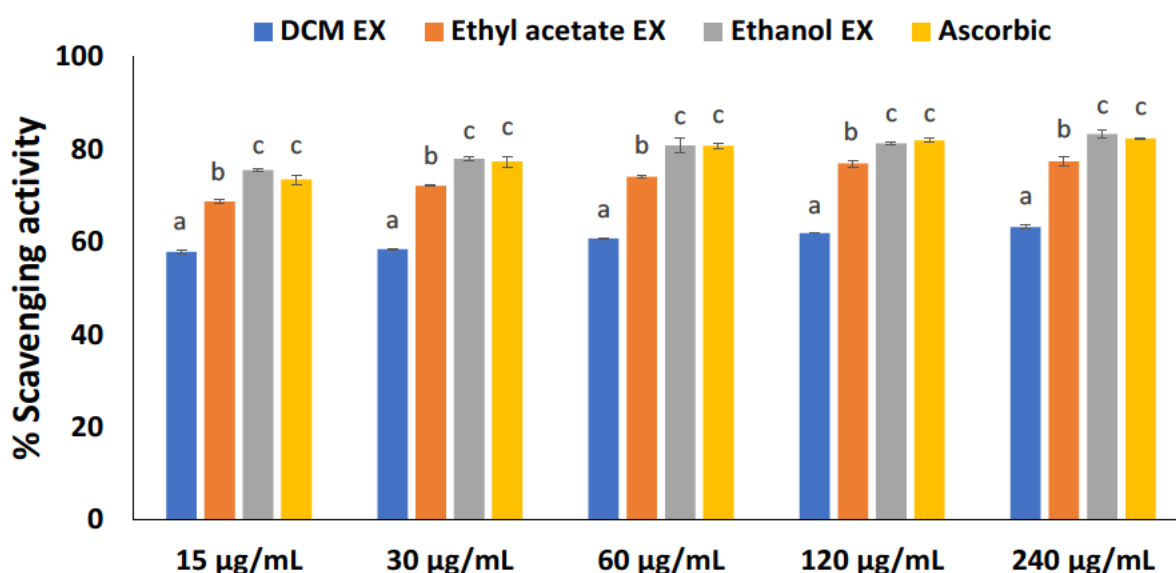


Figure 5.2.1: Total phenolic content of different extracts of jasmine green tea. Values represent mean \pm standard deviation ($n = 3$). ^{a-c}Different alphabets over the bars for a given extract represent significance of difference ($p < 0.05$). DCM = dichloromethane, EX = extract.

The DPPH radical scavenging activity of the different extracts of JGT compared to ascorbic acid are presented in **Figure 5.2.2**. All the extracts exhibited significant ($p < 0.05$) antioxidant activity in a dose-dependent pattern, with the ethanol extract had the highest scavenging activity ($IC_{50} = 0.11 \mu\text{g/mL}$), followed by standard drug, ascorbic acid ($IC_{50} = 0.72 \mu\text{g/mL}$).

Figure 5.2.2: DPPH radical scavenging activities of different extracts of jasmine green tea.



Values represent mean \pm standard deviation ($n = 3$). ^{a-e}Different alphabets over the bars for a given concentration for each extract represent significance of difference ($p < 0.05$). DCM = dichloromethane, EX = extract.

The ferric reducing antioxidant power (FRAP) of JGT different extracts were displayed in **Figure 5.2.3**. All the test samples displayed significant ($p < 0.05$) antioxidant power in a dose-dependent manner. The highest ferric reducing antioxidant power (FRAP) activity was exhibited in the ethanol extract, which followed by the standard drugs ascorbic acid and trolox as depicted by their IC_{50} values in **Table 5.1.1**.

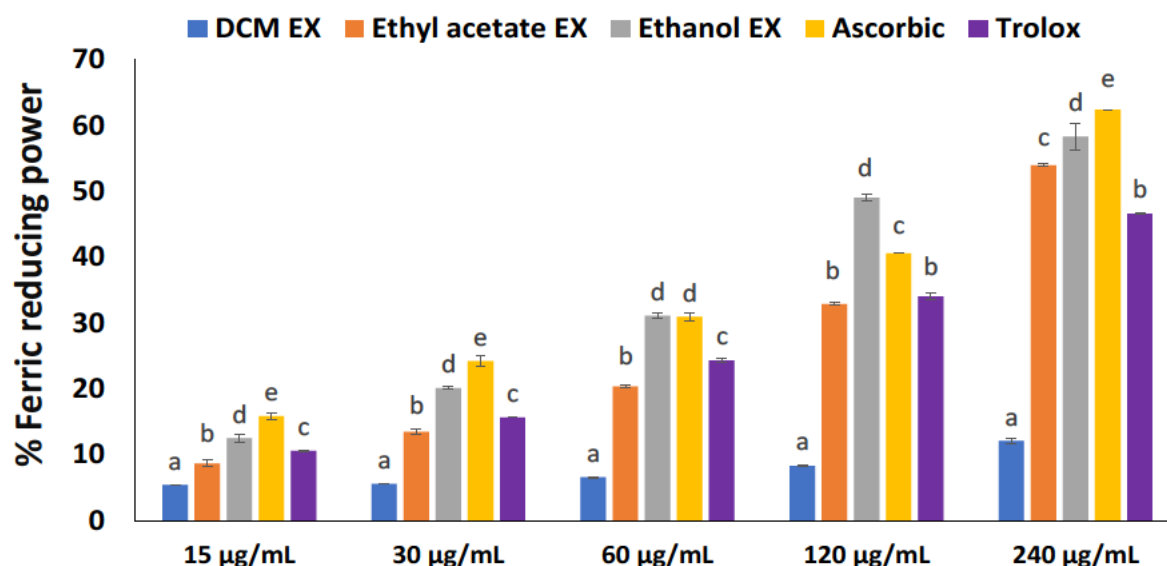


Figure 5.1.3: Ferric reducing antioxidant power (FRAP) activities of different extracts of jasmine green tea. Values represent mean \pm standard deviation ($n = 3$). ^{a-e}Different alphabets over the bars for a given concentration for each extract represent significance of difference ($p < 0.05$). DCM = dichloromethane, EX = extract.

The effects of the different extracts of JGT on α -glucosidase inhibitory activity were depicted in **Figure 5.2.4**. All test samples exhibited significantly ($p < 0.05$) inhibitory effects against α -glucosidase activity in a dose-dependent manner, with the standard drug acarbose ($IC_{50} = 0.12 \mu\text{g/mL}$) displayed the best activity followed by JGT ethanol extracts ($IC_{50} = 27.07 \mu\text{g/mL}$).

The test samples exhibited relatively high pancreatic lipase inhibitory activity but not in a remarkable dose-dependent manner (**Figure 5.2.5**). The standard drug orlistat had the highest inhibitory activity followed by ethyl acetate extract as portrayed by its IC_{50} value as depicted in **Table 5.1.1**.

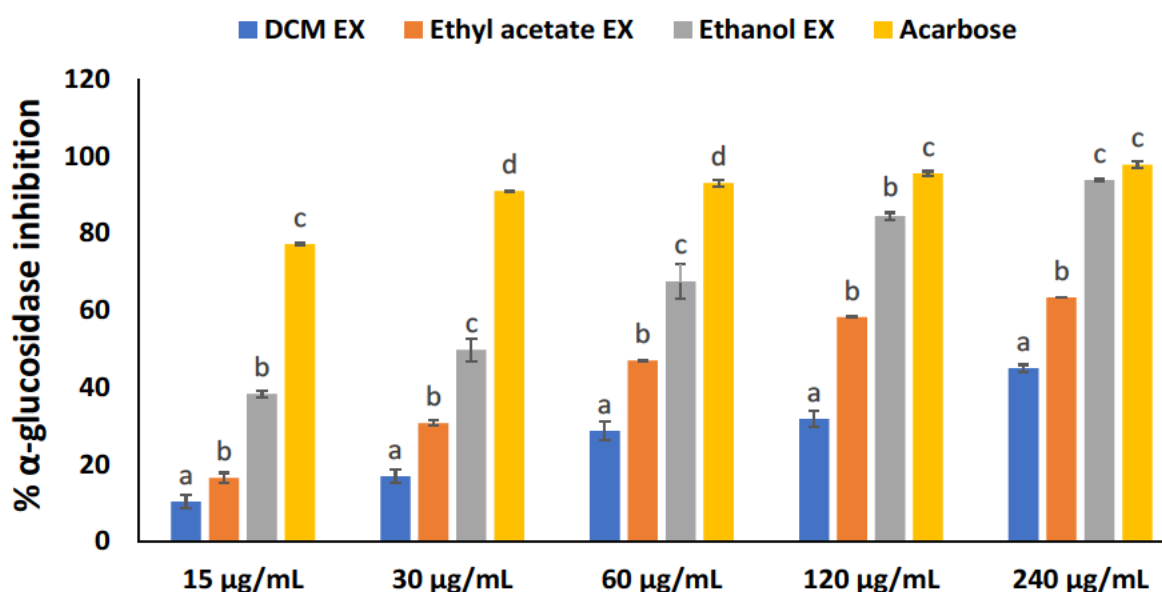


Figure 5.2.4: Inhibitory effect of different extracts of jasmine green tea on α -glucosidase activity. Data are presented as mean \pm SD. ^{a-c}Values with different letter above the bars for a given concentration are significantly ($p < 0.05$) different from each other. DCM = dichloromethane, EX = extract.

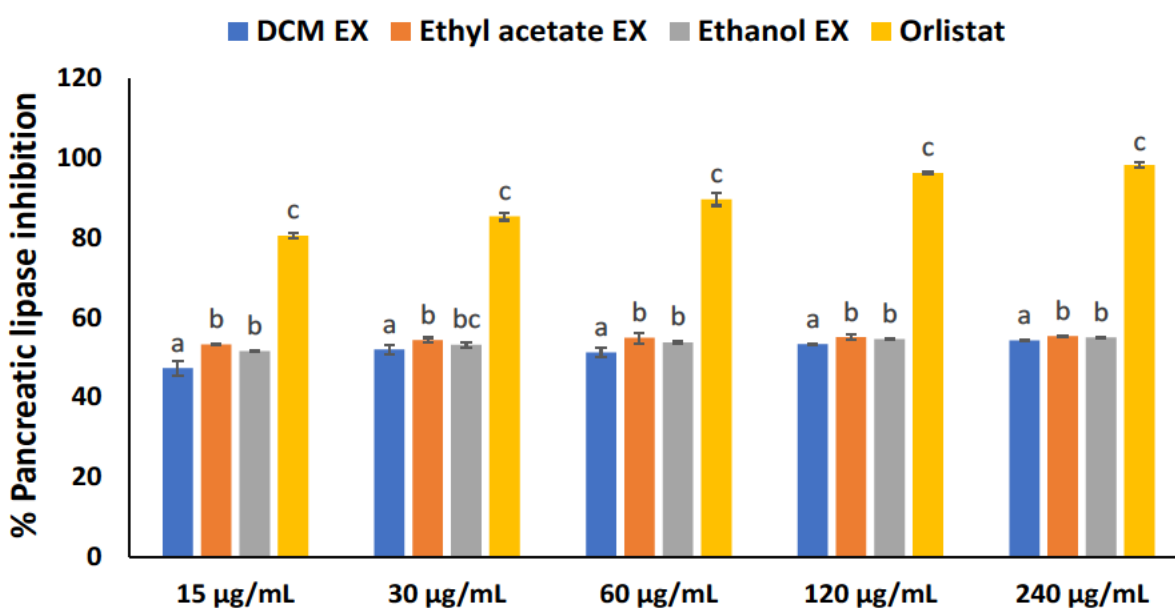


Figure 5.2.5: Inhibitory effect of different extracts of jasmine green tea on pancreatic lipase activity. Data are presented as mean \pm SD. ^{a-c}Values with different letter above the bars for a given concentration are significantly ($p < 0.05$) different from each other. DCM = dichloromethane, EX = extract.

The GSH levels of different JGT extracts in hepatic tissue were displayed in **Figure 5.2.6A**. A significantly ($p < 0.05$) decreased GSH level was exhibited in the untreated hepatic tissue.

Treatment with the standard drug ascorbic acid led to the highest GSH activity increase. Whereas the ethanol extract treatment displayed a higher increase compared to that of the DCM and ethyl acetate extracts. All the treatment with test samples increased GSH level in a dose-dependent effect with increasing concentration. The SOD activities significantly ($p < 0.05$) reduced in oxidative injured hepatic tissue as presented in **Figure 5.2.6B**. A dose-dependent effect on increasing SOD activity exhibited in all treatment with the standard drug ascorbic acid displaying the highest activity followed by ethanol extract of JGT as portrayed by its IC_{50} values of 7.18 $\mu\text{g/mL}$ and 8.67 $\mu\text{g/mL}$, respectively (**Table 5.1.1**).

The reduction of catalase activities exhibited in hepatic oxidative injured tissue was presented in **Figure 5.2.6C**. Treatment with the extracts and standard drug were significantly reversing the activity of catalase in tissues, with ascorbic acid displaying the best activity followed by ethanol extract of JGT. The significant ($p < 0.05$) increase in malondialdehyde (MDA) levels in FeSO_4 induced oxidative injured hepatic tissue was presented in **Figure 5.2.6D**. Treatment with the extracts and drug led to a significantly ($p < 0.05$) decreased of MDA levels in a dose-dependent pattern, with ascorbic acid displaying the best activity followed by the ethanol extract of JGT as portrayed by its IC_{50} values of 95.94 $\mu\text{g/mL}$ and 513.28 $\mu\text{g/mL}$, respectively (**Table 5.2.1**).

Table 5.2.1: IC_{50} values of different extracts of jasmine green tea activities

Activities	Dichloromethane Extract ($\mu\text{g/mL}$)	Ethyl Acetate Extract ($\mu\text{g/mL}$)	Ethanol Extract ($\mu\text{g/mL}$)	Ascorbic acid ($\mu\text{g/mL}$)	Acarbose ($\mu\text{g/mL}$)	Trolox ($\mu\text{g/mL}$)	Orlistat ($\mu\text{g/mL}$)
DPPH	3.87	3.44	0.11	0.72	-	-	-
FRAP	>1000	275.23	148.90	157.29	-	371.15	-
α -glucosidase	413.89	88.86	27.07	-	0.12	-	-
Anti-lipase	1.00	0.10	2.79	-	-	-	0.15
GSH	>1000	438.93	166.12	43.52	-	-	-
SOD	70.30	27.27	8.67	7.18	-	-	-
Catalase	>1000	>1000	>1000	>1000	-	-	-
LPO	>1000	991.89	513.28	95.94	-	-	-

IC_{50} , concentration to inhibit 50% activity. DPPH, 1,1-diphenyl-2-picrylhydrazyl; FRAP, ferric reducing antioxidant power; GSH, reduced glutathione; SOD, superoxide dismutase; LPO, lipid peroxidation.

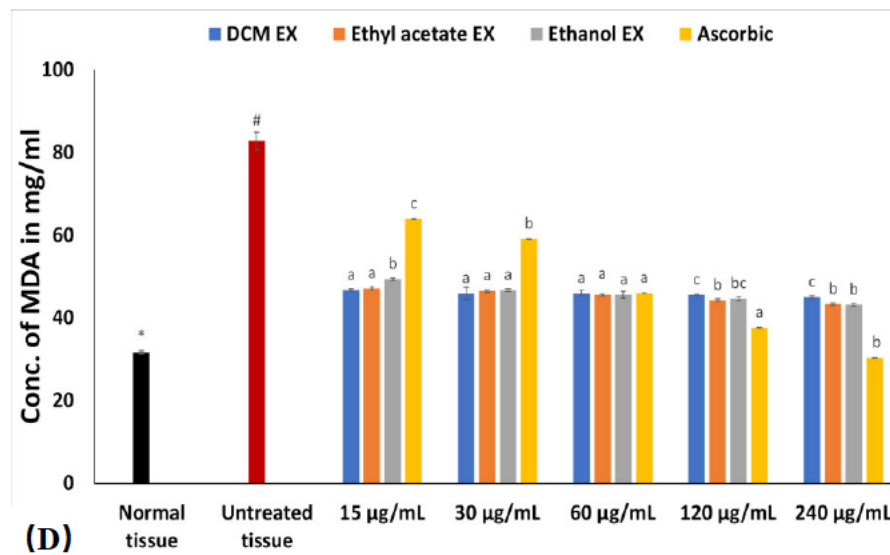
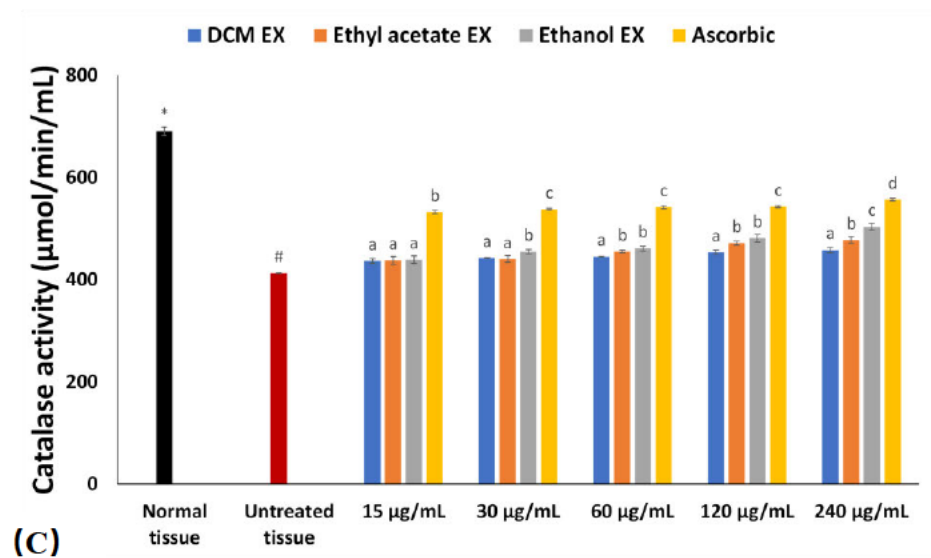
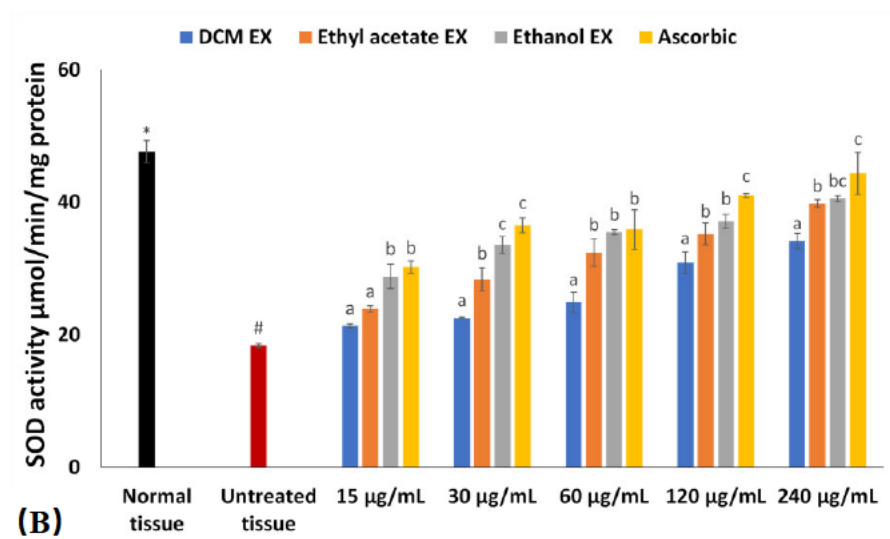
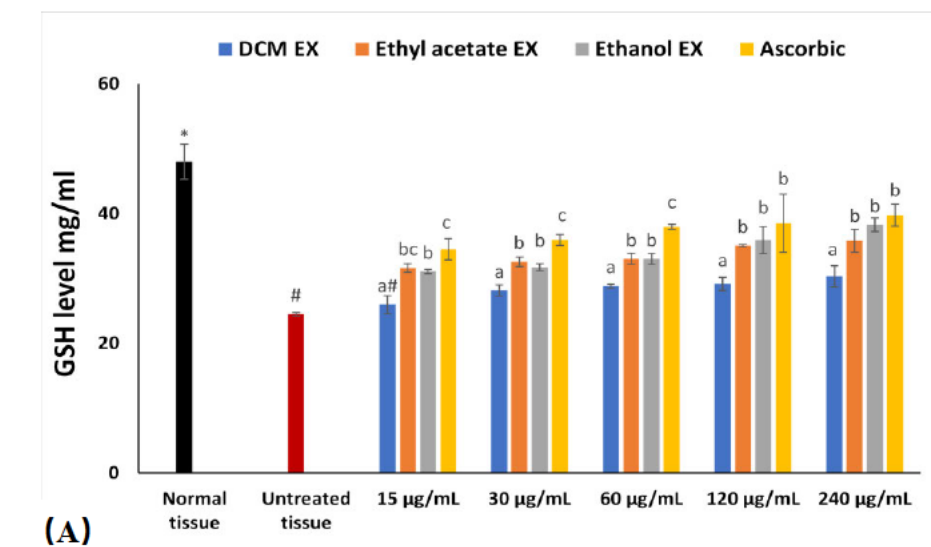


Figure 5.2.6: Effect of jasmine green tea on (A) GSH level, (B) SOD activity, (C) catalase activity, and (D) MDA level in oxidative hepatic injury. Values = mean \pm SD; n = 3. *Significantly different from untreated sample and #Significantly ($p < 0.05$) different from normal sample. ^{a-d}Values with different letter above the bars for a given concentration are significantly ($p < 0.05$) different from each other. DCM = dichloromethane, EX = extract.

The different extracts of JGT were subjected to GC-MS analysis to identify their possible bioactive compounds. The retention times, molecular mass, molecular formula, relative abundance, and the structures of identified phytochemicals are presented in **Table 5.2.2** and **Figure 5.2.7**. Phytochemicals were identified from the different extracts included caffeine, styrene, n-hexadecanoic acid, octadecanoic acid, tetradecanoic acid, 1-benzoyl-4,5-dihydro-4,4,5,5-tetramethyl-3-phenyl-1h-pyrazole, 1,2-propanediol, 3-benzyloxy-1,2-diacetyl.

Table 5.2.2: Compounds identified in DCM, ethyl acetate, ethanol extract of jasmine green tea by GC-MS

Compounds	Molecular formula	RT (min)	Molecular mass	Relative abundance %
DCM extract				
n-hexadecanoic acid	C ₁₆ H ₃₂ O ₂	31.68	256	25.13
octadecanoic acid	C ₁₈ H ₃₆ O ₂	35.41	284	8.08
Ethyl acetate extract				
n-hexadecanoic acid	C ₁₆ H ₃₂ O ₂	31.71	256	1.08
1-benzoyl-4,5-dihydro-4,4,5,5-tetramethyl-3-phenyl-1h-pyrazole	C ₂₀ H ₂₂ N ₂ O	32.21	306	2.05
1,2-propanediol, 3-benzyloxy-1,2-diacetyl	C ₁₄ H ₁₈ O ₅	39.96	266	0.33
Ethanol extract				
styrene	C ₈ H ₈	5.04	104	14.36
caffeine	C ₈ H ₁₀ N ₄ O ₂	29.18	194	2.92
n-hexadecanoic acid	C ₁₆ H ₃₂ O ₂	31.71	256	14.41
tetradecanoic acid	C ₁₄ H ₂₈ O ₂	35.42	228	4.80

The compounds presented in the table are those which matched similar compounds in the NIST library software.

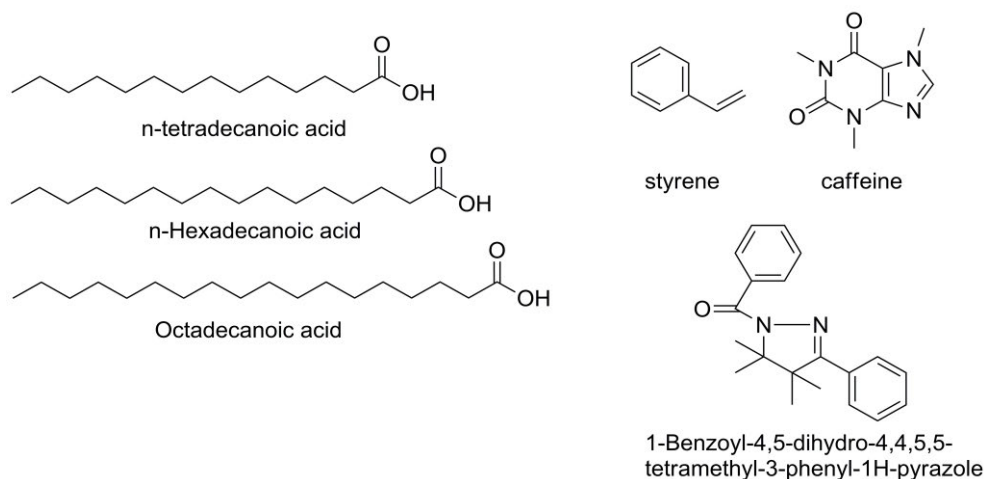


Figure 5.2.7: Compounds identified in the different extracts of jasmine green tea by GC-MS.

5.2.5 Discussion

DM is a chronic multifaceted condition that progressively gets worse if proper management strategies are not implemented. Hyperglycaemia and oxidative stress in diabetic patients work in tandem and result in severe incapacitating morbidities associated with diabetes (Jackson et al. 2012). The enhancement of the body's natural antioxidant defence system under oxidative stress conditions plays an important role in the management of diabetes (Birben et al. 2012). Various plant-derived secondary metabolites have been shown to have antioxidant capabilities that can have therapeutic advantages to people. These compounds include polyphenols, alkaloids, terpenoids, coumarins and saponins (Saxena et al. 2013, Chandrasekara and Shahidi 2018). In this study, the oxidative injury of hepatic tissue was induced *ex vivo* with FeSO₄ and treated with sequential extracts of JGT to investigate their antioxidant and antidiabetic potentials.

Tea polyphenols is an encompassing term for polyphenols in tea such as flavanols, anthocyanins, flavonoids, flavanols, and phenolic acids. Previous studies have reported that tea polyphenols have various beneficial therapeutic effects such as having antioxidant, anti-tumor, anti-cancer, antidiabetic, and antibacterial activities (Fu et al. 2017, Khan and Mukhtar 2019). Ethanol, the most polar solvent in this study, had significantly higher amounts of total polyphenolic content compared to dichloromethane and ethyl acetate. This strongly suggested that JGT contained more polar total polyphenols as compared to the less polar polyphenols. Furthermore, this high amount of polyphenol content may have strongly contributed to the ethanol extract exhibiting superior DPPH free radical scavenging activity, which further corresponding with the ethanol extract FRAP activities when compared with the DCM and

ethyl acetate extracts of JGT (**Figure 5.2.1 & 5.2.2, 5.2.3**). Previous studies frequently correlate relatively high total phenolic content with enhanced antioxidant activity including free radical scavenging activities and electron-donating potential (Saeed et al. 2012, Xiao et al. 2020). Exogenous dietary antioxidants are highly recommended to aid the endogenous antioxidant systems such as glutathione, SOD and catalase maintain a homeostatic oxidative status.

Reduced glutathione (GSH) is a cellular non-enzymatic antioxidant substance primarily found in the liver, which fights at the frontier in the body's endogenous antioxidant defence system (Tiwari et al. 2013, Mohamed et al. 2016). GSH is a tripeptide, γ -L-glutamyl-L-cysteinylglycine, the cysteine sulfhydryl (SH) group has a strong antioxidant capacity which can interact with the selenium-dependent glutathione peroxidase (GSH-Px) inhibiting oxidative stress by detoxifying foreign radicals, such as hydrogen peroxide, hydroxyl radicals, and peroxynitrite (Tsai et al. 2012). It is a biomarker of oxidative stress at the cellular level as illustrated by the decreased levels displayed in the untreated group (**Figure 5.2.6A**). Several studies have reported that T2D patients have relatively reduced GSH levels which is often linked to the perpetual hyperglycaemic induced oxidative stress (Tiwari et al. 2013). Similarly, the reduced GSH levels displayed in the untreated hepatic tissue group resulted from intensified impact of reactive oxygen species (ROS) formed due to incubation with FeSO_4 (pro-oxidant). Treatment of the hepatic tissue with the different JGT extracts, particularly the ethanolic extract, resulted significantly increased the GSH levels to near normal levels, therefore, reveal the anti-oxidative activity against Fe^{2+} -induced oxidative injury.

Superoxide dismutase (SOD) is an antioxidant enzyme that plays an important role in the balance of the body's redox reaction. It can quickly catalyse the dismutation of superoxide ($\text{O}_2^{\cdot-}$) generated from the electron transfer of the mitochondrial respiratory chain to hydrogen peroxide (H_2O_2) and oxygen, reducing the body's oxidative stress and protect cells from damage (Ighodaro and Akinloye 2018).

Catalase is one of the key enzymes in the endogenous antioxidant defence system, existing in red blood cells and peroxides in certain tissues, with high concentration in the liver (Krishnamurthy and Wadhvani 2012). It acts as the main regulator of H_2O_2 metabolism, decomposing the H_2O_2 to molecular oxygen and water and thus neutralizes it (Aguilar et al. 2016). As a result, to prevent the formation of more active hydroxyl radicals through the Fenton reaction (Patlevič et al. 2016). The significantly decreased activity of SOD and catalase enzyme

in the untreated hepatic tissue (**Figure 5.2.6B & C**) incubated with FeSO₄ further reveal the onset of oxidative injury which could be linked with the production of superoxide (O₂^{·-}) and hydroxyl (OH) radicals from iron overload (Davari et al. 2013). Increased activities in the treated group with the different solvent extracts of JGT (especially ethanol extract) indicate their anti-oxidative potential as well.

Iron plays a catalytic role in the onset of lipid peroxidation (Meli et al. 2013). H₂O₂ not neutralized by GSH and catalase interacts with Fe³⁺ to form hydroxyl through the Fenton reaction, which attacks the unsaturated fatty acids in the membrane, thereby triggering lipid peroxidation leading to the formation of the final product malondialdehyde (MDA) (Repetto et al. 2012). MDA is cytotoxic and can cause cross-linking polymerization of biological macromolecules such as proteins and nucleic acids and it is closely related to T2D and its complications (Guéraud et al. 2010, Saddala et al. 2013). In this study, the hepatic tissue incubated with FeSO₄ led to a significant evaluation in the MDA level, claim the onset of lipid peroxidation (**Figure 5.2.6D**), which also implied damage to the endogenous antioxidant defence system. The significantly decreased MDA level in the treated group further exhibits the antioxidative potential of the different solvent extracts (especially ethanol extract) corresponding to the exhibited increased GSH level, SOD and catalase enzyme activity (**Figure 5.2.6A - D**).

The main function of α-glucosidase is to catalyse the degradation of polysaccharides and oligosaccharides in the intestine into monosaccharides such as glucose. It plays a vital role in postprandial hyperglycaemic spikes associated with T2D (Dong et al. 2012). Inhibition of α-glucosidase activity retards the hydrolysis of carbohydrate into glucose thereby maybe used as an effective mechanism for the control postprandial blood glucose level rises (Kumar et al. 2011). The different JGT extracts had dose-dependent inhibitory activities against α-glucosidase with the ethanolic extract outperformed the dichloromethane and ethyl acetate extracts. It however did not surpass the inhibitory effect of acarbose (positive control) although it reached comparable extents particularly at the higher concentrations (**Figure 5.2.4**). Further isolation and identification of the α-glucosidase inhibitory bioactive compounds from the ethanolic extract of JGT may result in the development of antidiabetic therapeutic agents. This approach of using enzyme inhibiting compounds to help manage diabetes is widespread and may be applied for various kinds of enzymes within the gastro-intestinal tract.

Pancreatic lipase is an important enzyme found within the gastro-intestinal tract where it is responsible for the hydrolysis of dietary fat, which would otherwise be not directly absorbed

by the intestines (Guerra et al. 2012). Dietary fats must be hydrolysed by lipase into free fatty acids and glycerin that can be absorbed by the body (Wang et al. 2013). The pancreatic lipase inhibitor can inactivate lipase by forming a covalent bond with the active site serine of pancreatic lipase, thereby controlling lipolysis (Lunagariya et al. 2014). Therefore, diabetes induced by obesity resulting from a high-fat diet can be managed by inhibiting pancreatic lipase activity (Martins et al. 2010, Lunagariya et al. 2014). The pancreatic lipases inhibitory activity of the treatment (**Figure 5.2.5**) correlated further with the displayed α -glucosidase activity (**Figure 5.2.4**) exhibiting the antidiabetic potential of different solvent extracts of JGT in the control of T2D and its complications.

Different solvent system was used to extract JGT to get compounds with different polarity levels. GC-MS analysis of the extracts was undertaken to find out the presence of possible bioactive compounds that could be contributed to their antioxidant and antidiabetic activities. Caffeine, styrene, n-hexadecanoic acid, octadecanoic acid, tetradecanoic acid, 1-benzoyl-4,5-dihydro-4,4,5,5-tetramethyl-3-phenyl-1h-pyrazole, 1,2-propanediol, 3-benzyloxy-1,2-diacetyl were identified (**Figure 5.2.7 and Table 5.2.2**). Among them, n-hexadecanoic acid, tetradecanoic acid have the property of antioxidant, antimicrobial and other activities (Bodoprost and Rosemeyer 2007, Kala et al. 2011). Octadecanoic acid has the property of anti-inflammatory activity which was reported by the earlier researchers (Aparna et al. 2012). Caffeine is believed to have antioxidative and antidiabetic activities (Yamauchi et al. 2010, Jasiewicz et al. 2016). Thus, the exhibited antioxidative and antidiabetic activities of the different extracts can therefore be due to the synergistic effect of the above-mentioned phytochemicals.

5.2.6 Conclusions

These results indicate the antioxidant and antidiabetic activities of the different solvent extracts of Jasmine green tea. The ethanol extract exhibited the most potent activity overall. It had enhanced activity against free radicals and lipid peroxidation while concomitantly decreasing the activities of carbohydrate and lipid digesting enzymes. Furthermore, the ethanol extract exhibited hepatic oxidative injury protective effects as it augmented several antioxidant enzymes activities. These activities can be attributed to their total phenolic contents and the synergetic effect of the identified phytochemical compounds. However, further studies *in vivo* conditions are required to affirm the antidiabetic effects of ethanol extract of jasmine green tea along with molecular mechanisms before its usage for T2D management.

5.3 The *in vivo* antioxidant and antidiabetic activity of jasmine green tea (*Camellia sinensis*) extract in fructose-streptozotocin induced type 2 diabetic rats

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5.3.1 Abstract

The present study was carried out to evaluate the *in vivo* antidiabetic activity of concentrated hot water extract of Jasmine green tea (JGT). Male Sprague Dawley (SD) rats were employed to create a type 2 diabetes (T2D) model by feeding with fructose-containing diet (10% fructose solution *ad libitum*) for two weeks, followed by an intraperitoneal injection of streptozotocin (STZ) (40 mg/kg body weight (BW)). During the T2D intervention period, low and high dose (150 and 300 mg/kg BW) of JGT hot water extract and standard drugs (metformin, glibenclamide) in drinking water were orally administered to diabetic rats for five days per week for a five-week intervention period. Several blood and tissue parameters closely related to diabetes were analysed in the collected samples from rats. Hematoxylin-eosin staining was employed to analyse pancreatic morphological changes. Diabetic groups treated with JGT, particularly the high dose, exhibited impressive results in most parameters such as in ameliorating blood glucose level, oral glucose tolerance ability, improving serum insulin resistance, pancreatic β -cell function and recovering the morphological damages of the pancreatic tissue. JGT also increased liver glycogen and prevented oxidative stress mediated damages of organs when compared with untreated diabetic animals. Results from the present study indicate that JGT has promising antidiabetic activity and could be useful in the alleviation of T2D associated complications and it therefore can be exploited for the development of anti-T2D therapies.

Keywords: Type 2 diabetes, Rats, Jasmine green tea, Oxidative stress, Histopathology, Liver glycogen.

5.3.2 Introduction

Diabetes mellitus (DM) is one of the most global challenging health problems of the 21st century. The prevalence of DM is on the rise alarmingly, which has increased from 239 million in 2000 to approximately 463 million in 2019, and it is predicted to rise to 700 million by 2045 (IDF 2019). Meanwhile, T2D, the most prevalent type of DM, attributes to over 90% of all recorded diabetic cases. T2D is a complex and heterogeneous disease characterized by impaired pancreatic β -cell function and insulin resistance (Arredondo et al. 2018). Chronic hyperglycaemia resulted from insulin resistance has been implicated with the development of T2D due to it closely linked with the generation of free radicals (Erukainure et al. 2019). The increased production of these free radicals would overwhelm the defences of body's endogenous enzymatic and non-enzymatic antioxidant system resulting the onset of oxidative stress. Oxidative stress plays a major role in the pathogenesis and progression of many T2D complications affecting the small and large blood vessels and various organs (Oyebode et al. 2020). These complications (such as micro- and macrovascular complications) are common to diabetics including nephropathy, neuropathy, retinopathy, heart failure, myocardial infraction and stroke (Moodley et al. 2015). It is therefore essential to control blood glucose levels and oxidative stress for the treatment and management of T2D and its associated complications. Currently, T2D is predominantly managed with antidiabetic/pharmaceutical oral hypoglycaemic agents such as sulfonylureas, biguanides, α -glucosidase inhibitors, thiazolidinediones, glucagon-like peptide (GLP-1 analogues and DPP-4 inhibitors. However, the utilization of these drugs accompany with some unwanted side effects such as weight gain or loss, nausea, diarrhoea, increased risk of heart and liver failure (Choudhury et al. 2018).

In recent years, the exploitation of antidiabetic medications from tea (*Camellia sinensis*) and its extracts is increasingly receiving attention due to its perceived minimal side-effects (Fu et al. 2017). Besides daily consumed as the most drunk beverage in many parts of the world after water (Chang 2015), tea is used as one medicinal plant to modulate the risk of DM since time immemorial, due to its diverse biological actions such as anti-hyperglycaemic, antioxidant, hypolipidemic, anti-inflammatory, anti-cholesterol functions and antidiabetic activities (Alkhatib et al. 2017, Fu et al. 2017). These effects are attributed to the presence of its predominant phytochemicals including catechins, caffeine, amino acid, theaflavins and thearubigins (Chakravorty et al. 2019). Amongst such teas is JGT.

