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**Targeting selected diabetes-associated risks of atherogenesis
with *Bauhinia bowkeri* stem bark extracts**

By

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Submitted in fulfilment for the Master of Science by research
in the Faculty of Natural and Agricultural Sciences, Department of
Biochemistry, Genetics and Microbiology

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DEDICATION

I dedicate this thesis to myself and my future self, my closest friends and my family members.

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GENERAL ABSTRACT

Atherosclerosis is the main underlying cause of diabetes-associated cardiovascular disease mortalities. Persistent hyperglycemia upregulates other glucose metabolising pathways such as polyol and protein glycation pathways. These pathways contribute to the AGEs formation and ROS production. AGEs and ROS can modify key proteins, including low-density lipoprotein (LDL). LDL's oxidative modification is the major step in atherosclerosis pathogenesis. This study searched the potential of *Bauhinia bowkeri* extracts (hexane, dichloromethane and methanol) to inhibit the polyol pathway and LDL modification *in vitro*. The *in vitro* aldose reductase (ALR) and sorbitol dehydrogenase (SDH) inhibition assays were used to investigate the extracts' potential to inhibit the polyol pathway activity. Their inhibitory activity on protein glycation was investigated on LDL (and BSA) using fructose and methylglyoxal as glycating agents. The extracts' antioxidant activity was determined using the *in vitro* antioxidant assays like ferric reducing power, ABTS and DPPH radical scavenging assays. Their antioxidant activity was also investigated in the copper sulphate-induced LDL peroxidation. The selected major compounds' bioactivities were investigated using the same assays. *In silico* molecular docking against ALR and SDH was performed, and their ADMET properties were also studied.

FTIR and GC-MS results indicated the presence of various compounds. The crude extracts showed high inhibitory activity on ALR with IC_{50} values ranging from 75-90.8 $\mu\text{g/ml}$, and low on SDH ($IC_{50} \geq 185 \mu\text{g/ml}$). The extracts also showed 59.6 - 77.8 % inhibitory activity on LDL glycation. In addition to high reducing potential, the extracts chelated Fe^{2+} ions and scavenged DPPH and ABTS radicals. The extracts further showed antioxidant activity on LDL oxidation with a percentage range of 13 - 74 %. While all six selected compounds showed good ADMET properties, five showed good docking scores on ALR and SDH. Monoethylhexyl phthalate showed inhibitory activity on LDL glycation and oxidation, with the highest percentage inhibitions of 43.2 % and 54.13 %, respectively. The inhibition of the polyol pathway activity, and LDL glycation and oxidation, indicates the potential of *B. bowkeri* extracts and their compounds to be used as therapeutic agents in the amelioration of diabetes associated macrovascular complications, like atherosclerosis.

RESEARCH OUTPUT

Symposium contributions (Poster presentation)

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LIST OF ABBREVIATIONS USED

AA	Arachidonic acid
AA	Ascorbic acid
AAPH	2,2'-Azobis (2-amidinopropane) dihydrochloride
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette sub-family G member 1
ABTS	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
ADMET	Absorption distribution metabolism toxicity
AGEs	Advanced glycation end products
ALR	Aldose reductase
ASCVDs	Atherosclerotic cardiovascular disease
ATP	Adenosine triphosphate
BHA	Butylated hydroxy anisole
BME	Monoethylhexyl phthalate
BSA	Bovine serum albumin
CA	Citric acid
CD	Conjugated dienes
CVD	Cardiovascular disease
DCM	Dichloromethane
DE	Dichloromethane Extract
DNTB	5,5-dithio-bis-(2-nitrobenzoic acid)
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol

FRAP	Ferric reducing antioxidant ability
FTIR	Fourier transmission infrared spectroscopy
HE	Hexane Extract
HTS	High throughput screening
ICAM-1	Intercellular Adhesion Molecule 1
IL-6	Interleukin-6
LDL	Low density lipoprotein
LOX-1	Oxidized low-density lipoprotein receptor 1
M1	2-Thiobarbituric acid
M2	Trichloroacetic acid
MCP-1	Monocyte chemoattractant protein-1
MDA	Malonaldehyde
ME	Methanol Extract
MGO	Methylglyoxal
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NF-kB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidases
ORAC	Oxygen radical absorbance capacity
OxLDL	Oxidized low density lipoprotein
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factors

PMS	Phenazine methosulphate
RAGE	Receptors of advanced glycation end products
ROS	Reactive oxygen species
SDH	Sorbitol dehydrogenase
SEM	Standard error of the mean
sPLA2	Secretory phospholipase A2
SR-A1	Class A1 scavenger receptor
SR-B1	Scavenger receptor, class B type 1
TBA	2-Thiobarbituric acid
TCA	Trichloroacetic acid
TGF- β	Transforming growth factor beta
TNBSA	2,4,6-trinitrobenzene sulfonic acid
TNF- α	Tumor necrosis factor alpha
TPZT	2,4,6-Tris(2-pyridyl)-1,3,5-triazine
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
XO	Xanthine oxidase

Table of Contents

Acknowledgements	ii
General Abstract	iii
CHAPTER 1	1
Introduction and Literature review	1
1.1 Atherosclerosis: Background and Epidemiology	1
1.2 An overview of atherogenesis biochemical events	2
1.2.1 Endothelial dysfunction	2
1.2.2 Fatty streak formation	3
1.2.3 Foam cell formation.....	3
1.2.4 Fibrous plaque formation	4
1.3 Diabetic risks of atherogenesis	4
1.3.1 Hyperglycemia-induced oxidative stress	4
1.3.2 Activation of the polyol pathway	5
1.3.3 Formation of AGEs and atherogenesis	6
1.4 Current therapeutics and their targets.....	8
1.5 Potential of medicinal plants in the management of diabetes-related atherogenic complications	9
1.5.1 <i>Bauhinia bowkeri</i>	10
1.6 Strategies and techniques for discovering new drug candidates from natural products.....	11
1.7 Study rationale	12
1.8 Aim, Hypothesis and Objectives	13
1.9 Thesis layout	14
References.....	15
CHAPTER 2	22

2.1 Materials	22
2.1.1 Chemical and reagents	22
2.2 Methods	23
2.2.1 Plant collection and preparation	23
2.2.2 Phytochemical screening	24
2.2.2.1 FTIR analysis	24
2.2.2.2 GC-MS analysis	24
2.2.2.3 Total phenolic content	24
2.2.2.4 Total flavonoid content	25
2.2.3 In vitro antioxidants assays	25
2.2.3.1 2, 2-Diphenyl-1-Picrylhydrazyl scavenging activity	26
2.2.3.2 2, 2 azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) scavenging activity	26
2.2.3.3 Hydroxyl radical scavenging activity	27
2.2.3.4 Superoxide anion scavenging activity	27
2.2.3.5 Metal ion (Fe ²⁺) chelating activity assay	28
2.2.3.6 Oxygen Radical Absorbance Capacity assay	28
2.2.3.7 Ferric Reducing Antioxidant Power	29
2.2.4 Oxidation of LDL	29
2.2.4.1 Measurement of the conjugated dienes formation	29
2.2.4.2 Measurement of Thiobarbituric Acid Reactive Substances	30
2.2.5 Enzyme inhibition assays	30
2.2.5.1 Aldose Reductase inhibition Assay	30
2.2.5.2 Sorbitol Dehydrogenase inhibition Assay	31
2.2.5.3 Secretory phospholipase A ₂ inhibition activity	32
2.2.6 Antiprotein glycation activity	32
2.2.6.1 Fructose-induced protein glycation assay	32

2.2.6.2 Fructosamine content.....	33
2.2.6.3 Free amino group content	33
2.2.6.4 Congo red binding assay	34
2.2.6.5 Methylglyoxal-induced protein glycation assay	34
2.2.7 <i>In silicon</i> molecular docking and ADMET analysis	35
2.2.7.1 Molecular docking	35
2.2.7.2 ADMET Properties	35
2.2.8 <i>In vitro</i> enzyme inhibition, antiprotein glycation and antioxidant activities of the pure compound(s).....	36
2.2.9 Data analysis	36
References.....	37
CHAPTER 3	39
Abstract.....	39
3.0 Introduction	40
3.1 Materials and Methods.....	41
3.1.1 Reagents.....	41
3.1.2 Extraction of the plant material	41
3.1.3 Phytochemical screening	41
3.1.3.1 FTIR analysis	41
3.1.3.2 GC-MS analysis	41
3.1.3.3 Total phenolic content	41
3.1.3.4 Total flavonoid content	42
3.1.4 <i>In vitro</i> antioxidants assays	42
3.1.4.1 DPPH scavenging activity	42
3.1.4.2 ABTS scavenging activity.....	42
3.1.4.3 Hydroxyl radical scavenging activity	43

3.1.4.4 Superoxide anion scavenging activity	43
3.1.4.5 Metal ion chelating activity	43
3.1.4.6 Oxygen Radical Absorbance Capacity	44
3.1.4.7 Ferric Reducing Antioxidant Power	44
3.1.5 Oxidation of LDL	44
3.1.5.1 Measurement of the conjugated dienes formation	44
3.1.5.2 Measurement of thiobarbituric acid reactive substances.....	45
3.2. Results	45
3.2.1 Extraction	45
3.2.2 Phytochemical screening	45
3.2.2.1 FTIR analysis	45
3.2.2.2 GC-MS analysis	46
3.2.2.3 Total phenolic and flavonoid contents	49
3.2.3 Antioxidant activity	50
3.2.3.1 Free radical scavenging activity of the extracts.....	50
3.2.3.2 Oxygen radical absorbance capacity of the extracts	50
3.2.3.3 Reducing power and metal ion chelating activity of the extracts	51
3.2.4 Inhibition of LDL oxidation	52
3.3 Discussion.....	53
3.4 Conclusion	55
References.....	55
CHAPTER 4	59
Abstract.....	59
4.0 Introduction	60
4.1 Materials and Methods.....	61
4.1.1 Reagents.....	61

4.1.2 Extraction of plant material.....	61
4.1.3 Enzyme inhibition activity	61
4.1.3.1 Aldose reductase inhibition assay	61
4.1.3.2 Sorbitol dehydrogenase inhibition assay	61
4.1.3.3 Phospholipase A ₂ inhibitory activity	62
4.1.4 Antiprotein glycation activity	62
4.1.4.1 Fructose-induced protein glycation assay	62
4.1.4.2 Fructosamine content assay	62
4.1.4.3 Free amino group content assay	63
4.1.4.4 Congo Red Binding Assay	63
4.1.4.5 Methylglyoxal-induced protein glycation assay	63
4.2 Results	63
4.2.1 Inhibitory potential of the extracts on aldose reductase, sorbitol dehydrogenase and secretory phospholipase A ₂ activities	63
4.2.2 Effect of the extracts on fructose-induced protein glycation	65
4.2.3 Effect of the extracts on methylglyoxal-induced protein glycation	67
4.3 Discussion.....	68
4.4 Conclusion	70
References.....	70
 CHAPTER 5	 73
Abstract	73
5.0 Introduction	74
5.1 Materials and Methods	75
5.1.1 Reagents	75
5.1.2 Selected compounds for in silico studies	75
5.1.2.1 Molecular docking	76

5.1.2.2 ADMET properties	76
5.1.3 In vitro studies of the selected compound	76
5.1.3.1 Aldose reductase and sorbitol dehydrogenase inhibition assays	77
5.1.4 Anti-glycation activity of BME	77
5.1.4.1 Fructosamine content assay	77
5.1.4.2 Free thiol group content assay	77
5.1.5 In vitro antioxidant assays	78
5.1.5.1 Free radical scavenging activity, ORAC and reducing power of the compound	78
5.1.5.2 Oxidation of LDL	78
5.2 Results	78
5.2.1 Molecular docking study	78
5.2.2 ADMET properties of the compounds	79
5.2.3 Aldose reductase and sorbitol dehydrogenase inhibition	80
5.2.4 Anti-glycation activity of <i>BME</i>	81
5.2.4.1 Inhibitory activity of <i>BME</i> against fructose-induced LDL glycation	81
5.2.4.2 Inhibitory activity of <i>BME</i> against methylglyoxal-induced LDL glycation ..	83
5.2.5 In vitro antioxidant activity of BME	84
5.2.6 Inhibition of LDL oxidation	85
5.3 Discussion	86
5.4 Conclusion	88
References.....	88
CHAPTER 6	90
6.0 General Discussion.....	90
References.....	92
CHAPTER 7	94

General Conclusion	94
7.1 Summary and Conclusions	94
7.2 Recommendations for future studies	94
APPENDIX A	95
APPENDIX B	99
APPENDIX C	101
APPENDIX D	102
APPENDIX E	103

LIST OF TABLES

Table 3.1 Percentage yields of <i>B. bowkeri</i> extracts	45
Table 3.2 Bioactive compounds of <i>B. bowkeri</i> hexane extract.....	47
Table 3.3 Bioactive compounds of <i>B. bowkeri</i> DCM extract.....	48
Table 3.4 Bioactive compounds of <i>B. bowkeri</i> methanol extract.....	49
Table 3.5 Half maximal effective concentration (EC ₅₀) values of the extracts on DPPH, ABTS, Hydroxyl and superoxide anion radicals.....	50
Table 4.1 IC ₅₀ values of the extracts on aldose reductase and sorbitol dehydrogenase activities.	64
Table 5.1 Docking scores and glide energies (Kcal/mol) of the <i>B. bowkeri</i> compounds.	79
Table 5.2 ADMET properties of the major compounds of the <i>B. bowkeri</i> extracts	80
Table 5.3 The results of the inhibitory activity of the compound on aldose reductase	81
Table 5.4 The results of the inhibitory activity of the compound on sorbitol dehydrogenase	81

LIST OF FIGURES

Figure 1.1 Schematic representation of atherogenesis	2
Figure 1.2 Schematic representation of polyol pathway	6
Figure 1.3 Schematic representation of the formation of AGEs	7
Figure 1.4 A picture of <i>Bauhinia bowkeri</i>	11
Figure 2.1 Sequential diagrammatic representation of <i>B. bowkeri</i> stem bark extraction	23
Figure 3.1 FTIR spectra of hexane extract, methanol and DCM extract of <i>B.</i> <i>bowkeri</i>	46

Figure 3.2 The Total phenolic content and Total flavonoid content of the extracts of <i>Bauhinia bowkeri</i>	49
Figure 3.3 ORAC values of the <i>B. bowkeri</i> stem bark extracts	51
Figure 3.4. Ferric reducing power and metal ion chelating activity of <i>B. bowkeri</i> extracts.	51
Figure 3.5 The effect of <i>B. bowkeri</i> extracts on LDL and HDL oxidation	53
Figure 4.1 Inhibitory activity of the extracts on aldose reductase, sorbitol dehydrogenase and secretory phospholipase A ₂ activity.	65
Figure 4.2 Inhibitory activity of the extracts on fructose-induced LDL and BSA glycation	66
Figure 4.3 Inhibitory activity of the extracts on methylglyoxal-induced BSA glycation	67
Figure 5.1 Chemical structures of the selected abundant phytochemicals in <i>B. bowkeri</i> extracts.....	76
Figure 5.2 Inhibitory activity of BME on the fructose-induced glycation on LDL....	82
Figure 5.3 Inhibitory activity of BME on the methylglyoxal induced glycation on LDL	83
Figure 5.4 The antioxidant activity of BME.....	84
Figure 5.5 The BME inhibitory effect on LDL oxidation	85

CHAPTER 1

Introduction and Literature Review

1.1 Atherosclerosis: background and epidemiology

Atherosclerosis is defined as a complex inflammatory disorder characterized by the constriction of the arteries and plaque build-up consisting of lipid-laden macrophage cells, initiated by low-density lipoprotein (LDL) cholesterol accumulation in the arterial intima, which can lead to complications such as stroke and heart attack (Manish et al., 2024). The common complications of atherosclerosis include coronary artery, peripheral artery and aortic atherosclerotic disease, all commonly known as atherosclerotic cardiovascular diseases (ASCVDs). The characteristics associated with atherosclerosis are endothelial dysfunction, formation of fatty streaks, foam cell formation and fibrous plaque build-up. Atherosclerosis is associated with risk factors like physical inactivity, unbalanced diets, smoking, obesity, hypercholesterolemia and diabetes. Diabetes is a common and major independent risk factor of atherosclerosis (Michaeloudes et al., 2023). In a diabetic state, chronic hyperglycemia and hyperlipidemia directly or indirectly stimulate the atherogenic process that is clinically manifested as atherosclerosis. Chronic hyperglycemia upregulates other glucose metabolising pathways like the polyol pathway and protein glycation. These pathways produce advanced glycation end products (AGEs) and reactive oxygen species (ROS) (Papachristoforou et al., 2020). AGEs and ROS can directly glycate and oxidize LDL, which is a critical atherosclerosis step.