JGT is a scented tea, which uses green tea (*Camellia sinensis*) as a base tea for reprocessing (Shen et al. 2017). The Chinese often amalgamate fresh jasmine flower (*Jasminum sambac*)

with the base tea during the scenting processes to impart pleasant character. This is not only for the gastronomic role of the flower in flavouring the tea, but also for its synergistic effect with green tea to heighten their antioxidant capacities (Zayapor et al. 2019). JGT is the most welcomed scented tea in many countries including but not limited to China, Thailand, and Japan (Kunhachan et al. 2012). It is abundant in natural polyphenol antioxidants such as epigallocatechin gallate (EGCG), (-) epigallocatechin (EGC), (-) epicatechin gallate (ECG) and (-) epicatechin (EC). One recent study reported the anti-hyperglycaemic effects of jasmine tea at dose of 300 and 600 mg/kg BW in streptozotocin (STZ)-induced hyperglycaemic SD rats (Huang and Yang 2016). Some studies reported that jasmine tea exhibits its anti-obesity function, antioxidant activity *in vitro* and anticancer activity in breast cancer cells (MDA-MB-231) line (Okuda et al. 2001, Alappat et al. 2015). Some old studies demonstrated the hypocholesterolemia and anti-tumor effects of jasmine tea in some animal studies (Han and Xu 1990, Yang and Koo 1997). Additionally, two more old studies reported the consumption of JGT epicatechin isomers exhibited hypolipidemic in high fat and cholesterol fed male Syrian golden hamsters and free radical scavenging activity in male Sprague-Dawley rats (Zhang et al. 1997, Chan et al. 1999). However, the *in vivo* antioxidant and antidiabetic activity of JGT concentrated hot water extract have not been investigated.

This study investigated the *in vivo* antioxidant and antidiabetic effect of JGT aqueous extract in a fructose-fed streptozotocin (STZ)-induced T2D rat model.

5.3.3 Materials and methods

Please refer to the chapter 2 for details of the methods used for jasmine green tea (*Camellia sinensis*)

5.3.4 Results

DBC, JGT- and standard drugs- treated diabetic groups had significantly ($p < 0.05$) higher mean daily feed and water intake than NC and NJG groups, when DBC group exhibiting the highest feed and water intake among them as shown in **Figure 5.3.1**. DJGH group displayed significantly lower feed and water intake than that of DJGL and DBC groups. Meantime, DJGH had significantly lower feed intake compared to DBM group, but no statistically significant difference compared to DBG in terms of water intake.

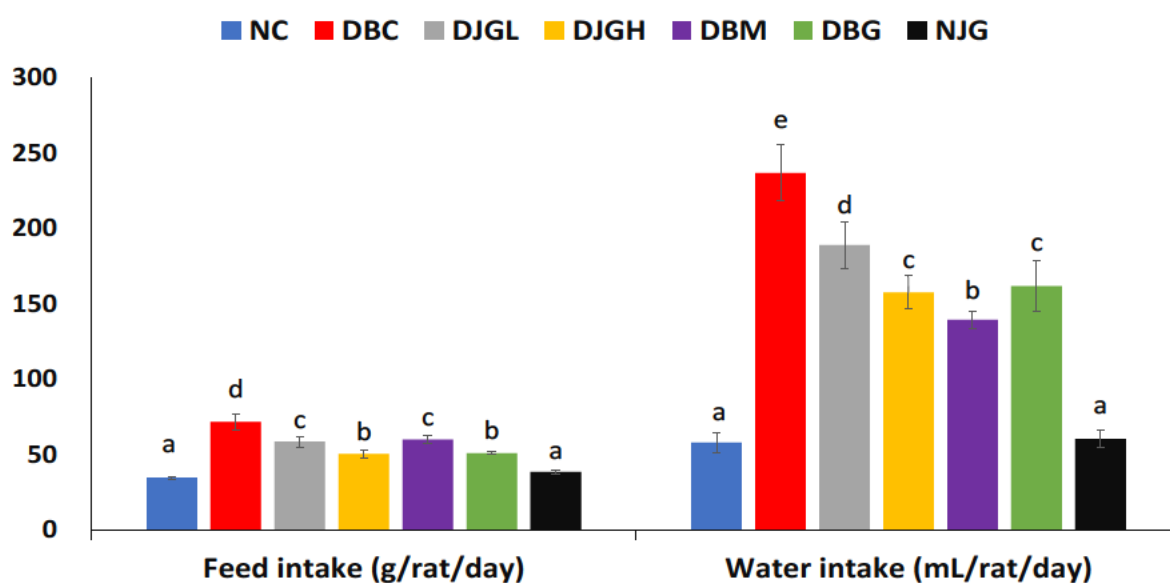


Figure 5.3.1: Feed and fluid intake in different animal groups during the intervention period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-e}Values with different letters over the bars for a given animal group represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NJG: Normal jasmine green tea (*Camellia sinensis*) high dose (toxicological control).

The weekly mean BW changes in different animal groups were presented in **Figure 5.3.2**. No significant difference of initial BW (week 0) was seen in the different animal groups; however, the BW gains of diabetic groups were significantly lower when compared to NC and NJG groups after the STZ injection. As the remaining experimental period (week 2 to week 6) proceeded, significant ($p < 0.05$) higher BW gains were displayed in all treatment groups compared to DBC group except for DBG, which exhibited a relatively higher BW increase than DBC. This was also replicated between DJGH and DJGL groups.

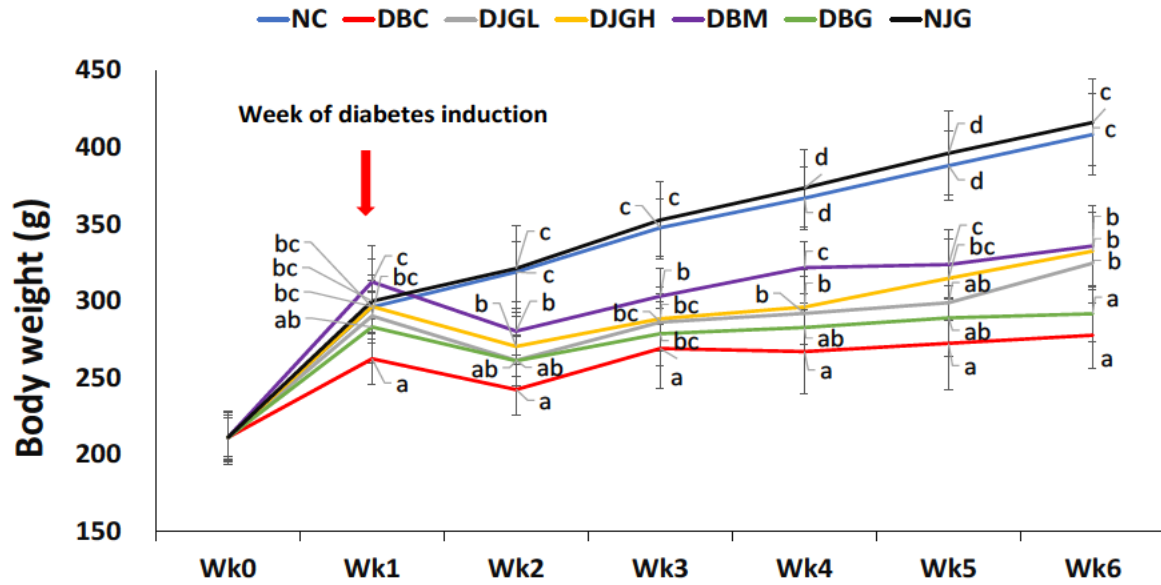


Figure 5.3.2: Mean body weight in different animal groups during the experimental period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-e}Values with different letters near the lines for a given week represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NJG: Normal jasmine green tea (*Camellia sinensis*) high dose (toxicological control)

The weight and relative weight of pancreas in all animal groups were displayed in **Figure 5.3.3**. A significantly lower pancreatic weight was seen in DBC group compared to normal groups. However, these were slightly reversed in JGT-and standard drug-treated groups, with DJGH having the best result among the treatment groups.

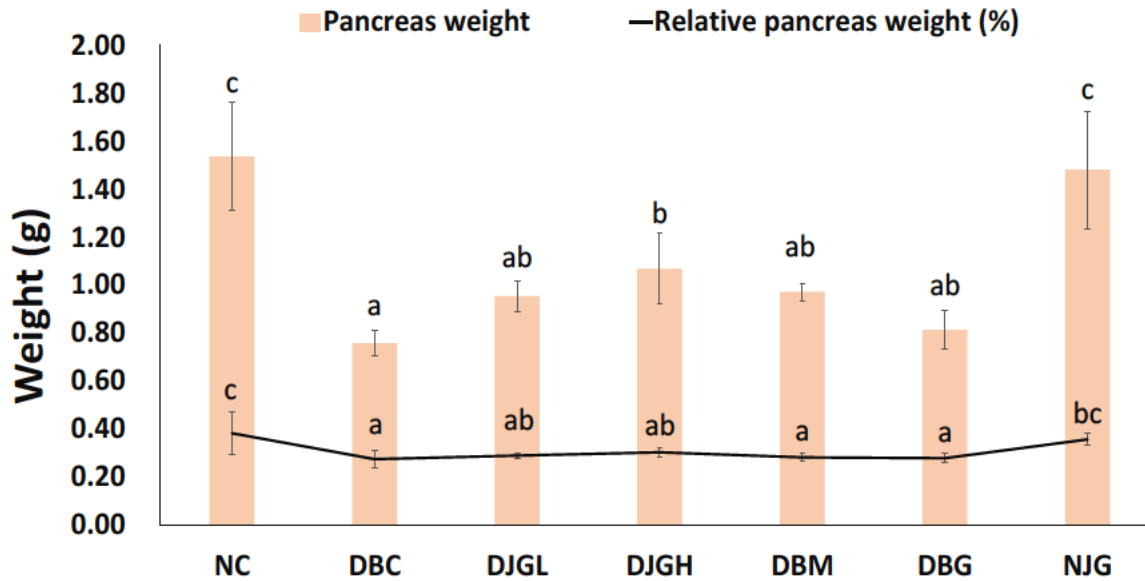


Figure 5.3.3: Weight and relative weight (relative to body weight) of pancreas in different animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-c}Values with different letters over the bars or near the line for a given animal group represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NJG: Normal jasmine green tea (*Camellia sinensis*) high dose (toxicological control)

Significantly ($p < 0.05$) higher blood glucose concentrations were observed in all diabetic groups compared to normal groups upon induction of T2D as indicated in **Figure 5.3.4**. Besides, all diabetic treatment groups displayed a reduction in the blood glucose levels when compared with DBC group, with DJGH showed a comparatively lower blood glucose level than that of DJGL group throughout the intervention period.

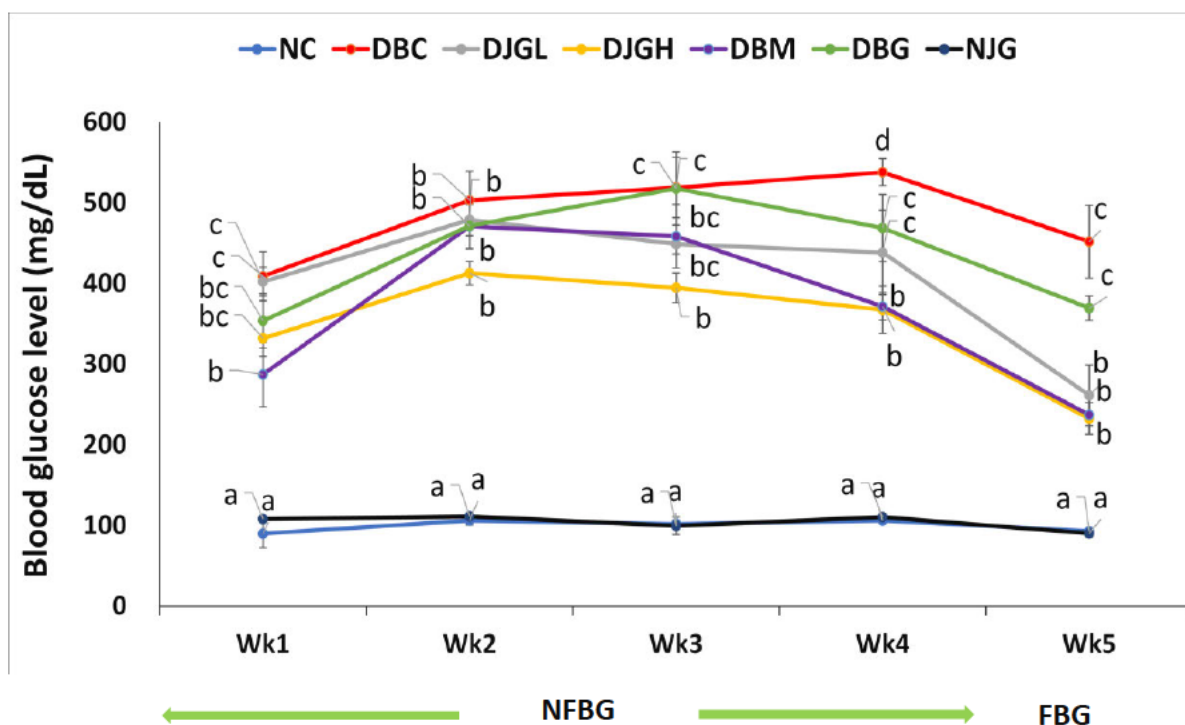


Figure 5.3.4: Weekly blood glucose concentrations of different animal groups during the intervention period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters near the lines for a given week represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NJG: Normal jasmine green tea (*Camellia sinensis*) high dose (toxicological control)

The glucose tolerance ability of normal groups was significantly ($p < 0.05$) higher than that of diabetic groups as presented in **Figure 5.3.5**. A significant ($p < 0.05$) elevation in blood glucose level at 30 minutes after an oral administration of a dose of 2 g/kg BW glucose was seen in all groups. Except for DBC, the blood glucose level of all diabetic rats' groups was peaked at 30 minutes and started declining till the end of the OGTT test period. Treated diabetic groups exhibited significantly ($p < 0.05$) lower blood glucose levels than that of DBC from 60 to 120 minutes. The diabetic DJGH group showed better glucose tolerance ability when compared with all other diabetic groups. The AUC of normal groups and other diabetic treated groups were significantly ($p < 0.05$) than that of DBC group, which again confirms their better glucose tolerance ability than DBC, with DJGH exhibiting better result than DJGL.

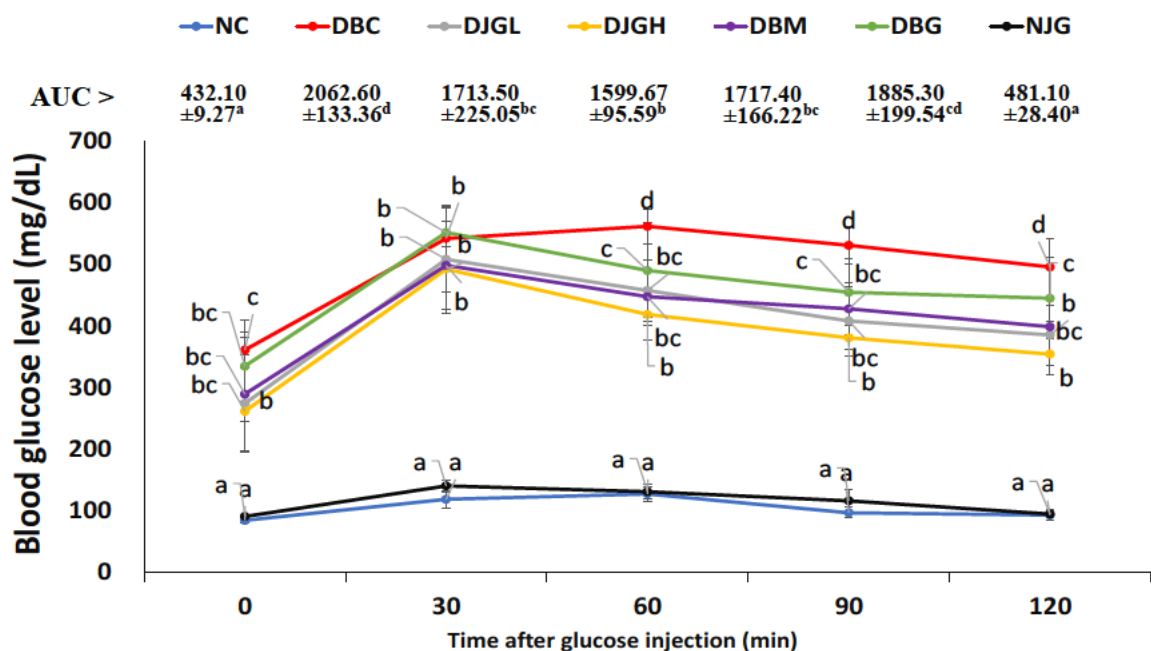


Figure 5.3.5: Oral glucose tolerance test (OGTT) with corresponding area under the curve (AUC) of different animal groups during the whole intervention period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters near the lines for a given time represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NJG: Normal jasmine green tea (*Camellia sinensis*) high dose (toxicological control).

The histopathological analysis of pancreatic tissues of all animal groups were shown in **Figure 5.3.6**. Microscopic examination of rats in NC (blue arrow) and NJG groups (black arrow) displayed regular pancreatic morphological characteristics with normal exocrine pancreatic acinar architecture and endocrine pancreatic islets when compared to diabetic groups. The islets of Langerhans in DBC (red arrow) rats had a distinctly collapsed structure, lysed tissues and a drastically reduced number of islet cells when compared with other groups. Whereas treatment of diabetic rats with JGT and standard drugs potentially improved this irregularity of pancreatic islets morphology in DBC, which included enhancing the regeneration of islets cells, recovering lysed tissues and nucleus, and restoring pancreatic islets structure with a relatively clear boundary. This improvement in DJGH (yellow arrow) exhibited better than that of DJGL (grey arrow). This was also repeated between in DBM (purple arrow) and DBG (green arrow) group.

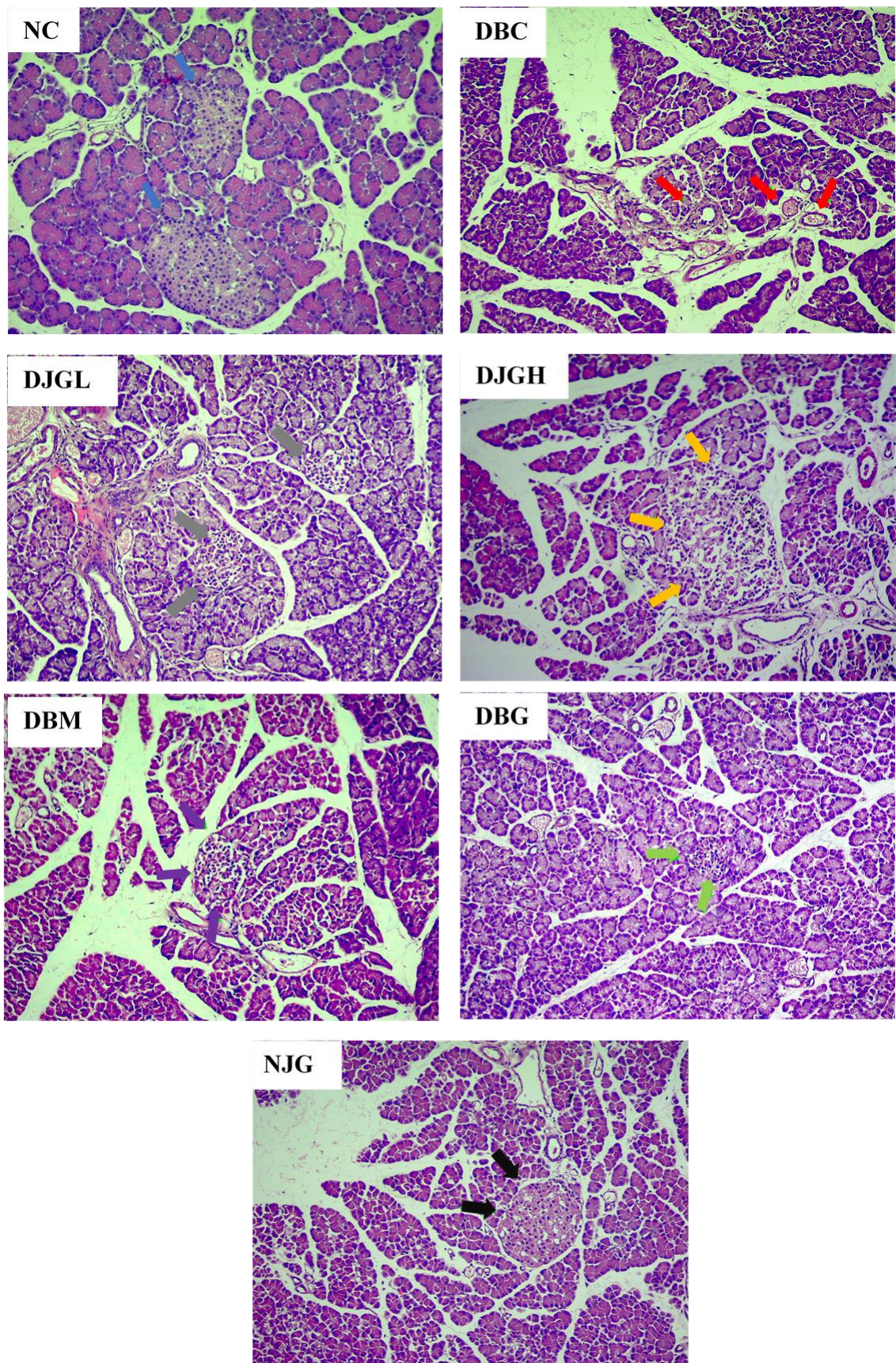


Figure 5.3.6: Histopathological examinations of the pancreas in different animal groups at the end of the experimental period. Magnification: 10x. NC: Normal control; DBC: Diabetic control; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NJG: Normal jasmine green tea (*Camellia sinensis*) high dose (toxicological control).

The concentrations of fasting serum lipids of all animal groups were presented in **Figure 5.3.7**. The total cholesterol and LDL-cholesterol concentrations in DBC group was significantly ($p < 0.05$) higher than the normal groups (NC and NJG). Likewise, all the diabetic groups with treatment exhibited a significant ($p < 0.05$) reduction of these cholesterol levels, with not significant difference among DJGL, DJGH and DBM groups. Furthermore, there was a concomitant significant decrease in HDL-cholesterol level in DBC when compared with normal groups, this however was significantly reversed in the treatment groups. The serum triglycerides in DBC were significantly ($p < 0.05$) higher compared to other groups after induction of T2D, while all diabetic treatment groups showed significant higher levels than normal groups, with DJGL group lower than standard drug groups but higher than DJGH group.

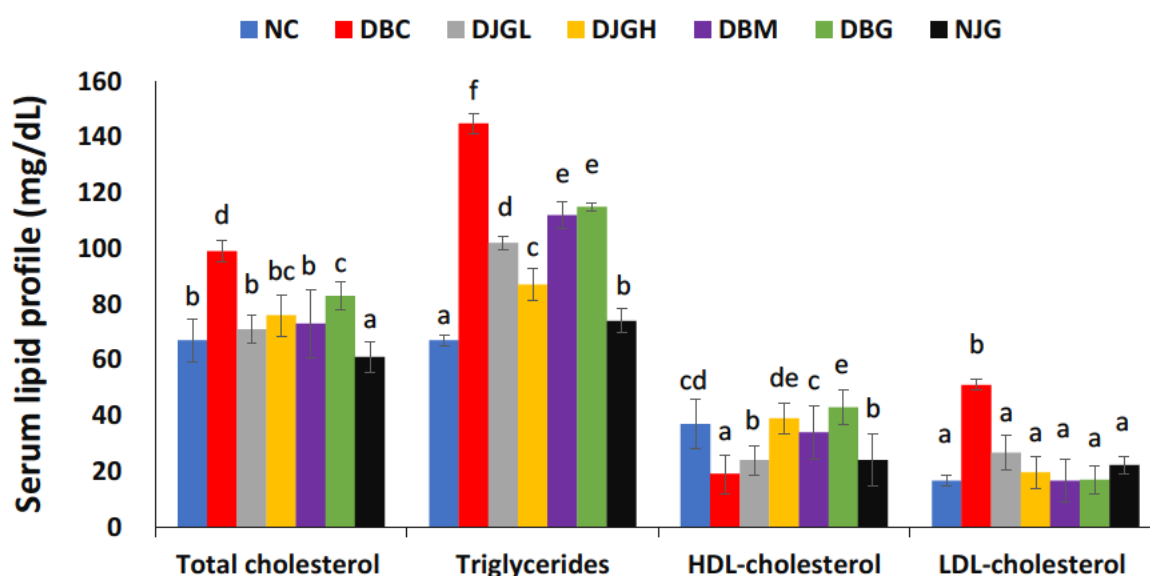


Figure 5.3.7: Serum lipid profile of different animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-f}Values with different letters over the bars for a given parameter represents significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NJG: Normal jasmine green tea (*Camellia sinensis*) high dose (toxicological control); HDL: High density lipoprotein; LDL: Low density lipoprotein.

The liver weight in DBC was significantly ($p < 0.05$) higher than the normal groups, while not statistically significant differences showed compared to other animal groups as displayed in **Table 5.3.1**. Nevertheless, the relative liver weight in DBC group was significantly ($p < 0.05$) higher than that of the NC, NJG and DJGH groups. Liver glycogen was showing the lowest level in DBC group. However, this was significantly reversed in all other groups, with not

significant difference between JGT- and DBM-treated groups, whereas the normal groups were showing higher liver glycogen level than diabetic groups.

Table 5.3.1: Liver weight, relative liver weight and liver glycogen level in the different animal groups at the end of the intervention period

Parameter	NC	DBC	DJGL	DJGH	DBM	DBG	NJG
Liver weights (g)	13.32±1.14 ^{cd}	11.13±1.23 ^{ab}	11.66±0.43 ^{abc}	10.48±0.26 ^a	12.41±1.48 ^{bcd}	11.87±0.89 ^{abcd}	13.67±0.65 ^d
Rel. liver weights (%)	3.18±0.07 ^a	4.03±0.59 ^{bc}	3.54±0.13 ^{ab}	3.30±0.11 ^a	3.59±0.24 ^{abc}	4.07±0.27 ^c	3.32±0.28 ^a
Liver glycogen (mg/g tissue)	6.26±0.23 ^d	2.25±0.27 ^a	4.52±0.28 ^c	5.11±0.30 ^c	4.89±0.16 ^c	3.66±0.67 ^b	5.99±0.10 ^d

Results are expressed as mean ± SD (n = 5 or 7), ^{a-d}Different superscript letters along a row indicate significance of difference (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$). NC: Normal control; DBC: Diabetic control; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NJG: Normal jasmine green tea (*Camellia sinensis*) high dose (toxicological control).

Table 5.3.2: Serum biochemical parameters in different animal groups at the end of the experimental period

Parameter	NC	DBC	DJGL	DJGH	DBM	DBG	NJG
AST (U/L)	84.20±13.33 ^a	156.20±14.96 ^d	99.83±5.42 ^{bc}	108.67±8.36 ^c	91.17±5.19 ^{ab}	96.60±15.93 ^{abc}	86.40±9.07 ^a
ALT (U/L)	84.20±2.17 ^a	93.40±3.65 ^c	88.33±1.75 ^b	86.33±3.44 ^{ab}	85.50±2.26 ^{ab}	88.67±4.73 ^b	87.40±1.67 ^{ab}
ALP (U/L)	72.40±2.30 ^a	364.60±15.98 ^e	196.67±12.03 ^c	164.50±10.41 ^b	173.33±11.48 ^b	351.00±6.32 ^d	72.00±5.15 ^a
Urea (mg/dL)	56.00±3.08 ^a	77.80±2.05 ^d	70.50±3.56 ^c	60.17±3.71 ^b	68.67±3.83 ^c	69.20±4.49 ^c	57.20±1.92 ^{ab}
UA (mg/dL)	2.60±0.55 ^a	7.17±3.89 ^b	4.20±1.15 ^a	3.81±1.48 ^a	3.21±1.12 ^a	4.17±3.38 ^a	2.93±1.13 ^a
Creatine (mg/dL)	1.81±0.39 ^{ab}	3.37±0.90 ^d	2.48±0.56 ^c	2.37±0.34 ^{bc}	2.19±0.32 ^{abc}	2.38±0.55 ^{bc}	1.71±0.39 ^a
CK-MB (U/L)	7.10±1.46 ^a	44.74±2.99 ^e	24.52±3.45 ^d	20.07±3.63 ^c	7.20±2.84 ^a	11.62±1.19 ^b	9.83±0.68 ^{ab}

Results are expressed as mean ± SD (n = 5 or 7), ^{a-e}Different superscript letters along a row indicate significance of difference (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$). NC: Normal control; DBC: Diabetic control; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NJG: Normal jasmine green tea (*Camellia sinensis*) high dose (toxicological control); AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; UA: Uric acid; CK-MB: Creatinine Kinase-myocardial band. ^{a-e}Different superscript letters along a row indicate the significance of difference (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$).

As shown in **Table 5.3.2**, significant ($p < 0.05$) increase in the serum levels of AST, ALT, ALP, urea, uric acid and CK-MB were displayed on induction of T2D. The DBC presented the highest level in all related parameters. However, significantly ($p < 0.05$) reversed levels of these parameters were exhibited in all diabetic treatment groups, with DJGH group displaying the most reduced levels in ALP and urea. While there was no significant difference among the treatment groups in the levels of AST and ALT. Treatment with JGT reduced the elevated creatine and CK-MB levels significantly, with DJGH having lower levels than DJGL.

All groups exhibited significantly ($p < 0.05$) higher level of serum insulin than DBC group except for DJGL group showing not statistically significant ($p > 0.05$) difference as presented in **Table 5.3.3**. The HOMA-IR score in DBC was significantly higher than that of other groups and the groups treated with JGT were able to decrease it significantly, with not statistically significant ($p > 0.05$) difference between the DJGL and DJGH groups. In addition, the HOMA- β score in DBC was significantly lower than that of normal groups, while diabetic groups treated with JGT displayed a significant ($p < 0.05$) increase in comparison to DBC. A significant ($p < 0.05$) higher serum fructosamine concentration was seen in DBC when compared with normal groups, this was however significantly improved in JGT- and standard drugs- treated diabetic groups.

Table 5.3.3. Serum insulin and fructosamine concentrations as well as computed HOMA-IR and HOMA- β scores in different animal groups at the end of the experimental period

	NC	DBC	DJGL	DJGH	DBM	DBG	NJG
Serum							
Insulin ($\mu\text{mol/L}$)	24.56 \pm 0.29 ^d	9.35 \pm 0.23 ^a	9.85 \pm 0.19 ^a	11.25 \pm 0.33 ^b	12.52 \pm 0.41 ^c	10.88 \pm 0.44 ^b	24.27 \pm 1.16 ^d
Fructosamine ($\mu\text{mol/L}$)	387.67 \pm 22.50 ^a	1210.33 \pm 60.00 ^d	813.33 \pm 47.50 ^c	587.00 \pm 62.00 ^b	574.67 \pm 58.45 ^b	546.33 \pm 33.13 ^a	400.67 \pm 0.53 ^a
HOMA-IR	0.80 \pm 0.01 ^a	1.73 \pm 0.04 ^e	0.88 \pm 0.22 ^b	0.90 \pm 0.03 ^b	1.02 \pm 0.03 ^c	1.38 \pm 0.06 ^d	0.76 \pm 0.04 ^a
HOMA- β	39.58 \pm 0.46 ^d	0.99 \pm 0.02 ^a	2.50 \pm 0.05 ^{bc}	3.34 \pm 0.10 ^c	3.62 \pm 0.12 ^c	1.78 \pm 0.07 ^{ab}	43.93 \pm 2.10 ^e

Results are expressed as mean \pm SD ($n = 5$ or 7). NC: Normal control; DBC: Diabetic control; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NJG: Normal jasmine green tea (*Camellia sinensis*) high dose (toxicological control); HOMA-IR: Homeostatic model assessment for IR (insulin resistance); HOMA- β : Homeostatic model assessment for β (β -cell function). ^{a-e}Different superscript letters along a row indicate the significance of difference (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$).

Significantly ($p < 0.05$) higher level of MDA was seen in the pancreas, liver, kidneys, and heart of the animals as portrayed in DBC group on induction of T2D when compared with the normal groups (NC and NJG) as indicated in **Figure 5.3.8**. The MDA level was significantly depleted in diabetic treated groups, this was also replicated in the different studied organs, with DJGH exhibiting significant lower MDA level than DJGL in heart, but not statistically different in pancreas, liver and kidneys.

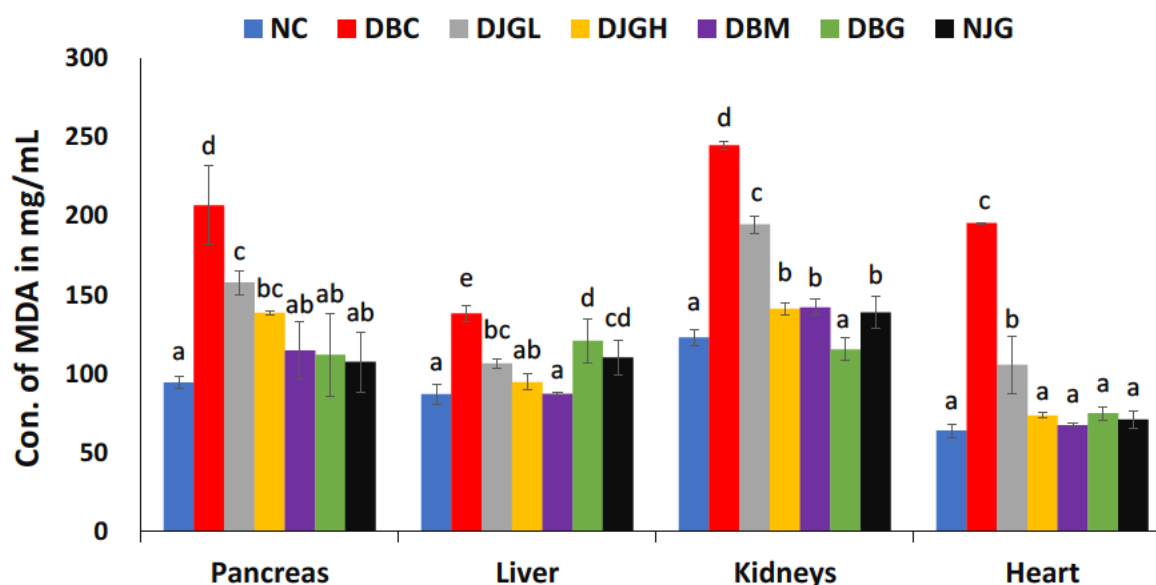


Figure 5.3.8: Thiobarbituric acid reactive substances (MDA equivalent) in the pancreas, liver, kidneys and heart of all animal groups at the end of the experimental period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-f}Values with different letters over the bars for a given sample represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NJG: Normal jasmine green tea (*Camellia sinensis*) high dose (toxicological control).

The SOD activities in the studied organs of all animal groups were presented in **Figure 5.3.9**. There was a significantly lower SOD activity in organs of DBC group compared with normal groups. However, this was significantly reversed in the pancreas, liver, heart of JGT- and standard drug- treated groups, with DJGH displaying significantly ($p < 0.05$) higher activity than DJGL. While in the kidneys, JGT high dose exhibited significantly lower activity than the low dose with only relatively higher activity than DBC group.

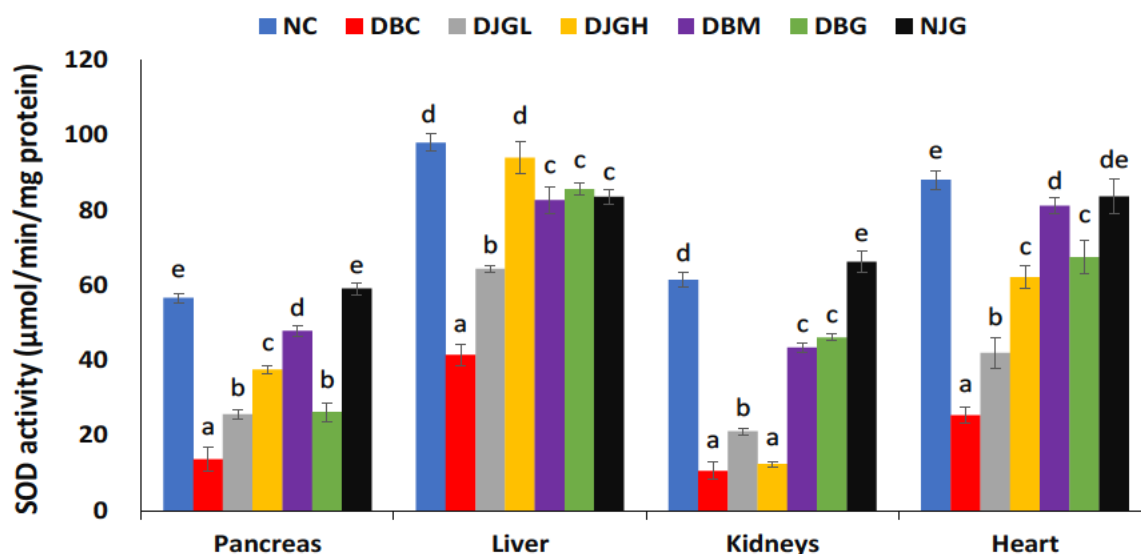


Figure 5.3.9: SOD activity in the pancreas, liver, kidneys and heart of all animal groups at the end of the experimental period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given organ represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NJG: Normal jasmine green tea (*Camellia sinensis*) high dose (toxicological control).

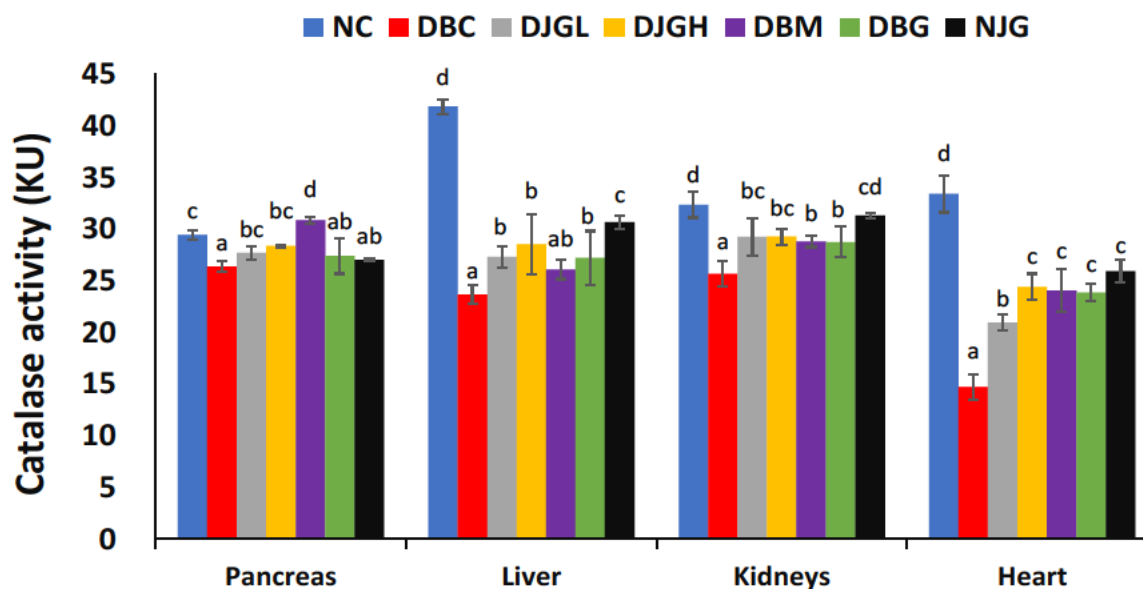


Figure 5.3.10: Catalase activity in the pancreas, liver, kidneys and heart of all animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given organ represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NJG: Normal jasmine green tea (*Camellia sinensis*) high dose (toxicological control).

The activities of catalase in organs of all animal groups were presented in **Figure 5.3.10**. The catalase activity in the liver, kidneys, and heart of DBC group was significantly ($p < 0.05$) lower than the normal groups and JGT-treated diabetic groups. In the pancreas, relatively higher catalase activity was shown in the NJG when compared to DBC group, however, significantly higher catalase activity was exhibited in the JGT-treated diabetic groups than DBC. DJGH was having significantly higher catalase activity than DJGL only in the heart.

The GSH levels of organs in all animal groups were shown in **Figure 5.3.11**. Significantly ($p < 0.05$) lower GSH level was seen in pancreas, liver, and kidneys in DBC group when compared with normal groups, this was replicated in heart of NC compared to DBC. GSH level of JGT- and standard drug treated groups was significantly increased in pancreas, liver, and kidneys when compared to DBC group, with DJGH significantly higher than DJGL in pancreas and kidneys. While in the heart, the increase of GSH level was not statistically ($p > 0.05$) different among all the diabetic animal groups.

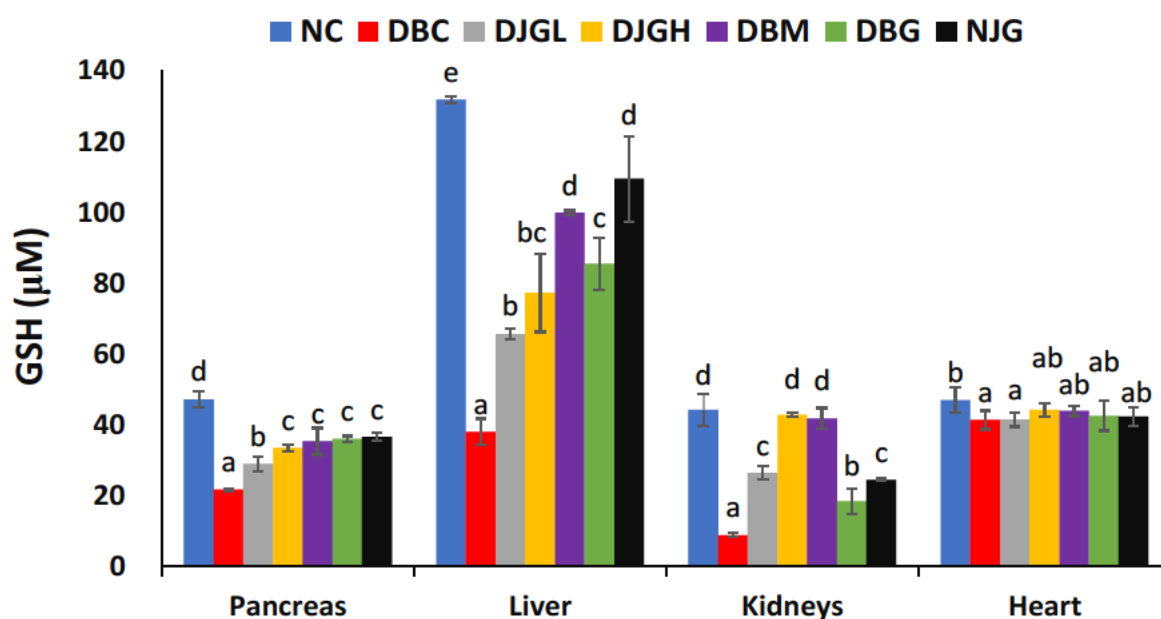


Figure 5.3.11: Reduced glutathione (GSH) levels in the pancreas, liver, kidneys and heart of all animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-f}Values with different letters over the bars for a given organ represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NJG: Normal jasmine green tea (*Camellia sinensis*) high dose (toxicological control).

The activity of GR in the organs of DBC group was significantly ($p < 0.05$) lower than normal groups as depicted in **Figure 5.3.12**. JGT- and standard drug- treated groups significantly improved the GR activity of pancreas, liver, and heart, with DJGH having significantly higher

GR activity than DJGL. While in the kidneys, oral administration of treatments to diabetic treated groups significantly improved the enzyme activity when compared with DBC group, except DJGH, which exhibited not statistically ($p > 0.05$) different when compared with DBC group.

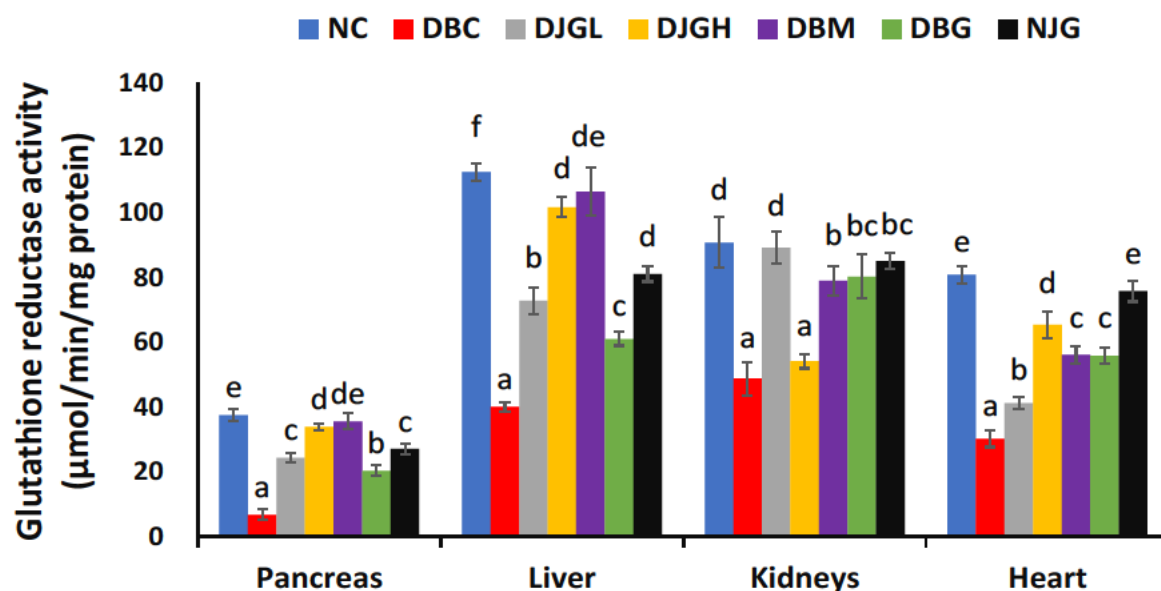


Figure 5.3.12: Glutathione reductase (GR) activity in the pancreas, liver, kidneys and heart of all animal groups at the end of the experimental period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-f}Values with different letters over the bars for a given organ represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NJG: Normal jasmine green tea (*Camellia sinensis*) high dose (toxicological control).

5.3.5 Discussion

Experimental T2D rat models mainly include simple high-fat diet-induced pathogenic rats and chemical drugs combined with high-calorie diet-induced pathogenic rats. Giving experimental rats a excessive high-sugar and high-fat diet can gradually increase the blood glucose of the rats and subsequently generate T2D symptoms such as impaired glucose tolerance and IR (Lozano et al. 2016). This model is similar in pathogenesis to human T2D. However, a small doses injection of STZ to destroy partial pancreatic β -cells and combining with a high-calorie and high-sugar diet to induce insulin resistance can shorten the T2D induction period and the clinical symptoms occurrence are more consistent with T2D (Zafar et al. 2009, Islam and Wilson 2012).

In this study, a 10% fructose diet was supplied for 2 weeks followed by a low dose streptozotocin (STZ) injection to induced T2D rat model to mimic the pathogenies of T2D in humans. Two major pathophysiology of T2D are insulin resistance and partial pancreatic β -cell dysfunction. Insulin resistance was created by feeding 10% fructose solution for two weeks (Wilson and Islam 2012).

Fatal symptom as ketoacid or non-ketoacidosis are caused by uncontrolled diabetes, while more common symptoms and signs of DM include polydipsia, polyphagia and concomitant loss of body weight (Islam 2011, Ibrahim et al. 2016). In our study, the significantly increased water and feed intake in diabetic groups indicated the T2D induction (**Figure 5.3.1**), the groups with JGT treatment though could not entirely reverse the STZ-induced T2D polydipsia, and polyphagia. Their effects ameliorated these parameters significantly better than the untreated diabetic group. Furthermore, the BW loss occurred in the diabetic groups also confirmed the T2D induction, which may attribute to the loss of muscle and tissue protein caused by increased protein glycation due to persistent hyperglycaemia (Eleazu et al. 2013). While the significantly better BW gain was displayed in the JGT treatment groups during most the intervention period revealed the gradual recovery of rats from diabetic condition (**Figure 5.3.2**). This may indicate a therapeutic effect of JGT against major symptoms of T2D, which may possibly result from the high phytochemicals of JGT. On the other hand, metformin and glibenclamide also could not completely reverse these parameters but metformin was better than JGT on polydipsia and body weight loss, while glibenclamide was better than JGT low dose and comparable to JGT high dose on polyphagia.

Chronic hyperglycaemia is the main pathological characteristic of T2D. It leads to defects in insulin secretion from pancreatic β -cells, increases β -cell apoptosis and decreases β -cell proliferation, which intensifies insulin resistance (IR) and glucose metabolism disorder (Nowotny et al. 2015). This corresponds with the elevated weekly blood glucose level (**Figure 5.3.3**), decreased serum insulin level, increased IR, reduced pancreatic β -cell function (**Table 5.3.3**) and pancreatic weight (**Figure 5.3.3**) in the DBC group. This also consistent with the depletion of β -cell cells as depicted by the pancreatic histological analysis (**Figure 5.3.6**). Blood glucose is the main indicator for the diagnosing of the onset and development of diabetes and its homeostatic maintenance ability is a very important parameter to evaluate the therapeutic effects of diabetic treatments (Alberti and Zimmet 1998, Szablewski 2011). The lower NFBG (week 1 to week 4) and FBG (at the last week) measured during the intervention

period in the treatment (JGT and standard drug) groups, especially in the high dose JGT treated rats, exhibits an anti-hyperglycaemic activity (**Figure 5.3.4**). This is in accordance with several reports on different types of tea (Islam 2011, Tang et al. 2013, Mostafa 2014).

The increased level of of serum insulin, ameliorated function of pancreatic β -cell depicted by HOMA- β score (**Table 5.3.3**), and the regeneration of pancreatic β -cells (**Figure 5.3.6**) together further portrays the anti-hyperglycaemic effect of JGT in the treatment and management of diabetes. Apart from pancreatic β -cell dysfunction, the insensitivity of cells and peripheral tissues such as liver, fat and skeletal muscles to insulin leads to the reduction of glucose uptake and utilization capabilities, resulting in increased blood glucose levels, which manifests as impaired glucose tolerance in T2D (Abdul-Ghani et al. 2006, Gastaldelli 2011, Kubota et al. 2011). OGTT is often used to assess the glucose tolerance level at diabetic condition (Etuk 2010, Erukainure et al. 2019, Sanni et al. 2019). The impaired glucose intolerance of DBC group further demonstrates the pancreatic β -cell dysfunction and insulin insensitivity of the untreated T2D rats. Moreover, the improved glucose tolerance in the JGT treated rats (DJGL and DJGH) furthermore support the anti-T2D effects of JGT (**Figure 5.3.5**). This corresponds with reported results of black tea extract and green tea extract in drinking water on glucose intolerance alleviation and anti-hyperglycaemic activity in T2D mouse model (Tang et al. 2013). However, the liver is the main organ responsible for insulin sensitivity, glucose and lipid metabolism. It reduces blood glucose concentration by promoting glycogen synthesis, glucose utilization and inhibiting gluconeogenesis in response to insulin action (Petersen et al. 2017).

The depletion of liver glycogen level shown in the DBC group (**Table 5.3.1**) could possibly be due to the increased glycogen phosphorylation or reduced glycogen synthase activity which are pathological features of STZ-induced T2D (Islam 2011). The significantly higher liver glycogen level in the JGT-treated diabetic group than that of the DBC group (**Table 5.3.1**) demonstrated the consumption of JGT to decrease the blood glucose level (**Figure 5.3.4**) and increase the glucose tolerance (**Figure 5.3.5**). This further revealed the antidiabetic activity of JGT attributing to its improvement of insulin sensitivity and hepatic glycogen synthesis.

Disordered lipid metabolism accompanied with impaired insulin secretion, is the primary pathophysiological changes in T2D, which leads to hyperglycaemia and IR (Savage et al. 2007, Vijayaraghavan 2010). Hyperlipidaemia is characterized by elevated levels of triglyceride and/or total cholesterol, increased LDL-cholesterol level, and decreased HDL-cholesterol level,

which is the major risk factor of T2D associated cardiovascular disease and atherosclerosis (Cannon 2008, Mooradian 2009). This meets with the serum lipid profile of DBC group (**Figure 5.3.7**). The significantly ($p < 0.05$) reduced levels of TG, TC, LDL-c and concomitant increased HDL-c level in the JGT-treated diabetic groups imply the anti-dyslipidemic and cardiac-protective potentials of JGT. This corresponds with previous studies on green tea extract and its polyphenols in alleviating the lipid metabolism disorders (Widowati et al. 2014, Ding et al. 2017). CK-MB (creatin kinase isoenzymes) mainly exists in cardiomyocytes and used a biomarker for coronary heart disease. The increased levels of CK (creatin) and CK-MB signals myocardial injury, such as acute myocardial infarction (Robinson and Christenson 1999, Patel 2014). However, the significantly ($p < 0.05$) reduced creatine and CK-MB levels in the groups of JGT treated rats compared to DBC group (**Table 5.3.2**) insinuates the cardiac-protective ability of JGT. This further corresponds with its anti-dyslipidemia potentials presenting cardiac-protective ability in T2D.

Serum levels of ALT, AST and ALP in DBC group were significantly ($p < 0.05$) higher than the normal groups (**Table 5.3.2**). These enzymes penetrate into blood stream may be due to hepatic inflammation, cholestasis, necrosis or cancer, thus their pathological increased serum levels used as biomarkers implying the onset of hepatic tissue injury (Erukainure et al. 2015, McGill 2016). Significantly decreased levels of these enzymes in the JGT treatment groups (**Table 5.3.2**) when compared to DBC group exhibits the hepatoprotective properties of JGT, which is in agreement with previous reports of Abolfathi et al. (2012) who reported the significantly decreased levels of these enzymes in green tea extract treated STZ-induced diabetic rats.

Serum urea and uric acid in DBC group were significantly ($p < 0.05$) higher than the normal groups (**Table 5.3.2**), they have causal relationship with the occurrence of diabetic nephropathy and been used as biomarkers of kidney failure (Hovind et al. 2011). However, their significantly decreased levels of these parameters in the JGT-treated groups compared to DBC group indicates renal-protective potentials of JGT, which is in line with previous reports on green and black aqueous extracts which were showing renal protective effects through lowering serum uric acid in hyperuricemic mice (Zhu et al. 2017).

Long-term hyperglycaemia has been closely related to diabetic micro- and macro-complications which associates with multiple cascades of reaction such as polyol pathway, hexosamine pathway, advanced glycation end product and activation of protein kinase C (King

and Loeken 2004, Giacco and Brownlee 2010). Fructosamine is a high-molecular ketoamine formed during the non-enzymatic saccharification of protein and glucose in plasma. The concentration of fructosamine is positively correlated with average serum glycaemic level and remains relatively stable. It has been therefore in some cases used as a glycaemic biomarker to assess early stage of diabetes control (Malmström et al. 2014, Sanni et al. 2019). Significantly ($p < 0.05$) higher level of fructosamine in rats of DBC compared to normal groups corresponds with its significantly higher blood glucose level than normal groups demonstrating the onset of diabetic complications and glycation cascade reaction (**Table 5.3.3**). However, the significantly reduced level of fructosamine in JGT-treated rats, indicates a recession of glycation cascade, which further support antidiabetic effect of JGT.

Likewise, long-term hyperglycaemia also increases the glycosylation reaction, which accelerate the production of oxygen free radicals and its accumulative effect causing lipid peroxidation (LPO) (Chenni et al. 2007). The progressively elevated lipid peroxidation (LPO) level disturbs the assembly of the membrane increasing vascular permeability, resulting in plasma proteins depositing on the basement membrane through endothelial cells, leading to the occurrence of vascular disease (Catalá 2009, Negre-Salvayre et al. 2010). MDA is one of the most important products of membrane lipid peroxidation which reflect the severity of lipid peroxidation and oxidative stress, and its generation can aggravate membrane damage (Suryawanshi et al. 2006, Gueraud et al. 2010). The significantly elevated MDA levels in DBC group than that of normal groups suggests an onset of lipid peroxidation on induction of T2D (**Figure 5.3.8**). While the significantly reduced MDA levels in JGT-treated groups compared to DBC group indicates anti-peroxidative potential of JGT.

Significantly lower SOD and catalase activity in DBC group than that of normal groups (NC and NJG) were depicted in **Figure 5.3.9 & 5.3.10**, which is similar to the findings of several recent studies of untreated STZ-induced T2D rats (Chukwuma and Islam 2017, Oyebode et al. 2020). SOD and catalase are two important endogenous antioxidant enzymes impeding harmful effect of free radicals. SOD specifically catalyses the disproportionation of superoxide anions to generate oxygen and hydrogen peroxide (H_2O_2). While catalase promotes the decomposition of the produced toxic hydrogen peroxide into harmless molecular oxygen and water (Ighodaro and Akinloye 2018). The significantly reversed activities of these enzymes in JGT-treated groups compared to DBC group as well as lower MDA level further indicates a potent *in vivo* antioxidant property of JGT (**Figure 5.3.8 – 5.3.10**).

As one of the most prominent non-enzymatic antioxidants, GSH can eliminate free-radical mediated damage, protect the sulfhydryl groups in molecules such as proteins and enzymes from being oxidized by harmful oxidants (Kabel 2014, Nurdiana et al. 2017). While GR is the major flavozyme maintaining the content of GSH in cells and organs, which plays an important role in preventing the oxidative decomposition of hemoglobin, maintaining the activity and reducibility of sulfhydryl proteins and the integrity of cells (Waggiallah and Alzohairy 2011, Gawlik et al. 2016). The significantly ($p < 0.05$) higher level of GSH and GR in JGT-treated rats than DBC group (**Figure 5.3.11 & 5.3.12**) again demonstrates the therapeutic effects of JGT in diminishing the *in vivo* antioxidative status of STZ-induced T2D.

5.3.6 Conclusion

In summary, the results of the present study demonstrate that the administration of aqueous jasmine green tea extract in drinking water exhibited anti-hyperglycaemic, anti-hyperlipidaemic, *in vivo* antioxidant activities, while ameliorated insulin resistance and improved pancreatic β -cell function, pancreatic morphology while exacerbating hepatic glycogen synthesis in STZ-induced T2D rats, thus suggesting its therapeutic potential and protective benefits against diabetes and its associated complications.

CHAPTER 6

THE ANTIOXIDANT AND ANTIDIABETIC ACTIVITIES OF GREEN ROOIBOS TEA *IN VITRO* AND *IN VIVO*

6.1 Background

Aspalathus linearis is a leguminous shrub in the *Fabaceae* family and the *Aspalathus* genus with bright green needle-shaped leaves and small yellow flowers (Ajuwon et al. 2018) (**Figure 6.1**). *A. linearis* is endemic to a restricted region called Cederberg, Western Cape province, South Africa (Erickson 2003, Krafczyk and Glomb 2008). The delicate stems and leaves of *A. linearis* are utilized to make herbal tea, rooibos or bush tea which is widely consumed globally (Muller et al. 2012). There are two forms of rooibos tea namely: fermented/traditional/red rooibos tea and unfermented/unoxidized/Green rooibos tea (Joubert et al. 2008).



Figure 6.1: The pictures of whole plant, flower of *Aspalathus linearis* (*Fabaceae*), copied without permission from Wikipedia, as well as the commercial fermented and unfermented product of rooibos tea that used in this study.

6.1.1 Ethnomedical uses

Aspalathus linearis is a medicinal plant native to South Africa (Joubert et al. 2008). Green rooibos tea (GRT) was first introduced to food, cosmetic, and functional beverage markets due to its higher phenolics levels than fermented rooibos tea (Joubert and de Beer 2011), which contribute to its exploration of pharmaceutical applications (Joubert and Schultz 2012) such as antidiabetic agents, medicament in the treatment of neurological and psychiatric disorders of the central nervous system, and topical application in preventing skin cancer (Joubert et al. 2008, Larsen et al. 2008, Frank and Dimpfel 2010).

6.1.2 Biological activities

Several studies corresponding to the pharmaceutical applications of GRT claimed its antidiabetic, anti-inflammatory, cardio-protective effects, and anti-mutagenic effects *in vitro*, *ex vivo*, *in vivo* and a few human studies (Snijman et al. 2007, Marnewick et al. 2011, Pantsi et al. 2011, Joubert et al. 2012, Muller et al. 2012, Mazibuko et al. 2013, Ajuwon et al. 2014, Kamakura et al. 2015, Xiao et al. 2020).

6.1.3 Phytochemistry

There are two distinctive flavonoids in *A. linearis* namely aspalathin, aspalainin (Shimamura et al. 2006, Iswaldi et al. 2011, Lee and Bae 2015) and some other different bioactive compounds such as nothofagin, luteolin, rutin, orientin, iso-orientin, vitexin and iso-vitexin (Krafczyk and Glomb 2008, Joubert et al. 2009, Marnewick 2009).

6.2 Chemical characterization of sequential extracts of green rooibos (*Aspalathus linearis*) tea, their antioxidative and antihyperglycemic potentials

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6.2.1 Abstract

This study characterized the phytochemicals of green rooibos tea (GRT) extracts of sequential extraction using increasing polarity solvents (dichloromethane, ethyl acetate, ethanol) and investigated their potential antioxidative and antihyperglycemic activities. The antioxidant activity of the extracts was determined *in vitro* by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP). The antihyperglycemic potentials of the extracts were determined by examining inhibitory effects on α -glucosidase and α -amylase enzymes. Furthermore, the extracts were subjected to gas chromatography-mass spectrometry (GC-MS) analysis to identify their possible bioactive components. GRT ethanol extract showed the highest level of total phenolics content and antioxidant capacity. This extract exhibited pronounced α -glucosidase and moderate α -amylase inhibitory activities compared to dichloromethane and ethyl acetate extracts. GC-MS analysis revealed the presence of n-hexadecenoic acid, octadecanoic acid, 1',4',2,6,6-pentamethyl-Spiro-1-(cyclohex-2-ene)-2'-(5'-oxabicyclo [2.1.0] pentane) and styrene. These results indicate the antihyperglycemic and antioxidative effects of the ethanol extract of GRT.

Keywords: Sequential extraction, Green rooibos tea, Antioxidant, Anti-hyperglycaemic, Type 2 diabetes

6.2.2 Introduction

Diabetes mellitus (DM) is a chronic disease with high morbidity, mortality, and medical costs, which affects the citizens of both developed and developing countries (Zimmet et al. 2016). It is one of the greatest threats to humans due to its micro- and macro-vascular complications such as nephropathy, neuropathy, retinopathy, cardiomyopathy, and even death (Chawla et al. 2016). According to the International Diabetes Federation (IDF), approximately 151 million people had diabetes in 2000, which increased to 463 million in 2019, with a projected increase to 700 million by 2045 (IDF 2019).

DM is a metabolic disorder caused by impaired insulin secretion from pancreatic β -cells or insulin resistance or both, which is characterized by chronic hyperglycaemia (ADA 2014). Unlike type 1 diabetes, type 2 diabetes (T2D) attributes to over 90% of the incidence of diabetes (IDF 2019). The persistent chronic hyperglycaemia of T2D causes damage to many cells and organs, leading to tissue metabolism disorders and even organs pathological changes in the body, resulting in the occurrence of diabetic complications (Campos 2012, Alam et al. 2014). Therefore, controlling hyperglycaemia is a major challenge to manage diabetes and its complications (Stolar 2010).

The management of hyperglycaemia as well as T2D relies currently mainly on oral hypoglycaemic drugs such as α -glucosidase inhibitors, sulfonylureas and biguanides (Inzucchil et al. 2012). These synthetic drugs have however numerous side effects and are prone to drug resistance besides high cost (Stein et al. 2013). Therefore, the search of new alternative therapies is becoming increasingly in demand, particularly from medicinal plants. Medicinal plants have been reported to be rich in bioactive phenolics with advantages of cheaper options, wider accessibility, multi-channel and multi-target actions, and reduced dissatisfactory symptoms and consequences in the possible treatment and management of various diseases including T2D (Ranilla et al. 2010, Arumugam et al. 2013).

GRT (*Aspalathus linearis*), also known as unfermented rooibos tea, an unoxidized form of traditional fermented rooibos tea. It is a broom-like member of the *Fabaceae* family, which is endemic to a restricted region called Cederberg, Western Cape province, South Africa (Erickson 2003, Krafczyk and Glomb 2008). GRT was first produced to achieve higher antioxidant levels than fermented rooibos tea to satisfy the demand of food, cosmetic, and functional beverage markets (Joubert and de Beer 2011). The higher levels of flavonoids that

have antioxidant properties combined with its caffeine-free status further aid its opportunities in exploration of pharmaceutical applications (Joubert and Schultz 2012).

GRT aqueous-based extracts or its bioactive compounds (aspalathin, aspalainin, and nothofagin) have been applied as antidiabetic agents, medicament in the treatment of neurological and psychiatric disorders of the central nervous system, and topical application in preventing skin cancer (Joubert et al. 2008, Larsen et al. 2008, Frank and Dimpfel 2010). Several more studies corresponding to the pharmaceutical applications of GRT claimed its antidiabetic, anti-inflammatory, and cardio-protective effects (Muller et al. 2012, Mazibuko et al. 2013, Kamakura et al. 2015). Although there are numerous reports mentioning the use of GRT, no direct scientific evidence exists demonstrating the hypoglycaemic and antioxidative effects of sequential extracts of Green rooibos used as a therapeutic strategy in the management of T2D.

Hence, the present study aimed to examine the antioxidative and anti-hyperglycaemic properties of different solvent extracts of GRT. Their phytochemicals were analyzed via GC-MS. Their antioxidant activities were tested using free radicals scavenging assays; and their inhibitory effects on the activities of enzymes linked to T2D, such as α -glucosidase and α -amylase were determined.

6.2.3 Materials and methods

Please refer to Chapter 2 for the detailed materials and methods for green rooibos tea (*Aspalathus linearis*).

6.2.4 Results

The total phenolic content of the extracts is portrayed in **Figure 6.2.1**. The ethanol extract of GRT displayed significantly ($p < 0.05$) higher phenol content when compared to the DCM and ethyl acetate extracts.

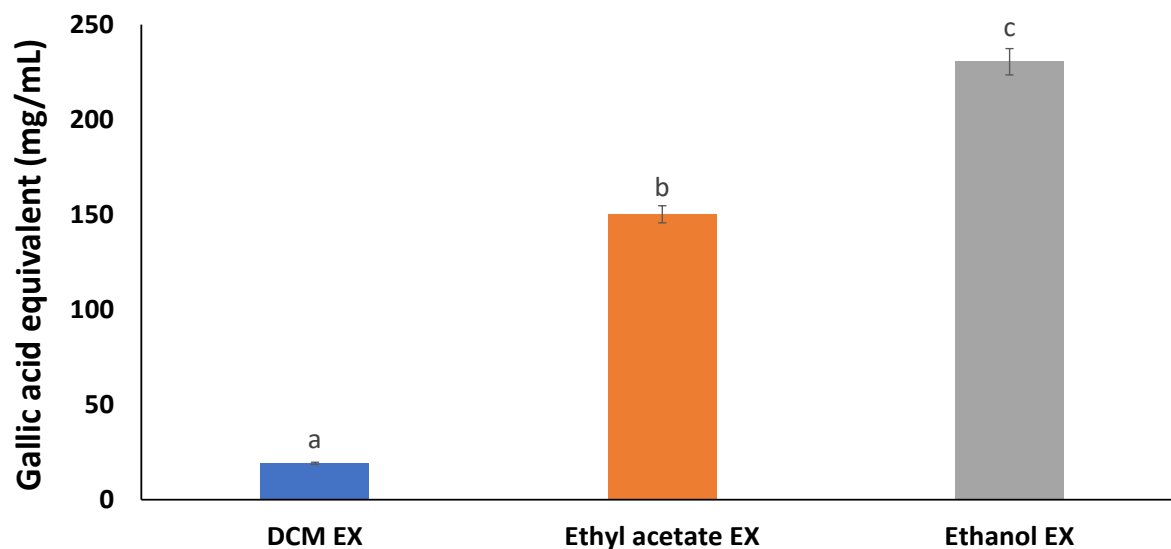


Figure 6.2.1: Total phenolic content of different extracts of green rooibos (*Aspalathus linearis*) tea. Values represent mean \pm standard deviation ($n = 3$). ^{a-c} Different alphabets over the bars for a given extract represent significance of difference ($p < 0.05$). EX = extract, DCM = Dichloromethane.

The DPPH free radical scavenging activities of different GRT extracts were displayed in **Figure 6.2.2**. All the extracts displayed scavenging capability at a dose-dependent manner, with the ethanol extract exhibiting the best activity compared to that of other extracts as portrayed by its lowest IC_{50} value of $0.36 \mu\text{g/mL}$ as seen in **Table 6.2.1**.

Table 6.2.1: IC_{50} values of green rooibos tea (*Aspalathus linearis*) activities

Activities	Dichloromethane extract ($\mu\text{g/mL}$)	Ethyl acetate extract ($\mu\text{g/mL}$)	Ethanol extract ($\mu\text{g/mL}$)	Ascorbic acid ($\mu\text{g/mL}$)	Acarbose ($\mu\text{g/mL}$)	Trolox ($\mu\text{g/mL}$)
DPPH	0.67	3.06	0.36	0.02	-	-
FRAP	>1000	566.90	193.66	131.98	-	256.65
α -amylase	>1000	>1000	>1000	-	>1000	-
α -glucosidase	329.31	961.79	49.15	-	0.12	-

IC_{50} , concentration to inhibit 50% activity, DPPH, 1,1-diphenyl-2-picrylhydrazyl; NO, nitric oxide; FRAP, ferric reducing antioxidant power.

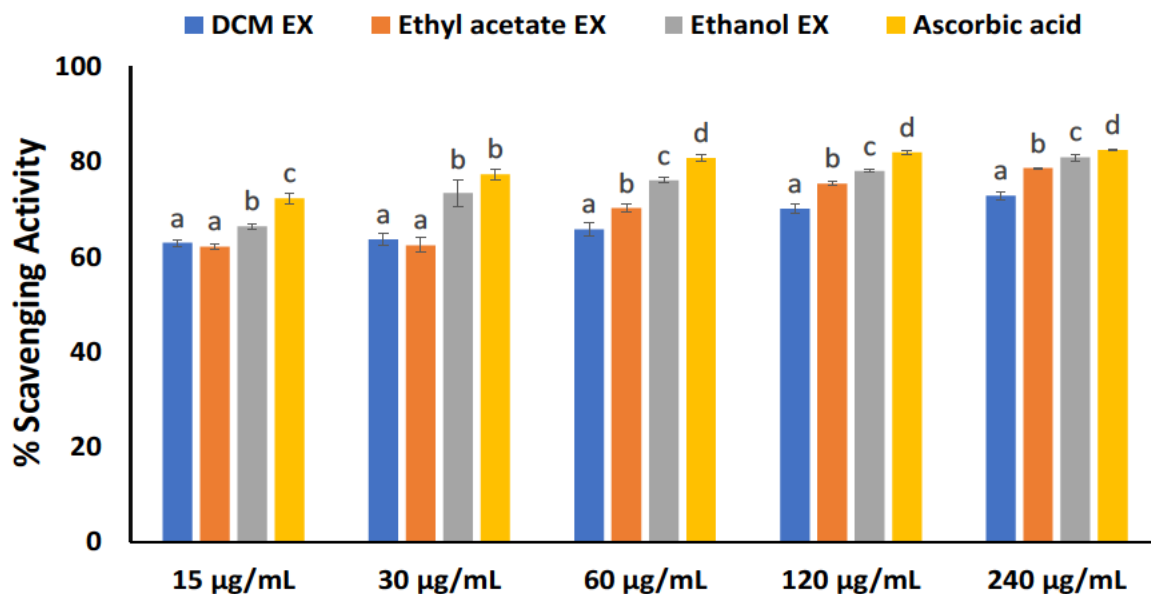


Figure 6.2.2: DPPH of different extracts of green rooibos (*Aspalathus linearis*) tea. Values represent mean \pm standard deviation ($n = 3$). ^{a-d} Different alphabets over the bars for a given concentration for each extract represent significance of difference ($p < 0.05$). DCM = dichloromethane, EX = extract.

The reducing power of different GRT extracts were displayed in **Figure 6.2.3**. All GRT extracts displayed FRAP activity in a dose-dependent manner with significant ($p < 0.05$) differences. The ethanol extract ($IC_{50} = 193.66 \mu\text{g/mL}$) exhibited the best activity compared to that of other extracts and one of the standard drugs trolox ($IC_{50} = 256.65 \mu\text{g/mL}$) as portrayed by IC_{50} values as seen in **Table 6.2.1**.

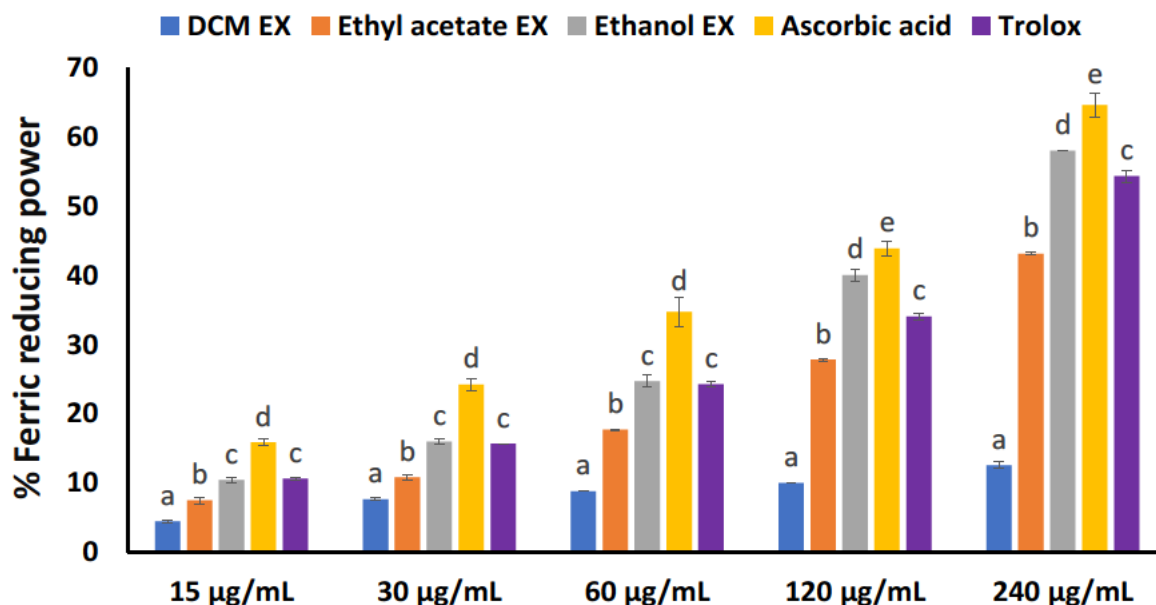


Figure 6.2.3: Ferric reducing antioxidant power (FRAP) activities of different extracts of green rooibos (*Aspalathus linearis*) tea. Values represent mean \pm standard deviation ($n = 3$). ^{a-e} Different alphabets over the bars for a given concentration for each extract represent significance of difference ($p < 0.05$). EX = extract, DCM = Dichloromethane.

All the GRT extracts significantly ($p < 0.05$) inhibited the activity of α -amylase in a dose-dependent manner, with the ethanol extract displaying a better activity compared to that of other extracts as presented in **Figure 6.2.4**. The α -glucosidase inhibitory activity of different extracts of GRT is portrayed in **Figure 6.2.5**. All GRT extracts significantly ($p < 0.05$) inhibited α -glucosidase activity in a dose-dependent pattern, with the ethanol extract exhibiting the highest activity except significantly lower than that of standard drug acarbose (**Figure 6.2.5**) as depicted by their IC_{50} values (**Table 6.2.1**).