The prevalence of atherosclerosis is at 17.9 million worldwide deaths yearly (Azevedo et al., 2022). Atherosclerosis is the major underlying cause of diabetes-associated cardiovascular disease (CVD) mortalities (Poznyak et al., 2020). Diabetes cases which are related to atherosclerosis account for over 33% of annual deaths globally. Over 30% of diabetic patients suffer from ASCVD, and more than half of them die from it (Roman and Pantea Stoian, 2021). It is thus crucial that the pathophysiology or biochemical events of atherosclerosis be understood.

1.2 An overview of atherogenesis biochemical processes

The atherogenesis process underlying the development and progression of atherosclerosis consists of various events, namely: dysfunctional endothelium, fatty streak formation, formation of foam cells and fibrous plaque formation (Figure 1.1). Endothelial dysfunction is one of the early events that contribute to arterial vasculature degeneration (Biswas and Khan, 2020). The dysfunctional endothelium increases the arterial wall permeability and allows the infiltration of low-density lipoprotein into the tunica intima. This is followed by a chain of events that lead to fibrous plaque formation. The rupture of this plaque is coupled with thrombotic events that are responsible for myocardial infarction. A proper understanding of the biochemical events of atherogenesis and risk factors thereof could be essential in the prevention and management of atherosclerosis and associated cardiovascular events.

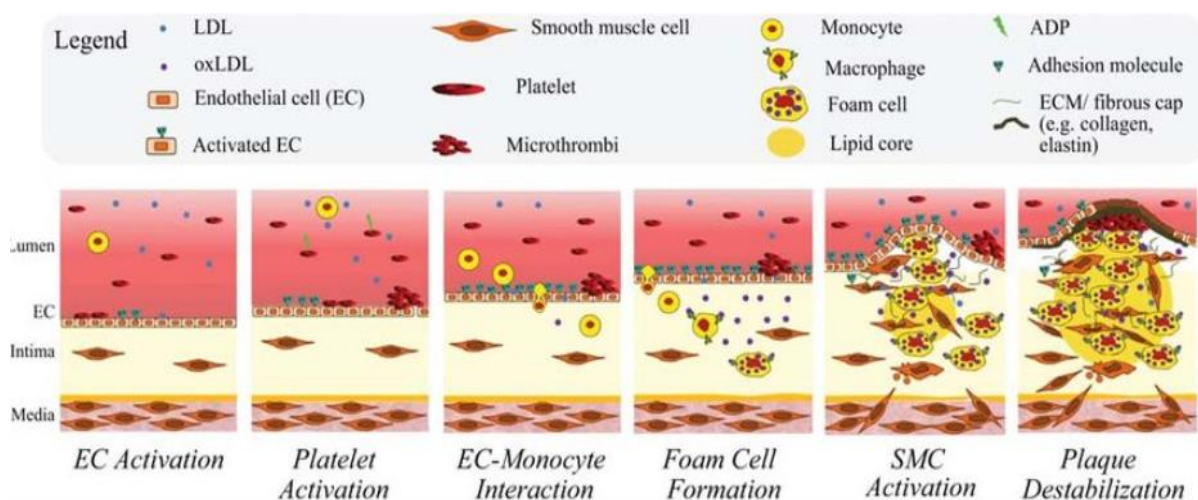


Figure 1.1: Schematic representation of atherogenesis. The endothelial cell is injured and accelerates the accumulation of oxidized LDL (oxLDL) into the tunica intima, which is engulfed by the macrophage cells and leads to foam cell formation. The Smooth Muscle Cells (SMC) are activated and generate the fibrous plaque, which can eventually rupture and lead to thrombotic events (De León et al., 2015).

1.2.1 Endothelial dysfunction

Endothelial dysfunction is described as a dysregulation in homeostasis which results in vasoconstriction and reduced vasodilation of blood vessels (Rastogi et al., 2024). It is characterized by reduced nitric oxide (NO) bioavailability, narrowing of the blood

vessels and pro-inflammatory state. Various factors like hypertension, hyperglycemia, smoking and hypercholesterolemia cause endothelial dysfunction (Scioli et al., 2020). These factors reduce the nitric oxide synthase (NOS) activity and consequent reduction in the NO production in the endothelium. These factors also produce reactive oxygen species (ROS) that stimulate the production of monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecules (VCAM-1), intercellular adhesion molecules (ICAM-1) and nuclear factor kappa-B (NF- κ B), tumour necrosis factor-alpha (TNF- α) is a major inflammatory mediator of endothelial dysfunction, which induces a pro-atherogenic environment and atherosclerotic plaque formation (Collado et al., 2021; Shirsath et al., 2021).

1.2.2 Fatty streak formation

Fatty streaks are lipid-rich containing foam cells in the innermost arterial wall. Fatty streak formation is caused by LDL accumulation and oxidation in the tunica intima. Pro-inflammatory mediators production is the initial stage of the fatty streak formation. The stimulated endothelial cells express several adhesion molecules like MCP-1, VCAM-1 and ICAM-1 which attract and facilitate the adhesion of the circulating monocytes into the endothelial space (Shirsath et al., 2021). Monocytes differentiate into macrophages. The accumulation of LDL in the intima increases the probability of LDL getting modified or oxidized (oxLDL). The anti-atherogenic effect of the macrophages detects and engulfs the oxLDL leading to foam cell formation.

1.2.3 Foam cell formation

Monocytes differentiate into pro- (M1) and anti- (M2) inflammatory macrophages. M1 macrophages further produce inflammatory cytokines, ROS, and further contribute to the propagation of monocyte recruitment and inflammatory response. Furthermore, macrophages express scavenger receptors, notably lectin-like oxidized LDL receptor 1 (LOX-1), CD36, scavenger receptor A (SRA-1), and engulf the oxLDL and lead to foam cell formation. Macrophages have SR-B1, ABCA1 and ABCG1 ATP-binding cassettes that play a role in cholesterol efflux. However, the proinflammatory environment in the macrophages impairs these transporters and leads to intracellular cholesterol accumulation and the formation of foam cells. OxLDL triggers the activity of the vascular smooth muscle cells (VSMCs) to migrate into the intima. VSMCs

express scavenger receptors that further allow the internalization of oxLDL and progression of foam cell formation.

1.2.4 Fibrous plaque formation

The rapid growth of the VSMCs causes the production of growth factors, including epidermal growth factor, insulin-like growth factor, platelet-derived growth factor (PDGF), transforming growth factor- β (TGF- β), fibroblast growth factor and vascular endothelial growth factor (VEGF) (Vacante et al., 2021). VSMCs migration and proliferation also accelerate the production of collagen, elastin and proteoglycans that take part in the generation and stabilization of a fibrous cap covering the atherosclerotic plaque and prevent its rupture (Jebari-Benslaiman et al., 2022).

1.3 Diabetic risks of atherogenesis

Chronic hyperglycemia that characterizes diabetes has been involved in micro-vascular and macro-vascular complications (Oguntibeju, 2019). Atherosclerosis is the most prevalent diabetes microvascular complication (Berbudi et al., 2020). In a diabetic state, chronic hyperglycemia and hyperlipidemia directly or indirectly stimulate the atherogenic process that is clinically manifested as atherosclerosis. Chronic hyperglycemia activates other glucose metabolizing pathways like the polyol pathway and the formation of AGEs (Mengstie et al., 2022). These pathways lead to overproduction of inflammatory mediators, ROS and decreased levels of endogenous antioxidants. The hyperglycemia-induced overproduction of ROS and inflammatory mediators consequently initiates the development and progression of atherogenesis (Agatemor et al., 2019).

1.3.1 Hyperglycemia-induced oxidative stress

Hyperglycemia induces overproduction of ROS by activating the electron transport chain enzymes. The key enzymes partaking in the production of ROS are NADPH oxidases (NOX) and xanthine oxidase (XO). The excessive ROS generation and production result in oxidative modification of lipids and proteins. These include LDL, a major protein involved in atherogenesis. The atherosclerotic lesions are initiated by an increase in oxLDL uptake by monocytes and macrophages through scavenger

receptor A1 (SR-A1), SR-B1 and CD36 (Shirsath et al., 2021). ROS alters the Apo B100 of LDL and leads to LDL modification and uptake by the macrophage cells, thus resulting in foam cell formation (Duan et al., 2023). ROS also induce the production of secretory phospholipase A₂ (sPLA₂), an enzyme involved in the metabolism of arachidonic acid. sPLA₂ releases arachidonic acid (AA) from phospholipids through catalysis. sPLA₂ further catalyses the hydrolysis of phospholipids in LDL, causing it to be proatherogenic and lead to foam cell formation and atherosclerosis (Murakami et al., 2023). Oxidative stress, being the major factor that can lead to various diabetes complications such as microvascular and macrovascular complications, it is of pivotal importance that it is prevented.

1.3.2 Activation of the polyol pathway

Hyperglycemic conditions of diabetes also activate the polyol pathway activity. This pathway comprises two biochemical reactions that are aldose reductase (ALR)-catalyzed and sorbitol dehydrogenase (SDH)-catalyzed reactions to reduce glucose to fructose. (Thakur et al., 2021). The NADPH-dependent ALR catalyzes the initial and rate-limiting step of the pathway (Figure 1.2) where glucose is reduced to sorbitol. While the accumulation of sorbitol is known to cause osmotic stress (Kazeem et al., 2020), NADPH depletion inhibits the formation of reduced glutathione (GSH). The depletion of intracellular GSH also favours increased production of ROS and oxidative modification of proteins and lipids, including LDL (Papachristoforou et al., 2020).

The product of ALR, sorbitol, further undergoes oxidation to fructose by the NAD⁺-dependent SDH. The increase in production of NADH through sorbitol oxidation to fructose promotes the production of powerful glycosylating agents, methylglyoxal and 3-deoxyglucosone, which react with proteins to produce AGEs. These AGEs are involved in the glycosylation of LDL, making it even more susceptible to oxidative modification and consequently contributing to early events of atherogenesis.

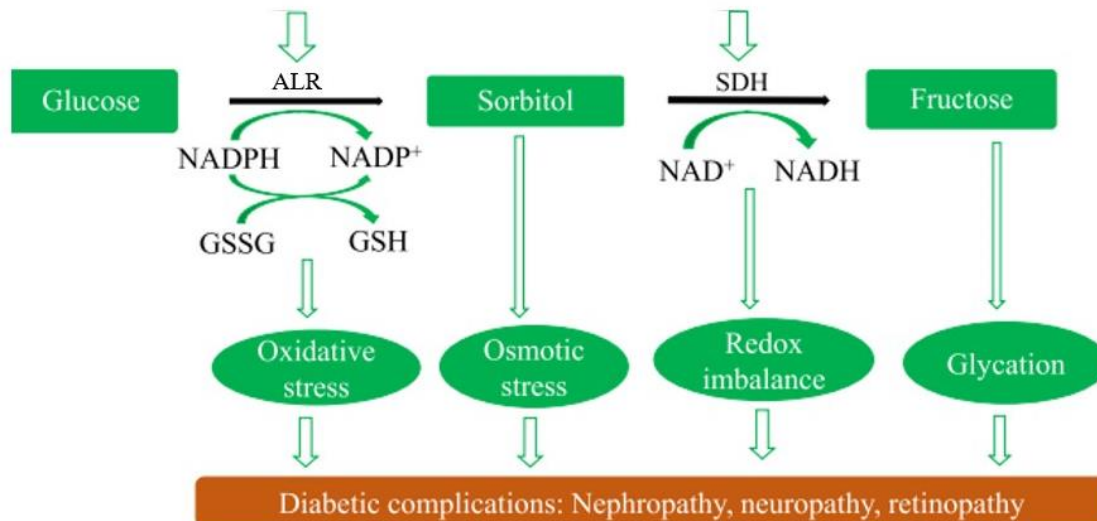


Figure 1.2: Schematic representation of polyol pathway. Glucose is converted to sorbitol by ALR. SDH oxidizes sorbitol to fructose. NADPH and NAD⁺ are used as coenzymes for ALR and SDH, respectively. NADPH depletion inhibits cellular regeneration of reduced glutathione, resulting in oxidative stress. Accumulation of fructose causes redox imbalance and glycation (Kazeem et al., 2020).

The polyol pathway enzymes, ALR and SDH, are the major targets in inhibiting the polyol pathway activity. Inhibition of ALR and/or SDH prevents the accumulation of sorbitol and fructose, and NADPH depletion (Kazeem et al., 2020). Literature evidence supports that ALR and SDH inhibitors prevent cellular oxidative stress, thus mitigating diabetic complications (Sheela et al., 2017; Kazeem et al., 2020; Jannapureddy et al., 2021). Hypoglycemic drugs can be used to prevent polyol pathway activity. However, the clinical limitations of the current antidiabetic drugs in controlling hyperglycemia stimulate the need to look for drugs that can target the polyol pathway.

1.3.3 Formation of AGEs and atherogenesis

AGEs naturally occur endogenously as part of the human body's metabolism. However, it can be pathogenic during hyperglycemic conditions and can result in diabetic complications, including ASCVDs (Karadaş, 2021). AGEs are formed through a non-enzymatic reaction between the amino and carbonyl groups of reducing sugars and proteins or lipids, respectively (Kuzan, 2021). This occurs through the Maillard reaction, which forms glycated products, termed Amadori products. Protein glycation occurs in 3 stages, namely: the initiation phase, the proliferation phase, and the

advanced proliferation phase (Liu et al., 2020). The reaction between a sugar's carbonyl group (-C=O) and a protein's amino group (-NH_2) is the initial step that produces aldimines or Schiff bases (Figure 1.3). This stage is followed by the proliferation stage, whereby Schiff bases further rearrange themselves into Amadori products. The last stage is the advanced proliferation phase of glycation, and it is an irreversible stage whereby the early Amadori products undergo several rearrangements, fragmentation and oxidative cleavage to generate irreversible reactive carbonyl intermediates including glyoxal and methylglyoxal (Gill et al., 2019; Kuzan, 2021). Amadori products can undergo autoxidation, and the reactive carbonyls can be further condensed with the available amine groups. This reaction produces a wide range of AGEs like pentosidine, $\text{N}\epsilon$ -(1-carboxyethyl)lysine (CEL) and $\text{N}\epsilon$ -(carboxymethyl)lysine (CML) (Twarda-Clapa et al., 2022).