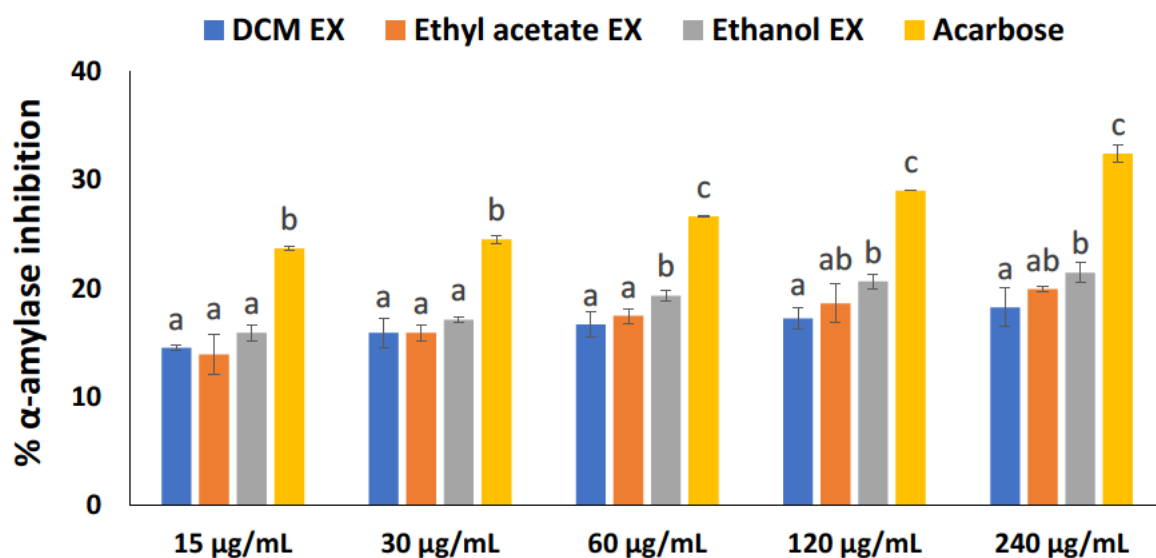


Figure 6.2.4: Inhibitory effect of different extracts of green rooibos (*Aspalathus linearis*) tea on α -amylase. Data are presented as mean \pm SD. ^{a-c}Values with different letter above the bars for a given concentration are significantly ($p < 0.05$) different from each other. DCM = dichloromethane, EX = extract.

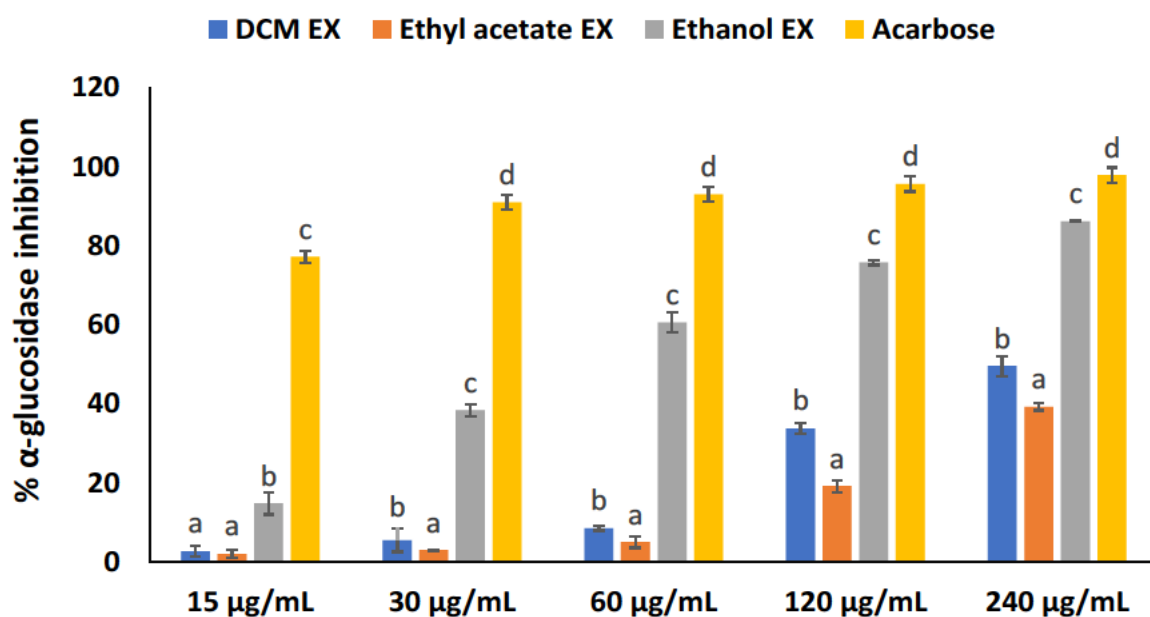


Figure 6.2.5: Inhibitory effect of different extracts of green rooibos (*Aspalathus linearis*) tea on α -glucosidase. Data are presented as mean \pm SD. ^{a-d}Values with different letter above the bars for a given concentration are significantly ($p < 0.05$) different from each other. EX = extract, DCM = Dichloromethane.

Possible bioactive compounds in the different extracts of GRT by GC-MS report. The GC-MS analysis revealed the presence of n-hexadecenoic acid, octadecanoic acid, 1',4',2,6,6-pentamethyl-Spiro-1-(cyclohex-2-ene)-2'-(5'-oxabicyclo [2.1.0] pentane), styrene. Above

these compounds shown in **Figure 6.2.6 & 6.2.7** and **Table 6.2.2** were identified based on the retention time, molecular mass, molecular formula, mass spectral data, as well as by using the database of National Institute Standard and Technology (NIST).

Table 6.2.2: Compounds identified in dichloromethane, ethyl acetate, ethanol extract of green rooibos tea (*Aspalathus linearis*) tea by GC-MS

Compounds	Molecular formula	RT (min)	Molecular mass	Relative abundance (%)
Dichloromethane extract				
n-hexadecanoic acid	C ₁₆ H ₃₂ O ₂	31.68	256	24.45
octadecanoic acid	C ₁₈ H ₃₆ O ₂	35.41	284	9.24
Ethyl Acetate extract				
1',4',2,6,6-pentamethyl-Spiro-1-(cyclohex-2-ene)-2'-(5'-oxabicyclo[2.1.0]pentane)	C ₁₄ H ₂₂ O	26.46	206	1.45
Ethanol extract				
styrene	C ₈ H ₈	5.05	104	16.78
n-hexadecanoic acid	C ₁₆ H ₃₂ O ₂	31.70	256	13.99

The compounds presented in the table are those which matched similar compounds in the NIST library software.

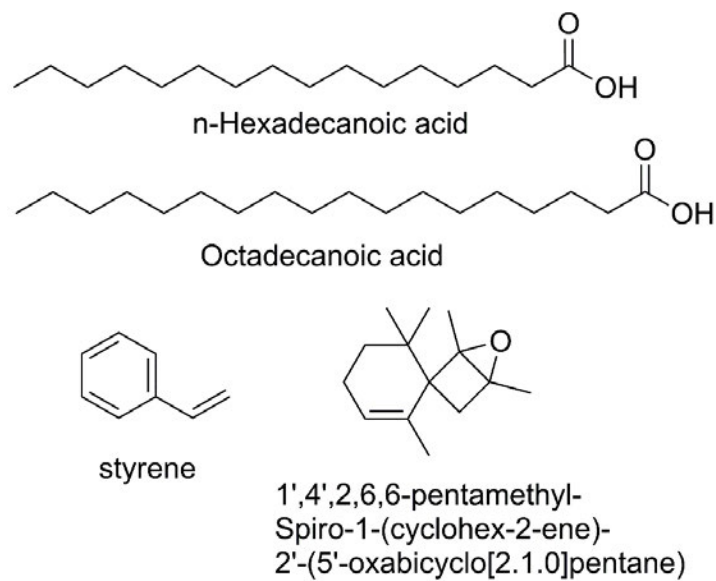
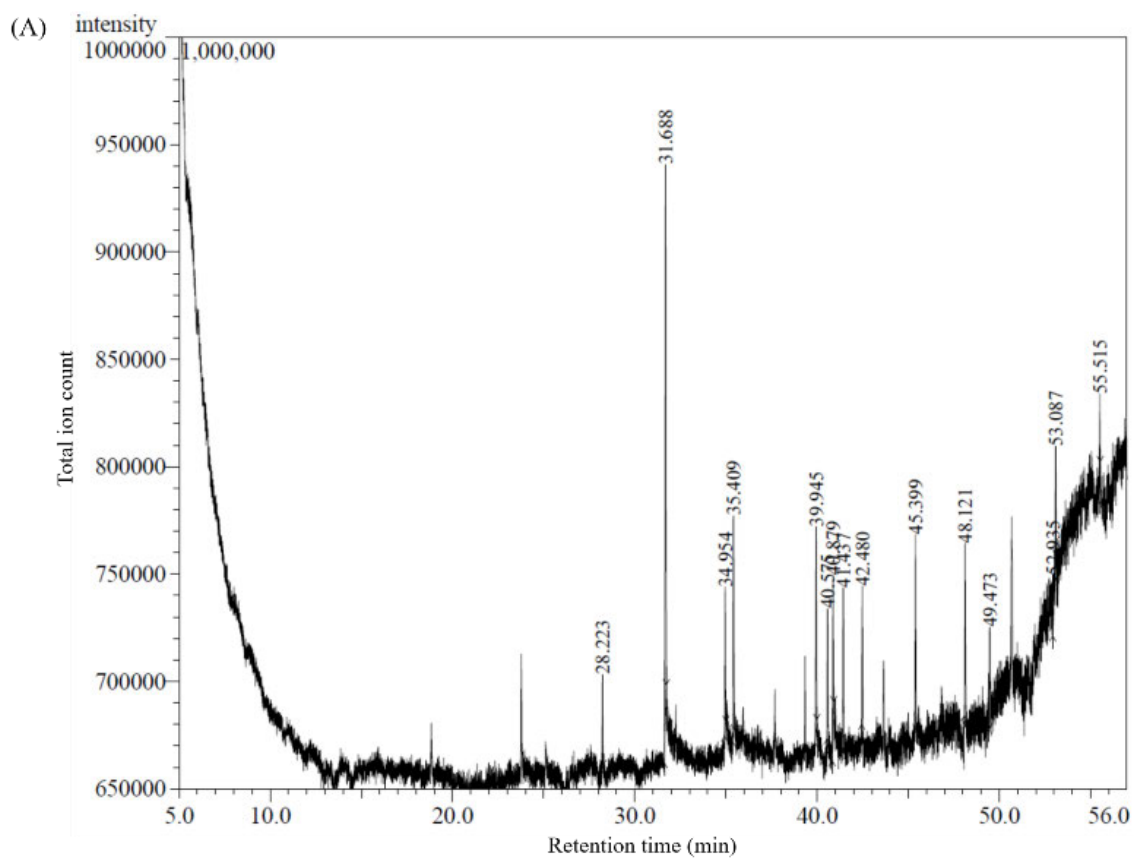


Figure 6.2.6: Compounds identified in the different extracts of green rooibos tea (*Aspalathus linearis*) by GC-MS.



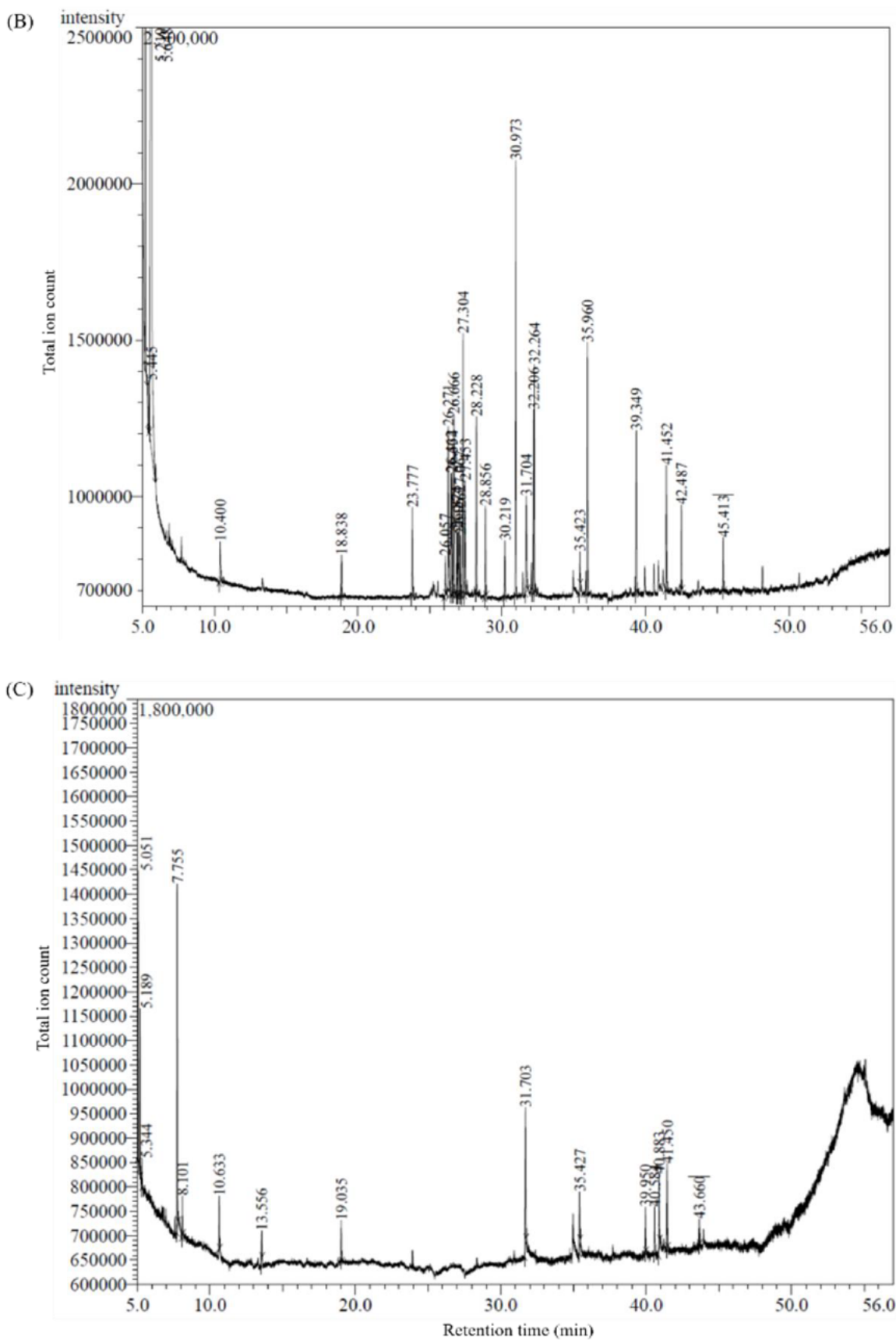


Figure 6.2.7: GC-MS chromatograms of green rooibos tea (*Aspalathus linearis*) tea extracts: A) DCM, B) Ethyl acetate, and C) Ethanol.

6.2.5 Discussion

Under diabetic condition, continued hyperglycaemia is a life-threatening risk factor (Wu et al. 2014). The stimulation of persistent hyperglycaemia may cause a decrease in insulin secretion and an increase in insulin resistance, resulting in glucose toxicity, causing β -cell dysfunction (Gupta et al. 2012). Meanwhile, oxidative stress in T2D corresponds to the hyperglycaemia-induced overproduction of reactive oxygen species (ROS) which leads to an imbalance of the endogenous antioxidant system (Aouacheri et al. 2015). There are strong indications that oxidative stress may be a key event in the progression of diabetic complications (Aouacheri et al. 2015). The antioxidant and antidiabetic properties of GRT aqueous extracts are well documented (Muller et al. 2012, Kamakura et al. 2015). In this study, the different GRT extracts obtained via sequential extraction were investigated for their antioxidative capacities through free radical scavenging abilities; and their anti-hyperglycaemic potentials through inhibitory effects on enzymes linked to carbohydrate digestion.

Bioactive substances such as polyphenols are widely distributed plant-derived dietary constituents, commonly known as natural antioxidants, have been implicated as the active components in herbal and traditional medicines (Chen et al. 2011). In this study, the ethanol extract of GRT showed the highest total phenolic content compared to other extracts (**Figure 6.2.1**). This indicates that GRT contains more polar total polyphenols than that of less polar polyphenols. The presence of phenolic ingredients possessing antioxidant activities of aqueous-based GRT as dihydrochalcones, flavonoids, phenolic acids have been well-documented (Krafczyk and Glomb 2008, de Beer et al. 2017). Furthermore, this may imply the antioxidant potentials of different extracts of GRT.

The biological effects of phenolics such as anti-inflammatory, anti-microbial, anti-HIV, and other pharmacological properties have been reported in various studies (Ghasemzadeh and Ghasemzadeh 2011, Kabera et al. 2014). The antioxidant activity of polyphenols may modulate the oxidative stress-induced glucose metabolic disorders from the headstream through free radical scavenging (Almajano et al. 2008). Whereas, the capacity of compound to scavenge free radicals is linked with its electron transfer ability (Sunmonu and Afolayan 2012). In this study, the free radical scavenging and ferric reducing abilities of different GRT extracts were determined *in vitro*. Several previous studies have claimed the free radical scavenging capacity as reflected by the total polyphenol content which corresponding to the results found in the present study (Joubert and de Beer 2012, Oyeboode et al. 2018). The ethanol extract displayed

the best capacity in scavenging activity against DPPH radicals and ferric-reducing antioxidant power (FRAP) than that of other extracts (**Figure 6.2.2 & 6.2.3**). Thus, met with the highest total phenolic content of the ethanol extract indicating its best antioxidant potency.

In the body, carbohydrates can only be absorbed into the small intestine by digestion into glucose and fructose (Jang et al. 2018). α -amylase and α -glucosidase are the two most critical enzymes for carbohydrate hydrolysis (Lordan et al. 2013). As a glycoside hydrolase, α -amylase is mainly secreted by the pancreas and salivary glands and is transported to the digestive tract. In the small intestine, α -amylase can degrade long-chain carbohydrates into glucose severing as the main metabolic fuels for the body (Luo et al. 2012, Varga 2012). While, α -glucosidase is located on the small intestinal mucosal cells and can hydrolyze the alpha-1,4 glycosidic bonds of disaccharides and starch to release glucose (Adeva-Andany et al. 2016, Lin et al. 2016). The inhibition of α -amylase and α -glucosidase activity plays an important role in regulating postprandial blood glucose levels by delaying the release rate of glucose (Tundis et al. 2010). It is one of the therapeutic approaches for type 2 diabetic patients through impeding digestion and absorption of carbohydrates (Chaudhury et al. 2017). Thus, the inhibitory activities of GRT extracts (especially ethanol extract) on α -amylase and α -glucosidase indicates antidiabetic potential as displayed by their anti-hyperglycemic efficacies (**Figure 6.2.4 & 6.2.5, Table 6.2.1**).

Different types of compounds were extracted from different types of sequential extracts of GRT due to different polarity of the solvents. GC-MS analysis of all the GRT extracts was undertaken to find out the presence of possible bioactive compounds that could be contributed to their antioxidant and anti-hyperglycemic activities. Octadecanoic acid, n-hexadecanoic acid, 1',4',2,6,6-pentamethyl-Spiro-1-(cyclohex-2-ene)-2'-(5'-oxabicyclo [2.1.0] pentane), and styrene were identified (**Figure 6.2.6 and Table 6.2.2**). Among them, n-hexadecanoic acid has antioxidant, antimicrobial and other activities (Kala et al. 2011). Octadecanoic acid was reported to possess anti-inflammatory activity (Lalitharani et al. 2009). However, there were no scientific papers describing the bioactivities of 1',4',2,6,6-pentamethyl-Spiro-1-(cyclohex-2-ene)-2'-(5'-oxabicyclo [2.1.0] pentane). Thus, isolation of this compound maybe necessary conducted to further understand its antioxidative and antidiabetic activities.

6.2.6 Conclusion

These results imply the antioxidant and anti-hyperglycaemic potentials of green rooibos tea sequential extracts (dichloromethane, ethyl acetate, ethanol). In particular, the ethanol-based

extract exhibited the overall most potent activity. It approved by free radical scavenging capacities, inhibitory effects on the key digestive enzymes α -glucosidase and α -amylase related to type 2 diabetes. Thus, the ethanol green rooibos tea extract could be used as an alternate plant source for the management of T2D. It should be noted that *ex vivo* and *in vivo* studies should also be conducted to confirm the reported *in vitro* results translate into activities that might support its therapeutic applications.

6.3 The *in vivo* antioxidant and ameliorative ability of green rooibos (*Aspalathus linearis*) tea hot water extract in fructose-streptozotocin induced type 2 diabetic rats

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6.3.1 Abstract

The present study is designed to examine the *in vivo* antioxidant and antidiabetic ability of green rooibos (*Aspalathus linearis*) tea concentrated hot water-infusion extract (GRT) in type 2 diabetes (T2D) model of rats. T2D was created in male Sprague Dawley (SD) rats through feeding 10% fructose solution *ad libitum* for 2 weeks followed by a single intraperitoneal injection of 40 mg/kg body weight (BW) streptozotocin. After the confirmation of T2D, diabetic rats in treatment groups were orally treated with low or high dose (150 and 300 mg/kg BW) of GRT in drinking water for 5 weeks (5 days/week). Metformin and glibenclamide were served as standard drugs. After 5 weeks intervention period, animals of untreated diabetic group (DBC) in comparison to normal animal groups demonstrated significantly ($p < 0.05$) higher blood glucose, serum lipids (TG, TC, and LDL-c), AST, ALT, ALP, urea, uric acid, creatinine, CK-MB, fructosamine, HOMA-IR score, MDA levels of organs (pancreas, liver, kidneys, and heart) and lower glucose tolerance ability, HDL-c, liver glycogen, serum insulin, HOMA- β score, SOD, catalase, GSH, GR in harvested organs. Histopathological examination of pancreas from different animal groups exhibited corresponding morphological changes in the islets and β -cells. While most of these elucidated parameters were notably reversed in a dose-dependent manner in GRT-treated diabetic groups. Results from this study indicate that Green rooibos tea (GRT) displayed promising antioxidant and antidiabetic ability in ameliorating and treating T2D and its associated complications.

Keywords: Green rooibos tea, Antioxidant, Antidiabetic, STZ, Type 2 diabetes rats.

6.3.2 Introduction

Diabetes mellitus (DM) is recognized as one of the most common global public health concerns in the 21st century, causing substantial morbidity, mortality, and long-term complications. According to the International Diabetes Federation (IDF), the prevalence of DM is increasing at an alarming rate. It has risen from 239 million in 2000 to approximately 463 million in 2019, and the figure is predicted to reach near 700 million people by 2045 (IDF 2019).

DM can be broadly divided into insulin-dependent and non-insulin-dependent diabetes, the former one refers to type 1 diabetes mellitus (T1D) which is characterized by the inability of insulin secretion due to the autoimmune-mediated destruction of pancreatic β cells. The later one is type 2 diabetes mellitus (T2D) which affects more than 90% of diabetes and is mainly characterized by chronic hyperglycaemia and insufficient compensation for peripheral insulin resistance. Furthermore, the impaired insulin secretion and/or insulin resistance will further cause the abnormalities in three major nutrients' metabolism, namely carbohydrate, lipid and protein (Savage et al. 2007, Ormazabal et al. 2018). Some evident risk factors are helping the spread of T2D such as obesity, aging, urbanization, physical inactivity and genetic predisposition (DeFronzo et al. 2015).

Under the diabetic state, the blood glucose concentrations levels remaining high due to the impairment of insulin-stimulated glucose uptake by fat and muscle, leading to an increase of glucose uptake by insulin-independent tissues (Nonogaki 2000, Zisman et al. 2000). The increased glucose flux will cause the overproduction of oxidant through several interacting pathways leading to the imbalance of the body's endogenous antioxidant defence system (RG 2005, Matough et al. 2012). Therefore, hyperglycaemia leads to the subsequent augmentation of reactive oxygen species (ROS) which has a causal role in the formation of oxidative stress. The generation of oxidative stress works as the key mediator in insulin resistance, impaired glucose tolerance as well as diabetes (Rains and Jain 2011). Hyperglycaemia-induced oxidative stress is also responsible for endothelial dysfunction, chronic inflammation, apoptosis and nitric oxide release, and all factors are interrelated in the pathophysiology of diabetic micro- and macro-vascular complications (Vanessa Fiorentino et al. 2013), such as nephropathy, neuropathy, retinopathy, heart failure, myocardial infraction and stroke (Moodley et al. 2015). Therefore, it is vital to control hyperglycaemia and oxidative stress to combat T2D and its associated complications.

Currently, there are different strategies for the treatment of T2D, which are essentially based on the control of hyperglycaemia (Corrales et al. 2020). It has been many years to use antidiabetic/pharmaceutical oral hypoglycaemic agents as the primary therapeutic for the treatment of T2D (Wu et al. 2020). However, such drugs including metformin, glucagon-like peptide (GLP-1 analogues), sulfonylureas and α -glucosidase inhibitors have various short- and/or long-term side effects, such as weight gain or loss, hypoglycemia, nausea, fluid retention, diarrhoea, heart and liver failure (Choudhury et al. 2018). In addition, approximately 80% of T2D patients live in low- and middle-income countries where adequate medical services are not available, which is another non-negligible factor limiting the use of such drugs (IDF 2019, Godman et al. 2020). Besides, weight control, dietary modifications, lifestyle and behavior norms changes are also reported exhibiting positive effects in the management of T2D (ADA 2019). Still, the compliance and acceptability of most patients weaken their efficacies (Beccuti et al. 2017). The visible dramatic rise in the global incidence of T2D implies the drawbacks of above-mentioned treatment strategies (Asrafuzzaman et al. 2017).

Therefore, it is necessary to develop alternative antidiabetic therapies and therapeutic strategies favoring diabetic patients with fewer side effects, higher accessibility and affordability to improve the present situation and prolong their lives. Medicinal plants or herbs are well-known sources of phytochemicals and natural antioxidants (Saxena et al. 2013, Tungmunnithum et al. 2018). It has been widely accepted that their bioactive compounds possessing ameliorative properties against oxidative stress-related chronic diseases with efficacy and safety (Martins et al. 2016, Sarrafchi et al. 2016). Thus, the pharmaceutical application of such medicinal plants/herbs to achieve multiple envisaged beneficial effect against T2D could be an attractive option. Among them is GRT.

GRT is an unfermented form of rooibos herbal (non-*Camellia sinensis*) teas, which is indigenous and exclusively located in the Cederberg mountainous regions in South Africa. It is made from delicate stems and leaves of a leguminous shrub with bright green needle-shaped leaves and small yellow flowers called *Aspalathus linearis* (Ajuwon et al. 2018). GRT is reported to exhibit better antioxidant ability than the fermented (oxidized; traditional) rooibos tea due to its non-fermentation process reserving higher levels of polyphenols and flavonoid (Marnewick 2009). The potential clinical use of GRT contributes to its commercial success around the world, which attributes to its diverse flavonoids including two distinctive flavonoids (aspalathin, aspalainin) (Shimamura et al. 2006) and other different bioactive compounds such as nothofagin, luteolin, rutin, orientin, iso-orientin, vitexin and iso-vitexin, (Krafczyk and

Glomb 2008, Joubert et al. 2009). While some of them has been reported to exhibit cardioprotective (Pantsi et al. 2011), anti-inflammatory (Ajuwon et al. 2014), antioxidant (Joubert et al. 2012, Xiao et al. 2020) and anti-mutagenic (Snijman et al. 2007) effects *in vitro*, *ex vivo*, *in vivo* and a few human studies (Marnewick et al. 2011). However, the activity and mechanistic properties of concentrated GRT hot water extract in a fructose-fed streptozotocin (STZ)-induced T2D model have so far not been investigated.

Therefore, the current study was carried out to examine the *in vivo* antioxidant and antidiabetic effect of concentrated GRT hot water extract in a fructose-fed streptozotocin (STZ)-induced T2D model of rats.

6.3.3 Materials and methods

Please refer to the chapter 2 for details of the methods used for green rooibos tea (*Aspalathus linearis*).

6.3.4 Results

All the diabetic groups had significant ($p < 0.05$) higher mean daily feed and water intake than NC and NGR groups, when DBC group exhibited the highest daily feed and water intake as shown in **Figure 6.3.1**. DGRH group displayed slightly lower feed intake and water intake than that of DGRL group (**Fig. 6.3.1**). Meanwhile, DBM had significantly lower feed and water intake than GRT-treated groups and DBG group. No statistically significant ($p > 0.05$) difference was observed between GRT-treated groups in daily feed and water intake.

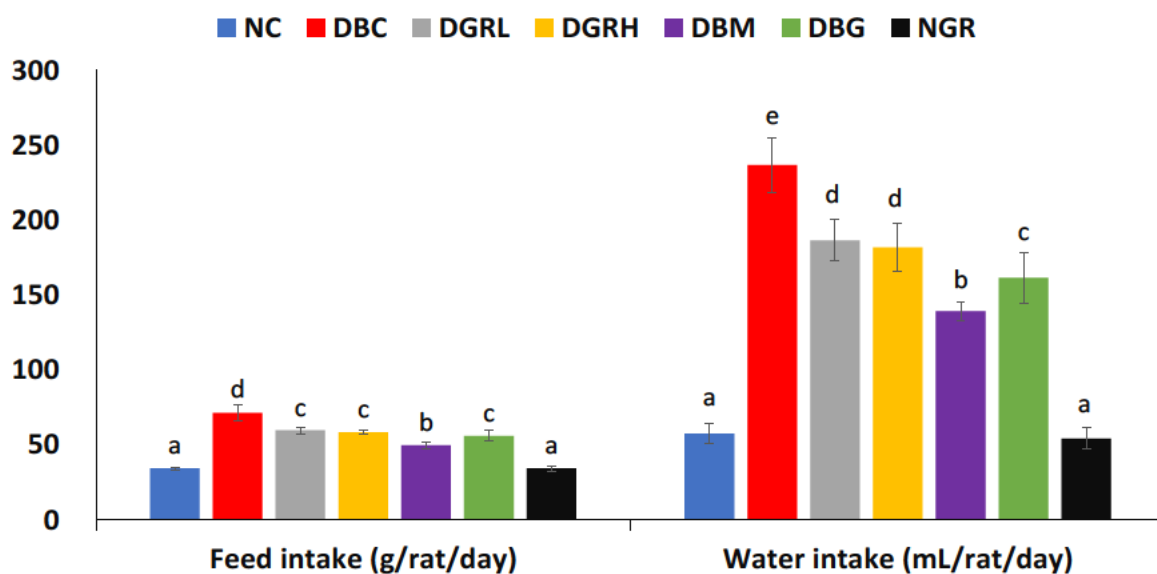


Figure 6.3.1: Mean feed and water intake in different animal groups during the experimental period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given parameter represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DGRL: Diabetic green rooibos tea (*Aspalathus linearis*) low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NGR: Normal green rooibos tea (*Aspalathus linearis*) high dose (toxicological control).

The weekly mean BW changes in all animal groups were shown in **Figure 6.3.2**. No significant difference of initial BW (week 0) was seen in different groups; however, the BW gains of diabetic groups were significantly ($p < 0.05$) lower than that of NC and NGR groups after the STZ injection. As the remaining experimental period (week 2 to week 6) proceeded, there were higher, but not significantly ($p > 0.05$) elevated BW was observed in the GRT- and glibenclamide- treated groups compared to DBC group. DBM exhibited a significantly ($p < 0.05$) higher BW gain than DBC group all through the intervention period. There was no significant ($p > 0.05$) difference between DGRL and DGRH groups.

The weight and relative weight of pancreas in all animal groups were displayed in **Figure 6.3.3**. A significantly lower weight was seen in DBC group when compared to normal groups. However, these were slightly reversed in GRT- and standard drug-treated groups, with DGRH group showing better pancreatic weight than that of DGRL group.

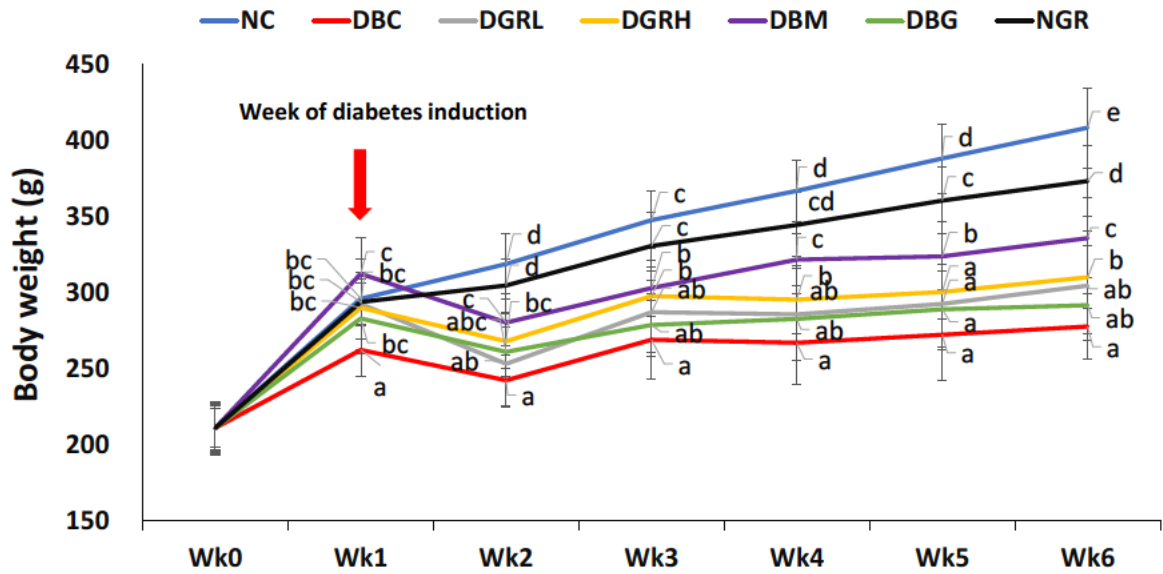


Figure 6.3.2: Mean body weight in different animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters near the lines for a given week represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DGRL: Diabetic green rooibos tea (*Aspalathus linearis*) low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NGR: Normal green rooibos tea (*Aspalathus linearis*) high dose (toxicological control).

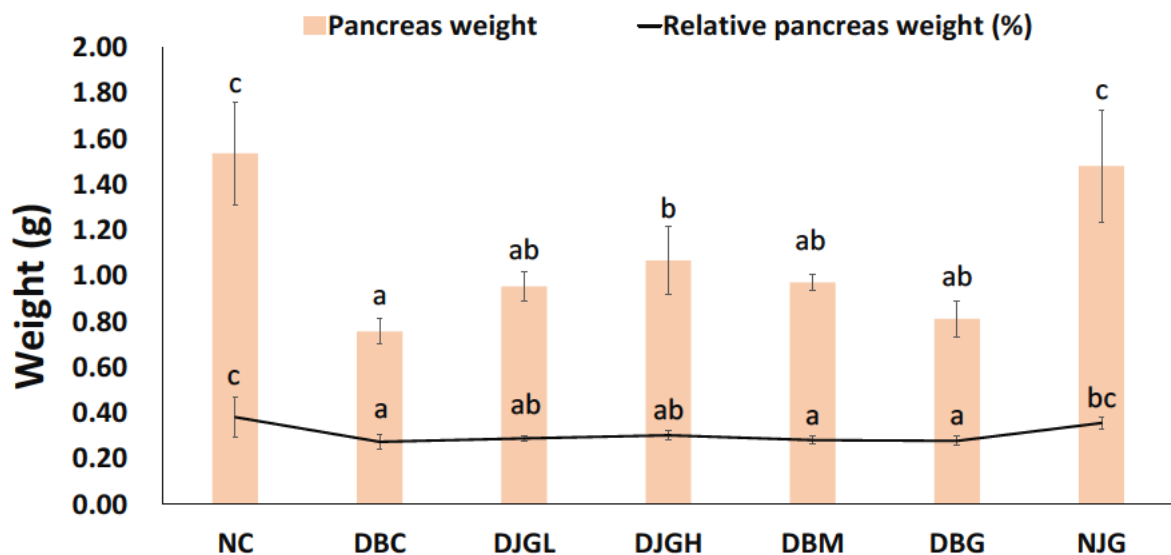


Figure 6.3.3: Weight and relative weight of pancreas in different animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars or near the line for a given animal group represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DGRL: Diabetic green rooibos tea (*Aspalathus linearis*) low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NGR: Normal green rooibos tea (*Aspalathus linearis*) high dose (toxicological control).

There were significantly ($p < 0.05$) higher blood glucose concentrations were observed in all diabetic groups when compared to normal groups upon induction of T2D as indicated in **Figure 6.3.4**. When all diabetic treatment groups displayed a reduction in the blood glucose levels compared to DBC group, the DGRH group showing lower blood glucose level than DGRL group.

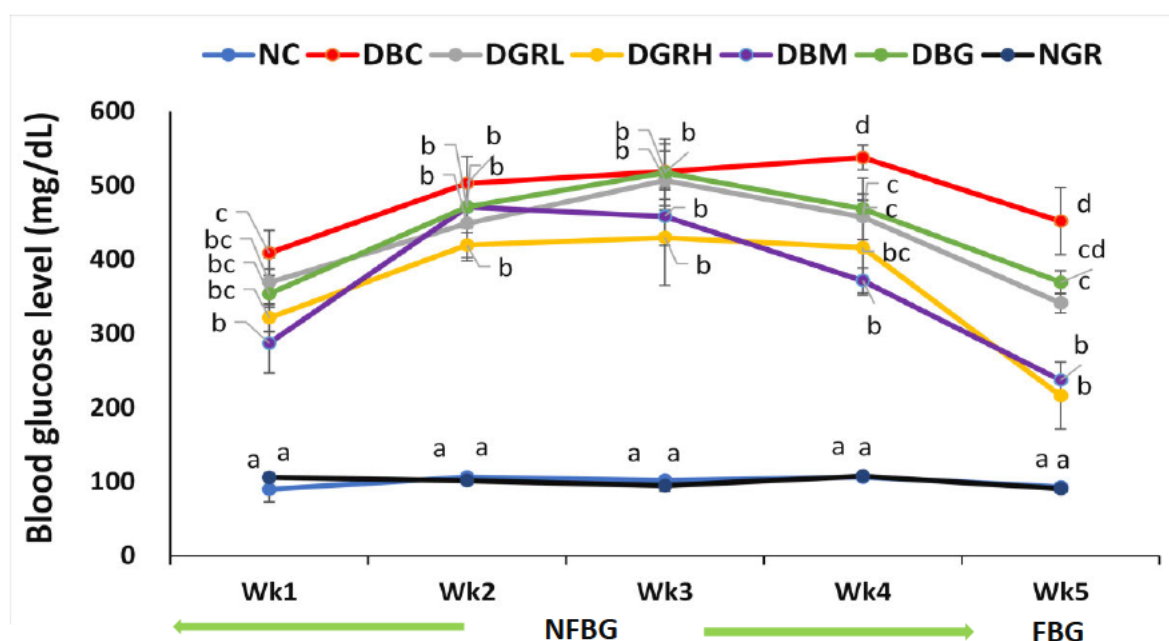


Figure 6.3.4: Weekly blood glucose concentrations of different animal groups. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters near the lines for a given week represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DGRL: Diabetic green rooibos tea (*Aspalathus linearis*) low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NGR: Normal green rooibos tea (*Aspalathus linearis*) high dose (toxicological control).

Significantly ($p < 0.05$) better glucose tolerance ability of the rats was seen in normal groups than that of the diabetic groups as depicted in **Figure 6.3.5**. There was a significant ($p < 0.05$) elevation of blood glucose level at 30 minutes for all groups after an oral administration of a dose of 2 g/kg BW glucose. Except for DBC, the blood glucose level of all diabetic groups was peaked at 30 minutes and started declining till the end of the OGTT test. All diabetic groups with treatment exhibited significantly ($p < 0.05$) lower blood glucose levels than DBC group at 60, 90- and 120-min, except DBG group at 120 minutes. The lowest blood glucose levels of all animal groups were recorded at 120 min. DGRH group displayed lower blood glucose than that of DGRL group during the entire period of the test. The AUC of normal groups and other diabetic treated groups were significantly ($p < 0.05$) lower than that of DBC, which again confirms their better glucose tolerance ability than DBC.

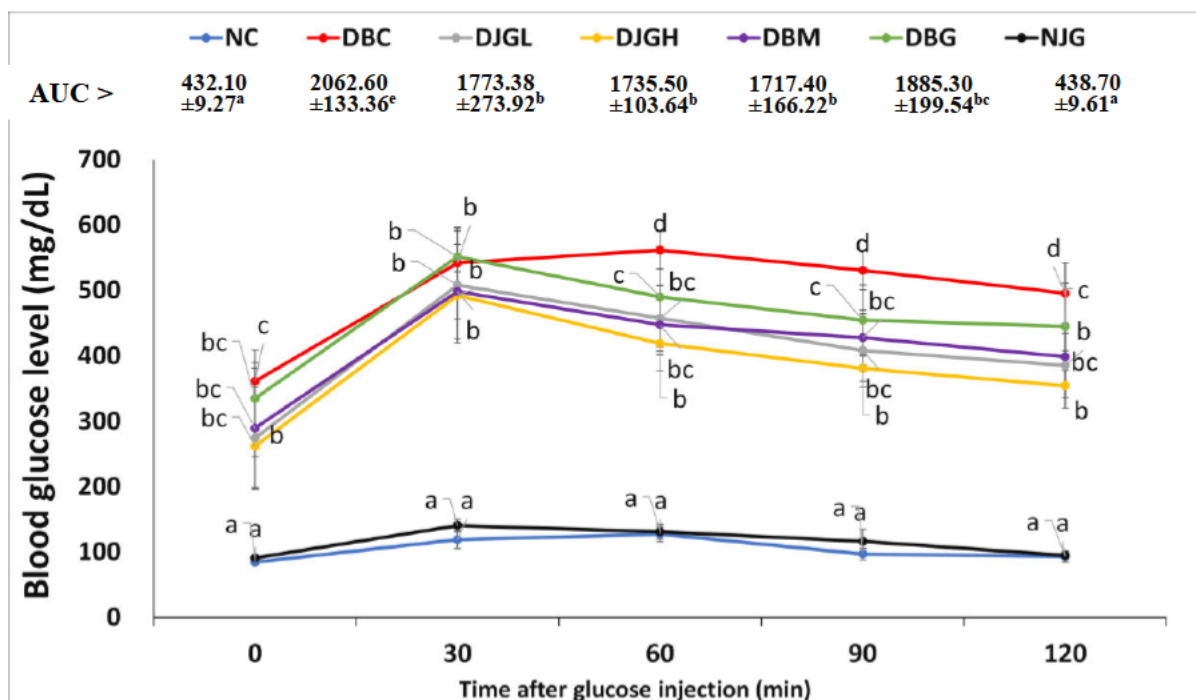


Figure 6.3.5: Oral glucose tolerance test (OGTT) with corresponding area under the curve (AUC) of different animal groups during the whole intervention period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters near the lines for a given time represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DGRL: Diabetic green rooibos tea (*Aspalathus linearis*) low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NGR: Normal green rooibos tea (*Aspalathus linearis*) high dose (toxicological control).

The histopathological analysis of pancreatic tissues harvested from all animal groups were shown in **Figure 6.3.6**. The parenchyma of rats in NC (blue arrow) displayed oval or circular cluster of cells in the lobules abundant in eosinophilic cytoplasm, which are pale staining compared to adjacent acini, with clear boundary between the endocrine and exocrine glands of pancreatic islets. When compared to NC and NGR (black arrow) groups, microscopic examination of the pancreas sections in DBC (red arrow) displayed a severe damage of pancreatic islets with collapsed structure, degeneration of endocrine cells and lysed tissues, implying a necrosis of pancreatic β -cells. While in the DGRL (grey arrow) and DGRHH (yellow arrow) treated groups, the number of islet cells in the pancreatic islets increased notably compared to DBC, appearing nearly as intact as the normal cells. However, this condition was also ameliorated metformin and glibenclamide treated diabetic groups, with DBM (purple arrow) showing a more promising regeneration of pancreatic islets than DBG (green arrow) group.

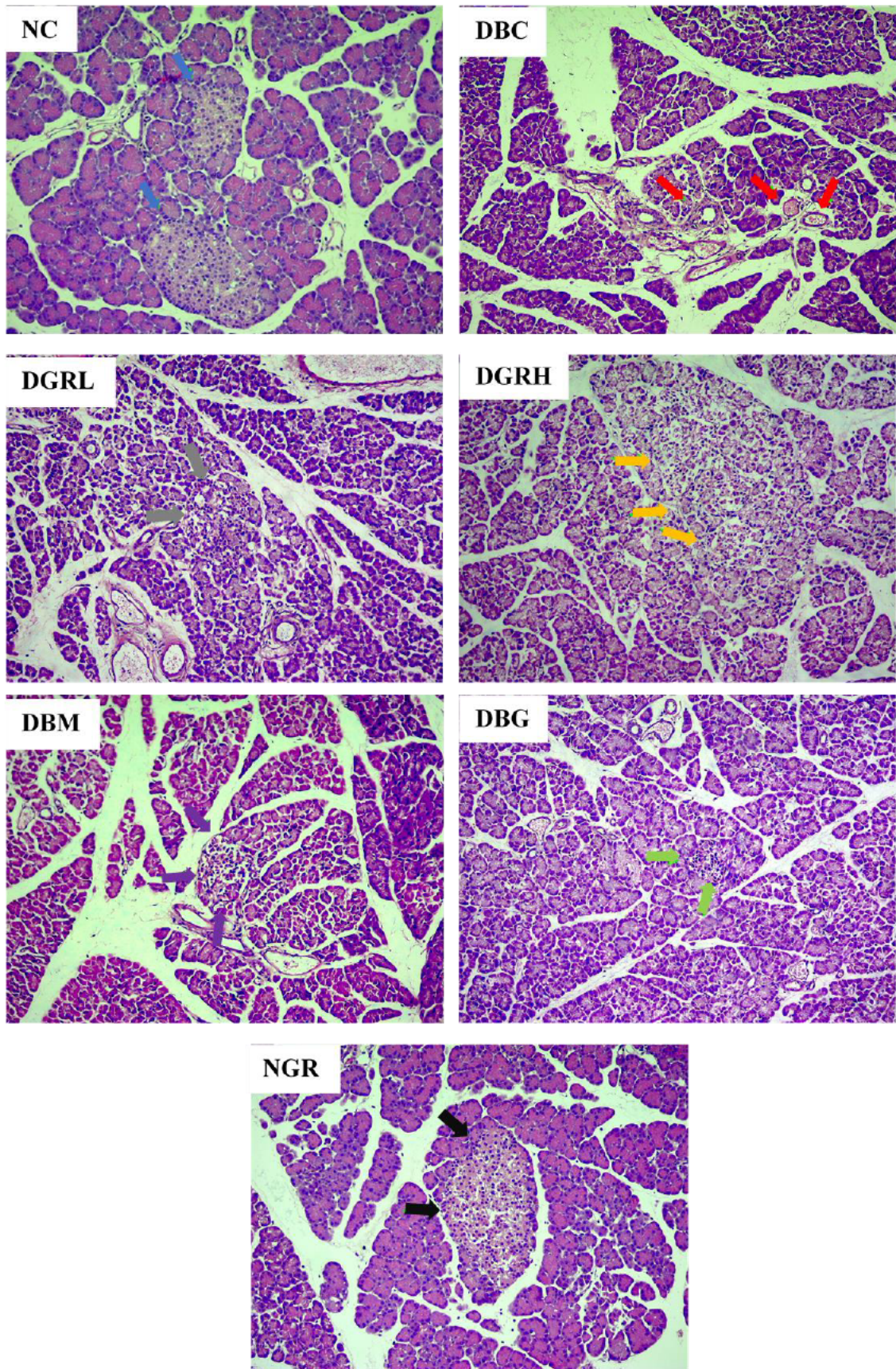


Figure 6.3.6. Histopathological examinations of the pancreatic tissue from different animal groups at the end of the experimental period. Magnification: 10x. NC: Normal control; DBC: Diabetic control; DGRL: Diabetic green rooibos tea (*Aspalathus linearis*) low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NGR: Normal green rooibos tea (*Aspalathus linearis*) high dose (toxicological control).

The data of the concentrations of fasting serum lipids at the end of the experimental period are presented in **Figure 6.3.7**. The concentrations of total cholesterol and LDL-cholesterol in DBC group was significantly ($p < 0.05$) higher than the normal groups. Likewise, all the diabetic groups with treatment exhibited a significant ($p < 0.05$) decrease in the levels of these cholesterols, with comparable lower level in DGRH group than that of DGRL group. Furthermore, there was a concomitant significant reduction in HDL-cholesterol level in DBC group when compared to the normal groups, this however was significantly reversed in the treatment groups, with DGRH group showing better result than DGRL group. The serum triglycerides in DBC group were significantly ($p < 0.05$) higher than that of other groups, while all diabetic treatment groups exhibited significantly ($p < 0.05$) higher levels when compared to normal groups, with DGRH group showing the lowest level among the treated diabetic groups.

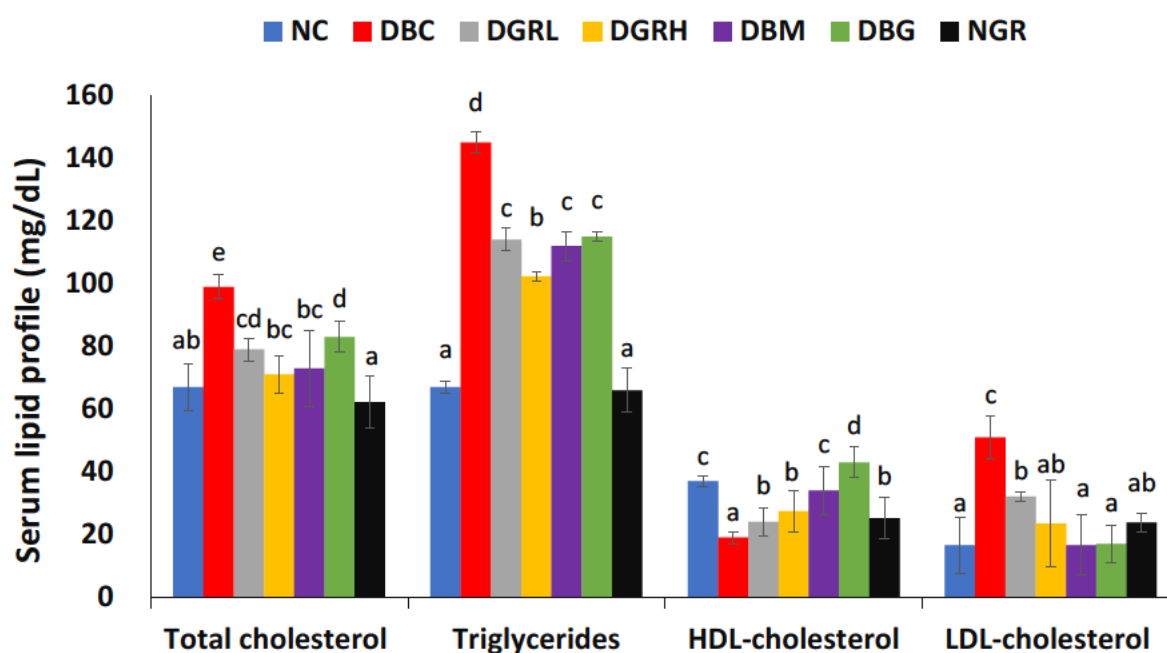


Figure 6.3.7: Serum lipid profile of different animal groups at the end of the experimental period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given parameter represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DGRL: Diabetic green rooibos tea (*Aspalathus linearis*) low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NGR: Normal green rooibos tea (*Aspalathus linearis*) high dose (toxicological control); HDL: High density lipoprotein; LDL: Low density lipoprotein.

Table 6.3.1: The effects of different doses of GRT on liver weight, relative liver weight and liver glycogen levels in the different animal groups at the end of the intervention period

Parameter	NC	DBC	DGRL	DGRH	DBM	DBG	NGR
Liver weights (g)	13.32±1.14 ^b	11.13±1.23 ^a	10.96±0.19 ^a	12.57±0.38 ^{ab}	12.41±1.48 ^{ab}	11.87±0.89 ^{ab}	11.95±0.20 ^{ab}
Rel. liver weights (%)	3.18±0.07 ^a	4.03±0.59 ^b	3.79±0.17 ^b	3.95±0.25 ^b	3.59±0.24 ^{ab}	4.07±0.27 ^b	3.25±0.18 ^a
Liver glycogen (mg/g tissue)	6.26±0.23 ^d	2.25±0.27 ^a	5.64±1.02 ^{cd}	5.95±0.85 ^{cd}	4.89±0.16 ^c	3.66±0.67 ^b	6.11±0.18 ^d

Data are expressed as mean ± SD (n = 5 or 7), ^{a-e}Different superscript letters along a row indicate the significance of difference (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$). NC: Normal control; DBC: Diabetic control; DGRL: Diabetic green rooibos tea (*Aspalathus linearis*) low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NGR: Normal green rooibos tea (*Aspalathus linearis*) high dose (toxicological control).

Table 6.3.2: Serum biochemical parameters in different animal groups at the end of the experimental period

Parameter	NC	DBC	DGRL	DGRH	DBM	DBG	NGR
AST (U/L)	84.20±1.33 ^a	156.20±14.96 ^e	111.00±5.44 ^d	105.00±2.85 ^{cd}	91.17±5.19 ^{ab}	96.60±15.93 ^{bc}	90.50±6.72 ^{ab}
ALT (U/L)	84.20±2.17 ^a	93.40±3.65 ^c	90.00±7.24 ^{bc}	84.00±1.59 ^a	85.50±2.26 ^{ab}	88.67±4.73 ^{ab}	87.00±0.74 ^{ab}
ALP (U/L)	72.40±2.30 ^a	364.60±15.98 ^e	132.00±7.35 ^c	86.00±6.63 ^b	173.33±11.48 ^d	351.00±6.32 ^d	81.00±1.87 ^{ab}
Urea (mg/dL)	56.00±3.08 ^a	77.80±2.05 ^d	74.00±5.76 ^{cd}	67.00±0.89 ^b	68.67±3.83 ^{bc}	69.20±4.49 ^{bcd}	73.00±6.96 ^{cde}
UA (mg/dL)	2.60±0.55 ^{ab}	7.17±3.89 ^d	5.11±0.27 ^{cd}	4.85±0.64 ^{bc}	3.21±1.12 ^{abc}	4.17±3.38 ^{abc}	2.29±0.34 ^a
Creatinine (mg/dL)	1.81±0.39 ^a	3.37±0.90 ^c	2.60±0.98 ^b	2.36±0.57 ^{ab}	2.19±0.32 ^{ab}	2.38±0.55 ^{ab}	2.01±0.26 ^{ab}
CK-MB (U/L)	4.90±1.46 ^a	44.74±2.99 ^d	17.90±0.47 ^c	16.70±0.68 ^c	7.20±2.84 ^a	11.62±1.19 ^b	6.60±1.75 ^a

Results are expressed as mean ± SD (n = 5 or 7). ^{a-e}Different superscript letters along a row indicate the significance of difference (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$). NC: Normal control; DBC: Diabetic control; DGRL: Diabetic green rooibos tea (*Aspalathus linearis*) low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NGR: Normal green rooibos tea (*Aspalathus linearis*) tea high dose (toxicological control); AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; UA: Uric acid; CK-MB: Creatinine Kinase-myocardial band.

DBC showed significant ($p < 0.05$) lower liver weight than that of NC group, but no significant ($p > 0.05$) difference with other groups as depicted in **Table 6.3.1**. DBC showed significant (p

< 0.05) lower liver weight than that of NC group, but no significant ($p > 0.05$) difference with other groups. The relative liver weight in DBC group was significantly ($p < 0.05$) higher than the normal groups (NC and NGR), while not statistically significant ($p > 0.05$) difference was observed among the diabetic groups. DBC group showed the lowest liver glycogen level compared to other groups, this was however significantly ($p < 0.05$) reversed by GRT treatments, with DGRH group showing slightly higher liver glycogen than DGRL group.

Significantly ($p < 0.05$) higher level of the serum AST, ALT, ALP, urea, uric acid, creatinine, and CK-MB in were seen in DBC group when compared to the normal groups (**Table 6.3.2**). A significantly ($p < 0.05$) higher level of serum AST, ALP, creatinine, and CK-MB in all diabetic groups, however these levels were significantly ($p < 0.05$) reversed in GRT- and standard drugs- treated diabetic groups compared to DBC group. The levels of serum ALT, uric acid, and urea in the DBC group were not significantly different compared to DGRL group. DGRH showed significantly lower level of serum ALT, ALP, urea than that of DGRL and DBC groups. There were no significant ($p > 0.05$) difference between DGRH and DGRL groups in terms of the levels of serum AST, creatinine, and CK-MB.

Table 6.3.3: Serum insulin and fructosamine concentrations as well as computed HOMA-IR and HOMA- β scores in different animal groups at the end of the experimental period

	NC	DBC	DGRL	DGRH	DBM	DBG	NGR
Serum							
Insulin ($\mu\text{mol/L}$)	24.56 \pm 0.29 ^d	9.35 \pm 0.23 ^a	9.52 \pm 0.56 ^a	10.42 \pm 0.23 ^b	12.52 \pm 0.41 ^c	10.88 \pm 0.44 ^b	29.29 \pm 0.24 ^e
Fructosamine ($\mu\text{mol/L}$)	387.67 \pm 22.50 ^a	1210.33 \pm 60.00 ^c	1133.33 \pm 91.56 ^c	1042.33 \pm 143.27 ^c	574.67 \pm 58.45 ^b	546.33 \pm 33.13 ^a	394.67 \pm 13.31 ^a
HOMA-IR	0.80 \pm 0.01 ^a	1.73 \pm 0.04 ^e	1.12 \pm 0.07 ^b	0.77 \pm 0.02 ^a	1.02 \pm 0.03 ^c	1.38 \pm 0.06 ^d	0.90 \pm 0.01 ^b
HOMA- β	39.58 \pm 0.46 ^d	0.99 \pm 0.02 ^a	1.72 \pm 0.10 ^b	3.42 \pm 0.07 ^c	3.62 \pm 0.12 ^c	1.78 \pm 0.07 ^b	54.43 \pm 0.45 ^e

Results are expressed as mean \pm SD (n = 5 or 7). ^{a-e}Different superscript letters along a row indicate the significance of difference (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$). NC: Normal control; DBC: Diabetic control; DGRL: Diabetic green rooibos tea (*Aspalathus linearis*) low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) tea high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NGR: Normal green rooibos tea (*Aspalathus linearis*) tea high dose (toxicological control); HOMA-IR: Homeostatic model assessment for IR (insulin resistance); HOMA- β : Homeostatic model assessment for β (β -cell function).

The level of serum insulin in DBC group exhibited significantly ($p < 0.05$) lower than other groups, however without statistically significant ($p > 0.05$) difference compared to DGRL group as presented in **Table 6.3.3**. There was significantly higher HOMA-IR score in DBC

group than that of other groups and the groups treated with GRT could reduce it significantly ($p < 0.05$), with DGRH exhibiting the best result. In addition, DBC group displayed significantly ($p < 0.05$) lower HOMA- β score than that of normal groups, while diabetic groups treated with GRT displayed a significant ($p < 0.05$) increase compared to DBC group. As for serum fructosamine, significantly higher concentration was displayed in DBC group than that of normal groups. While there were not statistically significant ($p > 0.05$) different between GRT-treated and untreated diabetic groups.

There was a significantly ($p < 0.05$) higher level of MDA in the pancreas, liver, kidneys, and heart of the animals after the induction of T2D as portrayed in DBC group when compared with the normal groups (NC and NGR) as indicated in **Figure 6.3.8**. The MDA level was significantly depleted in diabetic treated groups, which was also replicated in the different studied organs, with DGRH group exhibiting significantly ($p < 0.05$) lower MDA level than DGRL group.

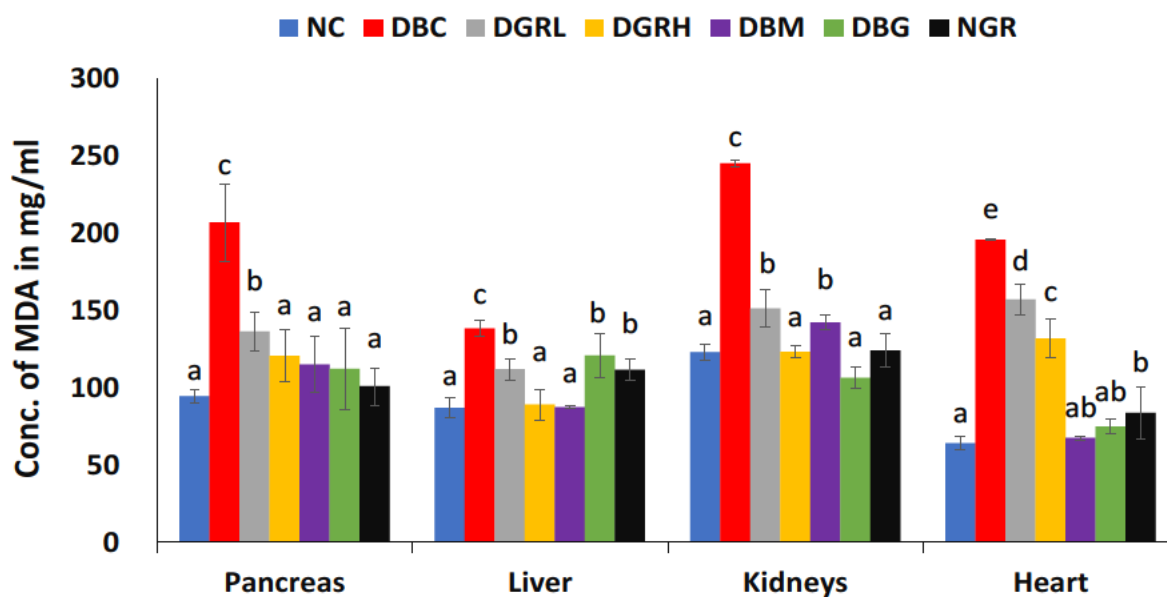


Figure 6.3.8: Thiobarbituric acid reactive substances (MDA equivalent) in the pancreas, liver, kidneys, and heart of all animal groups at the end of the experimental period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given organ represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DGRL: Diabetic green rooibos tea (*Aspalathus linearis*) low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NGR: Normal green rooibos tea (*Aspalathus linearis*) high dose (toxicological control).

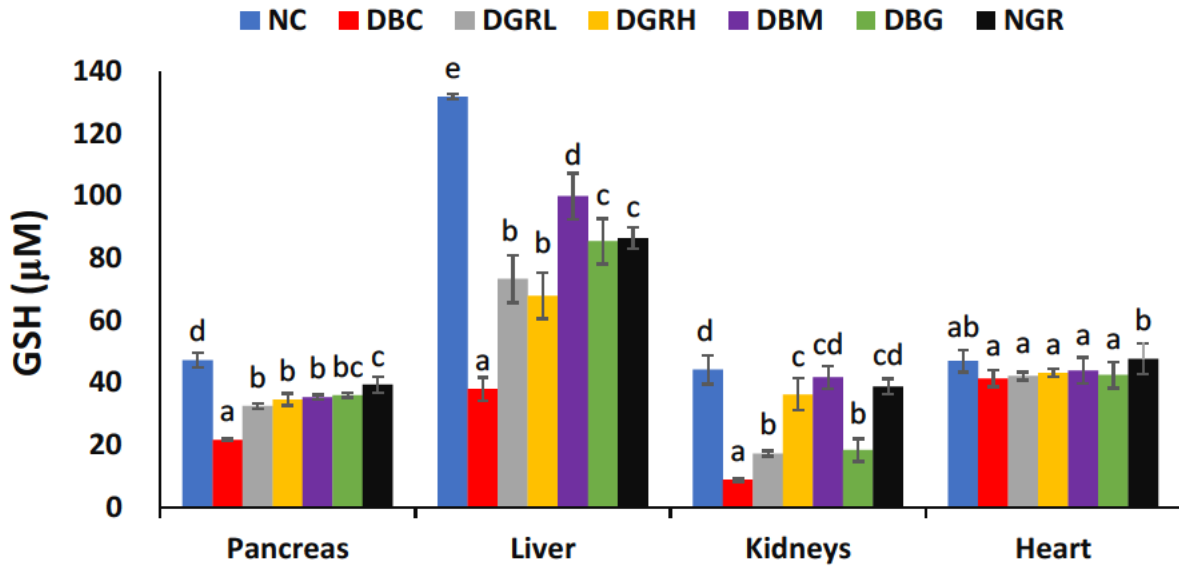


Figure 6.3.9: Reduced glutathione (GSH) levels in the pancreas, liver, kidneys and heart of all animal groups at the end of the experimental period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given organ represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DGRL: Diabetic green rooibos tea (*Aspalathus linearis*) low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NGR: Normal green rooibos tea (*Aspalathus linearis*) high dose (toxicological control).

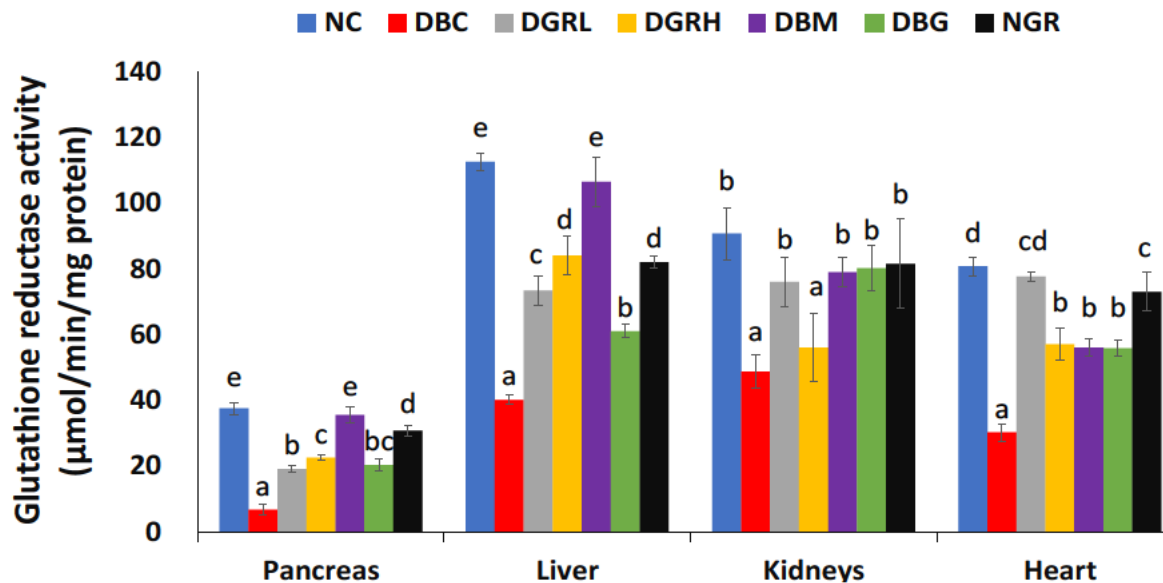


Figure 6.3.10: Glutathione reductase (GR) activity in the pancreas, liver, kidneys and heart of all animal groups at the end of the experimental period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given organ represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DGRL: Diabetic green rooibos tea (*Aspalathus linearis*) low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NGR: Normal green rooibos tea (*Aspalathus linearis*) high dose (toxicological control).

The GSH levels of organs in all animal groups were shown in **Figure 6.3.9**. Significantly ($p < 0.05$) lower GSH level was displayed in pancreas, liver, and kidneys in DBC group than that of normal groups. GSH level of GRT- and standard drugs- treated diabetic groups compared to DBC group was significantly increased in pancreas, liver, and kidneys, with DGRH group showing better result than DGRL group in kidneys and better results in DGRL group than DGRH group in pancreas and liver. While in the heart, there was no significant ($p > 0.05$) difference displayed among all diabetic groups.

The activity of GR in organs of DBC was significantly ($p < 0.05$) lower than normal groups as depicted in **Figure 6.3.10**. GRT- and standard drug- treated diabetic groups significantly improved the GR activity in organs, with DGRH group having significantly ($p < 0.05$) higher GR activity than DGRL group in liver and pancreas. While in the kidneys and heart, significantly ($p < 0.05$) higher GR enzyme activity was observed in the DGRL group than DGRH group.

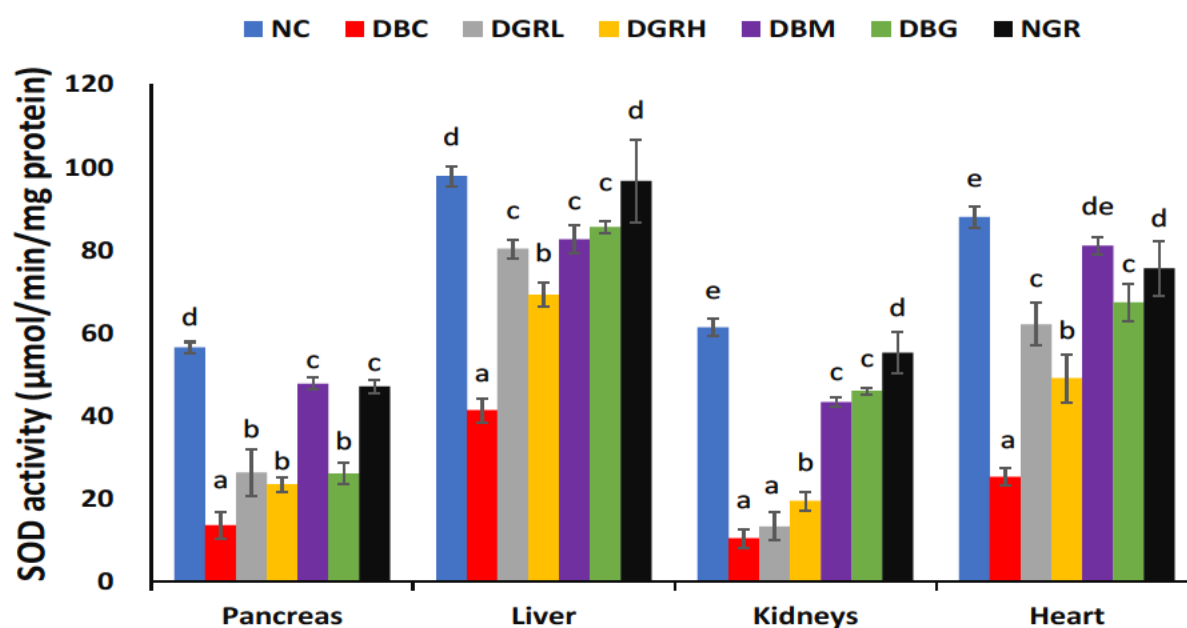


Figure 6.3.11: SOD activity in the pancreas, liver, kidneys, and heart of all animal groups at the end of the experimental period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given organ represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DGRL: Diabetic green rooibos tea (*Aspalathus linearis*) low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NGR: Normal green rooibos tea (*Aspalathus linearis*) high dose (toxicological control).

The SOD activities in pancreas, liver, kidneys, and heart of all animal groups were presented in **Figure 6.3.11**. There was a significantly ($p < 0.05$) lower SOD activity was observed in the organs of DBC group when compared with normal groups. However, this was significantly reversed in the pancreas, liver, kidneys, and heart of GRT- and standard drugs- treated groups with the exception in the kidneys of DGRL group. DGRH group displaying significantly ($p < 0.05$) higher SOD activity in the kidneys, while significantly ($p < 0.05$) lower SOD activity in the liver and heart, when compared to that of DGRL group.

The activities of catalase in organs of all animal groups are presented in **Figure 6.3.12**. The catalase activity in the pancreas, liver, kidneys, and heart of DBC group was significantly ($p < 0.05$) lower than that of the normal groups (NC and NGR). The liver and heart of GRT-treated diabetic groups displaying significantly higher catalase activity than DBC group, with DGRH group showing better result than DGRL group, and better results in DGRH than DGRL group were also seen in pancreas and kidneys. There was not statistically significant ($p > 0.05$) difference between DGRL and DBC groups in terms of pancreas and kidneys.

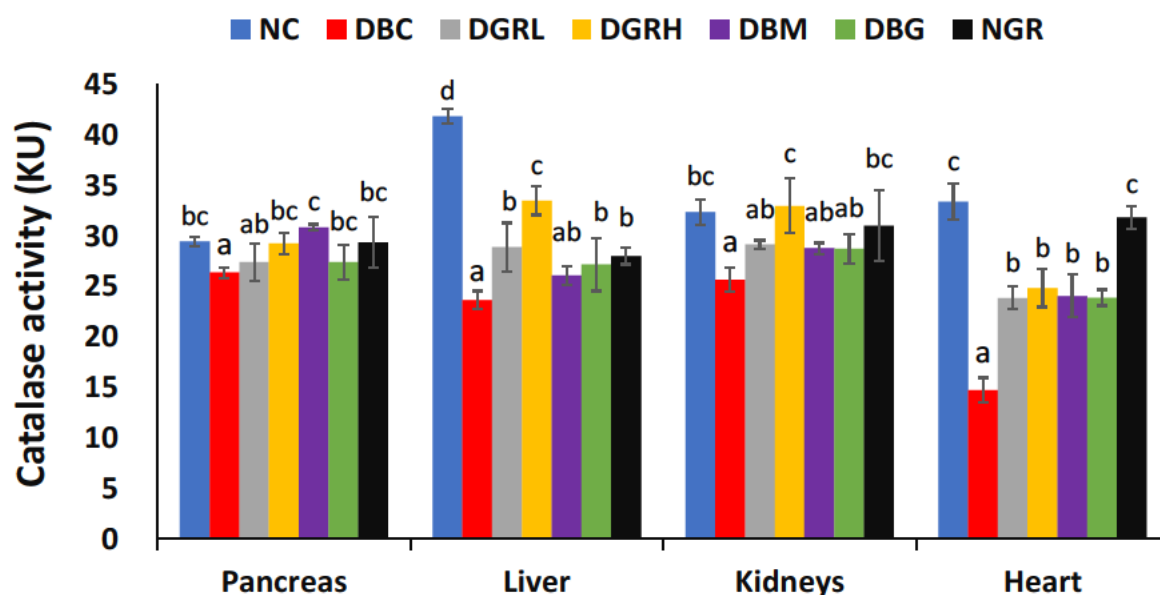


Figure 6.3.12: Catalase activity in the pancreas, liver, kidneys and heart of all animal groups at the end of the experimental period. Data are presented as the mean \pm SD of 5 or 7 animals. ^{a-d}Values with different letters over the bars for a given animal group represent significance of difference ($p < 0.05$). NC: Normal control; DBC: Diabetic control; DGRL: Diabetic green rooibos tea (*Aspalathus linearis*) low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) high dose; DBM: Diabetic metformin; DBG: Diabetic glibenclamide; NGR: Normal green rooibos tea (*Aspalathus linearis*) high dose (toxicological control).

6.3.5 Discussion

Consistently, rooibos tea has become more than a herbal tea, as a rich source of unique antioxidants being explored for their potential use as nutraceuticals, in particular for the prevention, treatment and management of T2D and its associated complications (Marnewick et al. 2011, Muller et al. 2018). Increasing *in vivo* experimental evidence supports the antidiabetic effects of GRT in diabetic animal models including STZ-induced diabetic Wistar rats, T2D diabetic ob/ob, KK-A^y mice, and high-fat-fed diabetic Vervet monkeys (Muller et al. 2012, Son et al. 2013, Kamakura et al. 2015, Orlando et al. 2017). In the current study, model rats of T2D were created by feed 10% fructose solution combining STZ to imitate the pathogenies of human T2D. Normal rats induced IR were gave a 10% fructose solution *ad libitum* for first two weeks. Partial pancreatic β -cell dysfunction was developed employing an intraperitoneal injection of STZ (40 mg/kg BW) after two weeks feeding of 10% fructose solution (Wilson and Islam 2012).