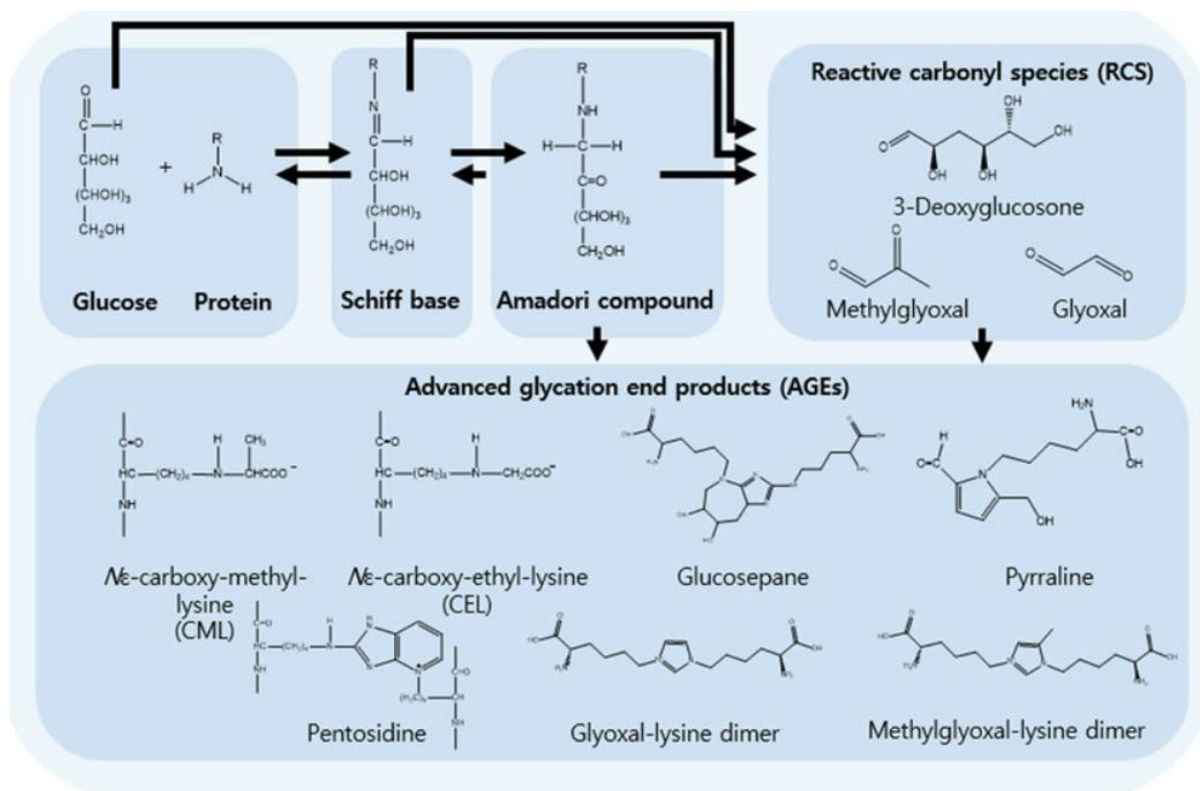


Figure 1.3. Schematic representation of the formation of AGEs (Cho et al., 2022).

AGEs formation is considered one of the important mechanisms that accelerates atherogenesis in diabetic patients. Hyperglycemia consequently stimulates the receptor's expression of the advanced glycated end products (RAGE). The binding of

AGEs to RAGE consequent to the production of intracellular ROS and it activates the redox-sensitive transcription factor NF- κ B. NF- κ B regulates the expression of several genes involved in inflammation and atherosclerosis, as well as interleukin-6 (IL-6), TNF- α , ICAM-I, VCAM-I, and MCP-I (Singh et al., 2020).

AGEs are also known to interfere with the conformational structure of LDL. AGEs induce the alteration of the phospholipids and apoprotein B of LDL, making it susceptible to oxidation (Moldogazieva et al., 2019). The glycosylation of LDL occurs on the positive residues of the LDL receptor binding domain which is critical for recognition by LDL receptors. The modified LDL molecules accumulate as the LDL-receptor-mediated uptake mechanism is impaired (Boren et al., 2020). The glycated LDL molecules are then recognized by scavenger receptors of the macrophage cells. The scavenger receptors engulf the modified LDL molecule in the sub-endothelial space which results in foam cell formation and atherosclerosis (Tian et al., 2020). The production of ROS and AGEs formation can be other key therapeutic targets in the prevention of macrovascular complications of diabetes.

1.4 Current therapeutics and their targets

Considering the increased prevalence of atherosclerosis in diabetic patients, hyperglycemia and hyperlipidemia remain the main therapeutic drug targets. Hyperglycemia, due to its ability to induce cell dysfunction and damage through various pathways, remains the primary target for the amelioration of diabetes and its complications. Metformin is one of the highly recommended drugs in the management of hyperglycemia. It is a drug that reduces the risks of diabetes complications by lowering the production of hepatic glucose (Piskovatska et al., 2019; Sansome et al., 2020). According to studies, Metformin has also been stated to lower blood levels of LDL cholesterol and thus decrease the risks of atherosclerosis (Amin et al., 2019).

The polyol pathway enzymes are also the main targets to prevent the overproduction of ROS and the AGEs generation and the related diabetic complications (Putta and Kilani, 2020). Aminoguanidine (AG) is currently used to impede the AGEs formation (Vasarri et al., 2020). AG reacts with the glycated protein's

Amadori carbonyl groups and dicarbonyl compounds, including methylglyoxal and 3-deoxyglucosone (Anwar et al., 2021).

Another target of diabetes-associated risk of atherogenesis is dyslipidaemia, which is distinguished by elevated LDL levels and low HDL levels in the blood. The oxidation of LDL is the first step of atherogenesis (Farooqui, 2021). Statins are cholesterol-lowering drugs that help to manage or prevent dyslipidemia. Statins inhibit a rate-limiting enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A (Liberale et al., 2020).

Regardless of the effectiveness of the currently available drugs to manage diabetes-associated atherosclerosis, these drugs have undesirable side effects which limit their use. Metformin is associated with nausea and diarrhoea; aminoguanidine is associated with liver function abnormalities, gastrointestinal disturbances and anaemia; and statins are associated with liver toxicity and muscle pains (Corcoran and Jacobs, 2018; Anwar et al., 2021; Paraskevas et al., 2022). The clinical limitations of the existing drugs indicate the necessity to look for alternative therapeutics, preferably from natural sources, with improved efficacy and safety profiles.

1.5 Potential of medicinal plants in the management of diabetes-related atherogenic complications

The use of medicinal herbs to manage and treat diseases has been used since the dates of immemorial till today. Medicinal plants in their crude form have been utilized to treat various ailments (Ahmad and Ahamad, 2020). Medicinal herbs are recommended for the management of various ailments because they possess phytoconstituents namely alkaloids, carotenoids, flavonoids, glycosides, terpenoids and saponins which are known to possess therapeutic properties. A large number of currently available pharmacologically active drugs have been derived indirectly or directly from plants (Salehi et al., 2019). These include Metformin, which is an anti-diabetic drug derived from *Galega officinalis* extract (Jugran et al., 2021); Ephedrine (from *Ephedra sinica*), Berberine (from *Coptis chinensis*) and Aspirin (from *Salix spp.*).

Medicinal plants, either as crude extracts and/or their isolated compounds, continue to offer exciting new opportunities in novel therapeutic drug discovery. Medicinal plants, such as crude extracts, have been reported to have various therapeutic activities. This includes *Carica papaya*, which has been reported by Kazeem et al. (2020) to have ALR and SDH inhibition activity, critical in oxidative stress prevention and glycation-mediated diabetic vascular complications. Mohd Dom et al. (2020) reported the activity of *Ficus deltoidea* extracts to have anti-glycation and antioxidant properties. The study of Sirichaiwetchakoon and Eumkeb (2024) further reported *Butea superba* extract to have antioxidant and anti-LDL oxidation activity in vitro. Acacetin and tilianin compounds found in *Agastache rugosa* species have also been said to have anti-atherogenic, anti-inflammatory and anti-hypertensive activities (Lam et al., 2020).

Among various medicinal plants, some South African traditional healers highly recommend and use stem bark extracts of *Bauhinia bowkeri* to manage Alzheimer's disease and diabetes-related health complications.

1.5.1 *Bauhinia bowkeri*

Bauhinia bowkeri Harv. belongs to the Fabaceae or Leguminosae family (Ahmed et al., 2012). It is generally known as *Kei Bauhinia*, *Kei White Bauhinia* (English), *umDlandlovu* (Xhosa) and *Keibeeskloou* (Afrikaans). *B. bowkeri* (Figure 1.4) is a tall, beautiful shrub with arching stems, available throughout seasons and blooms in spring or summer. It is commonly found next to rivers, but it is also drought-tolerant. *B. bowkeri* is an indigenous South African shrub located in the valley Bushveld of Eastern Cape and KwaZulu-Natal Province. In addition to diabetes-related health complications, the leaves and stem barks of *B. bowkeri* are traditionally used to induce vomiting, steaming and bathing for skin care (Sagbo and Mbeng, 2018). The acetone and methanol leaf extracts of *B. bowkeri* have reportedly been said to have anti-inflammatory and antioxidant activities (Ahmed et al., 2012). The *B. bowkeri* leaf extract scavenged ABTS and DPPH free radicals, and inhibited enzymes of the inflammatory pathway, 15-lipoxygenase and cyclooxygenase-1 (Ahmed et al., 2012; Sagbo and Mbeng, 2018). Free radicals and inflammation are most implicated in diabetes pathogenesis and its associated atherosclerosis. The ethanol extract of *B.*

bowkeri stem bark has also been indicated to possess anti- β -amyloid aggregation activity (Ojo et al., 2024).



Figure 1.4: A picture of *Bauhinia bowkeri* (pza.sanbi.org).

1.6 Strategies and techniques for discovering new drug candidates from natural products

Medicinal herbs have always been regarded as the primary source of new drugs and therapeutic agents. More than a thousand pharmaceutical drugs have been derived from medicinal plants (Süntar, 2020). During ancient times, natural products with therapeutic properties were discovered through trial and error. This trial-and-error method tests all potential bioactive compounds through biological assays till the desired compound is found. This method could be coupled with the advantage of quickly finding the desired compound if the right compound is tested, and it allows researchers to have a wide range of solutions to their scientific problems. However, it can also be time-consuming and very expensive. The isolation of bioactive compounds is associated with challenges, such as extraction, which is a time-consuming process including repeated steps of fractionation and biological activity tests (Stéphane et al., 2021; Zhang et al., 2020).

Due to the challenges of drug development, which is a time-consuming process, computer-based drug screening has been adapted to accelerate drug development. Before *in vitro* biological activity screening, plant compounds are pre-screened using a computer to decrease the quantity of screened compounds and enhance the

effectiveness of lead compound discovery. This saves samples, and time and lowers screening costs (Surabhi and Singh, 2018). Virtual screening methods such as molecular docking allow the compound to be tested against a protein of interest to predict its binding affinity. The compound is further predicted for its drug-likeness using the ADMET (absorption, distribution, metabolism, and excretion) predictor. Even though over the last two decades high throughput screening (HTS) technologies have significantly improved the speed, scale, and quality of the drug discovery process, it is still difficult to isolate and purify the minimal milligrams of the compounds needed for biological assays (Zhang et al., 2020). Since the biological assays are done *in vitro*, this approach has resulted in a very low success rate for new drug development, because both efficacy and toxicity must be validated *in vivo* or in animal trials which is very challenging and expensive (Steger-Hartmann and Raschke, 2020).

Despite the efficacy of computer-based drug screening, they are also coupled with false positive and false negative hits. This results in the isolation of inactive compounds to do the *in vitro* biological assays and exclude the compounds with the desired biological activities. This is also time-consuming as there will be a need to isolate new compounds again. Thus, it is advisable that all screening procedures such as *in silico*, *in vitro* and *in vivo* are done to increase the reliability of the results. In this study, both computer-based screening and *in vitro* biological assays have been used.

1.7 Study rationale

The chronic hyperglycemia and hyperlipidemia that characterize type II diabetes are responsible for the development and continuation of diabetes micro and macrovascular complications. Atherosclerosis has become the prevalent complication of diabetes that directly underlies the majority of cardiovascular events and death in diabetic patients. Despite the currently available antidiabetic drugs, there is a continuous and rapid increase in the prevalence of diabetes-related mortality cases (Sharma et al., 2023). Thus, it is a necessity to look for other drugs from natural sources like medicinal plants. Medicinal plants, due to their rich chemical diversity, offer new opportunities in drug discovery and development. Since some *Bauhinia bowkeri* crude extracts have been recorded to have anti-antioxidant and inflammatory activities (Sagbo and Mbeng, 2018), this indicates their potential to prevent diabetes-

associated risks of atherosclerosis. Thus, the proposed study assessed the potential of some crude extracts of *Bauhinia bowkeri* in mitigating some diabetes-associated risks of atherogenesis.

1.8 Aim, Hypothesis and Objectives

1.8.1 Aim

- To investigate the potential of *B. bowkeri* extracts to inhibit polyol pathway activity and LDL modification *in vitro*.

1.8.2 Hypothesis:

- H₁: The *B. bowkeri* extracts will significantly inhibit aldose reductase activity at 95% confidence.
- H₀: The *B. bowkeri* extracts will not significantly inhibit aldose reductase activity at 95% confidence.
- H₂: The *B. bowkeri* extracts will significantly inhibit the glycation of LDL at 95% confidence.
- H₀: The *B. bowkeri* extracts will not significantly inhibit the glycation of LDL at 95% confidence.

1.8.2 Objectives

- i. Extraction and phytochemical screening of the plant extracts.
- ii. *In silico* molecular docking of the plant extracts' major phytochemicals against aldose reductase and sorbitol dehydrogenase.
- iii. *In vitro* investigation of the plant extracts' inhibitory ability on ALR and SDH activities.
- iv. Determination of the antioxidant ability of the plant extracts *in vitro*.
- v. Determination of the inhibitory activity of the plant extracts against glycation and oxidation of LDL *in vitro*.

1.9 Thesis layout

Chapter 1: entails the background and literature review of the study.

Chapter 2: entails a list of all materials used for the study. It also describes the methods used to prepare crude plant extracts and perform all bioassays, including the *in silico* methods, required to answer the research questions of the study.

Chapters 3, 4 and 5 are written in a manuscript format. Each chapter consists of an introduction, materials and methods, results, discussion and conclusion.

Chapter 3: describes the *B. bowkeri* extracts' phytochemical screening.

The manuscript formatted chapter is titled "Phytochemical screening and antioxidant activity of the extracts from *Bauhinia bowkeri* stem bark".

Chapter 4: describes the inhibition of polyol pathway activity and protein glycation by *B. bowkeri* extracts.

The manuscript is titled "Potential of *Bauhinia bowkeri* extracts to inhibit polyol pathway activity and protein glycation *in vitro*".

Chapter 5: describes the inhibition of the polyol pathway and LDL modification by *B. bowkeri* bioactive compound.

The manuscript is titled "Phthalates from *Bauhinia bowkeri* extracts exhibit properties to inhibit the polyol pathway enzymes and low-density lipoprotein modification".

Chapter 6: entails the general discussion of the thesis.

Chapter 7: consists of a general conclusion and recommendations for future studies.

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CHAPTER 2

General Materials and Methodology

2.1 Materials

2.1.1 Chemicals and reagents

All the listed chemicals were bought from Merck and Sigma-Aldrich Co. Ltd

Potassium persulphate, Potassium ferricyanide, 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH), nicotinamide adenine dinucleotide phosphate (NADPH), Ascorbic acid (AA), 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS), Ferric chloride, Ethylenediaminetetraacetic acid (EDTA), Citric acid (CA), Deoxyribose, Hydrogen peroxide, Trichloroacetic acid (TCA), picrylsulfonic acid solution/2,4,6-Trinitrobenzenesulfonic acid solution (TNBSA), Sodium carbonate, 5,5-dithio-bis-(2-nitrobenzoic acid) (DNTB), Phenazine methosulfate (PMS), Trolox, nicotinamide adenine dinucleotide (NADH), Methylglyoxal solution, Congo red dye, Thiobarbituric acid (TBA), bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), tripyridyl-s-triazine (TPZT), nitroblue tetrazolium salt (NBT), sorbitol dehydrogenase, sorbitol, fluorescein, fructose, aminoguanidine, Low density lipoprotein (LDL), aldose reductase, DL-glyceraldehyde, varespladib, phenol red, phosphatidylcholine, 2,2'-Azobis (2-Amidinopropane) Dihydrochloride (AAPH), Quercetin, Copper sulphate (CuSO_4), High density lipoprotein (HDL), Folin-Ciocalteu reagent, 1-deoxymorpholino (DMF), Zopolrestat.

2.2 Methods

The method section is separated into two parts, Part A and Part B. Part A entails all the experimental work that was done using crude extracts of the plant. Part B entails the experimental work done using selected major bioactive compounds.

PART A

2.2.1 Plant collection and preparation

The *B. bowkeri* plant materials (stem bark) were purchased from the *muthi* market in Pongola, KwaZulu-Natal. The plant identity was confirmed by Dr Ntuli from the University of Zululand (Voucher specimen number: V12). The *B. bowkeri* stem barks were cleaned with running water, allowed to air-dry and finely chopped. The chopped plant material (506 g) was sequentially extracted (1:5 w/v) with hexane, dichloromethane (DCM) and methanol (Figure 2.1). The filtrates were obtained by filtering the three extracts using Whatman No.1 filter paper and concentrated under reduced pressure at 40°C to obtain crude hexane, DCM and methanol extracts using a rotary evaporator (Heidolph Laborota 4000). The obtained crude extracts were kept in the fridge at 4°C until they were required for use. The percentage (%) yields of the extracts were calculated using the formula below:

$$\% \text{ Yield} = \text{Mass of crude extract (g)} / \text{mass of dry plant material (g)} \times 100 \dots \dots \dots (2.1)$$

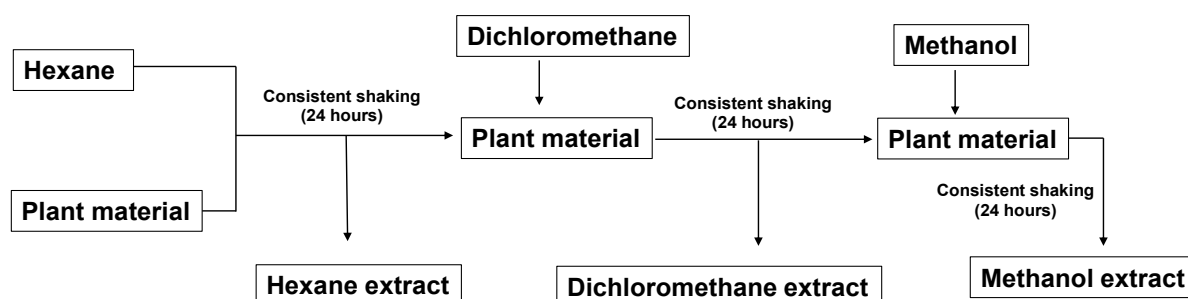


Figure 2.1. Sequential diagrammatic representation of *B. bowkeri* stem bark extraction. The plant material was sequentially extracted with hexane, dichloromethane (DCM) and methanol.

2.2.2 Phytochemical screening

The *B. bowkeri* extracts' phytochemical content was screened by qualitative and quantitative methods.

2.2.2.1 FTIR analysis of the plant extract

An FTIR spectrophotometer with a temperature (25- 28°C) and spectral range of 370-4000 cm⁻¹ was used to evaluate the *B. bowkeri* crude extracts' functional groups. Peak frequencies were compared to the correlation table of IR spectroscopy to determine functional groups.

2.2.2.2 GC-MS analysis of the plant extract

The volatile phytochemical constituents of *B. bowkeri* extracts were identified using the GC-MS and GC Clarus 500 system from Perkin-Elmer. GC-MS analysis was done by splitless injection of 2 µL of the samples. Helium was used as an eluent gas with 1 ml/min stable flow rate. The oven's temperature remained at 110 °C for 2 minutes before increasing by 10 °C/min until it reached 200°C, then by 5 °C/min to 280°C. The injector temperature remained at 250°C. The retention time and peak areas of the compounds of different extracts were recorded. The National Institute of Standards and Technology (NIST) was used to identify the names, structures and molecular weights of the compounds.

2.2.2.3 Total phenolic content

The assay principle is based on an oxidation/reduction reaction. Phenolic compounds reduce the Folin-Ciocalteu (FC) yellow reagent, which is detected by a blue colored product formation is colorimetrically measured at 765 nm. The method described by Aiyegoro and Okoh, (2010) was used to determine the extracts' total phenolic content (TPC) using the FC reagent. A volume of 2.5 ml of 10 % FC reagent and 2 ml of sodium carbonate (2% w/v) were added to 0.5 ml of the extract (1 mg/ml) dissolved in methanol. Gallic acid (GA) at varying concentrations (0.1, 0.2, 0.3, 0.4, 0.5 mg/ml) was used as the reference standard. A mixture consisting of methanol, FC reagent and sodium carbonate was a negative control. The resultant mixture was shaken for

15 min at 45°C while using a Platform shaker (Labcon). The SpectraMax Paradigm Multi-Mode Detection Platform (Softmax Pro 7.1) was used to read the absorbance of the samples at 765 nm. The mixture without the extract was used as a blank. The extracts' phenolic content was extrapolated from the gallic acid calibration curve (See Appendix A). The extracts' TPC was presented as milligrams (mg) of gallic acid equivalents (GAE) per gram of crude extract (mg GAE/g crude extract).

2.2.2.4 Total flavonoid content

The assay principle is based on aluminium chloride and flavonoids' reaction to form a flavonoid aluminium complex, which is measured by a yellow-colored product. This product can be colorimetrically measured at 420 nm.

The alcoholic aluminium chloride colorimetric method of Aiyegoro and Okoh (2010), was employed to evaluate the total flavonoid content (TFC) of the plant extracts. Aluminium chloride solution (10%) was prepared in ethanol, and plant extract (1 mg/ml) was prepared in methanol. The reaction mixture consisted of 3 ml of ethanol, 5.6 ml of distilled water, 0.2 ml of 1 M potassium acetate and 0.2 ml of 10% aluminium chloride and 1 ml of the extract (1 mg/ml). The reaction mixture was kept in an incubator for 30 minutes at room temperature. Quercetin at varying concentrations (0.1, 0.2, 0.3, 0.4, 0.5 mg/ml) served as the reference standard. The SpectraMax Paradigm microplate reader was used to read the absorbance of the reaction mixture at 420 nm. The above reaction mixture, consisting of methanol without the extract, served as a negative control. The TFC of the extracts was extrapolated from the quercetin calibration curve (See Appendix A). The total flavonoid content of the extracts was presented as milligrams (mg) of quercetin equivalents (QE) per gram of crude extract (mg QE/g crude extract).

2.2.3 *In vitro* antioxidant assays

The *in vitro* antioxidant capacity of the plant extracts was investigated against synthetic (DPPH and ABTS) and biological oxidants like superoxide anion and hydroxyl radicals. The Oxygen Radical Absorbance Capacity (ORAC), reducing power and metal ion chelating activity of the plant extracts were also evaluated. Unless otherwise stated, gallic acid (GA) and ascorbic acid (AA) served as positive controls. The percentage

free radical scavenging activity of the extracts was calculated using the following formula:

$$\text{Scavenging activity (\%)} = [(A_{\text{control}} - A_{\text{test}}) / (A_{\text{control}})] \times 100 \dots \dots \dots (2.2)$$

Where A_{control} was the sample's absorbance without an inhibitor, and A_{test} was the sample's absorbance in the presence of an inhibitor.

2.2.3.1 DPPH scavenging activity

The DPPH radical scavenging assay described by Brand-Williams (1995) was used to determine the scavenging activity of the plant extracts. Working solutions of both DPPH (0.135 mM) and extracts (20, 40, 60, 80 and 100 µg/ml) were made in methanol. To investigate the DPPH scavenging activity of the extracts, the same volumes of DPPH (0.135 mM) and the extract at different concentrations were mixed. Ascorbic acid (20, 40, 60, 80 and 100 µg/ml) and methanol were positive and negative controls, respectively. The reaction mixture was thoroughly vortexed using a Vortex (Lasec) and was kept in the dark at room temperature for 30 minutes. The SpectraMax Paradigm microplate reader was used to read the absorbance at 517 nm. Methanol represented a blank. The percentage of free radical scavenging activity of the extracts was calculated using formula **2.2**.

2.2.3.2 ABTS scavenging activity

The plant extracts' scavenging activity on ABTS was determined using the method of Re et al. (1999). Equal volumes of 2.4 mM potassium persulphate and 7 mM ABTS were mixed to prepare an ABTS radical. The reaction mixture was incubated for 16 hours at room temperature in the dark. ABTS stock solution (1 ml) was diluted with 60 ml of ethanol to make ABTS working solution. Equal volumes of the ABTS radical and the test extract's varied concentrations (4, 8, 16, 32 and 64 µg/ml) were mixed (1:1 v/v) and left at room temperature for 7 minutes. Ascorbic acid (4, 8, 16, 32 and 64 µg/ml) and butylated hydroxy anisole (4, 8, 16, 32 and 64 µg/ml) served as reference inhibitors, and a negative control was methanol. The 96-well microplate reader absorbance of the reaction mixture was read at 734 nm using a microplate reader. The percentage of free radical scavenging activity of the extracts was worked out using formula **2.2**.

2.2.3.3 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the extracts was determined following the method described by Khan et al. (2012) with slight modifications. Hydroxyl radical, generated from the Fe^{3+} -ascorbate- H_2O_2 (Fenton reaction), was investigated by deoxyribose degradation that produces thiobarbituric acid reactive substances (TBARS). Fe^{3+} gets reduced to Fe^{2+} by donating its electron to hydrogen peroxide (H_2O_2), resulting in hydroxyl radicals, which attack the deoxyribose and produce aldehydes such as malondialdehyde (MDA), which are measured by TBARS. The working solutions of deoxyribose (25 mM), ferric chloride (10 mM) and hydrogen peroxide (2.8 mM) were prepared in distilled water. Various extract concentrations (4, 8, 16, 32 and 64 $\mu\text{g/ml}$), 25 mM deoxyribose, 100 mM ascorbic acid, 2.8 mM hydrogen peroxide, 10 mM ferric chloride and 10 mM phosphate buffer (pH 7.4) made up the reaction mixture. Ascorbic acid and butylated hydroxy anisole served as positive controls, and methanol as a negative control. The reaction mixture was kept at 37°C for 1 hour. Thereafter, 1 ml of 1% thiobarbituric acid (TBA) and 1 ml of 3% trichloroacetic acid (TCA) were added to the reaction mixture and heated using a Bunsen burner at 100°C for 20 minutes, the temperature was monitored by a thermometer. The TBARS produced were measured spectrophotometrically at 535 nm using the microplate reader. The free radical scavenging activity percentage was calculated by formula **2.2**.

2.2.3.4 Superoxide anion scavenging activity

The plant extracts' superoxide anion scavenging activity was determined by the protocol of Yamaguchi et al. (2000). The working solutions of the reagents phenazine methosulfate (60 μM), NBT (200 μM) and NADH (468 μM) were made in 0.1 M phosphate buffer pH 7.4. The reaction mixture had extracts (100 and 500 $\mu\text{g/ml}$), 60 μM of phenazine methosulfate and 20 μM of NADH. Methanol and quercetin were used as negative and positive controls, respectively. The reaction mixture was incubated at 25°C for 5 minutes, and the absorbance was read using the microplate reader at 560 nm. The percentage of free radical scavenging activity of the extracts was calculated using formula **2.2**.

2.2.3.5 Metal ion (Fe^{2+}) chelating activity assay

The plant extracts' Fe^{2+} chelating activity was determined following the protocol described by Decker and Welch (1990). Ferrous chloride (2 mM) dissolved in distilled water was mixed with the various test extract concentrations (4, 8, 16, 32 and 64 $\mu\text{g/ml}$). After 30 to 60 seconds, 5 mM ferrozine was added to initiate the reaction. The reaction mixture was mixed and left at room temperature for 10 minutes. The absorbance of the mixture was measured spectrophotometrically using the Paradigm microplate reader at 562 nm. Ethylenediaminetetraacetic acid (EDTA) and citric acid (CA) were used as positive controls, and methanol as a negative control. The percentage of metal chelating activity of the test extracts was calculated using the following formula:

$$\text{Chelating activity (\%)} = [(A_{\text{control}} - A_{\text{test}}) / (A_{\text{control}})] \times 100 \dots\dots\dots (2.3)$$

Where A_{control} was the absorbance of the sample without an inhibitor, and A_{test} was the absorbance of the sample with an inhibitor.

2.2.3.6 Oxygen Radical Absorbance Capacity assay

The method reported by Álvarez et al. (2012) was adapted to investigate the plant extracts' Oxygen Radical Absorbance Capacity (ORAC). The 2,2-Azobis(2-amidinopropane) dihydrochloride (AAPH) and fluorescein were used as peroxyl radical generators and the fluorescent probe, respectively. Concentrations of AAPH (250 mM) and fluorescein (1 M) were made in 75 mM phosphate buffer solution at pH 7.4. The reaction mixture containing the extract at various concentrations (12.5, 25, 50, 100 and 200 $\mu\text{g/ml}$) and 1 M fluorescein (1:6) was incubated in black 96-well microplates at 37 °C for 30 minutes. Afterwards, AAPH (250 mM) at 1:7 ratio in phosphate buffer was added to the mixture. Trolox (0.625, 1.25, 2.5, 5 and 10 $\mu\text{g/ml}$) was used as a standard. The SpectraMax Paradigm microplate reader was used to measure fluorescein's fluorescence intensity at a 485 nm excitation wavelength and 520 nm emission wavelength every minute for 120 minutes. The differences of areas under the decay curves (See Appendix A) were used to calculate relative ORAC values, and the results were presented as nmol Trolox equivalents per gram of sample (nmol TE/g).