The result of significantly ($p < 0.05$) higher water and feed intake and body weight loss in DBC group compared to normal rats indicated the successful induction of T2D, which was in consistent with major common symptoms of STZ-induced T2D including polydipsia, polyphagia and body weight loss (Islam 2011, Ibrahim et al. 2016). Najafian (2016) reported that the oral administration of aspalathin exhibited a weight loss control and food and water intake reduction in STZ-induced diabetic rats. There are similar findings was found by Sezik et al. (2005) who reported BW loss prevention after the consumption of another phytochemical of rooibos tea, isoorientin, (15 and 30 mg/kg BW) for 30 days in STZ-induced diabetic rats. Although significantly improved water and feed intake and relatively ameliorated BW loss in DGRL and DGRH groups (**Figure 6.3.1**) compared to DBC group, the treatment could not reverse the STZ-induced T2D condition to normal, which may be due to a short intervention period. It still revealed a potent antidiabetic effect of GRT against the major symptoms of T2D when polyphenols from GRT might be responsible for these effects.

Persistent hyperglycaemia was displayed in DBC group compared to NC group after the injection of STZ, which further confirmed the successful induction of T2D (**Figure 6.3.4**). Meanwhile, significantly higher HOMA-IR score in DBC group compared to NC group (**Table 6.3.3**) demonstrated the onset of IR and significantly lower HOMA- β score (**Table 6.3.3**) in DBC compared to NC indicated the induction partial pancreatic β -cell dysfunction, which are the primary pathogenesis of T2D (Bergman et al. 2002). However, the NFBG and FBG in

DGRL and DGRH groups suggested a dose-dependent effect of GRT in reducing blood glucose level (**Figure 6.3.4**), when significant ($p < 0.05$) reducing effects were observed particularly during week 4 and week 5. The potential blood glucose improvement ability was further supported by the OGTT result (**Figure 6.3.5**), high blood glucose concentrations during the whole test indicated the impaired glucose tolerance in DBC group, while the significantly lower blood glucose in DGRL and DGRH groups after 30 min exhibiting the ability of GRT in improving glucose intolerance in a dose-dependent manner. These are in line with Muller et al. (2012) who reported that an aspalathin-enriched unfermented rooibos extract (25 mg/kg BW, 30 mg/kg BW) modulated glucose metabolism via reducing blood glucose and increasing glucose tolerance in STZ-induced diabetic Wistar rats. This is also evident by Mikami et al. (2015) who reported an overload carbohydrate induced non-diabetic rat model displaying GRT effect of counteracting the elevation of blood glucose.

Moreover, Son et al. (2013) and Kamakura et al. (2015) reported a glucose tolerance improving activity in type 2 diabetic ob/ob and KK- A^y mice model, respectively, which further highlighted the antidiabetic potential of GRT extract and its novel flavonoid aspalathin. Additionally, one recent study by Orlando et al. (2017) reported that the supplementation of aspalathin-enriched GRT extract to demonstrate its antidiabetic potential through glucose tolerance improvement in high-fat-fed diabetic Vervet monkeys. Two old papers reported that another phenolic present in rooibos tea, isoorientin, displayed its antidiabetic effects in STZ-induced diabetic rats through improving hyperglycaemia (Andrade-Cetto and Wiedenfeld 2001, Sezik et al. 2005). This could be attributed to the potent effects of GRT and its phenolics on inhibiting the activities of carbohydrate digesting key enzymes linked to T2D, as well as suppressing intestinal glucose absorption as reported previously (Ademiluyi and Oboh 2013, Kachidza 2014, Mikami et al. 2015, Xiao et al. 2020). Furthermore, the management of hyperglycaemia of GRT further correspond with its effects on alleviating major common T2D symptoms exhibits its pharmaceutical interest, as anti-hyperglycaemia is one of the main effects of current synthetic oral hypoglycaemic agents (Hung et al. 2012).

Chronic uncontrolled hyperglycaemia leads to insulin secretion defects in pancreatic β -cells, with subsequent increasing β -cell apoptosis and decreasing β -cell proliferation and β -cell number and function has been well-documented (Bonner-Weir and O'Brien 2008, Vetere et al. 2014, Nowotny et al. 2015). This manifested as the reduced serum insulin level, impaired pancreatic β -cell function and decreased pancreatic weight (**Figure 6.3.3 and Table 6.3.3**) in

the DBC group. Nevertheless, the consumption of GRT heightened serum insulin levels, improved IR and pancreatic β -cell function and restored the pancreatic morphology in diabetic groups (**Table 6.3.3 and Figure 6.3.6**). In one of our previous papers, the concentrated hot water GRT demonstrated *in vitro* antioxidant activity in addition to other studies working on aqueous extracts of unfermented and fermented rooibos tea or their bioactive compounds (Joubert et al. 2005, Almajano et al. 2008, Li et al. 2009, Mathijs et al. 2014, Xiao et al. 2020). This indicates that GRT may protect pancreatic islets from morphology through the antioxidant properties since oxidative stress plays a major causal role in the T2D pancreatic β -cell damage (Montane et al. 2014). Furthermore, serum fructosamine is accepted as a useful tool in order to understand the level of hyperglycaemia for a considerable period of time, the significantly higher concentration of fructosamine in DBC group compared to normal groups (**Table 6.3.3**) suggested an increase of protein glycosylation, which mainly contributes to the generation of advanced glycation end products in the diabetic state (Mittman et al. 2010). However, there was no significant ($p > 0.05$) alterations of the serum fructosamine level in GRT-treated groups which seems to be in line with some published data (Vural et al. 2001, Baydas et al. 2002, Ulicna et al. 2006). This suggested that free radicals not necessarily the only causative reason for the development of STZ-induced T2D (Szkudelski 2001).

Mazibuko-Mbeje et al. (2019) reported that aspalathin-enriched GRT extract has strong potential to ameliorate hepatic insulin resistance through improving insulin sensitivity in palmitate-exposed C3A liver cells and obese insulin-resistant rats. The liver is the main organ responsible for insulin sensitivity; it reduces blood glucose concentration via suppressing gluconeogenesis and promoting glycogen synthesis in response to insulin action (Petersen et al. 2017). The significantly ($p < 0.05$) reduced liver glycogen level shown in the DBC rats (**Table 6.3.1**) has been reported in STZ-induced T2D (Oyebode et al. 2020). However, this effect was significantly reversed to near normal in the DGRL and DGRH groups and was even better than DBG and DBM groups (**Table 6.3.1**). This may indicate that GRT modulates glycogen biosynthesis by improving hepatic insulin sensitivity through reducing glycogen phosphorylation or increasing glycogen synthase activity (Islam 2011). This hepatic glycogen synthesis and storage activity may also contribute to the consumption of GRT, lowering the blood glucose level (**Figure 6.3.4**) in comparison to DBC group, thus further revealed its antidiabetic activity. This was also supported by its antihyperlipidemic effects (**Figure 6.3.5**).

It is believed that dyslipidaemia in T2D has been implicated with IR, which may stimulate lipolysis from the fat depots and eventually elevating the serum levels of TG and TC (De Silva

and Frayling 2010, Vijayaraghavan 2010). In one earlier study, treatment of aspalathin-enriched GRT extract (90 mg/kg) to high-fat-fed diabetic Vervet monkeys for 28 days displayed antihyperlipidemic activity by improving total cholesterol due to reducing the level of LDL-c (Orlando et al. 2017). In another study, consumption of aqueous rooibos tea extract (10 g/L) for 14 weeks to hyperlipidaemic mice exhibited potent activity in reducing serum levels of TC, TG and fatty acid concentrations (Beltrán-Debón et al. 2011) which is consistent with partial hypocholesterolaemia effect in similar models (Ziaee et al. 2009). However, in our study, the significantly improved serum lipid profile in DGRL and DGRH groups compared to the DBC groups exerted antihyperlipidemic effects of GRT with reducing levels of TG, TC, LDL-c and increasing HDL-c level, which could be attributed to the reduction of IR in GRT treated animals (**Figure 6.3.7**).

Ayeleso, Oguntibeju et al. (2015) reported the hepato-protective ability of fermented rooibos tea extract (20 g/L) consumed by intramuscular injected STZ-induced diabetes for 49 days exhibiting significantly ($p < 0.05$) lower serum ALT, AST, and ALP when compared to diabetic control animals. Additionally, Ulicna et al. (2006) reported that the oral administration of rooibos tea (5 mg/kg) for eight weeks and oral administration of alkaline rooibos tea extract (300 mg/kg BW) for eight weeks only slightly decreased the concentration of plasma urea and activity of AST, ALP, ALT, but significantly ($p < 0.05$) decreased the concentration of creatinine. This was partially in agreement with our study. Treated groups of low dose and high dose (150 mg/kg and 300 mg/kg) of concentrated hot water of GRT exhibited notable reductions of serum AST, ALT, ALP, urea, uric acid, creatinine, and CK-MB compared to the untreated diabetic group (**Table 6.3.2**), when the effects of DGRH group was significantly ($p < 0.05$) better than DGRL group, particularly in improving serum ALT, ALP and urea near to normal. This result demonstrated the cardiac-protective, hepato-protective, and renal-protective properties of GRT.

The antioxidant activity of rooibos tea in models of STZ-induced diabetes has been reported in several previous studies (Ulicna et al. 2006, Ayeleso et al. 2014, Dlodla et al. 2017). Ulicna et al. (2006) reported aqueous and alkaline extracts of fermented rooibos tea (2.5% *ad libitum*) in STZ-induced diabetic Wistar rats when significantly decreased MDA level was observed in the liver, kidney, lens, and plasma. MDA is a biomarker of severity of membrane lipid peroxidation and oxidative stress (Gueraud et al. 2010). The improved lipid peroxidation state in different organs showed in GRT-treated diabetic groups indicates the anti-peroxidative potential of GRT

through significantly decreasing MDA levels compared to DBC, particularly in a high dose group of GRT (**Figure 6.3.8**).

GSH is a prominent non-enzymatic antioxidant (Nurdiana et al. 2017), while GR activity attributes to GSH content, protein activity and integrity of cells and organs (Gawlik et al. 2016). The significantly ($p < 0.05$) higher level of GSH in pancreas, liver and kidneys (**Figure 6.3.9**) and GR activity in organs of DGRL and DGRH groups when compared to DBC group (**Figure 6.3.10**) again portrays the *in vivo* antioxidant property of GRT in STZ-induced T2D. This is backed up by the consumption of six cups of fermented rooibos tea per day in adults human volunteers with risk of cardiovascular disease, exhibiting increased antioxidant status and decreased oxidative stress through decreasing lipid peroxidation and increasing GSH level and GSH/GSSG ratio (Marnewick et al. 2011).

Waisundara and Hoon (2015) reported that different concentrations of rooibos tea exhibiting its stimulation of the catalase and SOD enzyme activities in a dose-dependent manner in an *in vitro* diabetes model. SOD and catalase are important endogenous antioxidant enzymes scavenging free radicals. The former one specifically catalyses superoxide anions to oxygen and hydrogen peroxide (H_2O_2), while the latter one converts H_2O_2 into oxygen and water (Ighodaro and Akinloye 2018). The significantly reverted activities of these enzymes in most of the organs of animals in DGRL and DGRH groups compared to the animals in DBC group (**Figure 6.3.11 & 6.3.12**), further collaborate the decreased MDA level (**Figure 6.3.8**) supporting the *in vivo* antioxidant effects of GRT. Therefore, these data indicated the antidiabetic effect of concentrated hot water GRT in organs of high fat-fed STZ- induced T2D rats were mediated by increasing in the activity of SOD, catalase, GR, and the level of GSH, meanwhile decreasing the level of MDA.

6.3.6 Conclusion

In conclusion, oral administration of green rooibos tea concentrated hot water extract (150 and 300 mg/kg BW) have demonstrated *in vivo* antioxidant and antidiabetic activity through improving the major symptoms of T2D, reducing hyperglycaemia, alleviating glucose tolerance ability, improving insulin resistance, ameliorating pancreatic β -cell histology and β -cell functions and hyperlipidaemia, improving some biomarkers of T2D associated complications, suppressing organs oxidative stress in a fructose-fed STZ-induced T2D model of rats. Hence, green rooibos tea can be used not only as antidiabetic drink but also as an antidiabetic food supplements in diabetic food and food products.

CHAPTER 7

7.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 General discussion

In the 21st century, diabetes mellitus (DM) is one of the global epidemics with one of the highest prevalence, which has shown a worldwide rapid growth trend with a prevalence of 9.3% (approximately 463 million) in 2019 and estimated prevalence around 10.9% (over 700 million) in 2045 (WHO 2016, IDF 2019). Among them, the prevalence of diabetes in China has reached 10.9% (about 116 million), which is accounting for around 25% of the total number of global diabetics. On the other hand, the prevalence of diabetes in South Africa reaches 12.8% (about 4.58 million), which is around 24% of the total number of diabetics in Africa. Therefore, China is the focus of global diabetes, while South Africa is the focus in Africa. There are a lot of synthetic drugs that are currently employed to control and treat DM, but with undesired side effects and less accessible to diabetics in developing countries. Tea is the most worldwide common beverage just after water that can be categorized into traditional tea or herbal tea, which are made from *Camellia sinensis* and non-*Camellia sinensis* teas or tisanes, respectively. In China, *Camellia sinensis* has more than 5000 years of cultivation and application history since time immemorial, with mounting evidence of beneficial to diabetics. While in South Africa, the antidiabetic effect and various health-promoting functions of rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* species) tea on DM make them increasingly popular worldwide (Van Wyk and Gericke 2000, Van Wyk 2008).

Five teas were selected based on their daily consumption and literature review in the treatment of DM and its associated complications. These teas are jasmine green tea (*Camellia sinensis*), zhengshanxiaozhogn black tea (*Camellia sinensis*), red honeybush tea (*Cyclopia genistoides*), red and green rooibos tea (*Aspalathus linearis*). Sequential extraction and concentrated hot-water infusion extracts of these teas were assessed in this study to support further explorations of these teas in the pharmaceutical industry either in their crude form or standard formulations. Their antioxidant, antidiabetic properties, mechanisms of actions, and possible bioactive phytochemicals responsible for these activities were investigated through *in vitro*, *ex vivo* and *in vivo* tests. Moreover, their antioxidant and antidiabetic activities were compared to give guidance to those tea drinkers.

In vitro and *ex vivo* comparative studies on phytochemicals, antioxidative and antidiabetic activities were conducted between *Camellia sinensis* (ZSXZ black tea) and *Aspalathus linearis*

(red rooibos tea) (**Section 3.1**). The total phenol content of these two teas concentrated infusions was determined with red rooibos tea exhibited significantly higher content than black tea. Concentrated infusions of the teas demonstrated antioxidant potentials via displaying significant free radical scavenging activities *in vitro*. Their antioxidant activities in FeSO₄-induced oxidative hepatic tissue was further demonstrated by their abilities of significantly increasing the reduced glutathione level, enzyme activities of superoxide dismutase and catalase, while concomitantly depleting the level of malondialdehyde. Moreover, the teas also significantly inhibited intestinal glucose absorption in the isolated rat jejunum and α -amylase activities, and elevated glucose uptake in the isolated psoas muscle. The LC-MS phytochemical profiling revealed the presence of hydroxycaffeic acid, l-threonate, caffeine, vanillic acid, n-acetylvaline, and spinacetin 3-glucoside in *C. sinensis*. While quinolinic acid, coumestrol, phloroglucinol, 8-hydroxyquercetagenin, umbelliferone, and ajoene in *A. linearis*. Based on the results from this study, these teas may thus be employed as functional foods in the management of the disease, with *A. linearis* (rooibos tea) showing better activity than *C. sinensis* (ZSXZ black tea).

Comparative studies on antioxidative, antiobesogenic and antidiabetic activities of concentrated infusions of *C. sinensis* (jasmine green tea) and *A. linearis* (green rooibos tea) were examined *in vitro* and *ex vivo* in this study (**Section 3.2**). *C. sinensis* exhibited higher total polyphenolic content, better *in vitro* and *ex vivo* antioxidant ability than *A. linearis*. *C. sinensis* also showed better α -amylase and α -glucosidase inhibitory activities. While for anti-lipase activity, *A. linearis* was higher than *C. sinensis*. The glucose absorption in the small intestine was significantly lower, and glucose uptake in the peripheral tissues was significantly higher in *C. sinensis* than *A. linearis*. The LC-MS analysis revealed the presence of trifoliol, catechin, L-theanine, vanillic acid, epigallocatechin, and caffeine in *C. sinensis*, while vanillic acid, chrysoeriol, *p*-coumaric acid, dihydroferulic acid, luteolin and quercetin in *A. linearis*. These results highlighted their usage in antidiabetic actions.

Red honeybush tea (*Cyclopia genistoides*) was sequentially extracted with different solvents (dichloromethane, ethyl acetate, ethanol, and aqueous) following increasing polarity index on scientific purposes to assess their antioxidant, antidiabetic and antiobesogenic potentials *in vitro* and *ex vivo*. The aqueous and ethyl acetate extracts were the most potent in free radical scavenging and α -glucosidase enzymes activity. The antioxidant activity of all extracts was further affirmed by the ability to increase CAT and SOD activities, glutathione level and lipid

peroxidation elimination in Fe³⁺-induced oxidative pancreatic tissue (**Section 4.2**). Their GC-MS analysis revealed the presence of n-hexadecanoic acid, *cis*-6-octadecenoic acid, 4-hydroxybenzoic acid, shikimic acid, propanoic acid, 2-methylpropyl ester, hexadecanoic acid methyl ester, 9,12-octadecadienoic acid, methyl ester, 9,12-octadecadienoic acid (*Z,Z*)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester, pentadecyl acrylate, n-nonadecanol-1, tyrosol, phytol, 3-n-butylthiolane, sitosterol, vitamin E, 3-O-methyl-D-glucose, 4-(4-hydroxyphenyl)butan-2-one, 4-(4-hydroxy-3-methoxyphenyl)-2-butanone, and stigmastan-3,5-diene. A low dose of 150 and a high dose of 300 mg/kg body weight of concentrated hot-water infusion extract of *Cyclopia genistoides* in drinking water was subjected to *in vivo* analysis in type 2 diabetes model of rats (**Section 4.3**). After 5 weeks intervention, the improved condition in streptozotocin-induced diabetic rats of the treatments exhibiting the antihyperglycemic, antihyperlipidemic, antioxidant effects of *Cyclopia genistoides*, therefore contributing to its outstanding potential as a nutraceutical in ameliorating type 2 diabetes and some of its associated complications.

For jasmine green tea (*Camellia sinensis*), varying polarity solvents (dichloromethane, ethyl acetate, ethanol) were utilized to yield its sequential extracts, which were then subjected to assess their antioxidant and antidiabetic activities *in vitro* and *ex vivo* models (**Section 5.2**). Among them, the ethanol extract displayed the best antioxidant activity *in vitro* and the best inhibitory potential on α -glucosidase activity. At the same time, the ethyl acetate extract exhibited the best inhibitory potential on pancreatic lipase activity. Their antioxidant activity was further elucidated in oxidative hepatic tissue through promoting catalase activity, superoxide dismutase activity, and reduced glutathione levels with concomitantly decreasing levels of malondialdehyde. All the extracts were subjected to gas chromatography-mass spectroscopy (GC-MS) analysis for possible bioactive compounds identification. Caffeine, styrene, n-hexadecanoic acid, octadecanoic acid, tetradecanoic acid, 1-benzoyl-4,5-dihydro-4,4,5,5-tetramethyl-3-phenyl-1h-pyrazole and 1,2-propanediol, 3-benzyloxy-1,2-diacetyl were revealed. Concentrated hot-water infusion extract of Jasmine green tea in drinking water were orally administered to diabetic rats five days per week for five weeks (**Section 5.3**). Diabetic treatment groups exhibited impressive results in a dose-dependent manner in most parameters such as ameliorating blood glucose level, increasing oral glucose tolerance ability, improving serum insulin resistance and pancreatic β -cell function, and prevented oxidative stress-mediated damages of organs compared to the untreated diabetic animals. In general, Jasmine green tea could play a positive role in exploiting antidiabetic alternative therapies.

The dichloromethane, ethyl acetate, ethanol crude extracts of green rooibos tea (*Aspalathus linearis*) was investigated for their potential antioxidant and antihyperglycemic activities (**Section 6.2**). *In vitro* assays including 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) were utilized to determine the antioxidant activity of all the extracts. The ethanol extract showed the highest level of total phenolics content and antioxidant capacity. While the antihyperglycemic potentials of the extracts were determined by examining inhibitory effects on α -glucosidase and α -amylase enzymes, with the ethanol extract exhibiting pronounced enzyme inhibitory activities compared to dichloromethane and ethyl acetate extracts. Furthermore, gas chromatography-mass spectrometry (GC-MS) analysis of all the extracts revealed the presence of n-hexadecanoic acid, octadecanoic acid, 1',4',2,6,6-pentamethyl-spiro-1-(cyclohex-2-ene)-2'-(5'-oxabicyclo [2.1.0] pentane) and styrene. However, there were no reports about the bioactivities of 1',4',2,6,6-pentamethyl-spiro-1-(cyclohex-2-ene)-2'-(5'-oxabicyclo [2.1.0] pentane), further work is needed to be done to confirm its antioxidant or antidiabetic ability. These results suggest an antihyperglycemic and antioxidative effect of green rooibos tea, particularly its ethanol extract. In addition, the antioxidant and antidiabetic ability of green rooibos tea concentrated hot water-infusion extract was examined in a type 2 diabetes animal model (**Section 6.3**). Oral administration of the low and high dose of green rooibos tea treatment demonstrated antioxidant and antidiabetic activity through reducing hyperglycaemia, alleviating glucose tolerance ability, improving insulin resistance, ameliorating pancreatic β -cell histology and β -cell functions and hyperlipidaemia, suppressing diabetes-induced oxidative stress. Results from this study indicate that green rooibos tea displayed promising antioxidant and antidiabetic ability in ameliorating and treating T2D and its associated complications.

7.2 General conclusion

Five teas investigated in this study rendering promising antioxidant and antidiabetic activity *in vitro* and *ex vivo* models. Besides, three of them namely: jasmine green tea (*Camellia sinensis*), red honeybush tea (*Cyclopia genistoides*), green rooibos tea (*Aspalathus linearis*) exhibiting outstanding antidiabetic activity in fructose-fed streptozotocin-induced T2D model of rats employed in the current study (**Table 7.1**). According to the scoring table, high dose of jasmine green tea showing the best activity followed by the high dose of red honeybush tea, low dose of jasmine green tea, high dose of green rooibos tea, low dose of red honeybush tea, when lowest activity was observed for the low dose green rooibos tea. Based on these results, jasmine green tea and red honey bush tea extracts of concentrated hot water have been found the most

effective teas from Chinese and South African origin, respectively. However, further studies are warranted in human subjects in order to confirm the results of this study.

Table 7.1: Scoring value of jasmine green tea, green rooibos tea, and red honeybush tea for different diabetes-related parameters during the intervention period

Diabetes-related parameters		Score value for effects of treatment groups compared to DBC					
Category	Assay or measurement	DRHL	DRHH	DJGL	DJGH	DGRL	DGRH
Calorie and weight gain	Food intake	+1	+	+1	+2	+1	+1
	Mean body Weight at week 6	+0	+0	+1	+1	0	+1
	Net effect score	1	1	2	3	1	2
Glycaemic and insulin profile	NFBG at week 4	+1	+1	+1	+2	+1	+1
	FBG at week 5	+1	+1	+1	+1	+1	+2
	AUC of OGTT	+2	+2	+1	+2	+1	+1
	Serum insulin	+1	+3	+3	+3	0	+1
	Net effect score	5	7	6	8	3	5
Serum lipid profile	Total cholesterol	+1	+2	+2	+2	+1	+2
	Triglyceride	+2	+2	+2	+3	+1	+2
	HDL-cholesterol	+2	+4	+1	+2	+1	+1
	LDL-cholesterol	+1	+2	+1	+1	+1	+1
	Net effect score	6	10	6	8	4	6
Total net effect score		12	18	14	19	8	13

Values 0 to 3 means either no significant difference (0) or first to third level (1 to 3) of significant difference ($p < 0.05$) of treatment groups compared to the diabetic control groups. Positive (+) score values mean that the effect of treatments was potentially beneficial (+) relative to diabetic untreated group (DBC). DBC: Diabetic control; DRHL: Diabetic red honeybush (*Cyclopia genistoides*) tea low dose; DRHH: Diabetic red honeybush (*Cyclopia genistoides*) tea high dose; DJGL: Diabetic jasmine green tea (*Camellia sinensis*) low dose; DJGH: Diabetic jasmine green tea (*Camellia sinensis*) high dose; DGRL: Diabetic green rooibos (*Aspalathus linearis*) tea low dose; DGRH: Diabetic green rooibos tea (*Aspalathus linearis*) high dose.

7.3 Recommendation

The data of these study suggest that while all teas from both Chinese and South Africa origins have potential antioxidative and antidiabetic effects, jasmine green tea from Chinese origin and red honeybush tea from South African origin have been found to be most effective at their high dosages. It is also better to mention that no adverse effects were observed in animal models with the high dosages of these teas in our study so these teas can be used as potential antidiabetic food supplements. However, detailed studies are recommended to carry out on metabolomics, molecular and gene expression studies of the key proteins related to type 2 diabetes to understand the mechanisms behind the actions of these teas fully. Human diabetic subjects could be applied to confirm the antidiabetic activity of these teas. The identified phytochemicals can also be isolated and subject to further studies regarding developing novel antidiabetic drugs.

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APPENDIX A ANIMAL ETHICS APPROVAL LETTER



13 August 2019

Mr Xin Xiao (217082112)
School of Life Sciences
Westville Campus

Dear Mr Xiao,

Protocol reference number: AREC/020/019D

Project title: Comparative antidiabetic effects and mechanisms of actions of some widely consumed Chinese and South African indigenous teas.

Full Approval – Research Application

With regards to your revised application received on 07 August 2019. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 06 August 2020.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....
Dr Sanil D Singh, PhD
Chair: Animal Research Ethics Committee

/kr

cc Supervisor: Prof MS Islam
Cc Registrar: Mr Simon Mokoena

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APPENDIX B PUBLICATIONS FROM THIS THESIS

J Food Sci Technol
https://doi.org/10.1007/s13197-020-04471-w



ORIGINAL ARTICLE

Phytochemical properties of black tea (*Camellia sinensis*) and rooibos tea (*Aspalathus linearis*); and their modulatory effects on key hyperglycaemic processes and oxidative stress

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Abstract The comparative phytochemicals, antioxidative and antidiabetic activities of *Camellia sinensis* (black tea) and *Aspalathus linearis* (rooibos tea) were studied in vitro and ex vivo. Concentrated infusions of the teas showed significant free radical scavenging activities in vitro. They significantly increased the glutathione level, superoxide dismutase and catalase enzyme activities in oxidative hepatic injury, while concomitantly depleting malondialdehyde level. The teas significantly inhibited intestinal glucose absorption and α -amylase activities, and elevated muscle glucose uptake. LCMS phytochemical profiling revealed the presence of hydroxycaffeic acid, l-threonate, caffeine, vanillic acid, n-acetylvaline, and spinacetin 3-glucoside in *C. sinensis*. While quinolinic acid, coumestrol, phloroglucinol, 8-hydroxyquercetagenin, umbelliferone, and ajoene were identified in *A. linearis*. These results portray the antioxidant and antidiabetic potencies of both teas, with *A. linearis* showed better activity compared to *C. sinensis*. These teas may thus be used as functional foods in the management of diabetes and other oxidative stress related metabolic disorders.

Keywords Antioxidants · Antidiabetics · Black tea · Rooibos tea · Type 2 diabetes

Introduction

Diabetes mellitus (DM) is the most endemic of all metabolic diseases, as it was reported to affect over 425 million people in 2017 (IDF 2018). This depicts a 2.4% rise in prevalence from 415 million in 2015 (IDF 2016) and it is expected to increase by 48% to 629 million in 2045, with an upsurge of 156% expected for Africa (IDF 2018).

Diabetes mellitus is characterized by increased blood glucose level (hyperglycemia) owing to disorder in the metabolism of carbohydrate, protein and lipids (Erukainure et al. 2013), which is caused by failure of the pancreatic β -cell to secrete insulin, and/or failure of the cells to use the secreted insulin (Erukainure et al. 2018a, b, c). The former is referred to as type 1 diabetes (T1D), while the latter is often referred to as type 2 diabetes (T2D) and the most prevalent of all diabetes types as it is responsible for over 90% of morbidity and mortality due to DM (IDF 2016, 2018). Hyperglycemia leading to oxidative stress is the major trigger of T2D pathogenesis, that leads to micro- and macro-vascular complications such as retinopathy and neuropathy (Chukwuma and Islam 2017; Constantino et al. 2013; Erukainure et al. 2017a, b; Tiwari et al. 2013). Oxidative stress occurs in T2D as a result of increased generation of reactive oxygen species (ROS) from increased glucose oxidation, which overwhelms the cell's endogenous antioxidative system (Maritim et al. 2003; Sanni et al. 2018). Increased activities of carbohydrate hydrolyzing enzymes particularly α -glucosidase and α -amylase have also been reported to contribute to hyperglycemia owing to rapid breakdown of dietary

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carbohydrate leading to postprandial rise in blood glucose level (Oyebode et al. 2018).

Camellia sinensis is a well known medicinal plant commonly referred to as tea and has been consumed as beverage from time immemorial. Its origin has been ascribed to Asia and it has been described as the most globally consumed beverage second to water (Macfarlane and Macfarlane 2004). *Camellia sinensis* is commercially available in most countries as black, green and white teas. Several studies have reported its antidiabetic and antioxidant properties (Bhatt et al. 2010; Dufresne and Farnworth, 2001; Fu et al. 2017; Kumar and Rizvi 2015), which has been attributed to its phytochemical constituents particularly the catechins and alkaloids (Frei and Higdon 2003; Han et al. 2016; Williamson et al. 2011).

Aspalathus linearis is a medicinal plant native to South Africa, belonging to the Fabaceae family and the *Aspalathus* genus. Its leaves are utilized in the production of the herbal tea, rooibos or bush tea which is widely consumed globally (Joubert et al. 2008). Its medicinal properties have been widely studied and has been reported for antidiabetic and antioxidant activities (Joubert et al. 2008; Marnewick et al. 2003; Patel et al. 2016). These medicinal properties have been attributed to its reported high ascorbic acid content as well as polyphenols such as the flavones and dihydrochalcones particularly aspalathin and nothofagin (Iswaldi et al. 2011; Lee and Bae 2015).

Camellia sinensis and *A. linearis* constitute the most common teas consumed in Southern Africa, and often used singly or combined in the management of various ailments including DM. However, there is a dearth in their comparative studies. This study thus aims at comparing the phytochemical, antidiabetic and antioxidative properties of *C. sinensis* (black tea) and *A. linearis* (rooibos tea) by investigating their ability to promote hypoglycaemic processes vis-à-vis muscle stimulation of glucose uptake, inhibiting intestinal glucose absorption and activities of major carbohydrate digestive enzymes, as well as improving antioxidant enzymes activities.

Materials and methods

Tea bags

Commercial *C. sinensis* and *A. linearis* tea bags were purchased from local malls at Fuzhou, China and Durban, South Africa respectively. Five bags (10 g) of each product were infused in 100 mL of boiled water and allowed to stand for 2 h. The infusions were decanted into a weighed beaker and concentrated at $50\text{ }^\circ\text{C}$ in a water bath. After concentrating, the beaker was reweighed, and the yielded concentration was calculated to be 4.2 and 3.6 g of

C. sinensis and *A. linearis* samples respectively. They were stored in airtight vials until further analysis.

A stock solution (1 mg/mL) was prepared from each sample using distilled water, from which different working concentrations (15, 30, 60, 120 and 240 $\mu\text{g/mL}$) were prepared for further studies.

Total phenolic content

The total phenolic content of the samples was analyzed via the Folin–Ciocalteu reagent assay and expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (Liu and Yao 2007).

In vitro antioxidant activity

In vitro antioxidant activities were determined for the teas using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity (Braca et al. 2002) and the ferric reducing antioxidant power (FRAP) assay (Benzie and Strain 1996).

For DPPH activity, 100 μL of each sample concentrations were incubated with 50 μL of 0.3 mM DPPH solution (in methanol) for 30 min in the dark. Absorbance was read at 517 nm.

For FRAP, 100 μL of each sample concentration was incubated with equal volumes of sodium phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide at $50\text{ }^\circ\text{C}$ for 30 min. 100 μL of 10% trichloroacetic acid was used in acidifying the reaction mixture, 100 μL of distilled water and 200 μL of 0.1% FeCl_3 were then added. Absorbance was read at 700 nm.

Ascorbic acid was used as standard drugs for both activities.

Enzyme inhibitory activity

The teas were assayed for their antidiabetic activities by determining their inhibitory effect on pancreatic α -amylase (Shai et al. 2010). Briefly, 50 μL of each tea concentration or acarbose was incubated with equal volume of porcine pancreatic amylase (2 U/mL; in 100 mM phosphate buffer, pH 6.8) for 10 min at $37\text{ }^\circ\text{C}$. After which, an equal volume of 50 μL of 1% starch solution in 100 mM phosphate buffer (pH 6.8) was added to the reaction mixture and further incubated for 10 min at $37\text{ }^\circ\text{C}$. A 100 μL of the dinitrosalicylate (DNS) color reagent was added to the mixture and boiled for 10 min. Absorbance was read at 540 nm.

Animals

Five male albino rats of Sprague Dawley strain and weighing about 200–250 g were procured for the

experiment from the Biomedical Research Unit (BRU), University of KwaZulu-Natal, Durban, South Africa. The rats were sacrificed by euthanizing with halothane, after overnight fasting (12 h). Their small intestines (jejunum), muscles and liver were harvested, rinsed in 0.9% NaCl solution to remove blood stains and used immediately for *ex vivo* studies comprising of glucose absorption and uptake, and anti-oxidative stress activities.

The animals were maintained under the guidelines approved by the Animal Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa (Protocol approval number: AREC/067/017D).

Ex vivo anti-oxidative activity

After homogenizing in 50 mM sodium phosphate buffer (pH 7.5; with 10% Triton X-100), the harvested livers were centrifuged at 15,000 rpm at 4 °C for 10 min. The supernatants were decanted and stored in 2 mL Eppendorf tubes.

A 100 µL of each tea concentration was incubated with a reaction mixture containing 100 µL of the liver homogenates and 30 µL of 0.1 mM FeSO₄ for 30 min in 5% CO₂ incubator. A reaction mixture without any tea sample or standard drug served as negative control (untreated). Ascorbic acid was used as the standard drug.

The incubated samples were then analyzed for reduced glutathione (GSH) level (Ellman 1959), catalase (Chance and Maehly 1955) and superoxide dismutase (SOD) (Kakkar et al. 1984) activities, and malondialdehyde (MDA) level (Chowdhury and Soulsby 2002).

Glucose absorption in isolated rat jejunum

The inhibitory activity of the teas on intestinal glucose absorption was assayed by incubating the different concentrations of the teas with 5 cm of the isolated rat jejunum in Krebs buffer (118 mM NaCl, 5 mM KCl, 1.328 mM CaCl₂·2H₂O, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃ and 11.1 mM glucose) (Chukwuma and Islam 2015). Glucose concentration were determined before and after the incubation with an Automated Chemistry Analyzer (Labmax Plenno, Labtest Inc., Costa Brava, Brazil). Glucose absorption was calculated with the following formula:

$$\text{Glucose absorption/cm of rat jejunum} = \frac{GC1 - GC2}{\text{Jejunum length (cm)}}$$

where GC1 and GC2 are glucose concentrations (mg/dL) before and after incubation respectively.

Glucose uptake in isolated rat psoas muscles

The ability of the teas to exacerbate glucose uptake in the isolated rat psoas muscles was assayed by determining the glucose concentration after a 2 h incubation of 0.5 g of isolated rat psoas muscle with different concentrations of the test samples in 8 mL of Krebs buffer (Chukwuma and Islam 2015). Metformin (Sigma Aldrich, South Africa) was used as a positive control. Glucose concentration were measured before and after the incubation with an Automated Chemistry Analyzer (Labmax Plenno, Labtest Inc., Lagoa Santa, Brazil). The intestinal glucose absorption was calculated with the following formula:

$$\text{Glucose uptake} = \frac{GC1 - GC2}{\text{Muscle weight (g)}}$$

where GC1 and GC2 are glucose concentrations (mg/dL) before and after the incubation respectively.

Liquid Chromatography-Mass Spectrometric (LC-MS) Analysis

The tea samples were subjected to LC-MS analysis using Shimadzu LCMS-2020 Single Quadrupole Liquid Chromatograph Mass Spectrometer (LC-MS). A HP-5MS capillary column was used (30 m × 0.25 mm ID, 0.25 µm film thickness, 5% phenylmethylsiloxane). The LC stop time was set at 4.00 min. The PDA sampling frequency was set at 1.5625 Hz and the operating mode was on low pressure gradient. Other operating parameters were as follows: Pump A: LC-2030 Pump, Flow rate: 0.2000 mL/min, Mobile Phase B Conc.: 95.0%; C Conc.: 0.0%; D Conc.: 0.0%; A: Water; Mobile Phase B: Methanol; Start Wavelength: 190 nm; End Wavelength: 800 nm; Cell Temp.: 40 °C; Start Time: 0.00 min; End Time: 4.00 min; Acquisition Mode: Scan Polarity: Positive; Event Time: 1.00 s; Detector Voltage: + 1.00 kV; Threshold: 0; Start m/z: 50.00; End m/z: 1700.00; Scan Speed: 1667 u/sec. Compounds were identified by direct comparison of mass spectral data with those in the <https://foodb.ca/spectra/ms/search>.

Statistics

Data were presented as mean ± SD, and significance of difference was established at $p < 0.05$ using one-way analysis of variance (ANOVA). Statistical analyses were carried out using IBM Statistical Package for the Social Sciences (SPSS) for Windows, version 23.0 (IBM Corp, Armonk, NY, USA). The difference between the treated and untreated samples was used in calculating the IC₅₀ values for each tea using their respective regression lines, where $x = 50$ (Erukainure et al. 2017a, b).