2.2.3.7 Ferric Reducing Antioxidant Power

The ferric reducing antioxidant power (FRAP) assay described by Benzie and Strain (1999) was adapted to investigate the reducing power of the plant extracts. In 40 mM HCL, the working solutions of 10 mM tripyridyltriazine (TPZT), 20 mM FeCl₃ hexahydrate and 300 mM sodium acetate buffer solution at pH 3.6 were prepared. The FRAP reagent was prepared by mixing 1 part of 10 mM tripyridyltriazine (TPZT), 1 part of 20 mM FeCl₃ hexahydrate and 10 parts of 300 mM sodium acetate buffer solution at pH 3.6. The reaction mixture was constituted by adding 0.2 ml of the plant extract (20 and 100 µg/ml) to 3.8 ml of the FRAP reagent. The resulting mixture was incubated for 30 minutes at 37 °C. Ascorbic acid functioned as a reference standard, and methanol as a negative control. The absorbance was read at 593 nm by the microplate reader. The FRAP of the extracts was extrapolated from the FeSO₄ calibration curve (See Appendix A). The data obtained was displayed in milligram equivalents of FeSO₄ per gram of dry weight of extract.

2.2.4 Oxidation of LDL

The extracts' antioxidant activity was also determined in copper sulfate-induced LDL (and HDL) oxidation. The lipid peroxidation inhibitory activity of the plant extracts was determined by the measurement of conjugated dienes (CD) formation and TBARS. Gallic acid served as a known inhibitor.

2.2.4.1 Measurement of the Conjugated Dienes formation

Final concentrations of 200 µg protein/ml of LDL (or HDL), in 10 mM phosphate buffer (pH 7.4) medium, were mixed with 64 µg/ml of the plant extract and 10 µM of CuSO₄. Gallic acid (64 µg/ml) was a positive control, and methanol was a negative control. The reaction mixture was incubated at 37 °C for 2 hours. While incubating, conjugated dienes formation was continuously observed at 2-minute intervals by reading the absorbance at 234 nm using the SpectraMax Paradigm microplate reader.

2.2.4.2 Measurement of Thiobarbituric Acid Reactive Substances

Final concentration of 500 µg protein/ml of LDL (or HDL), in 10 mM phosphate buffer (pH 7.4) medium, was mixed with the extract at different concentrations (4, 64 and 192 µg/ml) and 10 µM of CuSO₄. Gallic acid (4 and 64 µg/ml) was a positive control, and methanol was a negative control. The reaction mixture was incubated at 37 °C for 5 hours. After the incubation period, 0.5 mM of EDTA was added to terminate the reaction. Subsequently, 20% of trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA) solutions in 0.05 M NaOH were added. A water bath with adjusted temperature to 90 °C was used to heat the reaction mixture for 20 minutes. The samples were allowed to cool down at 4°C and centrifuged at 3000 rpm for 15 minutes using a Lasec centrifuge. After centrifugation, the supernatant was collected, and the absorbance was read at 532 nm utilizing the SpectraMax Paradigm microplate reader. The malondialdehyde (MDA) (See Appendix A) was used to determine the total quantity of TBARS formed. The data wereshown as nanomoles (nmol) of MDA equivalents per mg protein.

2.2.5 Enzyme inhibition assays

The enzyme inhibition assays of the extracts were done on Aldose reductase (ALR), Sorbitol dehydrogenase (SDH) and secretory phospholipase A₂ (sPLA₂).

2.2.5.1 Aldose Reductase Inhibition Assay

Aldose reductase is a rate-limiting enzyme of the polyol pathway that catalyzes the reduction of glucose into sorbitol by using NADPH as a cofactor. The assay measures the ability of ALR to oxidize NADPH to NADP⁺, which is measured by a decline in absorbance at 340 nm.



The aldose reductase inhibitory activity of the extracts was investigated by the technique of Fatmawati et al. (2015) with slight changes. The working solutions of 10 mM DL-glyceraldehyde and 0.15 mM NADPH were prepared in distilled water. The

extracts (50, 100, 150 and 200 µg/ml) and Zopolrestat (100, 200, 300 and 400 µg/ml) were prepared in methanol.

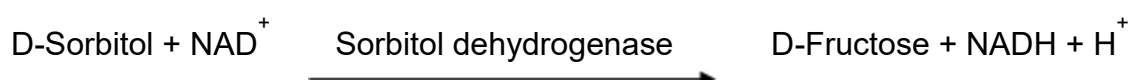
A mixture consisting of 100 mM sodium phosphate buffer (pH 6.2), 0.15 mM NADPH, 10 mM DL-glyceraldehyde and the extract at varying concentrations (50-200 µg/ml) was pre-incubated for 5 minutes at 25°C. The reaction was initiated by adding 5 µL of 100 µg/ml aldose reductase. All the assay components were at their final concentration in the reaction mixture, compared to zopolrestat (standard) and methanol (negative control). The change in absorbance was measured at 340 nm, at 2-minute intervals for 10 minutes by the SpectraMax Paradigm microplate reader. The percentage (%) enzyme inhibitory activity of the extracts was calculated using the following formula:

$$\text{Inhibition rate (\%)} = [(A_{\text{control}} - A_{\text{test}}) / A_{\text{control}}] \times 100 \dots \dots \dots (2.4)$$

Where A_{control} was the sample's absorbance without an inhibitor, and A_{test} was the sample's absorbance with an inhibitor.

2.2.5.2 Sorbitol Dehydrogenase Inhibition Assay

Sorbitol dehydrogenase is a polyol pathway enzyme that converts sorbitol to fructose using NAD^+ as a cofactor. The assay is based on the SDH ability to reduce NAD^+ to NADH, which is measured by an increase in absorbance at 340 nm.



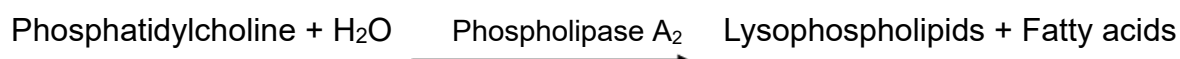
The working solutions of 100 mM sorbitol, 0.5 mM NAD^+ and 100 µg/ml SDH were prepared in distilled water. The different concentrations of the plant extracts and quercetin (50, 100, 150 and 200 µg/ml) were prepared in methanol.

The method of Kobayashi et al. (2002) was used to determine the inhibitory activity of the plant extracts on sorbitol dehydrogenase activity. The reaction mixture consisted of 100 mM Tris-HCl buffer (pH 9.0), 0.5 mM NAD^+ , 0.5 µL of 100 µg/ml sorbitol dehydrogenase, 100 mM sorbitol (substrate) and plant extract at varying concentrations (50, 100, 150 and 200 µg/ml). The addition of NAD^+ was to initiate the reaction. Quercetin acted as a positive control, and methanol as a negative control. The SpectraMax Paradigm microplate reader was used to measure the rate of change

in the absorbance of the mixture at 340 nm. The sorbitol dehydrogenase inhibitory activity of the plant extract was calculated as percentage inhibition using equation **2.4**.

2.2.5.3 Secretory phospholipase A₂ inhibition activity

Secretory phospholipase A₂ (sPLA₂) is an enzyme involved in inflammation that is responsible for releasing arachidonic acid from phospholipids. The assay is based on the sPLA₂ to hydrolyse phosphatidylcholine, which is detected by color change and measured at 558 nm.



The plant extracts' inhibitory activity on secretory phospholipase A₂ (sPLA₂) was investigated using the protocol described by de Araujo and Radvanyi (1987). Briefly, 10 mM phosphatidyl-choline, 100 mM NaCl, and 10 mM CaCl₂ were used to make the sPLA₂ substrate, and 0.055 mM phenol red as a colorimetric indicator in 100 ml H₂O. Just before use, the pH of the reaction mixture was adjusted to 7.6. A volume of 5 µL of the sPLA₂ enzyme (0.05 µg/ml) solution was incubated with 5 µL of extract (192 µg/ml) for 10 minutes at room temperature. Varespladib (64 µg/ml) was used as a positive control, and methanol was used as a negative control. Then, 190 µL of the freshly prepared substrate was added to start the reaction. The kinetic enzyme hydrolysis was monitored at 1-minute intervals for 20 minutes by reading the optical density at 558 nm using the SpectraMax Paradigm microplate reader. The enzyme inhibitory activity of the plant extract was calculated.

2.2.6 Antiprotein glycation activity

The antiprotein glycation activity of the plant extracts was evaluated on BSA and LDL using fructose and methylglyoxal as the protein glycating agents.

2.2.6.1 Fructose-induced protein glycation assay

The *in vitro* antiglycation activity of the plant extracts was investigated using Fructose-LDL and Fructose-BSA glycation systems using the method of Bhatwadekar and Ghole (2005) with modifications. A working concentration of 0.25 mg/ml of LDL (or 10 mg/ml of BSA) prepared in phosphate-buffered saline (PBS) was mixed, in 0.1 M PBS

(pH 7.4), with extract (100 and 500 µg/ml) in methanol, 0.25 M fructose and 0.002% sodium azide dissolved in distilled water. The reaction mixture was incubated at 37°C in the dark for seven days (four weeks for BSA). Aminoguanidine (100 and 500 µg/ml) dissolved in methanol functioned as a known inhibitor, and a negative control used was methanol. The SpectraMax Paradigm Multi-Mode Detection Platform (Softmax Pro 7.1) was used to measure the fluorescence intensity of AGEs formation at an excitation wavelength of 335 nm and emission wavelength of 385 nm. The percentage inhibition of AGEs was calculated using the following formula:

$$\%AGE \text{ inhibition} = [(A_{\text{control}} - A_{\text{test}}) / A_{\text{control}}] \times 100 \dots \dots \dots (2.5)$$

Where A_{control} was the sample's absorbance without an inhibitor, and A_{test} was the sample's absorbance with an inhibitor.

The extracts' antiglycation activity was further confirmed by investigating the contents of fructosamine, free amino group and protein aggregates in the glycated samples.

2.2.6.2 Fructosamine content

The fructosamine content of the glycated protein samples was determined using the NBT assay described by Ayyappan et al. (2015). A working solution of 0.7 mM NBT was prepared in 1 M sodium carbonate buffer, pH 10.35. To determine the fructosamine content of the protein samples, 130 µL of 0.7 mM was mixed with 70 µL of the glycated protein sample. The reaction mixture was incubated at 37°C for 30 minutes, and the absorbance was measured at 540 nm using the Paradigm microplate reader. The fructosamine content was determined from the 1-deoxymorpholino (DMF) standard curve and expressed as nanomoles (nmol) of DMF equivalents per well (nmol/well).

2.2.6.3 Free amino group content

The 2,4,6-Trinitrobenzene Sulfonic Acid (TNBSA) assay reported by Sattarahmady et al. (2007) was adapted to determine the free amino group content of the glycated protein samples. The working solution of 0.01% (w/v) TNBSA was prepared in distilled water. To determine the free amino group content, 70 µL of the glycated protein sample was mixed with 35 µL of 0.01% (w/v) TNBSA and the reaction mixture was

incubated at 37°C for 1 hour. The reaction was stopped by adding 10% SDS and 1 M HCl. The absorbance was read at 340 nm. A standard curve of L-arginine was used to determine the free amino group concentration. The results were expressed as nmol equivalence of L-arginine amino group content per well (nmol/well).

2.2.6.4 Congo red binding assay

Congo red was used to determine amyloid fibril aggregates in the glycated BSA (Klunk et al., 1989). A volume of 500 µL of glycated BSA was combined with 100 µL of Congo red (100 µM). The mixture was incubated for 5 minutes at room temperature. The absorbance at 530 nm was read by utilizing the SpectraMax Paradigm Multi-Mode Detection Platform (Softmax Pro 7.1). The content of amyloid fibril aggregates was presented as absorbance (nm). The absorbance is directly proportional to the content of amyloid fibril aggregates.

2.2.6.5 Methylglyoxal-induced protein glycation assay

The extracts' *in vitro* antiprotein glycation activity was also investigated using methylglyoxal as a glycating agent on BSA (Bhatwadekar and Ghole, 2005). LDL could not be used in this experiment due to reagent limitations. A working concentration of 10 mg/ml of BSA dissolved in phosphate-buffered saline (PBS) was mixed, in 0.1 M PBS (pH 7.4), with extract (100 and 500 µg/ml) dissolved in methanol, 0.1 M MGO and 0.002% sodium azide prepared in distilled water. The solution was incubated at 37°C in the dark for 2 weeks. Aminoguanidine (100 and 500 µg/ml) dissolved in methanol served the purpose of a standard, and methanol was a negative control. The fluorescence intensity of AGEs formation was measured using a Paradigm microplate reader at an excitation wavelength of 360 nm and an emission wavelength of 430 nm. The percentage inhibition of AGEs was calculated using equation **2.5**.

The extracts' antiglycation activity was further confirmed by determining the presence of protein aggregates and free amino group contents of the glycated samples as described in **2.2.6.3** to **2.2.6.4**.

PART B

2.2.7 *In silico* molecular docking and ADMET analysis

Selected *B. bowkeri* compounds from GC-MS analysis data were subjected to an *in silico* molecular docking study. The ADMET properties of the selected compounds were also determined.

2.2.7.1 Molecular docking

Molecular docking predicts binding affinity between a ligand and target proteins, interaction of potential drugs and their targets, further predicting the drug inhibition activity, computationally expediting drug discovery and development.

The 3-dimensional structures of different receptors Aldose reductase (3G5E), Sorbitol dehydrogenase (3QE3) and Secretory phospholipase A2 (1TC8) were obtained from RCSB Protein Data Bank (PDB). The selected *B. bowkeri* compounds were subjected to a molecular docking study against 3G5E, 3QE3 and 1TC8 using Maestro software from Schrodinger (2022 1). The structures of compounds were drawn using the ChemDraw Ultra 6.0 software, and the structures were transformed into 3-dimensional formats. All the conversions needed for the docking study were done using an open-source chemical toolbox. The Maestro software from Schrodinger (version 12.8) was used to prepare the ligands and proteins. The docking calculations were done using the Maestro software from Schrodinger into the 3-dimensional structure of the protein's catalytic and allosteric sites. The binding free energy was calculated using Maestro software (Ugwu et al., 2018).

2.2.7.2 ADMET Properties

Absorption, distribution, metabolism, excretion, toxicity or ADMET is used to determine drug interaction with the body, efficacy and safety profile, which is crucial for drug development.

The selected compounds' ADMET properties were determined using the pkCSM online tool (<https://biosig.lab.uq.edu.au/pkcsml/>). The pkCSM uses graph-based signatures to evaluate and predict small-molecule pharmacokinetic properties to evaluate drug efficacy.

2.2.8 *In vitro* enzyme inhibition, antiprotein glycation and antioxidant activities of the pure compound(s)

The *in vitro* inhibition activity of the pure compound(s) on aldose reductase, sorbitol dehydrogenase, LDL glycation and antioxidant activities was performed as described for the extracts with modifications on the compound concentrations used. Different concentrations of the pure compound(s), ranging from 0.1 mM to 1 mM, were used for the determination of antioxidant activity. The enzyme inhibitory activity of the compound was tested at 0.1, 0.2, 0.3, 0.4 and 0.5 mM. The antiprotein glycation activity of the compound was tested at 0.1, 0.3 and 0.5 mM.

2.2.9 Data analysis

The experiments were done in triplicate with three (3) independent biological repeats. The data obtained was stated as mean $\pm$ standard error of the mean (SEM). Statistical comparisons between the results were performed by one-way analysis of variance (ANOVA) followed by Dunnett post hoc test using GraphPad Prism 8. The statistical difference was considered significant where $p \leq 0.05$.