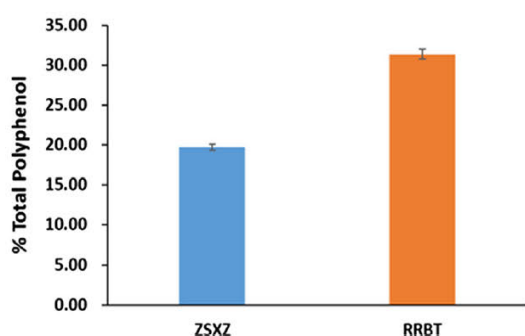


Fig. 1 Total phenolic contents of *C. sinensis* and *A. linearis*. Data are presented as mean \pm SD; n = 3. ZSXZ: *C. sinensis*; RRBT: *A. linearis*

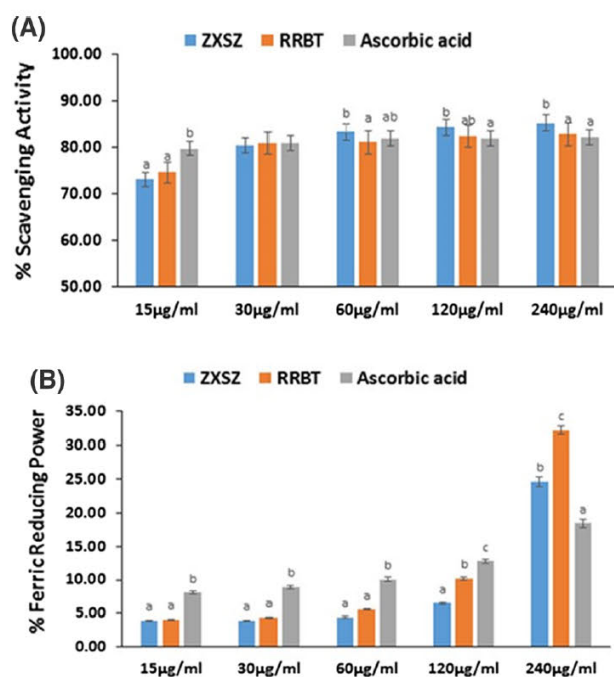


Fig. 2 a DPPH scavenging and b Ferric reducing antioxidant power (FRAP) activities of *C. sinensis* and *A. linearis*. Data are presented as mean \pm SD. ^{abc}Values with different letter above the bars for a given concentration are significantly ($p < 0.05$) different from each other. ZSXZ: *C. sinensis*; RRBT: *A. linearis*

Results

As shown in Fig. 1, both tea samples had moderate phenolic contents. The phenolic content of *A. linearis* was significantly ($p < 0.05$) higher than that of *C. sinensis*.

Both teas significantly ($p < 0.05$) scavenged DPPH, with *A. linearis* displaying the best scavenging activity as shown in Fig. 2a and Table 1. Both teas showed a dose-dependent FRAP activity as shown in Fig. 2b, with the highest activity observed at the highest concentration (240 $\mu\text{g}/\text{mL}$).

Both teas moderately inhibited the activities of α -amylase but were significantly ($p < 0.05$) lower when compared to the standard drug, acarbose as depicted in Fig. 3.

Incubation of hepatic tissue homogenates with FeSO_4 led to significant ($p < 0.05$) depletion of GSH level, SOD and catalase activities, while significantly ($p < 0.05$) increasing MDA level as depicted in Fig. 4a–d. Incubation with the teas significantly ($p < 0.05$) increased the GSH level, SOD and catalase activities, and concomitantly depleted MDA level. The ability of both teas to increase the SOD activities were dose-dependent, with *A. linearis* showing the best activity (Fig. 4b). Based on the IC_{50} values (Table 1), *A. linearis* had the best activities except for MDA depletion.

Incubation of isolated rat jejunum with the teas significantly ($p < 0.05$) inhibited intestinal glucose absorption as depicted in Fig. 5a, with *C. sinensis* showing a dose dependent activity. The low IC_{50} value of *A. linearis*, indicates a better activity compared to *C. sinensis*.

Incubation of isolated psoas muscle with the teas led to significant ($p < 0.05$) increase in muscle glucose uptake as shown in Fig. 5b. Both teas showed dose-dependent activities, with *C. sinensis* having the best activity.

LCMS analysis led to the identification of hydroxycaffeic acid, l-threonate, caffeine, vanillic acid, n-acetylvaline, and spinacetin 3-glucoside in *C. sinensis* as depicted in Fig. 6a, while quinolinic acid, coumestrol, phloroglucinol, 8-hydroxyquercetagenin, umbelliferone, and ajoene were identified in *A. linearis* as shown in Fig. 6b.

Table 1 IC_{50} values of *C. sinensis* and *A. linearis* activities

Activities	<i>C. sinensis</i>	<i>A. linearis</i>	Ascorbic acid	Acarbose
DPPH	0.03 $\mu\text{g}/\text{mL}$	0.01 $\mu\text{g}/\text{mL}$	0.05 $\mu\text{g}/\text{mL}$	–
FRAP	> 1000 $\mu\text{g}/\text{mL}$	> 1000 $\mu\text{g}/\text{mL}$	> 1000 $\mu\text{g}/\text{mL}$	–
α -amylase	> 1000 $\mu\text{g}/\text{mL}$	> 1000 $\mu\text{g}/\text{mL}$	> 1000 $\mu\text{g}/\text{mL}$	> 1000 $\mu\text{g}/\text{mL}$
GSH	112.02 $\mu\text{g}/\text{mL}$	84.10 $\mu\text{g}/\text{mL}$	90.34 $\mu\text{g}/\text{mL}$	–
SOD	> 1000 $\mu\text{g}/\text{mL}$	87.27 $\mu\text{g}/\text{mL}$	797.56 $\mu\text{g}/\text{mL}$	–
Catalase	1.71 $\mu\text{g}/\text{mL}$	1.62 $\mu\text{g}/\text{mL}$	2.21 $\mu\text{g}/\text{mL}$	–
MDA	1.55 $\mu\text{g}/\text{mL}$	2.6 $\mu\text{g}/\text{mL}$	3.52 $\mu\text{g}/\text{mL}$	–
Glucose absorption	162.22 $\mu\text{g}/\text{mL}$	85.82 $\mu\text{g}/\text{mL}$	–	–
Glucose uptake	242.64 $\mu\text{g}/\text{mL}$	383.63 $\mu\text{g}/\text{mL}$	–	–

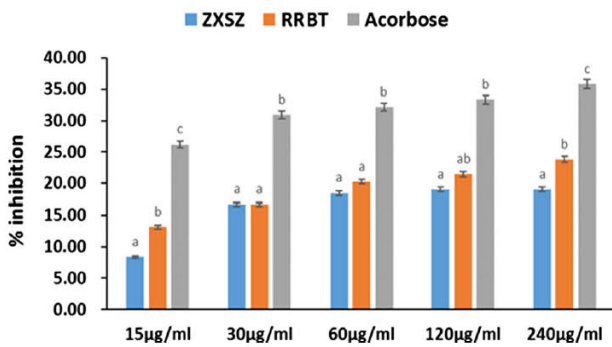


Fig. 3 Inhibitory effect of *C. sinensis* and *A. linearis* on α -amylase activity. Data are presented as mean \pm SD. ^{abc}Values with different letter above the bars for a given concentration are significantly ($p < 0.05$) different from each other. ZXSZ: *C. sinensis*; RRBT: *A. linearis*

Discussion

Tea drinking culture has been in practice from time immemorial, with Yunnan in western China said to be the birthplace of this culture (Kumakura 2002). Aside *C.*

sinensis, there have been in increase in other types of tea notably herbal teas which also enjoy worldwide consumption (Joubert et al. 2008). Though often taken as recreational beverages and food, teas have been reported for their medicinal properties (Sharma et al. 2007; Siddiqui et al. 2004). This study reports the ability of *C. sinensis* and *A. linearis* teas to scavenge free radicals and to inhibit the activities of major carbohydrate catabolic enzymes linked to type 2 diabetes as well as their phytoconstituents.

The total phenolic contents of both teas were rather very low (Fig. 1) which corroborates previous studies by Ane-sini et al. (2008), Pal et al. (2012) and Bhebe et al. (2015) which reported low total phenolic content for *C. sinensis* and *A. linearis*. This however contradicts previous reports that both teas were had rich contents of phenolics. Pereira et al. (2014) reported high phenolic contents for black, green and white *C. sinensis* and correlated them with the antioxidant properties of the studied teas. Damiani et al. (2019) also reported high phenolic properties for *A. linearis* and also correlated the antioxidant activity of the tea to the phenolic content. Although the present study reports low

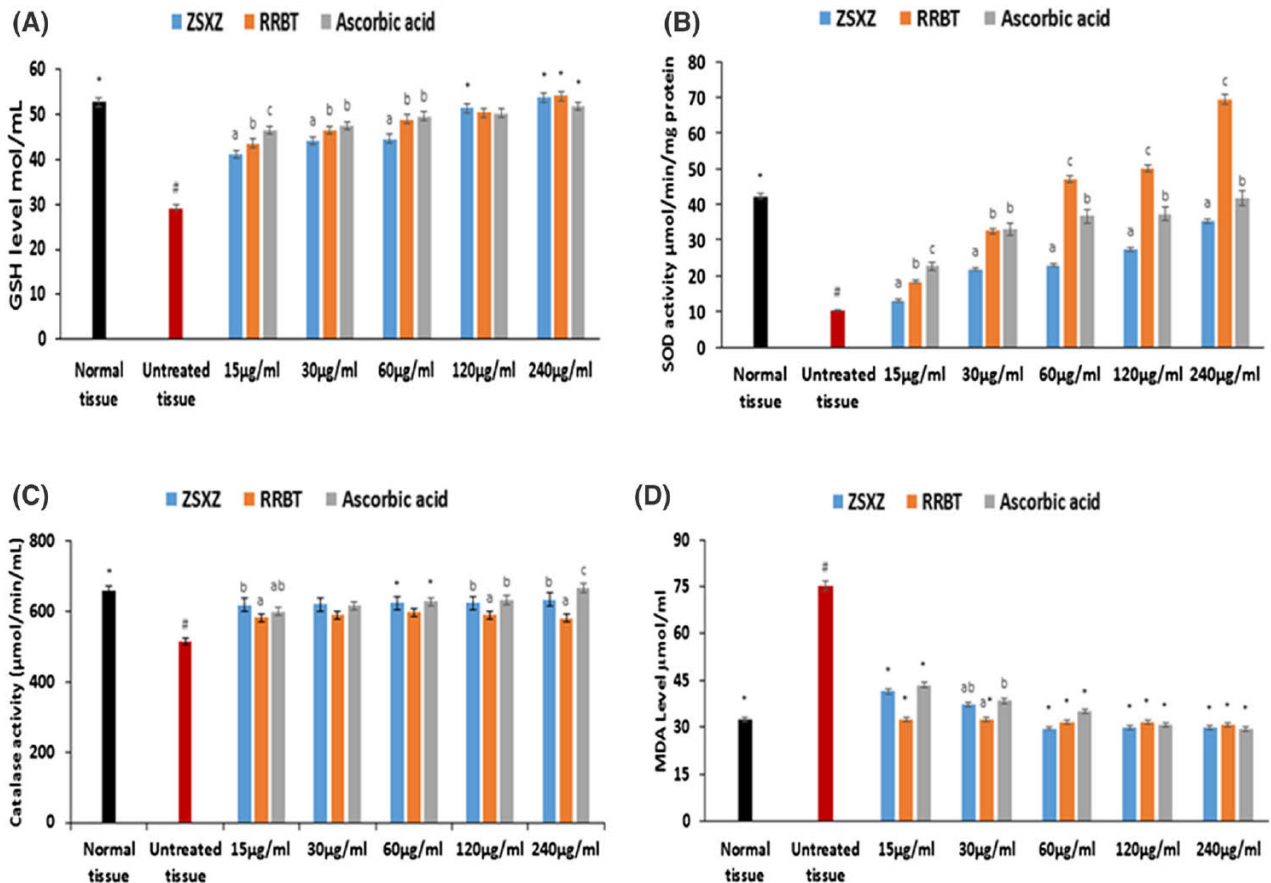
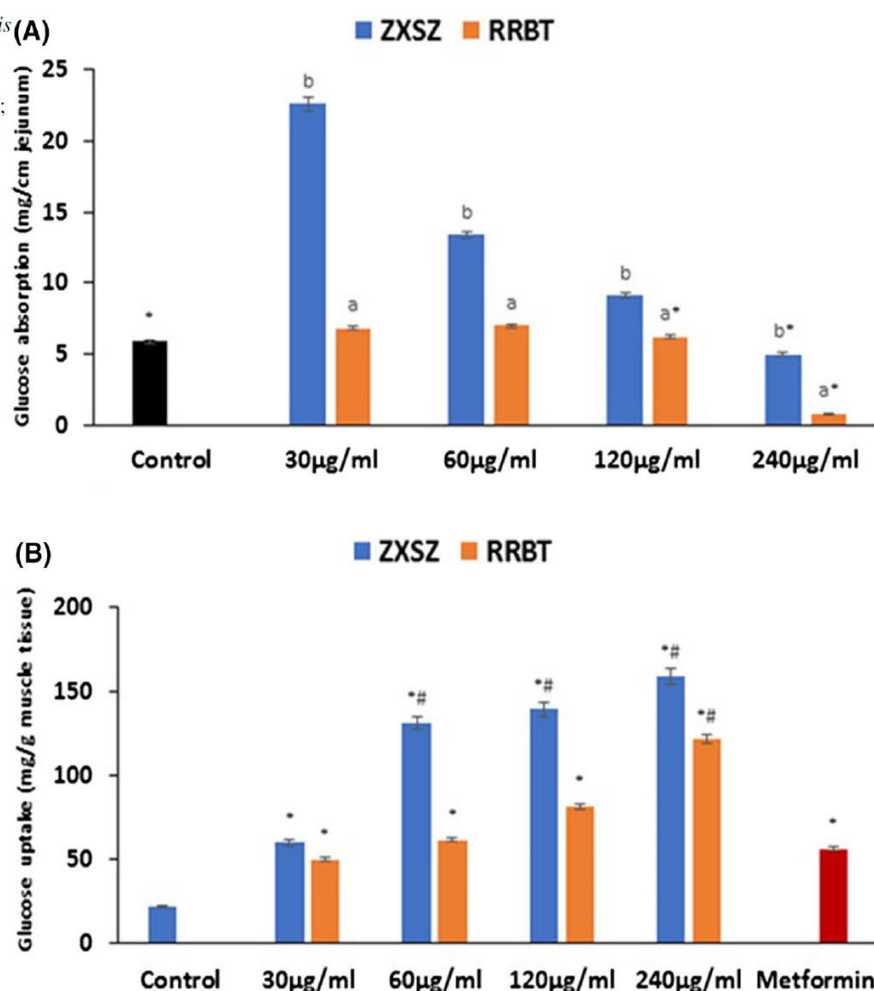


Fig. 4 Effect of *C. sinensis* and *A. linearis* on (a) GSH level, (b) SOD activity, (c) catalase activity, and (d) MDA level in oxidative hepatic injury. Data are presented as mean \pm SD; n = 3. *Significantly

different from untreated sample and #Significantly ($p < 0.05$) different from normal sample. ZXSZ: *C. sinensis*; RRBT: *A. linearis*

Fig. 5 Effects of *C. sinensis* and *A. linearis* on glucose (a) absorption in isolated rat jejunum and (b) uptake in isolated psoas muscle. Data are presented as mean \pm SD; n = 3. *Significantly different from untreated sample and #Significantly ($p < 0.05$) different from normal sample. ZXSZ: *C. sinensis*; RRBT: *A. linearis*



phenolic contents for both teas, they however contribute to the antioxidant properties of the teas as depicted by their ability to scavenge DPPH and reduce Fe^{3+} (Fig. 2a, b).

The influence of oxidative stress in the pathogenesis of type 2 diabetes and its complications due to hyperglycemia induced increased production of free radicals are well documented (Erukainure et al. 2012; King and Loeken 2004; Tiwari et al. 2013). These free radicals have been shown to attack cellular proteins, DNAs, membrane lipids which may subsequently lead to cell death (Maritim et al. 2003). Increased lipid peroxidation owing to suppressed GSH level, SOD and catalase activities is a major oxidative mechanism. The high DPPH scavenging and FRAP activities (Fig. 2a, b) of the teas indicates their free radical and reducing power properties. This corroborates previous reports on the potent antioxidant properties of *C. sinensis* and *A. linearis* (Pereira et al. 2014; Damiani et al. 2019). This is further depicted by the ability of both teas to elevate the levels of GSH, SOD and catalase activities, while suppressing lipid peroxidation in oxidative hepatic injury (Fig. 4a–d). These potencies may be attributed to the LC–MS identified compounds of the teas (Fig. 6), particularly

the phenolics which are well known antioxidants (Heleno et al. 2015; Zhao et al. 2014).

Inhibition of major carbohydrate digestive enzymes has been reported in several studies to be effective in the treatment and management of type 2 diabetes (Van 2006). The inhibition of α – amylase by the teas *C. sinensis* and *A. linearis* (Fig. 3) corroborates previous studies (Gao et al. 2013; Mikami et al. 2015; Muller et al. 2012; Ramírez et al. 2012; Vinholes and Vizzotto 2017) and further portrays their antidiabetic properties. These studies attributed the enzyme inhibitory properties to the phytoconstituents of both teas (Dludla et al 2017; Gao et al. 2013; Muller et al. 2012; Wang, et al. 2012), thereby implying that the total phenol content (Fig. 1) and identified compounds (Fig. 6a, b) may play a synergetic role regarding this activity.

Inhibition of and/or delayed intestinal glucose absorption can also lead to decreased postprandial elevation of blood glucose level, thus can be employed in the treatment and management of T2D (Chukwuma and Islam 2015). Studies have reported the ability of plant extracts to suppress glucose absorption in the intestine mostly at the first quarter jejunal and duodenal regions (Erukainure et al.

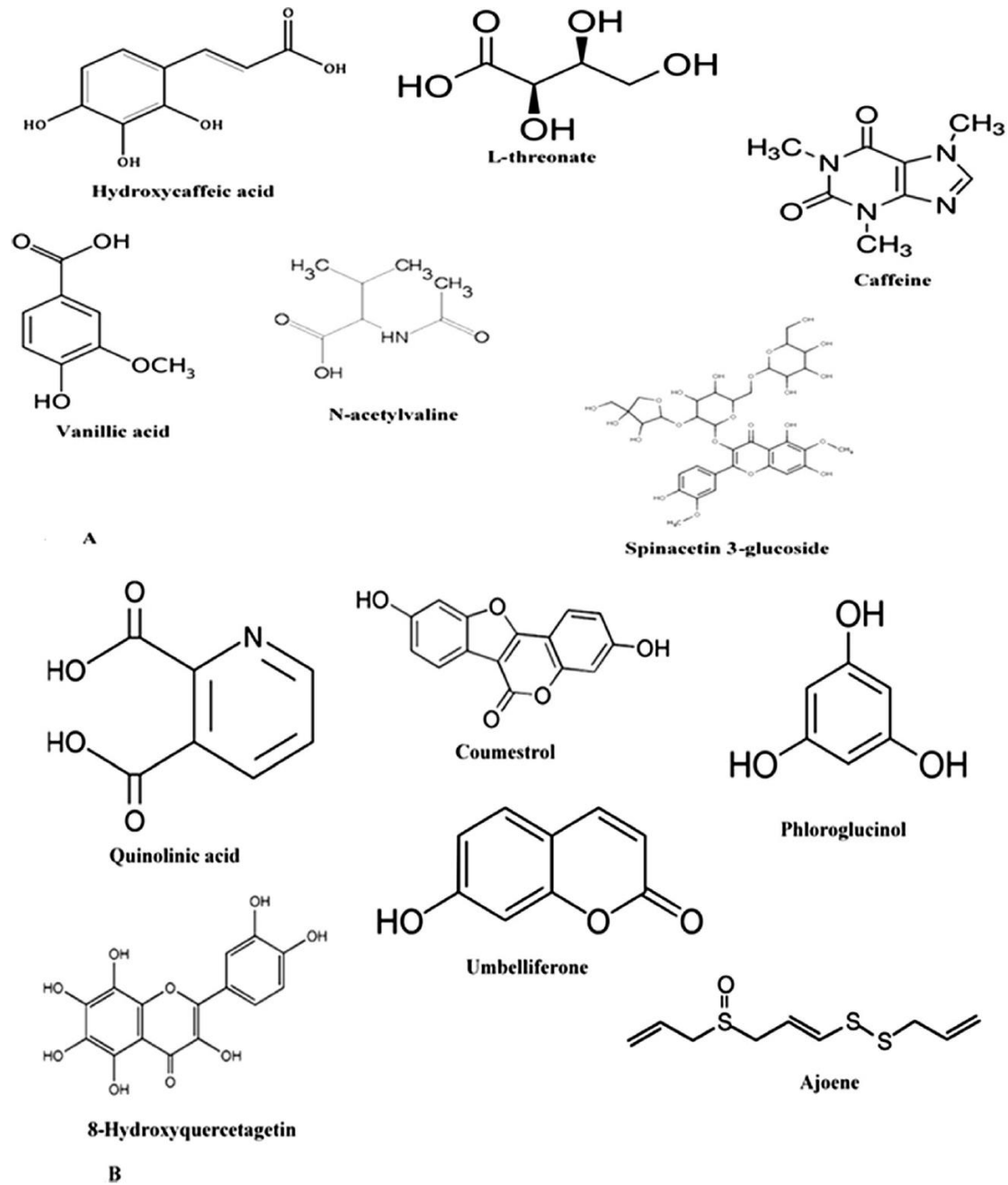


Fig. 6 LCMS identified compounds in (a) *C. sinensis* and (b) *A. linearis*

2018a, b, c, 2019; Oyeboade et al. 2018). The inhibitory effects of the studied teas (Fig. 5a) demonstrates their ability to delay the intestinal absorption of dietary glucose, thus preventing postprandial elevation of blood glucose level. This also corroborates with their ability to inhibit α -amylase activity (Fig. 3).

The role of skeletal muscle in carbohydrate metabolism have been well documented (Oyeboade et al. 2018; Sinacore and Gulve 1993). This can be attributed to their richness in the glucose transporter, GLUT-4 which facilitates glucose uptake (Oyeboade et al. 2018; Satoh 2014). Some

commercial antidiabetic drugs such as metformin exhibit their antidiabetic activity by triggering muscle glucose uptake (Natali and Ferrannini 2006). Thus, the ability of the studied teas to stimulate muscle glucose uptake (Fig. 5b) further insinuates their antidiabetic potentials. This may also portray an improved insulin sensitivity, as insulin resistance has been implicated in the defects in muscle glucose uptake (Satoh 2014; Sinacore and Gulve 1993).

Phytochemicals have been reported for their antioxidant and antidiabetic activities (Alasalvar and Bolling 2015;

Chukwuma et al. 2019). The studied biological activities of *C. sinensis* and *A. linearis* may be attributed to the identified phytochemicals (Fig. 6a, b), thus depicting a synergistic effect. The presence of the phenolics, hydroxycaffeic acid, vanillic acid, and n-acetylvaline as well as the phenolic glycoside, spinacetin 3-glucoside in *C. sinensis* portrays a strong antioxidant potency as phenolics are well known for their antioxidant and antidiabetic properties (Chukwuma et al. 2019; Erukainure et al. 2018a, b, c). The presence of caffeine may also contribute to the antidiabetic activity of *C. sinensis*, as the hypoglycemic activity of caffeine has been reported in non-diabetics, pre-diabetics and diabetics (Bhaktha et al. 2015; Lane 2011; Lee et al. 2016). Similarly, the presence of phloroglucinol, 8-hydroxyquercetagenin, and umbelliferone in *A. linearis* (Fig. 6b) may also contribute to its antioxidant and antidiabetic activities. Phytoestrogen, coumestrol and ajoene have also been shown to possess antioxidant and antidiabetic properties (Bhathena and Velasquez 2002; Hattori et al. 2005; Yuk et al. 2011), and may also contribute to the biological activities of *A. linearis*.

Conclusion

These results depict the antioxidative and antidiabetic potencies of *C. sinensis* and *A. linearis* as demonstrated by their ability to scavenge free radicals, suppress lipid peroxidation, inhibit α -amylase enzyme activity and intestinal glucose absorption, and concomitant increase in antioxidant enzymes activities and muscle glucose uptake. Thus, further affirming the utilization of these teas for managing T2D and its complications, with *A. linearis* being more potent compared to *C. sinensis*. Hence, they may be employed as functional foods in the management of diabetes and other oxidative stress related metabolic disorders.

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Compliance with ethical standards

Conflict of interest The authors report no conflict of interest.

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Sequential extracts of red honeybush (*Cyclopia genistoides*) tea: Chemical characterization, antioxidant potentials, and anti-hyperglycemic activities

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Abstract

The antioxidant, antidiabetic, and anti-obesogenic potentials of different extracts (dichloromethane, ethyl acetate, ethanol, and aqueous) of the red honeybush (*Cyclopia genistoides*) tea were investigated in vitro and ex vivo. All extracts exhibited significant scavenging and reducing power activities, with the aqueous and ethyl acetate extracts being the most potent. In vitro antidiabetic analysis revealed the extracts to be potent inhibitors of α -glucosidase and lipase activities. All extracts increased catalase and SOD activities, and glutathione level in oxidative pancreatic injury. GC-MS analysis revealed the presence of fatty acids, fatty acid ester, phytols, sterols, saccharide, ketones, and triterpenes. These results imply that the sequential extracts of honeybush tea (particularly the aqueous and ethyl acetate extracts) may not only exhibit antioxidant potentials but also mediate anti-hyperglycemia activities by inhibiting lipid and carbohydrate digestion.

Practical applications

Red honeybush tea is enjoyed widely in South Africa and around the world due to its no caffeine and very low tannin content, as well as many healthcare attributes. There are however no scientific reports for its sequential extraction of different solvents on antidiabetic effects. The different extracts of honeybush tea (particularly the aqueous and ethyl acetate extracts) inhibited lipid and carbohydrate digestive enzymes linked to type 2 diabetes (T2D), as well as modulate oxidative pancreatic injury. These findings will promote its utilization as a potential nutraceutical in the management of diabetes and its complications.

KEYWORDS

antioxidants, honeybush tea, sequential extraction, type 2 diabetes

1 | INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases whose hallmark characteristic is chronic hyperglycemia. It is caused by disorders

of carbohydrate, protein, and fat metabolism resulting from defects in insulin secretion and/or action (ADA, 2014; WHO, 1999). Diabetes is associated with long-term damage, dysfunction, and even failure of multiple organs in severe circumstances. Currently, around 90%

or more of all patients with diabetes worldwide are type 2 diabetes (T2D), or non-insulin-dependent diabetes mellitus (NIDDM).

T2D may result due to the inability of insulin responsive cells to utilize insulin or relatively low insulin levels being secreted by islets of Langerhans β -cells. Due to the mild nature of T2D especially in the early stages the first line management strategies do not involve the injection of insulin disease (Chatterjee, Khunti, & Davies, 2017). Nowadays, the clinical management of T2D mainly involves the use of oral pharmacological agents that stimulate β -cell insulin secretion, increase sensitivity of insulin to effector cells, and retard postprandial assimilation into the blood. These strategies are focused on achieving good glycaemic control to ultimately reduce the risk of development or progression of diabetes complications (Marín-Peñalver, Martín-Timón, Sevillano-Collantes, & del Cañizo-Gómez, 2016; Stein, Lamos, & Davis, 2013). Although oral antidiabetic drugs have exhibited some success in the management of hyperglycemia, their effects are not always sustained and may be associated with undesirable side effects such as hypoglycemic shock risk, weight gain, and other comorbidities (Chaudhury et al., 2017; Khardori & Griffing, 2011). Therefore, looking for therapeutic agent with reduced side effects will be of great significance in the management and possible treatment of diabetes. Medicinal plants have been reported for their uses in the management of diabetes. They are postulated to have superior benefits over synthesized drugs as they are believed to have less potential undesirable side effects, more affordable, and readily available (He, Chen, & Li, 2019; Hung, Qian, Morris-Natschke, Hsu, & Lee, 2012).

Honeybush tea, which is indigenous to South Africa and grows exclusively in the Cederberg Mountains north of Citrusdal to the Cape Peninsula in the South and Port Elizabeth in the Eastern Cape region (Joubert, Gelderblom, & De Beer, 2009; Kokotkiewicz & Luczkiewicz, 2009; Marnewick, 2009). About 23–24 *Cyclopia* species have been found till now but only three species are vastly commercially made into honeybush tea namely, *Cyclopia intermedia*, *Cyclopia subternata*, and *Cyclopia genistoides* (Joubert et al., 2009; Kokotkiewicz & Luczkiewicz, 2009). Honeybush tea is characterized by sweet honey-like aroma, which is made from the leaves, stem, and flowers of the *Cyclopia* species (Kokotkiewicz & Luczkiewicz, 2009). The effects of honeybush tea on antimutagenic, antioxidant, cancer modulating, and cardiovascular has been shown in vitro and in vivo studies (Marnewick et al., 2011; Pantsi, Marnewick, Esterhuysen, Rautenbach, & Van Rooyen, 2011).

The methanolic extracts of 22 *Cyclopia* species were screened by De Nysschen, Van Wyk, Van Heerden, and Schutte (1996) who reported that the xanthone, mangiferin, the flavanone, hesperidin, and isosakuranetin were the major flavonoids common to all the species. Along with more recent research by Joubert, Richards, Merwe, De Beer, Manley, & Gelderblom (2008) showed that mangiferin, isomangiferin, and hesperidin are also existing in all *Cyclopia* species. Although the phytochemicals of methanolic extracts of honeybush tea have been reported, the antioxidative and antidiabetic potentials of different extracts of red honeybush (*Cyclopia genistoides*) tea are still poorly documented.

Therefore, this study was designed not only to analyze the phytochemical constituents of different extracts of red honeybush tea through GC-MS analysis but also to investigate their effects on key enzymes linked to T2D vis-à-vis α -glucosidase and lipase, as well as their mitigative effect on oxidative pancreatic injury, ex vivo.

2 | MATERIALS AND METHODS

2.1 | Chemicals and instrumentation

1,1-diphenyl-2-picrylhydrazyl radical (DPPH), acarbose, ascorbic acid, gallic acid, and potassium ferricyanide were obtained from Sigma-Aldrich (Germany). Deuterated chloroform, deuterated methanol, dinitrosalicylic acid (DNS), Folin-Ciocalteu reagent, ferric chloride, Griess reagent, starch, sodium nitroprusside, and thiobarbituric acid were purchased from Merck (Germany). Ethylenediaminetetraacetic acid (EDTA), orlistat, morpholinepropanesulphonic acid (MOPS), *p*-nitrophenyl- α -D-glucopyranoside (*p*NPG), *p*-nitrophenyl butyrate (*p*NPB), and yeast α -glucosidase were attained from Sigma-Aldrich (Germany).

Büchi Rotavapor II (Büchi, Switzerland), GC-MS 6890 series (Agilent Technologies, USA), and Synergy HTX Multi-mode Reader (BioTek Instruments Inc, USA) were used.

2.2 | Tea sample

Commercial red honeybush tea bags (young leaf tips of organic honeybush; manufacturer: SPAR Group Ltd) were obtained from a local food shopping mall at Durban, South Africa.

2.3 | Preparation of sequential extracts

Fifty gram of honeybush tea from tea bags were sequentially extracted with dichloromethane (DCM), ethyl acetate, ethanol, and water to yield the respective extracts at room temperature after 48 hr per extraction. The extracts were filtered with Whatmann (No. 1) filter paper and concentrated at 40°C under reduced pressure with a rotary evaporator except for aqueous extract. Aqueous extract was concentrated by placing in a water bath set at 50°C. All the concentrated samples were stored in glass vials at 4°C until when needed.

A stock solution of 1 mg/ml was prepared from different extracts, from which various working concentrations at 15, 30, 60, 120, and 240 μ g/ml were prepared for biological activities.

2.4 | Estimation of total phenolic content (TPC)

With the protocol described by Antolovich, Prenzler, Patsalides, McDonald, and Robards (2002) method, the TPC of sequential extracts of the tea were determined. Briefly, 200 μ l of (concentration

at 240 µg/ml) tea extracts were incubated with 1 ml of 10 times diluted Folin-Ciocalteu reagent and 800 µl of 0.7 M Na₂CO₃ at room temperature for 30 min. Then, the absorbance was measured at 765 nm. The results were indicated in milligrams of gallic acid equivalent (GAE) per gram dry weight.

2.5 | In vitro antioxidant activities

2.5.1 | Free radical scavenging (DPPH) assay

To determine the DPPH activity, the method described by (Bukhari, Simic, Siddiqui, & Ahmad, 2013) was utilized with slight modifications. Briefly, 50 µl of 0.3 mM DPPH solution (in methanol) was added to a 96-well plate contained 100 µl of each solvent extracts of various working concentrations (15–240 µg/ml), followed by incubating in the dark for 30 min at room temperature. Then, the absorbance was recorded at 517 nm for the blank without the extracts.

2.5.2 | Ferric reducing antioxidant power (FRAP) assay

The reducing power was determined via a slightly modified method described by (Ademiluyi & Oboh, 2013a). Briefly, a volume of 100 µl of each extract mixed with 100 µl of sodium phosphate buffer (0.2 M, pH 6.6) and 100 µl of 1% potassium ferricyanide were incubated at 50°C for 30 min. Then, 100 µl of 10% trichloroacetic acid was used for the acidification of reaction mixture followed by 100 µl of distilled water and 200 µl of 0.1% FeCl₃ were sequentially added. The absorbance was recorded at 700 nm. The standard ascorbic acid was utilized in this assay.

2.5.3 | Nitric oxide (NO) radical scavenging assay

Nitric oxide radical scavenging activity was determined via a slightly modified method as described by Balakrishnan, Panda, Raj, Shrivastava, and Prathani (2009). Briefly, 100 µl of 10 mM sodium nitroprusside in phosphate buffered saline was added to 100 µl of each tea extract at various working concentrations (15–240 µg/ml) and incubated at 37°C for 2 hr. Then, a 100 µl of Griess reagent was added. Sample without tea extract was used as a control, while quercetin was used as the standard drug. A 150 µl of the reaction mixture was then transferred to a 96-well plate and read at 546 nm.

2.6 | Inhibitory activities of sequential extracts

2.6.1 | α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity was determined according to a previously published method with slight modifications (Ademiluyi &

Oboh, 2013b). Briefly, a volume of 250 µl of each tea extract or acarbose at different working concentrations (15–240 µg/ml) was mixed with 500 µl of 1.0 U/ml α-glucosidase solution in phosphate buffer (100 mM, pH 6.8) and incubated at 37°C for 15 min. Then, a volume of 250 µl of pNPG solution (5 mM) dissolved in the same phosphate buffer was added. The reaction mixture was further incubated at 37°C for another 20 min. The absorbance was recorded at 405 nm and the inhibitory activity was expressed as percentage of the control without the inhibitors.

2.6.2 | Pancreatic lipase inhibitory activity

The inhibitory activity of pancreatic lipase was determined according to an earlier reported method (Kim et al., 2010). Briefly, a solution of porcine pancreatic lipase (2.5 mg/ml) was mixed with 10 mM MOPS (morpholinepropanesulphonic acid) and 1 mM pH 6.8 EDTA to prepare an enzyme containing buffer. A volume of 100 µl of each solvent tea extracts at the test concentration (15–240 µg/ml) was mixed with 169 µl of Tris buffer (100 mM Tris-HCl and 5 mM CaCl₂, pH 7.0). Then, 20 µl of the prepared enzyme buffer was added to the mixture and incubated for 15 min at 37°C. A 5 µl of the substrate solution (10 mM pNPB in dimethyl formamide) was added and further incubated at 37°C for 30 min. The lipase inhibitory activity was determined by recording the hydrolysis of p-NPB to *p*-nitrophenol at 405 nm.

2.7 | Ex vivo antioxidative studies

2.7.1 | Induction of tissue oxidative stress and treatment

Induction of isolated pancreatic tissue oxidative stress was conducted as described by Fraga, Leibovitz, and Tappel (1988) with slight modification. Briefly, a 100 µl aliquot from each tea solvent extract at test concentrations (15–240 µg/ml) was incubated with a reaction mixture containing 100 µl of the pancreatic homogenates and 30 µl of 0.1 mM FeSO₄ in an incubator at 5% CO₂ and 37°C for 30 min. A reaction mixture without tea extract or standard drug served as a negative control (untreated). Ascorbic acid was utilized as a standard drug.

Animal tissues were handled in accordance with the approved protocol of the Animal Ethical Committee of the University of KwaZulu-Natal, Durban, South Africa (protocol number: AREC/020/019D).

2.7.2 | Determination of superoxide dismutase (SOD) enzyme activity

The SOD enzyme activity of each tea extracts was measured according to a previously published method described by Kakkar, Das, & Viswanathan (1984) with slight modifications. Briefly, a volume of

170 μl of 0.1 mM diethylenetriaminepentaacetic acid (DETAPAC) and 15 μl of the incubated samples were added in a 96-well plate. Then, the reaction mixture was mixed with 15 μL of freshly prepared 1.6 mM 6-hydroxydopamine (6-HD) and gently tapping four sides of the 96-well plate quickly. The absorbance was measured for 3 min at 1 min interval at 492 nm.

2.7.3 | Measurement of lipid peroxidation

Lipid peroxidation was determined according to an earlier method with slight modifications described by Fraga et al. (1988). Briefly, a volume of 200 μl of the incubated samples was mixed with 200 μl of 8.1% SDS solution, 750 μl of 20% acetic acid, 2 ml of 0.25% thiobarbituric acid (TBA) solution, and 850 μL of milliQ water. Then, the reaction mixture was boiled in a water bath for 1 hr. After cooling, 200 μl of the reaction mixture was transferred to a 96-well plate. The absorbance was determined at 532 nm.

2.7.4 | Determination of catalase (CAT) activity

The CAT activity was measured according to the method of Aebi (1984) with slight modification. Briefly, 10 μl of the incubated samples was mixed with 340 μl of sodium phosphate buffer (50 mM, pH 7.0). Then, 150 μl of 2 M hydrogen peroxide (H_2O_2) was added. The absorbance of the reaction mixture was determined at 240 nm for 3 min at 1 min interval.

2.7.5 | Determination of Reduced Glutathione (GSH) concentration

The reduced GSH level activity was determined according to the method described by Ellman (1959). Briefly, 300 μl of the incubated samples was mixed with 100 μl 10% TCA and then, centrifuged for 10 min at 15,000 rpm. Then, 80 μl of the supernatants was collected into a 96-well plate, 40 μl of 0.5 mM DTNB, and 200 μl sodium phosphate buffer (0.2 M, pH, 7.8) were sequentially added. The absorbance was recorded at 415 nm after incubation for 15 min.