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CHAPTER 3

Phytochemical screening and antioxidant activity of the extracts from *Bauhinia bowkeri* stem bark

Abstract

Oxidative stress continues to be implicated in various diseases, including diabetes-related atherosclerotic cardiovascular complications. There is thus an increasing need to discover and develop novel drugs with antioxidant properties to treat oxidative stress-related diseases. This study aimed to screen the phytochemical content and antioxidant activity of the *Bauhinia bowkeri* stem bark extracts (hexane, dichloromethane, and methanol). The crude extracts were screened for their phytochemical composition. The antioxidant activities of the plant extracts were evaluated by various assays, including the DPPH, ABTS, ORAC and low-density lipoprotein (LDL) inhibition assay. FTIR analysis indicated the presence of functional groups of phenols and flavonoids. GC-MS analysis confirmed the presence of various bioactive compounds, including phenolics. The highest TPC was displayed in the methanol extract when compared with the other extracts. The extracts demonstrated free radical scavenging activity in a concentration-dependent manner with EC₅₀ values ranging from 44 to 60 µg/ml on DPPH and ABTS. The methanol extract exhibited the highest antioxidant activity of the other extracts, with an ORAC value of 189 (µmol TE/g). The extracts further exhibited the potential to inhibit the oxidative modification of LDL. It was the methanol extract that also showed more inhibitory activity of LDL oxidation by up to 61%. The obtained results indicate that the *Bauhinia bowkeri* extracts have the potential as natural alternatives to manage oxidative stress-linked diseases.

3.0 Introduction

Oxidative stress continues to be implicated in numerous diseases like diabetes, neurodegenerative diseases, cancer, and cardiovascular diseases diabetes-related atherosclerotic cardiovascular complications (Hilal et al., 2024). Free radicals are involved in the LDL's oxidative modification, which is a major step in the atherosclerosis development (Poznyak et al., 2021). An endogenous overproduction of free radicals and suppression of antioxidants, oxidize DNA, lipids and proteins, consequently altering cellular functions or damaging cells. There is thus an increasing necessity to discover and develop novel drugs with antioxidant properties to treat oxidative stress-related diseases.

Medicinal plants have long been known for their therapeutic properties since ancient times, and continue to be a useful source of therapeutics for various diseases. Medicinal plants are known to have therapeutic activity because of their phytochemicals or secondary metabolites such as terpenoids, flavonoids, saponins, polyphenols, alkaloids, and carotenoids. Medicinal properties of various plants have been linked to their antioxidant and anti-inflammatory activities (Rodríguez-Yoldi, 2021). Phenols and flavonoids are the most common and popular phytochemicals widely known for plants' antioxidant and anti-inflammatory properties (Adeyemo et al., 2022). Medicinal plants can be used as crude extracts or derivatives of drugs to manage several diseases. The screening of the medicinal plants' phytochemicals is of major importance in discovering new therapeutics (Ahlidja et al., 2024).

Traditional healers recommend *Bauhinia bowkeri*, also referred to as *Kei Bauhinia* (English), from the Fabaceae family. It is distributed in the Eastern Cape and KwaZulu Natal province of South Africa. This plant is highly recommended by Zulu traditional healers for the management of various disorders, including diabetes-related complications. The leaf extracts of this plant have been reported to have antioxidant and anti-inflammatory activities (Sagbo and Mbeng, 2018). This study aimed at screening the phytochemical composition and potential antioxidant activity of *Bauhinia bowkeri* stem bark extracts.

3.1 Materials and Methods

3.1.1 Reagents

All the reagents used in this chapter were the same as the reagents described in 2.1.1.

3.1.2 Extraction of the plant material

The stem barks of *B. bowkeri* (Voucher specimen number V12) were washed with tap water, air dried and cut into small pieces. Thereafter, the plant material (305.24 g) was extracted sequentially (1:5 w/v) with hexane, dichloromethane (DCM) and methanol. The filtrates were individually concentrated under reduced pressure at 40°C using a rotary evaporator (HeidolphóLaborota 4000) to obtain the crude extracts of hexane, DCM and methanol. The yields (%) of the extracts were calculated using the formula 2.1.

3.1.3 Phytochemical screening

3.1.3.1 FTIR analysis

The extracts were exposed to FTIR spectral analysis to determine the functional groups present (Oliveira et al., 2016). A drop of liquid extract was used to analyze the spectrum range of 4000-500 cm⁻¹ using the FTIR spectrophotometer (Spectrum Two, PerkinElmer, USA).

3.1.3.2 GC-MS analysis

GC-MS analysis of the extracts was performed to determine volatile compounds using the Perkin-Elmer GC Clarus 500 system and gas chromatography. The names, structures and molecular weights of the bioactive compounds were confirmed using the National Institute of Standards and Technology (NIST).

3.1.3.3 Total phenolic content

The Folin-Ciocalteu reagent method was followed to determine the total phenolic content of the plant extracts (Aiyegoro and Okoh, 2010). To 0.5 ml of each extract (1 mg/ml), 2.5 ml of 10% Folin-Ciocalteu reagent and 2 ml of sodium carbonate (2% w/v)

were added. The resulting mixture was incubated at 45°C for 15 minutes. The absorbance of the samples was measured at 765 nm on the SpectraMax Paradigm Multi-Mode Detection Platform (Softmax Pro 7.1, Molecular Devices LLC, USA). The total phenolic content of the extracts was expressed as milligrams of gallic acid equivalents per gram of crude extract (mg GAE/g crude extract).

3.1.3.4 Total flavonoid content

The total flavonoid content of the extracts was also determined following the alcoholic aluminium chloride method described by Aiyegoro and Okoh (2010). The reaction mixture contained 1 ml of extract (1 mg/ml), 3 ml of ethanol, 0.2 ml of 10% aluminium chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water. The reaction mixture was allowed to stand at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 420 nm. The total flavonoid content of the extracts was expressed as milligrams of quercetin equivalents per gram of crude extract (mg QE/g crude extract).

3.1.4 *In vitro* antioxidant assays

3.1.4.1 DPPH scavenging activity

The DPPH radical scavenging assay was followed to determine the free radical scavenging activity of the plant extracts (Brand-Williams, 1995). Briefly, DPPH (0.135 mM) was mixed (1:1 v/v) with the extract (20-100 µg/ml). Ascorbic acid and methanol were used as positive and negative controls, respectively. The reaction mixture was left in the dark at room temperature for 30-60 minutes. The absorbance was measured using the SpectraMax Paradigm microplate reader at 517 nm. The percentage free radical scavenging activity of the extracts was calculated using formula **2.2**.

3.1.4.2. ABTS scavenging activity

The method of Re et al. (1999) was adapted to determine the ABTS scavenging activity of the plant extracts. ABTS radical of equal volumes and the test extract (100-800 µg/ml) were mixed well. The mixture was allowed to stand for 7 minutes at room temperature. Ascorbic acid and methanol were used as positive and negative controls

respectively. The absorbance was read at 734 nm. The percentage free radical scavenging activity of the extracts was calculated using formula **2.2**.

3.1.4.3 Hydroxyl radical scavenging activity

The scavenging activity of the plant extracts on hydroxyl radicals was determined using the method described by Khan et al. (2012). The reaction mixture containing 25 mM deoxyribose, 10 mM FeCl₃, 100 mM ascorbic acid, 2.8 mM H₂O₂, 10 mM phosphate buffer (pH 7.4) and plant extract (100-800 µg/ml) was incubated at 37°C for 1 hour. Ascorbic acid and butylated hydroxy anisole were used as positive controls. The thiobarbituric acid reactive substances (TBARS) produced were measured spectrophotometrically at 535 nm. The percentage free radical scavenging activity of the extracts was calculated using formula **2.2**.

3.1.4.4 Superoxide anion scavenging activity

The superoxide anion scavenging activity of the plant extracts was measured with the phenazine methosulfate assay (Yamaguchi et al., 2000). The reaction mixture consisted of the plant extract (100-800 µg/ml), 60 µM of phenazine methosulfate, 200 µM NBT and 468 µM NADH. Quercetin was used as a positive control. The reaction mixture was incubated at 25°C for 5 minutes, and the absorbance was read at 560 nm. The percentage free radical scavenging activity of the extracts was calculated using formula **2.2**.

3.1.4.5 Metal ion chelating activity

The metal ion (Fe²⁺) chelating activity of the extracts was determined following the method described by Decker and Welch (1990). Ferrous chloride (2 mM) was mixed with the extract (100-800 µg/ml). The reaction was initiated by adding 5 mM ferrozine. After 10 minutes at room temperature, the absorbance of the mixture was measured at 562 nm. EDTA and citric acid (CA) were used as standards. The percentage of metal chelating activity of the extracts was calculated using formula **2.3**.

3.1.4.6 Oxygen Radical Absorbance Capacity

The antioxidant capacity of the extracts was determined by the Oxygen Radical Absorbance Capacity (ORAC) assay (Álvarez et al., 2012). Briefly, a mixture (1:6 v/v) of the extract (12.5-200 µg/ml) and fluorescein (1 M) was pre-incubated at 37 °C for 30 minutes. Subsequently, 250 mM AAPH was added to the mixture at a 1:7 ratio. Trolox was used as a standard. The fluorescence was measured every minute at an excitation wavelength of 485 nm and an emission wavelength of 520 nm for 120 minutes. The relative ORAC values of the samples were expressed as nmol Trolox equivalents per gram of sample (µmol TE/g).

3.1.4.7 Ferric Reducing Antioxidant Power

The ferric reducing antioxidant power (FRAP) assay described by Benzie and Strain (1996) was modified to determine the reducing power of the plant extracts. Briefly, 0.2 ml of the extract (20 and 100 µg/ml), was mixed with 3.8 ml of FRAP reagent. The resulting mixture was incubated at 37°C for 30 minutes. The absorbance was measured at 593 nm. Ascorbic acid was used as a positive control and the results were expressed in milligram equivalents of FeSO₄ per gram of dry weight.

3.1.5. Oxidation of LDL

The copper sulfate-mediated LDL oxidation assay described by Kim et al. (2019) was adapted to determine the inhibitory activity of the plant extracts on LDL (and HDL). The antioxidant activity of the extracts was determined by the measurement of conjugated dienes (CD) formation and TBARS produced. Gallic acid was used as a standard.

3.1.5.1 Measurement of the conjugated dienes formation

To measure the amounts of dienes formed, HDL or LDL (each at 200 µg protein/ml) were independently incubated with CuSO₄ (final concentration, 10 µM with and without the plant extract (64 µg/ml), in a medium containing 10 mM phosphate buffer (pH 7.4). The formation of CDs was monitored at 37 °C for 2 hours at 2-minute intervals by measuring the absorbance at 234 nm.

3.1.5.2 Measurement of thiobarbituric acid reactive substances

To measure the amounts of TBARS produced, LDL or HDL (each at 500 µg/mL) was incubated with CuSO₄ in the presence of the extract (0, 4, 64, and 192 µg/ml). The reaction mixture was incubated for 5 h at 37 °C. EDTA (0.5 mM) was added to terminate the reaction. Afterwards, 20% of TCA and TBA solutions were added and the mixture heated in a water bath at 90 °C for 20 minutes. After cooling at 4°C and centrifuging at 3000 rpm for 15 minutes, the absorbance of the supernatant was measured at 532 nm. The amount of TBARS formed was expressed as nmol MDA equivalents per mg protein.

3.2 Results

3.2.1 Extraction

The plant material was sequentially extracted with hexane, DCM and methanol. The extraction yields are shown in Table 3.1. The methanol extract showed a higher percentage yield than the hexane and DCM extracts.

Table 3.1 Percentage yields of *B. bowkeri* stem bark extracts.

Extract	Crude extract weight (g)	Yield (%)
Hexane	2.37	0.77
DCM	3.35	1.09
Methanol	4.5	1.47

3.2.2 Phytochemical screening

3.2.2.1 FTIR analysis

The FTIR spectrum was measured between 4000 and 500 cm⁻¹ to determine the functional groups present in the extracts. The results displayed peaks ranging between 1500 and 800 cm⁻¹ and 3500 and 2800 cm⁻¹ (Figure 3.1). The three extracts showed a similar pattern of spectra at the same wavenumbers. The peaks ranging between 3500 and 2800 cm⁻¹ showed the presence of the functional groups O-H, N-H, and C-H stretch. The peaks in the region between 1500 and 800 cm⁻¹ showed the presence of the phosphate and C=O groups (Thummajitsakul et al., 2020).

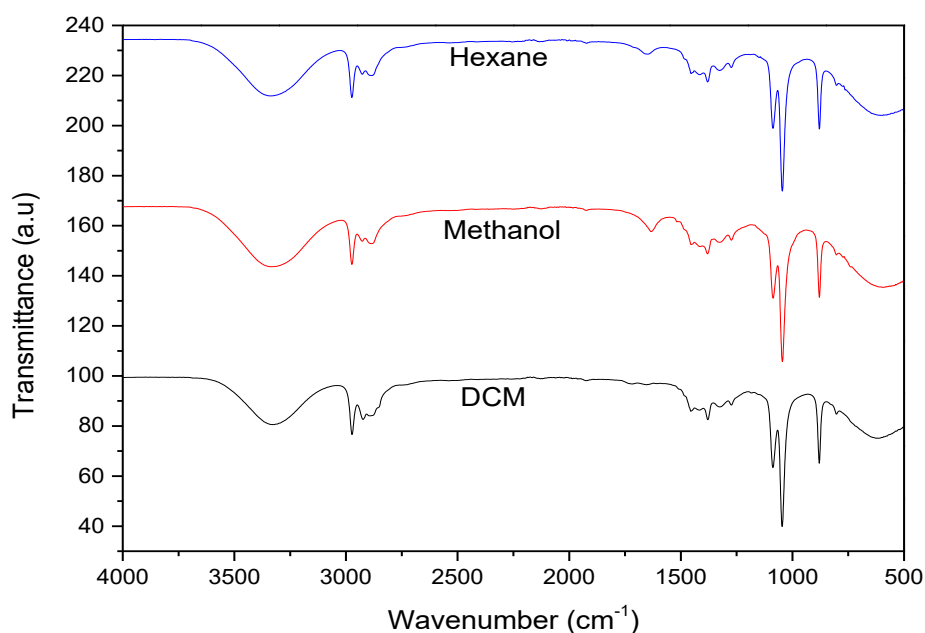


Figure 3.1. FTIR spectra of (A) hexane extract, (B) methanol and (C) DCM extract of *B. bowkeri*. The spectra were recorded at a wavenumber between 4000 and 500 cm^{-1} .

3.2.2.2 GC-MS analysis

The results of the GC-MS analysis of the stem bark extracts of *B. bowkeri* are presented in Tables 3.2-3.4. A total of more than 50 bioactive compounds were recorded from the extracts of the plant (See full spectra in Appendix A). The chromatogram of *B. bowkeri* extracts indicated 23 peaks for the hexane extract (Table 3.2), more than 23 peaks for the DCM extract (Table 3.3) and 6 peaks for the methanol extract (Table 3.4). For this study, the compounds with the highest peak area were considered abundant, and they were recorded. For hexane extract, the abundant compounds that have a peak area of 90% and above included Phenol, 2,5-bis(1,1-dimethylethyl)-, Hexadecane, Tetradecane, Phenol, 3,5-bis(1,1-dimethylethyl)-, Nonadecane, 1-Octadecanesulphonyl chloride, Phenol, 2,4-bis(1,1-dimethylethyl)-, Nonadecane, Hexadecane, Tetradecane, 1-Heptadecene, and Octadecane, 1-chloro- For the DCM extract, the abundant compounds (peak area $\geq 4\%$) included Heneicosane, Heptadecane, 2-methyl-, Diisooctyl phthalate, and 2-(3,3-Dimethyl-but-1-ynyl)-1,1-dimethyl-3-methylene-cyclopropane. In the methanol extract, the abundant compounds (peak area $\geq 70\%$) included Monoethylhexyl phthalate (BME), 1,2-Benzenedicarboxylic acid, diisooctyl ester and Phthalic acid, cyclohexyl 2-pentyl ester.

Table 3.2. Bioactive compounds of the hexane extract of *B. bowkeri* stem bark.

Peak number	Retention time (min)	Peak area (%)	Name of the compound	Chemical formula
1	13.736	83	Nonane, 3,7-dimethyl-	C ₁₁ H ₂₄
2	13.736	72	Dodecane, 1-iodo-	C ₁₂ H ₂₅ I
3	13.931	97	Phenol, 2,4-bis(1,1-dimethylethyl)-	C ₁₄ H ₂₂ O
4	13.931	91	Phenol, 2,5-bis(1,1-dimethylethyl)-	C ₁₄ H ₂₂ O
5	13.931	90	Phenol, 3,5-bis(1,1-dimethylethyl)-	C ₁₄ H ₂₂ O
6	14.882	94	Hexadecane	C ₁₆ H ₃₄
7	14.882	91	Tetradecane	C ₁₄ H ₃₀
8	14.882	90	Nonadecane	C ₁₉ H ₄₀
9	14.882	90	1-Octadecanesulphonyl chloride	C ₁₈ H ₃₇ ClO ₂ S
10	16.028	94	Nonadecane	C ₁₉ H ₄₀
11	16.028	91	Hexadecane	C ₁₆ H ₃₄
12	16.028	90	Tetradecane	C ₁₄ H ₃₀
13	17.058	95	1-Heptadecene	C ₁₇ H ₃₄
14	17.058	90	Octadecane, 1-chloro-	C ₁₈ H ₃₇ Cl
15	17.058	83	E-14-Hexadecenal	C ₁₆ H ₃₀ O
16	18.069	87	10-Methylnonadecane	C ₂₀ H ₄₂
17	18.350	59	Phthalic acid, 2-ethylcyclohexyl isobutyl ester	C ₂₀ H ₂₈ O ₄
18	18.350	50	Phthalic acid, 4-chloro-3-methylphenyl propyl ester	C ₁₈ H ₁₇ ClO ₄
19	18.996	72	Sulfurous acid, octadecyl 2-propyl ester	C ₂₁ H ₄₄ O ₃ S
20	18.996	52	Sulfurous acid, butyl heptadecyl ester	C ₂₁ H ₄₄ O ₃ S
21	20.599	62	6-Octadec enoic acid	C ₁₈ H ₃₄ O ₂
22	20.599	55	Oleic Acid	C ₁₈ H ₃₄ O ₂
23	23.951	53	1,2-Benzenedicarboxylic acid, dipropyl ester	C ₁₄ H ₁₈ O ₄

Table 3.3. Bioactive compounds of the DCM extract of *B. bowkeri* stem bark

Peak number	Retention time (min)	Peak area (%)	Name of the compound	Chemical formula
1	5.161	0.004514	Propanoic acid, 2-methoxy-, methyl ester	C ₅ H ₁₀ O ₃
2	5.275	0.077642	Ether, 2-chloro-1-methylethyl isopropyl	C ₆ H ₁₃ ClO
3	6.56	0.071293	Ethylbenzene	C ₈ H ₁₀
4	6.76	0.34371	p-Xylene	C ₈ H ₁₀
5	12.1	0.061605	Decane	C ₁₀ H ₂₂
6	14.0	0.18803	Undecane	C ₁₁ H ₂₄
7	17.42	2.4548	Tetradecane	C ₁₄ H ₃₀
8	18.8	0.1179	2,4-Di-tert-butylphenol	C ₁₄ H ₂₂ O
9	18.98	1.058	Hexadecane	C ₁₆ H ₃₄
10	21.22	0.10172	Benzene, 1,2,3,4-tetramethoxy-5-(2-propenyl)-	C ₁₃ H ₁₈ O ₄
11	23.18	9.4881	Nonadecane	C ₁₉ H ₄₀
12	24.93	1.2163	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂
13	25.65	7.0003	Heneicosane	C ₂₁ H ₄₄
14	27.91	4.0837	Heptadecane, 2-methyl-	C ₁₈ H ₃₈
15	29.98	2.1618	Dotriacontane	C ₃₂ H ₆₆
16	31.04	17.801	Diisooctyl phthalate	C ₂₄ H ₃₈ O ₄
17	31,09	2.8049	Bumetrizole	C ₁₇ H ₁₈ ClN ₃ O
18	38.14	2.0877	Ethyl iso-allocholate	C ₂₆ H ₄₄ O ₅
19	38.15	2.0877	á-Sitosterol	C ₃₅ H ₆₀ O ₆
20	39.03	1.1843	Lupeol	C ₃₀ H ₅₀ O
21	42.11	4.3405	2-(3,3-Dimethyl-but-1-ynyl)-1,1-dimethyl-3-methylene-cyclopropane	C ₁₂ H ₁₈
22	37.51	0,78735	Stigmasterol	C ₂₉ H ₄₈ O
23	40.78	0.37799	Friedelan-3-one	C ₃₀ H ₅₀ O

Table 3.4. Bioactive compounds of the methanol extract of *B. bowkeri* stem bark.

Peak number	Retention time (min)	Peak area (%)	Name of the compound	Chemical formula
1	9.981	64	Phenol, 3-methyl-	C ₇ H ₈ O
2	9.981	64	Phenol, 2-methyl-	C ₇ H ₈ O
3	9.981	64	Phenol, 4-methyl	C ₇ H ₈ O
4	23.842	87	Monoethylhexyl phthalate	C ₁₆ H ₂₂ O ₄
5	23.842	83	1,2-Benzenedicarboxylic acid, diisooctyl ester	C ₂₄ H ₃₈ O ₄
6	23.842	72	Phthalic acid, cyclohexyl 2-pentyl ester	C ₁₉ H ₂₆ O ₄

3.2.2.3 Total phenolic and flavonoid contents

The TPC and TFC contents of the crude extracts were determined, and the results are shown in Figure 3.3. The results showed that the *B. bowkeri* extracts had appreciable amounts of phenols and flavonoids. There was a statistically significant difference among the tested extracts. While the highest TPC was observed in the methanol extract (ME) (Figure 3.3A), the highest TFC was observed in the hexane extract (HE) (Figure 3.3B), in contrast to the other extracts.

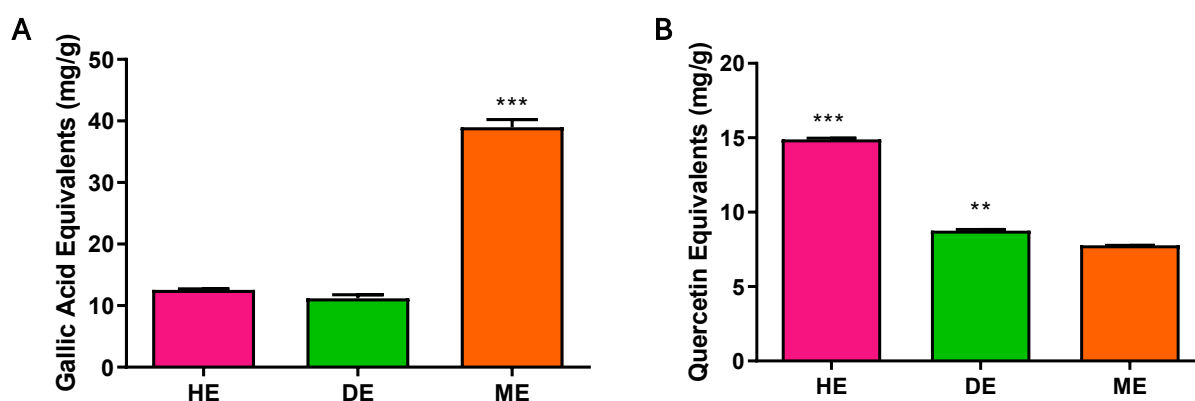


Figure 3.2. The Total phenolic content (A) and Total flavonoid content (B) of the extracts of *B. bowkeri*. HE- hexane extract, DE- DCM extract, ME- methanol extract. The data was expressed as mean $\pm$ SEM (n = 3), **p < 0.01 and ***p < 0.001.

3.2.3 Antioxidant activity

3.2.3.1 Free radical scavenging activity of the extracts

The extracts' free radical scavenging activity was determined on DPPH, ABTS, hydroxyl and superoxide anion radicals. The plant extracts showed free radical scavenging activity that is dependent on concentration (Figure 3.4). The ME exhibited the highest scavenging activity in contrast to the hexane and DCM extracts, with the lowest EC₅₀ values of 60 and 44 µg/ml on DPPH and ABTS, respectively, and 75% scavenging activity on superoxide anion. However, the hexane extract exhibited the maximum activity on hydroxyl radical with the lowest EC₅₀ value of 3.2 µg/ml.

Table 3.5. Half maximal effective concentration (EC₅₀) values of the extracts on DPPH, ABTS, hydroxyl and superoxide anion radicals.

	EC ₅₀ (µg/ml)			
	DPPH	ABTS	Hydroxyl radical	Superoxide
Hexane	>100	>100	3.2	>500
DCM	>100	61 ± 4.4	3.5 ± 1.1	>500
Methanol	70.5 ± 0.03	44 ± 5.3	3.4 ± 0.82	320 ± 2.11
Ascorbic acid	29 ± 0.004	6.9 ± 1.2	3.2 ± 1.5	-
Butylated hydroxy anisole	-	3.2 ± 1.5	2.9 ± 0.4	-
Quercetin	-	-	-	84 ± 0.8

Data are expressed as mean ± SEM, (n=3).

3.2.3.2. Oxygen radical absorbance capacity of the extracts

The ORAC activity of the plant extracts was also investigated, and the findings are presented in Table 3.5. It was observed that the extracts showed ORAC. The ME also showed the highest oxygen radical absorbance capacity with an ORAC value of 189 µmol TE/g. The HE showed the lowest activity with an ORAC value of 181 µmol TE/g.

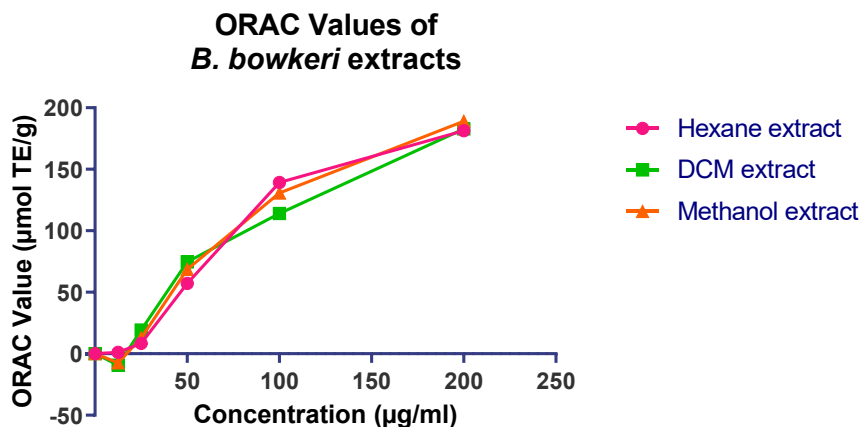


Figure 3.3. ORAC values ($\mu\text{mol TE/g}$) of the *B. bowkeri* stem bark extracts. Data are expressed as mean $\pm$ SEM, (n=3).

3.2.3.3 Reducing power and metal ion chelating activity of the extracts

The ferric reducing power and metal ion chelating activity of the extracts were also determined. The results further showed that the plant extracts exhibited concentration-dependent ferric reducing power and metal ion chelating activity (Figure 3.4). The results showed that the methanol extract (at 100 $\mu\text{g/ml}$) exhibited the highest reducing power when compared to the other extracts, which showed a lower reducing power. The hexane extract showed the highest metal ion (Fe^{2+}) chelating activity (55 %), while the methanol and DCM extracts showed 50% chelating activity.

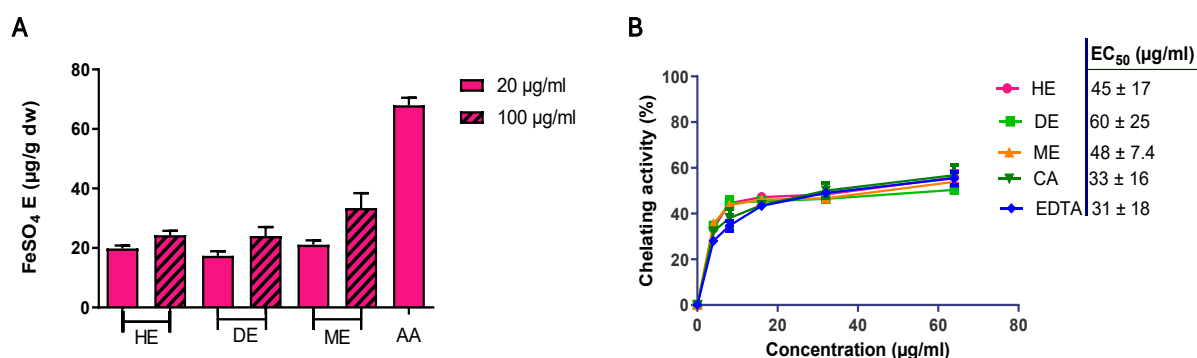


Figure 3.4. Ferric reducing power (A) and metal ion (Fe^{2+}) chelating activity (B) of *B. bowkeri* extracts. The data was expressed as mean $\pm$ SEM (n = 3), **p < 0.01 and ***p < 0.001. HE- hexane extract, DE- DCM extract, ME- methanol extract, AA- ascorbic acid, and CA- citric acid

3.2.4 Inhibition of LDL oxidation

The plant extracts' antioxidant capacity was also investigated in the copper sulfate-induced LDL and HDL oxidation. In the LDL incubated with copper sulfate only, an increase in CD formation was observed. A similar observation was made in the LDL incubated with copper sulfate with DCM extract. However, incubation of the LDL with copper sulfate with the hexane or methanol extracts resulted in no detectable CD formation (Figure 3.5A). This observation was similar to that of the normal control (LDL only) and gallic acid used as the positive control.

While an increase in the MDA levels was also observed in the LDL incubated with copper sulfate only, the plant extracts also reduced the MDA levels of the oxLDL, dependent on concentration (Figure 3.5B). The methanol extract (at 192 µg/ml) showed significantly better (74%) inhibition of MDA formation than the hexane and DCM extracts, which showed 57% and 13% inhibitions, respectively. However, GA (used as a positive control) reduced the MDA content of the oxLDL better than any of the extracts. Gallic acid showed up to 91% inhibition of MDA formation in the oxLDL (Figure 3.5C).

Furthermore, the extracts showed very little inhibition of HDL oxidation. The extracts showed an MDA concentration comparable to that of the oxidized HDL, except for the methanol extract (64 µg/ml), which showed less MDA concentration as compared to the hexane and DCM extracts. Gallic acid showed to have a reduced MDA concentration than all three extracts (Figure 3.5D). However, the methanol showed better inhibition at 64 µg/ml (24.4%) than the hexane and DCM extracts (Figure 3.5E), but its inhibitory activity was less than that of gallic acid (63%), the standard inhibitor.