2.8 | GC-MS analysis of the different tea extracts

The phytochemical constituents of the tea extracts were analyzed using GC-MS. The analysis was carried out with an Agilent technologies 6.90 Series GC coupled with (an Agilent) 5973 Mass Selective detector and driven by Agilent Chemstation software (Agilent Technologies, USA). A HP-5MS capillary column (30 m \times 0.25 mm ID, 0.25 μm film thickness, 5% phenylmethylsiloxane) was utilized. The ultra-pure helium was utilized as a carrier gas at a flow rate of 1.0 ml/min and linear velocity of 37 cm/sec. The injector and oven

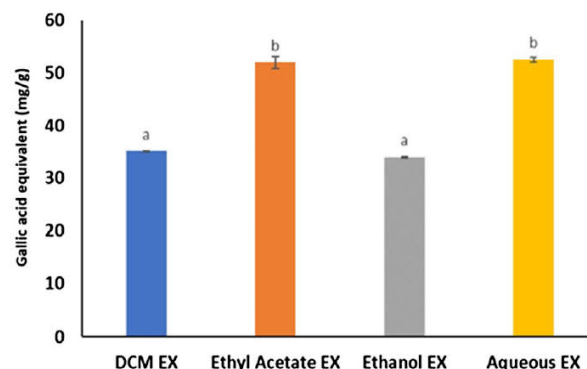


FIGURE 1 Total phenol content of different extracts of red honeybush (*C. genistoides*) tea. Values represent mean \pm SD; $n = 3$. ^{ab}Different alphabets over the bars for a given extract represent significance of difference ($p < .05$). DCM, dichloromethane; EX, extract

temperature were set at 250°C and 60°C, respectively. The oven was programed to 280°C at the rate of 10°C/min with a hold time of 3 min. Injections of 1 μl test sample were made in splitless mode. The mass spectrometer was operated in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230°C, quadrupole temperature 150°C, solvent delay 4 min, and scan range 50–70 amu. Compounds were identified by direct comparison of their mass spectral data with those in the NIST library.

2.9 | Statistics

Data are presented as mean \pm standard deviation (SD). Statistical analysis was performed using IBM Statistical Pack for the Social Sciences (SPSS) for Windows, version 25.0 (IBM Corp, Armonk, NY, USA) using Tukey's-HSD multirange *post hoc* test. The significant difference was established by one-way analysis of variance (ANOVA) at $p < .05$.

3 | RESULTS

3.1 | Total phenolic content

The TPC of the extracts are presented in Figure 1. The aqueous and ethyl acetate extracts showed the highest phenol content compared to the ethanol and DCM extracts with significant difference.

3.2 | In vitro antioxidant activity

The results of the DPPH free radical scavenging activities of the extracts are presented in Figure 2a. All the extracts showed a dose-dependent activity, where the aqueous showing the most

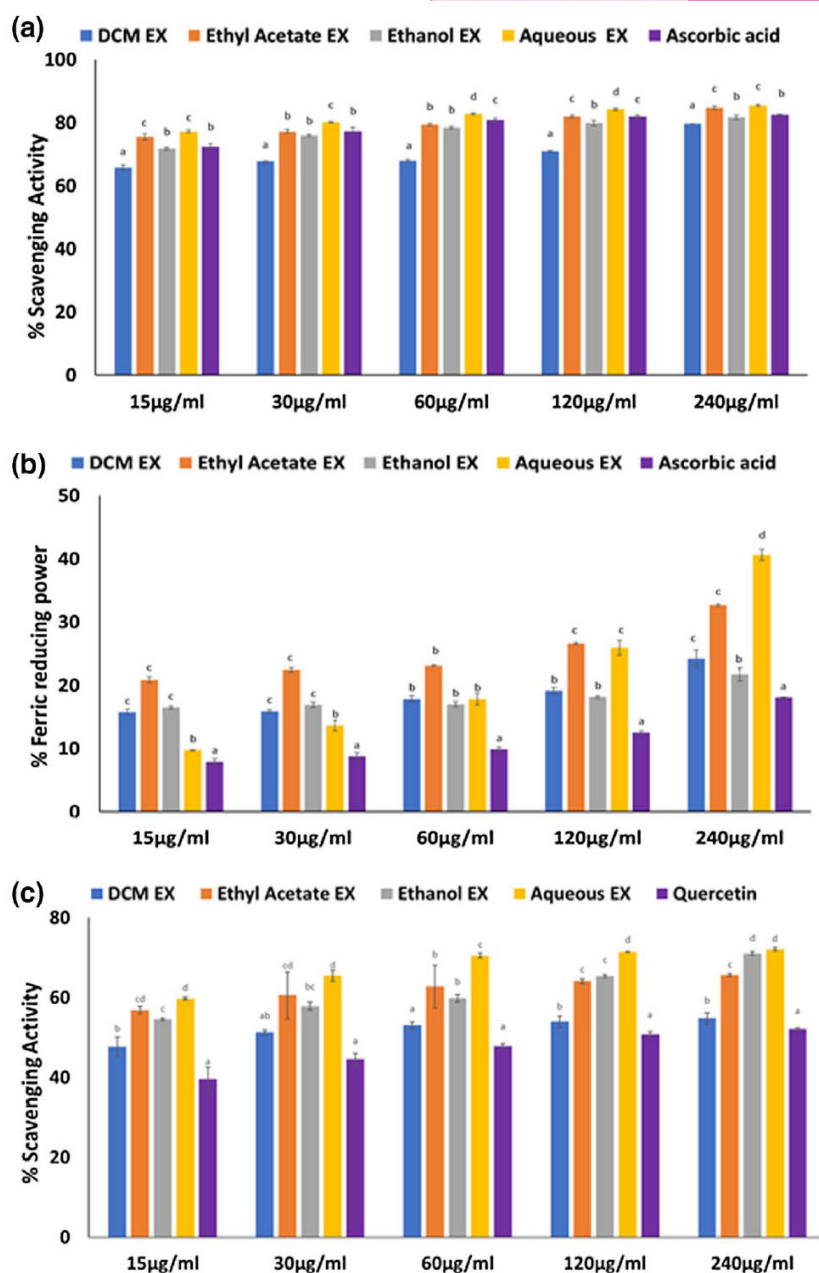


FIGURE 2 (a) DPPH scavenging activity; (b) Ferric reducing antioxidant power (FRAP); and (c) NO scavenging activity of different extracts of red honeybush (*C. genistoides*) tea. Values represent mean \pm standard deviation ($n = 3$). ^{a-d}Different alphabets over the bars for a given concentration for each extract represent significance of difference ($p < .05$). DCM, dichloromethane; EX, extract

potent activity ($IC_{50} = 0.12 \mu\text{g/ml}$). The activity of aqueous, ethyl acetate ($IC_{50} = 0.72 \mu\text{g/ml}$), and ethanol ($IC_{50} = 1.90 \mu\text{g/ml}$) extracts were comparable to the positive control, ascorbic acid ($IC_{50} = 1.93 \mu\text{g/ml}$). The aqueous extract exhibited a significantly higher ($p < .05$) activity than other extracts except at concentration of $240 \mu\text{g/ml}$ (Figure 2a).

The reducing power of the extracts are presented in Figure 2b. All the extracts showed a dose-dependent activity, with the aqueous extract being the most potent as depicted by its IC_{50} value of $861.00 \mu\text{g/ml}$. The other extracts had IC_{50} values over $1,000 \mu\text{g/ml}$. However, the reducing power of all extracts were

significantly higher ($p < .05$) than the ascorbic acid standard with the aqueous extract showing significantly higher ($p < .05$) activity than the other extracts at concentration of 120 and $240 \mu\text{g/ml}$ (Figure 2b).

The NO radical scavenging activity of the extracts are presented in Figure 2c. All the extracts showed a dose-dependent activity and a significantly ($p < .05$) higher activity against NO-radicals than quercetin, with the aqueous extract showing the highest activity compared to other extracts except at the concentration of $240 \mu\text{g/ml}$ as depicted in Figure 2c. The ethyl acetate extract showed the most efficient activity, as depicted by its IC_{50} value of $1.07 \mu\text{g/ml}$

TABLE 1 IC₅₀ values of different extracts of red honeybush (*Cyclopia genistoides*) tea

Activities	Dichloromethane Extract (µg/ml)	Ethyl Acetate Extract (µg/ml)	Ethanol Extract (µg/ml)	Aqueous Extract (µg/ml)	Ascorbic acid (µg/ml)	Quercetin (µg/ml)	Acarbose (µg/ml)	Orlistat (µg/ml)
DPPH	6.13 ± 0.30	0.72 ± 0.61	1.90 ± 0.53	0.12 ± 0.30	1.93 ± 0.70	-	-	-
FRAP	>1,000 ± 0.64	>1,000 ± 0.29	>1,000 ± 0.48	861 ± 0.80	>1,000 ± 0.36	-	-	-
NO	24.64 ± 2.45	1.14 ± 2.62	8.01 ± 0.63	1.07 ± 0.55	-	116.65 ± 1.24	-	-
α-glucosidase	91.70 ± 4.69	63.26 ± 0.88	90.12 ± 1.41	59.05 ± 1.70	-	-	0.12 ± 1.08	-
Anti-lipase	9.01 ± 0.32	6.29 ± 2.23	4.84 ± 2.18	5.86 ± 3.16	-	-	-	0.15 ± 0.98
GSH	552.85 ± 5.12	105.95 ± 2.28	132.71 ± 5.2	87.32 ± 2.04	3.9 ± 3.17	-	-	-
SOD	5.83 ± 3.11	3.85 ± 2.05	4.74 ± 2.17	1.28 ± 2.08	2.96 ± 3.09	-	-	-
Catalase	>1,000 ± 5.01	>1,000 ± 2.33	>1,000 ± 5.25	>1,000 ± 5.07	>1,000 ± 5.45	-	-	-
LPO	12.67 ± 0.90	10.36 ± 0.75	12.42 ± 0.96	9.23 ± 2.35	7.87 ± 2.12	-	-	-

compared to that of the positive control, quercetin (116.65 µg/ml) (Table 1).

3.3 | Carbohydrate digesting enzymes inhibitory activity

The results of α-glucosidase inhibitory activity of extracts are presented in Figure 3. All the extracts significantly ($p < .05$) inhibited the activities of α-glucosidase in a dose-dependent manner, with the aqueous extract showing the highest activity except significantly lower than acarbose standard (Figure 3). The pancreatic lipase inhibitory activity of all extracts is shown in Figure 4. All extracts inhibited lipase activity in a dose-dependent manner. However, the activities were significantly lower ($p < .05$) than the orlistat standard (Figure 4).

3.4 | GSH level

The induction of oxidative injury in the pancreatic tissue with FeSO₄ caused significant ($p < .05$) decrease in the levels of reduced GSH as depicted in Figure 5a. Treatment with the aqueous and ethyl acetate extracts exhibited a significant increase in a dose-dependent effect, where the aqueous extract showing a better activity and lower IC₅₀ value (Table 1).

3.5 | SOD activity

There was a significant ($p < .05$) reduction in the SOD activity on induction of oxidative injury in the untreated pancreatic tissues. Treatment with the different extracts led to a dose-dependent restorative effect, with the aqueous extract exhibiting the highest activity compared to other treatments (Figure 5b) as depicted by its low IC₅₀ value in Table 1.

3.6 | Catalase activity

As shown in Figure 5c, induction of oxidative stress in the untreated pancreatic tissues led to decreased CAT activity. The activity of the honeybush tea extracts treated groups was significantly ($p < .05$) reversed.

3.7 | Lipid peroxidation

The inhibitory effect of the honeybush tea extracts on Fe²⁺-induced lipid peroxidation in isolated pancreatic tissue is presented in Figure 5d. There was an elevated level of MDA following the induction of oxidative injury. All extracts significantly reduced the MDA levels, with the aqueous extract being the most potent but exhibited

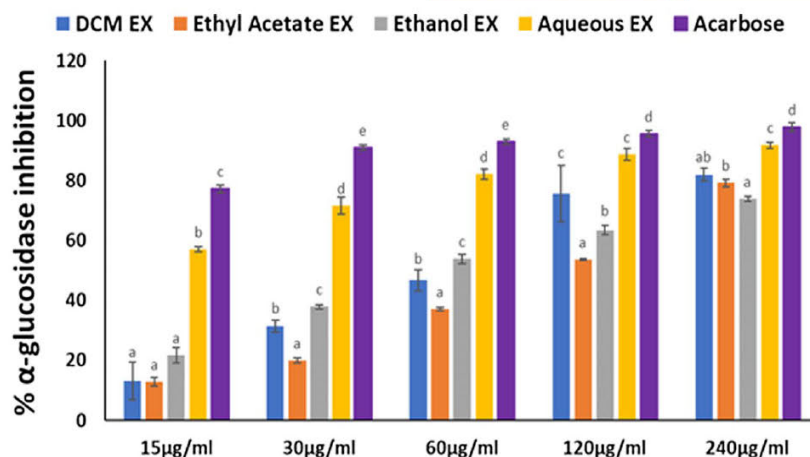


FIGURE 3 Inhibitory effect of different extracts of red honeybush (*C. genistoides*) tea on α -glucosidase activity. Data are presented as mean \pm SD. ^{a-c}Different alphabets over the bars for a given concentration are significantly ($p < .05$) different from each other. DCM, dichloromethane; EX, extract

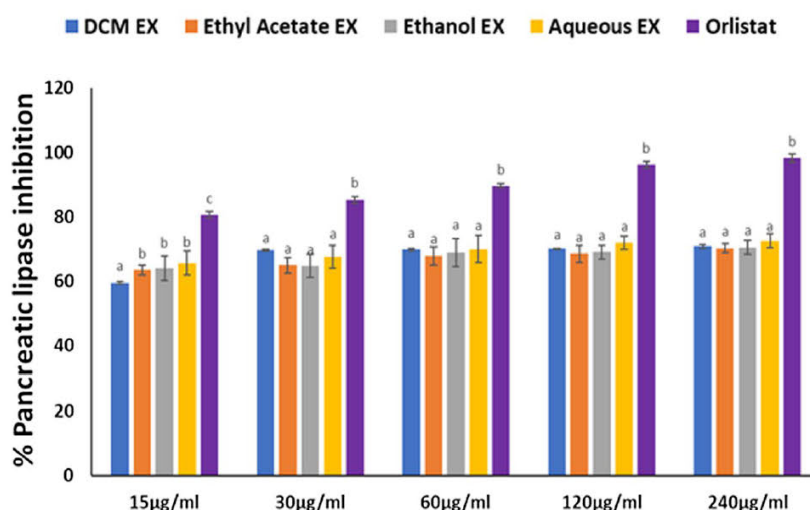


FIGURE 4 Inhibitory effect of different extracts of red honeybush (*C. genistoides*) tea on pancreatic lipase activity. Data are presented as mean \pm SD. ^{a-c} Different alphabets over the bars for a given concentration are significantly ($p < .05$) different from each other. DCM, dichloromethane; EX, extract

less activity compared to that of ascorbic acid as depicted by their IC_{50} values in Table 1.

3.8 | Gas chromatography–mass spectrometric (GC–MS) analysis

The identification of some major bioactive compounds of different honeybush tea extracts were carried out using GC–MS analysis. The retention time (RT), molecular formula, molecular mass, and relative abundance are shown in Table 2 and Figure 6. The structures of the possible compounds presented in Figure 6, 19 phytochemicals were identified from the different extracts, including seven major classes of compounds: fatty acid (*n*-hexadecanoic acid, *cis*-6-Octadecenoic acid, 4-hydroxybenzoic acid, shikimic acid), fatty acid ester (propanoic acid, 2-methylpropyl ester, hexadecanoic acid methyl ester, 9,12-octadecadienoic acid, methyl ester, 9,12-Octadecadienoic

acid (Z,Z)-,2-hydroxy-1-(hydroxymethyl)ethyl ester, pentadecyl acrylate), phytols (*n*-nonadecanol-1, tyrosol, phytol, 3-*n*-butylthiolane), sterols (sitosterol, vitamin E (α -tocopherol)), saccharide (3-O-Methyl-D-glucose), ketones (4-(4-hydroxyphenyl)butan-2-one, 4-(4-hydroxy-3-methoxyphenyl)-2-butanone), and triterpenes (stigmastan-3,5-diene).

4 | DISCUSSION

The global prevalence of diabetes has reached a record high and continues to rise. This has prompted the search for cost-effective novel antidiabetic agents. Traditional medicinal plants which have been employed for therapeutic purposes since time immemorial have emerged as readily available options providing templates for development of antidiabetic pharmacological agents. The therapeutic nature of plants emanates from groups of secondary metabolites

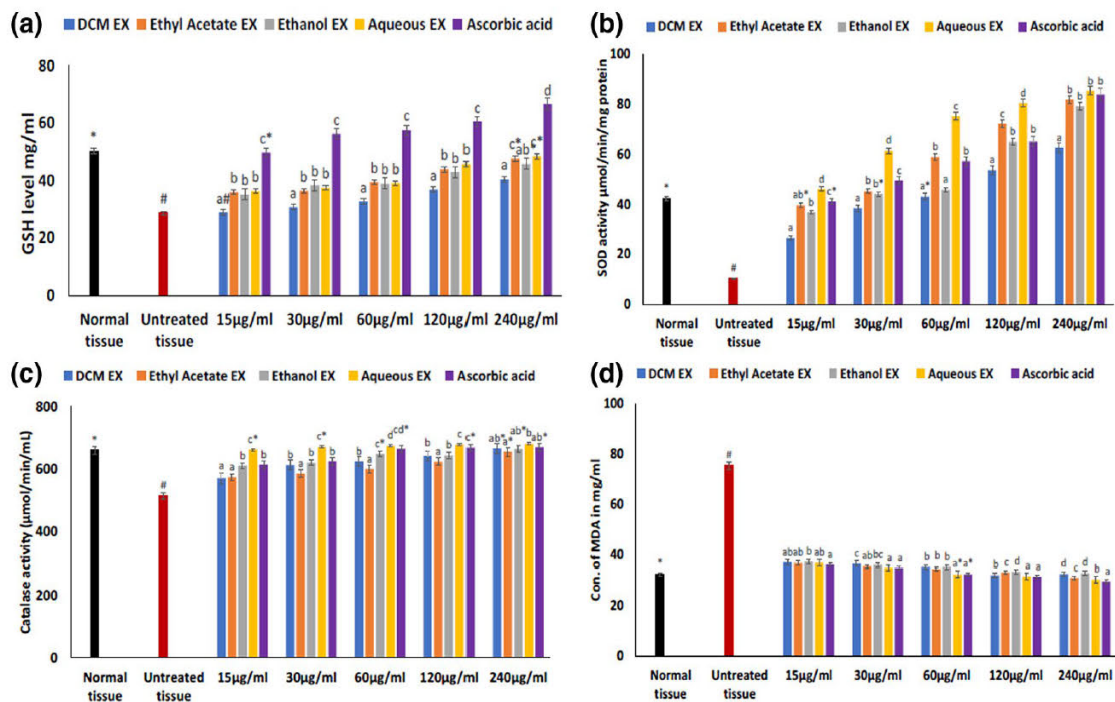


FIGURE 5 Effect of red honeybush (*C. genistoides*) tea on (a) GSH level, (b) SOD activity, (c) catalase activity, and (d) MDA level in oxidative pancreatic injury. Values = mean \pm SD; $n = 3$. *Significantly different from untreated sample and #significantly ($p < .05$) different from normal sample. ^{a-d} Different alphabets over the bars for a given concentration are significantly ($p < .05$) different from each other. DCM, Dichloromethane; EX, extract

they contain such as terpenoids, saponins, alkaloids, and polyphenols. Polyphenols are well-known for their beneficial health effects (Bazzano et al., 2002; Wang, Hu, & Xiao, 2008). They mainly act as antioxidants due to the presence of hydroxyl substituents and aromatic ring structures (Kefalas, Kallithraka, Parejo, & Makris, 2003). The moderately high total phenol contents of the ethyl acetate and aqueous extracts the tea (Figure 1), depicts the presence of phenolics in the tea, which insinuates its beneficial health properties.

The ability of the different extracts of honeybush tea to scavenge free radicals was determined using the DPPH method. This colorimetric assay is based on the color change that occurs when an unstable form of the DPPH (hydrazyl-purple) is converted to a more stable form (hydrazine-yellow). This conversion is facilitated by the coupling of the lone pair of electrons in this synthetic free radical to those from an antioxidant compound (Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007). In vitro DPPH analysis of the different solvent extracts (Figure 2a and Table 1) showed dose-dependent DPPH scavenging activity. The ethyl acetate and aqueous extracts exhibited good DPPH radical scavenging activity with IC_{50} values of 0.72 µg/ml and 0.12 µg/ml, respectively. These extracts marginally outperformed ascorbic acid (positive control) which had an IC_{50} value of 1.93 µg/ml.

Furthermore, the antioxidant capacity of the extracts in terms of their electron donating potential was analyzed by the FRAP assay (Benzie & Strain, 1996). In this assay, extracts that are capable of electron donation result in the formation of a Prussian colored ferric-ferrocyanide complex (Vijayalakshmi & Ruckmani, 2016). The deepness of the resulting blue solution is directly proportional to the electron

donating ability of the extract. The aqueous extract resulted in the best ferric ion reducing activity with an IC_{50} of 861 µg/ml while the rest had IC_{50} values greater than 1,000 µg/ml including ascorbic acid (positive control) (Figure 2b). Both the DPPH and FRAP are robust routine antioxidant assays that are used to determine the hydrogen and electron donating capabilities of plant-derived extracts. Previous studies have shown a high level of correlation between the amount of TPC and antioxidant activity (Oyebode et al., 2018; Xiao, Erukainure, Sanni, Koorbanally, & Islam, 2020). A similar trend was observed in the current study where the aqueous extract which had the highest TPC had the best activity in both the DPPH and FRAP assays. Phenolic compounds are rich in delocalized electrons on their π ring structure that can be readily donated especially to unstable electron deficient radicals. The enhanced antioxidant activity of the aqueous extract observed in this study corroborates previous reports on the antioxidant activities of fermented *C. genistoides* (Joubert et al., 2008). Exogenous antioxidants help supplement the endogenous antioxidant capacity within the body thereby aid to prevent the harmful effects of oxidative stress.

Under conditions of oxidative stress, there is overproduction of NO which is an important secondary messenger under homeostatic conditions. The excess NO may subsequently interact with superoxide anion to form a much more powerful oxidant peroxynitrite ($ONOO^-$), which is implicated in the induction β cell apoptosis (Oyadomari et al., 2001; Pacher, Beckman, & Liaudet, 2007). Moieties that retard the production of excess NO may, therefore, play a protective function in the afore-mentioned β cell destruction. Interestingly, all the extracts assayed in the current study showed

TABLE 2 Compounds identified in different extracts of red honeybush (*Cyclopia genistoides*) tea by GC-MS

Compounds	Molecular formula	Retention Index	Molecular mass	Relative abundance (%)
DCM Extract				
Propanoic acid, 2-methylpropyl ester	C ₇ H ₁₄ O ₂	5.05	130	1.94
<i>n</i> -Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	18.82	256	8.80
Hexadecanoic acid methyl ester	C ₁₈ H ₃₆ O ₂	19.13	284	0.68
<i>n</i> -Nonadecanol-1	C ₁₉ H ₄₀ O	20.02	284	0.49
<i>cis</i> -6-Octadecenoic acid	C ₁₈ H ₃₄ O ₂	20.54	282	10.10
4-(4-hydroxyphenyl)butan-2-one	C ₁₀ H ₁₂ O ₂	14.32	164	0.33
4-(4-hydroxy-3-methoxyphenyl)-2-butanone	C ₁₁ H ₁₄ O ₃	15.46	194	0.73
Phytol	C ₂₀ H ₄₀ O	20.25	296	0.48
Vitamin E (α -tocopherol)	C ₂₉ H ₅₀ O ₂	28.31	430	0.85
sitosterol	C ₂₉ H ₅₀ O	30.21	414	0.87
Ethyl Acetate Extract				
<i>n</i> -Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	18.82	256	11.33
<i>n</i> -Nonadecanol-1	C ₁₉ H ₄₀ O	19.15	284	1.89
9,12-Octadecadienoic acid, methyl ester	C ₁₉ H ₃₄ O ₂	20.49	294	17.52
<i>cis</i> -6-Octadecenoic acid	C ₁₈ H ₃₄ O ₂	20.54	282	13.71
Pentadecyl acrylate	C ₁₇ H ₃₂ O ₂	21.93	268	1.09
tyrosol	C ₈ H ₁₀ O ₂	12.70	138	1.58
4-hydroxybenzoic acid	C ₇ H ₆ O ₃	13.64	138	0.45
4-(4-hydroxyphenyl)butan-2-one	C ₁₀ H ₁₂ O ₂	14.33	164	0.37
4-(4-hydroxy-3-methoxyphenyl)-2-butanone	C ₁₁ H ₁₄ O ₃	15.46	194	0.36
Phytol	C ₂₀ H ₄₀ O	20.25	296	0.46
stigmastan-3,5-diene	C ₂₉ H ₄₈	28.14	396	0.76
Vitamin E (α -tocopherol)	C ₂₉ H ₅₀ O ₂	28.32	430	0.64
Ethanol Extract				
Shikimic acid	C ₇ H ₁₀ O ₅	17.02	174	1.21
3-O-Methyl-D-glucose	C ₇ H ₁₄ O ₆	16.04	194	2.38
<i>n</i> -Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	18.81	256	12.12
6-Octadecenoic acid	C ₁₈ H ₃₄ O ₂	20.54	282	17.18
9,12-Octadecadienoic acid (Z,Z)-,2-hydroxy-1-(hydroxymethyl)ethyl ester	C ₂₁ H ₃₈ O ₄	25.11	354	1.19
Aqueous Extract				
3- <i>n</i> -Butylthiolane	C ₈ H ₁₆ S	15.85	144	2.14
3-O-Methyl-D-glucose	C ₇ H ₁₄ O ₆	15.925	194	2.19
<i>n</i> -Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	18.82	256	9.80
9,12-Octadecadienoic acid, methyl ester	C ₁₉ H ₃₄ O ₂	20.51	294	22.76
<i>cis</i> -6-Octadecenoic acid	C ₁₈ H ₃₄ O ₂	20.55	282	13.94
9,12-Octadecadienoic acid (Z,Z)-,2-hydroxy-1-(hydroxymethyl)ethyl ester	C ₂₁ H ₃₈ O ₄	25.11	354	3.40

Note: The compounds presented in the table are those which matched similar compounds in the NIST library software.

remarkable NO production inhibition capabilities (Figure 2c and Table 1), especially the ethyl acetate and aqueous extract with IC₅₀ values of 1.143 μ g/ml and 1.067 μ g/ml.

Frequent consumption of high carbohydrate or high glycemic index foods such as refined grains and processed sugary drinks has been implicated as a major risk of diabetes (Willett, Manson,

& Liu, 2002). Ingested dietary complex carbohydrates are successively degraded into simpler forms by an array of glycosidic bond cleaving enzymes. The α -glucosidase enzyme is one such enzyme that is located on the intestinal cell membrane where it facilitates the final hydrolysis of oligosaccharides, trisaccharides, and disaccharides to various monosaccharides including glucose. Absorption of

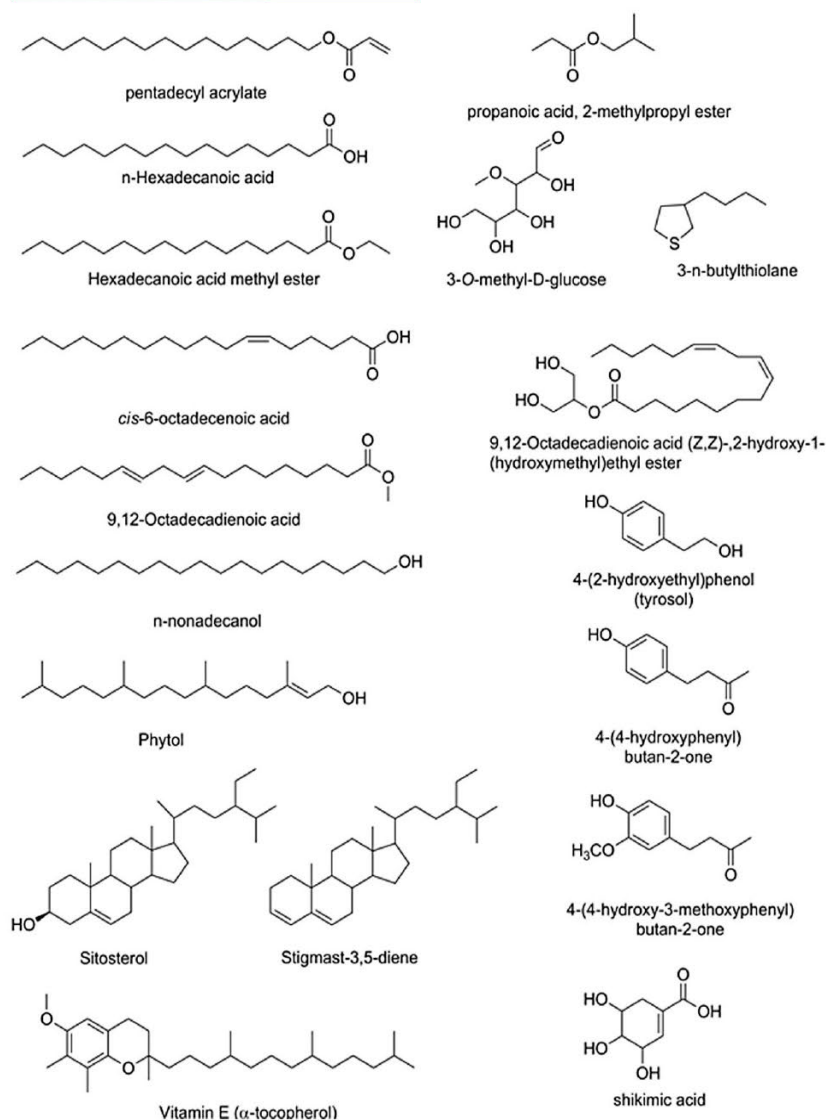


FIGURE 6 Chemical structures of compounds identified in the DCM, ethyl acetate, ethanol, and aqueous extract of red honeybush tea (*C. genistoides*) by GC-MS. GC-MS = gas chromatography-mass spectroscopy

these simple sugars is responsible for the postprandial blood glucose spikes observed after a carbohydrate rich meal. In the current study among all extracts assayed, the aqueous extract of honeybush tea (Figure 3), showed the best α -glucosidase inhibitory activity with an IC_{50} value of 59.05 μ g/ml (Table 1). However, the extract did not surpass the inhibitory activity of acarbose (positive control) which had an IC_{50} value of 0.12 μ g/ml. The significant difference in inhibitory activity between the aqueous extract of honeybush tea and acarbose may be attributed to the former being a crude mixture of compounds while the latter is a pure compound. Identification and isolation of the active compound in the honeybush tea may help to enhance its activity. Compounds with α -glucosidase inhibitory activity have been recognized as potent antidiabetic agents, because they can effectively retard the digestion of complex carbohydrates and their subsequent absorption, thereby reducing the postprandial blood glucose spikes (Hara & Honda, 1990). Such activity has been shown in previous studies (Beelders et al., 2014). Thus, persuades us to suggest that the ethyl acetate and aqueous extracts of honeybush

tea can be exploited as potent candidates for isolation of α -glucosidase inhibitors which might be effective as therapeutic agents against T2D. The results in the present study further corroborates previous reports on the ability of honeybush teas to inhibit α -glucosidase activities in vitro (Ajuwon, Ayeleso, & Adefolaju, 2018; Miller, Malherbe, & Joubert, 2020).

The excessive intake of calories in the diet together with a sedentary lifestyle may cause obesity. Obesity is the accumulation of body fat causing one to be overly overweight and is associated with several negative health effects. Dietary triglycerides serve as a high-calorie energy source for the body but are often not utilized and are, therefore, usually stored as fats in the adipose tissue (Rial, Karelis, Bergeron, & Mounier, 2016). Pancreatic lipase is an enzyme that catalyzes the hydrolysis of triglycerides in the gastrointestinal tract. Therefore, inhibiting pancreatic lipase activity can prevent triglyceride absorption, thus contributes to the management of obesity and its related diseases. In the current study, it was shown that the ethanol extract of honeybush tea had the highest pancreatic lipase

inhibitory activity (Table 1), followed by the aqueous extract with IC_{50} values of 4.84 $\mu\text{g/ml}$ and 5.86 $\mu\text{g/ml}$, respectively. Thus, corroborating previous reports on the *in vitro* antiobesogenic activity of honeybush tea (Raaths, 2016).

Oxidative stress occurs when the bodies antioxidant defense systems are overwhelmed by the amount of reactive species being produced during normal tissue metabolism. Diabetes is known to aggravate the extent to which oxidative stress occurs in tissues throughout the body. However due to the low expression of antioxidant enzymes such as CAT and SOD in the pancreatic tissues, β cells are particularly prone to damage by oxidative stress (Limón-Pacheco & Gonsebatt, 2009). In the current study, FeSO_4 was employed as a prooxidant as it is known to generate substantial amounts of oxidized free radicals through the Haber-Weiss reaction (Schulze & Hu, 2005). This reaction was employed to mimic conditions of elevated oxidative stress that is particularly observed in diabetic patients. The incubation of the pancreatic tissue homogenate with FeSO_4 in the current study was shown to significantly decrease of GSH levels and SOD, CAT enzyme activities while concomitantly the increasing of lipid peroxidation with respect to the untreated group (Figure 5a-d). This observation coincides with previous studies on the effects of oxidative stress on antioxidant enzyme activity and lipid peroxidation (Erukainure, Mopuri, Oyeboode, Koorbanally, & Islam, 2017; Sanni, Erukainure, Oyeboode, Koorbanally, & Islam, 2018).

GSH is an important antioxidant in the body, it serves as a direct free-radical scavenger, as a substrate for GSH peroxidase activity, and as a cofactor for many enzymes (Aslani & Ghobadi, 2016). The increased level following treatments with the extracts (Figure 5a) therefore, depicts an improved antioxidant activity.

Superoxide dismutase (SOD) is among the endogenous antioxidant enzymes. SOD consists of active protein peptides with metal ion groups which can specifically catalyzes the disproportionation reaction of superoxides generated in the metabolism to hydrogen peroxide (Mirończuk-Chodakowska, Witkowska, & Zujko, 2018). The products of this reaction are effectively decomposed to water and oxygen in a reaction catalyzed by CAT (Raza et al., 2017). The marked increase in the activity of CAT and SOD enzymes after the treatment with different concentrations (Figures 5b-d) demonstrated the antioxidant activity of honeybush tea solvent extracts.

Lipid peroxidation is a multistep chain of reactions which includes initiation, propagation, and termination in which oxidants attack the polyunsaturated fatty acids. Various secondary products such as malondialdehyde (MDA) are formed in this reaction. Reaction of MDA and thiobarbituric acid is routinely used to spectrophotometrically to determine lipid peroxidation (Halliwell & Chirico, 1993). In the current study, there is significant reduction of lipid peroxidation after the treatment with different concentrations of honeybush tea, shows the antioxidant potential of the aqueous and ethyl acetate extracts (Figure 5d). As lipids are the basic biomolecules on which membrane structure is built. They also perform other functions such as chemical messengers and as prerequisites for the cholesterol and vitamin formation. Therefore, extracts such as those from red honeybush tea that retard their destruction through peroxidation have

a therapeutic function in curbing the development of various degenerative complications that result from extreme oxidative stress conditions. These may furthermore assist the endogenous antioxidant enzymes that normally counter the harmful effects of reactive oxygen species.

These antioxidative activities by the extracts corroborates previous reports on the ability of honeybush to exacerbate GSH level, SOD, and CAT activities, while suppressing lipid peroxidation in oxidative-mediated tissue injuries (Marnewick, Joubert, Swart, van der Westhuizen, & Gelderblom, 2003; Ros-Santaella, Kadlec, & Pintus, 2020; Van der Merwe, De Beer, Swanevelder, Joubert, & Gelderblom, 2017).

GC-MS analysis of the extracts was done in order to identify the presence of possible bioactive compounds that could be responsible for the observed antioxidant and antidiabetic activities. A total of 19 phytochemicals were identified from this plant (Figure 6 and Table 2), which included seven major classes of compounds: fatty acids, fatty acid esters, ketones, sterols, phytols, saccharide, and triterpenes, many of them have been reported to possess various medicinal properties including antioxidant and antidiabetic activity (Islam et al., 2018; Orhan et al., 2012; Sosa, Bagi, & Hameed, 2016). Hexadecanoic acid, ethyl ester, and vitamin E have been reported for their potent antioxidant activities (Garg & Bansal, 2000; Kumar, Kumaravel, & Lalitha, 2010). The antidiabetic activities of 4-(4-hydroxy-3-methoxyphenyl)-2-butanone, sitosterol, and shikimic acid have also been reported (Ahmad et al., 2015; Gupta, Sharma, Dobhal, Sharma, & Gupta, 2011; Orhan et al., 2012). The high content of 9,12-octadecadienoic acid, methyl ester, and cis-6-octadecenoic acid in the aqueous extract may contribute synergistically with other identified phytochemicals to its high activities as unsaturated fatty acids and their esters have been reported for their potent antioxidant activities (Erukainure et al., 2018; Henry, Momin, Nair, & Dewitt, 2002). These identified compounds may thus be responsible for the studied antioxidative and antidiabetic activities of honeybush tea.

5 | CONCLUSION

These results demonstrate the antioxidant and antidiabetic activities of the different extracts of honeybush tea, especially the ethyl acetate and aqueous. Their antioxidative and antidiabetic potentials are evident by free radical scavenging, lipid peroxidation elimination, decrease of α -glucosidase and lipid digesting enzymes activities, as well as the increase of antioxidant enzymes activities. These activities may be attributed to the TPC and the synergistic effect of the identified phytochemicals. Further *in vivo* studies in diabetic models are however recommended to decipher the molecular mechanism by which these extracts exhibit their antidiabetic effects.

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CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

AUTHOR CONTRIBUTION

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Conceptualization; Methodology; Writing-review & editing: Beseni

Data curation; Investigation; Writing-review & editing: Koorbanally

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