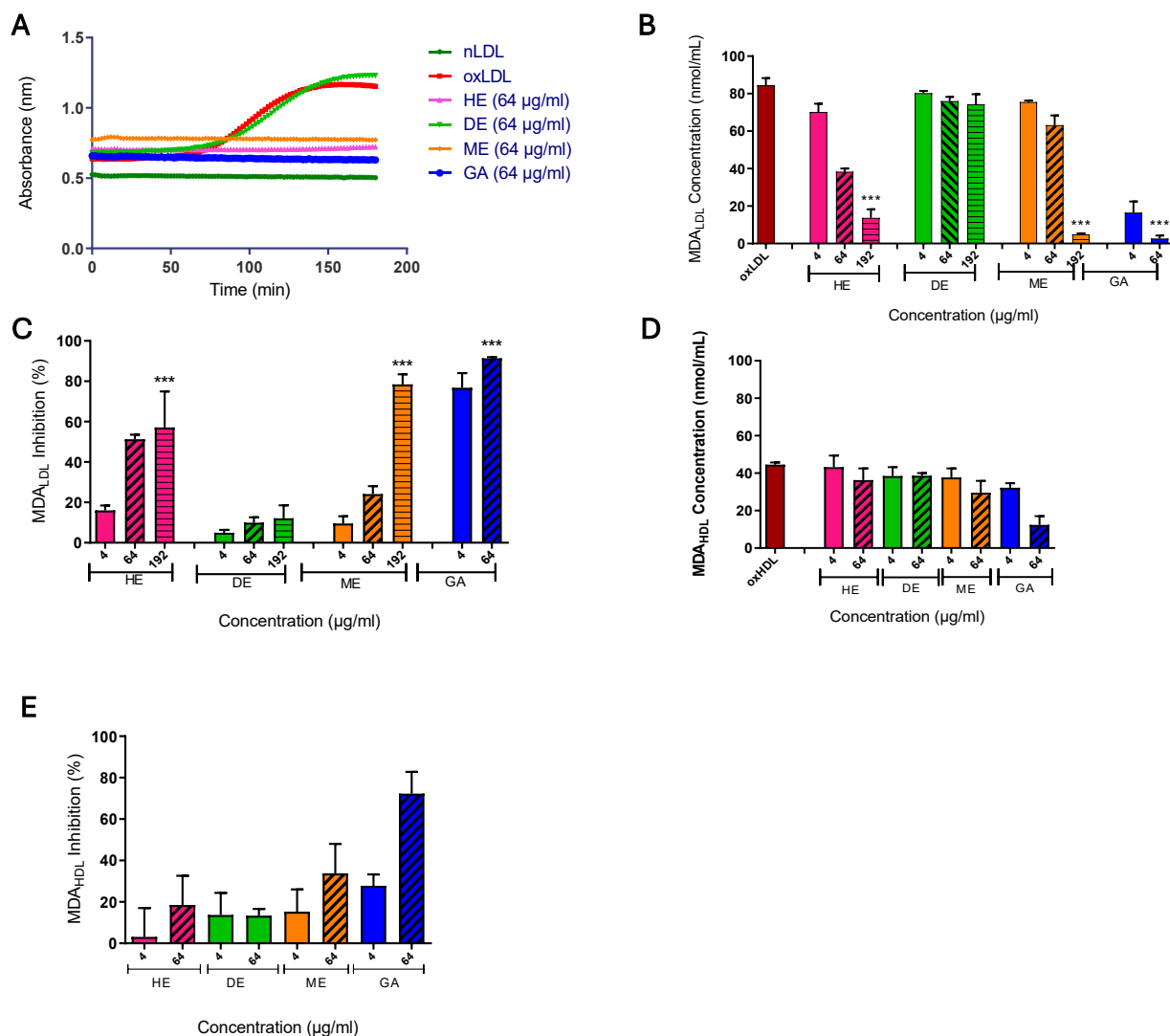


Figure 3.5. The effect of *B. bowkeri* extracts on LDL and HDL oxidation. **(A)** Inhibition of CD formation in LDL, **(B)** LDL-MDA concentration, **(C)** LDL-MDA percentage inhibition, **(D)** HDL-MDA concentration and **(E)** HDL-MDA inhibition. LDL (or HDL) was incubated with CuSO₄ for 3 hours at 2-minute intervals, and the CD formation and MDA formation were measured at 234 nm and 532 nm, respectively. The data was expressed as mean $\pm$ SEM (n = 3), *p < 0.05, **p < 0.01 and ***p < 0.001.

3.3 Discussion

Medicinal plants are recognized for their therapeutic properties because of their phytochemical composition. These phytochemicals include terpenoids, saponins, carotenoids, alkaloids, flavonoids, and polyphenols. In this study, the phytochemical constituents of *B. bowkeri* plant extracts were screened. The presence of functional groups such as O-H (hydroxyl group), N-H (amine group), C-H, phosphate, and C=O

(carbonyl group) as revealed by FTIR analysis (Figure 3.1) indicated the presence of phytochemicals like the flavonoids and other phenols. GC-MS analysis further revealed that the plant extracts have more than 50 bioactive compounds, which include Phenol, 2,5-bis(1,1-dimethylethyl)-, 1-Octadecanesulphonyl chloride, 1,2-Benzenedicarboxylic acid, diisooctyl ester and Monoethylhexyl phthalate. These compounds have been reported to have antioxidant and anti-inflammatory activities (Khan et al., 2019; Asaduzzaman et al., 2020; El-Shahir et al., 2022; Wiraswati et al., 2023).

Since numerous diseases are linked to oxidative stress, including atherosclerosis, the antioxidant activity of the plant extracts was investigated. The ability of the extracts to scavenge free radicals (Figure 3.4), reduce ferric iron, and chelate ferrous iron (Figure 3.5 A and 3.5 B) indicated their antioxidant activities. The antioxidant activity of *B. bowkeri* stem bark extracts could be because of the TPC and TFC of the extracts (Figure 3.3). Phenols and flavonoids are well-known to have antioxidant activities (Ji et al., 2020; Phuyal et al., 2020). Previous studies have also indicated a strong correlation between plant extracts' antioxidant activity and their phenolic content (Bittar et al., 2024; Mehmood et al., 2022; Olvera-Aguirre et al., 2022). The presence of antioxidant compounds such as Monoethylhexyl phthalate, 1,2-Benzenedicarboxylic acid, diisooctyl ester and Phenol, 2,5-bis(1,1-dimethylethyl)- as revealed by the GC-MS analysis could have also contributed to the antioxidant activity of the *B. bowkeri* extracts.

The antioxidant activity of the extracts was further confirmed in the copper sulfate-mediated LDL peroxidation. Lipid peroxidation consists of an initiation phase, which includes conjugated diene (CD) formation, and a propagation phase, which consists of further oxidation and malonaldehyde (MDA) production. The inhibitory activity shown by the extracts (hexane and methanol, Figure 3.5 A) on the formation of CDs and MDA further indicated their antioxidant activity. DCM extract's inhibitory activity on the MDA formation but not the CD formation indicates that the extract only inhibits lipid peroxidation in the secondary stage (propagation) of lipid peroxidation, where MDA is formed, but not in the primary stages where CDs are formed. The extracts could have inhibited the lipid peroxidation by chelating the Cu^{2+} ions used to initiate the oxidation reaction. This apparent mechanism is supported by the metal ion

chelating activity and ferric reducing power exhibited by the extracts (Figure 3.5 A and 3.5 B). The highest anti-LDL oxidation activity exhibited by the methanol extract may be attributed to its higher phenolic content. According to Hari et al. (2024), there is a strong correlation between TPC and the plant extracts' antioxidant activity.

The anti-LDL oxidation activity of the extracts could be vital in the prevention of atherosclerosis and related cardiovascular complications. LDL oxidation is the major step in the development of atherosclerosis.

3.4 Conclusion

This study was based on phytochemical screening and antioxidant activity of *B. bowkeri* stem bark extracts. It is concluded that the *B. bowkeri* stem bark extracts (hexane, methanol and DCM) contain various phytochemicals which include flavonoids and other phenolics. The extracts also exhibited antioxidant activity. This study indicated that there is a strong correlation between the phytochemicals and the antioxidant activity of the plant extracts. The results imply that the *B. bowkeri* extracts can be used in the management of ailments associated with oxidative stress. Further studies are recommended to investigate the extracts' activity on other pathways that further contribute to oxidative stress. Such pathways include the polyol and protein glycation pathways. This is the focus of the next chapter.

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CHAPTER 4

Potential of *Bauhinia bowkeri* extracts to inhibit polyol pathway activity and protein glycation *in vitro*

Abstract

Hyperglycemia-induced activation of pathways like the polyol pathway and protein glycation are involved in diabetes development of micro- and macro-vascular complications. This study investigated the inhibitory potential of *Bauhinia bowkeri* stem bark extracts (hexane, dichloromethane and methanol) on the polyol pathway activity and protein glycation *in vitro*. The ALR and SDH assays were used to investigate the extracts' potential to inhibit the polyol pathway activity. The plant extracts' antiglycation activity was examined on fructose-induced LDL (or BSA) glycation. The protein was incubated with fructose with and without extracts (100 and 500 µg/ml) for 7 days. The extracts showed inhibitory activity on ALR with IC₅₀ values of 75-90.8 µg/ml, and SDH (IC₅₀ ≥ 185 µg/ml). The extracts further exhibited inhibitory activity against BSA and LDL glycation. The methanol extract exhibited the highest inhibitory activity of up to 61% on the LDL glycation. The obtained results indicate that the *B. bowkeri* extracts have therapeutic potential against diabetes-related vascular complications.

4.0 Introduction

Chronic hyperglycaemia that characterizes diabetes is involved in micro-vascular and macro-vascular complications (Oguntibeju, 2019). Chronic hyperglycaemia activates other glucose metabolizing pathways like the polyol pathway and the formation of AGEs (Lee et al., 2022). The polyol pathway is initiated by the NADPH-dependent ALR activity, an enzyme that reduces glucose to sorbitol. The sorbitol is converted to fructose by NAD⁺-dependent SDH. NADPH is also a cofactor in the formation of reduced glutathione (GSH); the aldose reductase reaction depletes intracellular GSH levels. The depletion of GSH favours increased production of ROS and oxidative stress (Papachristoforou et al., 2020). These ROS oxidize proteins and lipids, including low-density lipoprotein (LDL). LDL oxidation is a critical step in atherogenesis, resulting in atherosclerosis (Poznyak et al., 2021).

Excess blood glucose can also react with proteins, including LDL, through the Millard reaction, to produce Schiff bases, which further reposition themselves to produce Amadori products (Kuzan, 2021). These Amadori products undergo a series of reactions, including irreversible reactions that generate highly reactive carbonyls such as methylglyoxal and glyoxal. These reactive dicarbonyls further produce AGEs, which alter proteins, DNA and lipoproteins (Sarmah et al., 2022). AGEs can further glycate LDL and make it susceptible to oxidative modification by altering its apoprotein B. Furthermore, AGEs react with the RAGE, which also produces ROS, oxidize LDL, and stimulates the overexpression of pro-inflammatory cytokines and macrophage infiltration (Vekic et al., 2023). The listed factors stimulate atherogenesis, leading to macrovascular complications such as atherosclerosis in diabetics.

Polyol pathway inhibition activity and protein glycation can help ameliorate vascular complications of diabetes. The currently used antidiabetic drugs have side effects (Sarmah et al., 2022), and this fuels the need to find an alternative treatment that has fewer side effects. Medicinal plants are recognized to have phytochemicals responsible for therapeutic activities. In the previous study, *B. bowkeri* extracts were screened for their phytochemical composition and were found to consist of various types of phytochemicals. These include phenolics, terpenoids and alkaloids. Polyphenols have various bioactivities like anti-inflammatory, antioxidant, and anti-

diabetic activities (Scarpa et al., 2024). Thus, this study investigated the *B. bowkeri* stem bark extracts' inhibitory potential on the polyol pathway and protein glycation *in vitro*.

4.1 Materials and Methods

4.1.1 Reagents

The chemical reagents utilized in this chapter were similar to those mentioned in section 2.1.1.

4.1.2 Extraction of plant material

The *B. bowkeri* stem bark extracts were prepared as described in Chapter 3 to obtain the hexane, dichloromethane and methanol crude extracts.

4.1.3 Enzyme inhibition activity

4.1.3.1 Aldose reductase inhibition assay

The aldose reductase inhibitory assay was performed following the method of Fatmawati et al. (2015). The reaction mixture containing 0.15 mM NADPH, 10 mM DL-glyceraldehyde, and the extract (50-200 µg/ml) in 100 mM sodium phosphate buffer (pH 6.2), was pre-incubated at 25°C for 5 minutes. The reaction was initiated by adding 5 µl of 100 µg/ml aldose reductase. Zopolrestat was used as a positive control, and methanol was used as a negative control. The absorbance of the reaction mixture was read at 340 nm every 2 minutes for 10 minutes. The enzyme inhibitory activity (%) of the plant extracts was calculated using formula **2.4**.

4.1.3.2 Sorbitol dehydrogenase inhibition assay

The inhibitory activity of the plant extracts on sorbitol dehydrogenase activity was determined following the method described by Kobayashi et al. (2002). The assay mixture contained 100 mM Tris-HCl buffer (pH 9.0), 100 µg/mL sorbitol dehydrogenase, 100 mM sorbitol (substrate) and the extract (4-64 µg/ml). The reaction was initiated by adding 0.5 mM NAD⁺. Quercetin was used as a positive control, and methanol was used as a negative control. The rate of change in the absorbance of the

mixture was measured at 340 nm. The inhibitory activity of the plant extract was calculated using formula **2.4**.

4.1.3.3 Phospholipase A₂ inhibitory activity

The plant extracts' inhibitory activity on secretory phospholipase A₂ (sPLA₂) was determined using phosphatidylcholine as a substrate (de Araujo and Radvanyi, 1987). Briefly, a volume of 5 µl of sPLA₂ (100 µg/ml) solution was pre-incubated with 5 µl of the extract (192 µg/ml) for 10 minutes at room temperature. The reaction was initiated by adding 190 µl of the freshly prepared enzyme substrate [10 mM phosphatidylcholine, 100 mM NaCl and 10 mM CaCl₂]. Phenol red (0.055 mM) was used as a colorimetric indicator. Varespladib was used as a standard. The enzyme activity was monitored at 1-minute intervals for 20 minutes by reading the optical density at 558 nm. The enzyme inhibitory activity of the extract was calculated using formula **2.4**.

4.1.4 Antiprotein glycation activity

The antiprotein glycation activity of the plant extracts was investigated on BSA and LDL using fructose and methylglyoxal (MGO) as the protein glycating agents.

4.1.4.1 Fructose-induced protein glycation assay

The antiprotein glycation activity of the plant extracts was determined in fructose-induced protein glycation (Bhatwadekar and Ghole, 2005). The reaction mixture in 0.1 M phosphate-buffered saline (PBS, pH 7.4), consisted of 0.25 mg/ml of LDL (or 10 mg/ml of BSA), the extract (100 and 500 µg/ml), 0.25 M fructose, and 0.002% sodium azide. Aminoguanidine was used as a reference standard. The reaction mixture was incubated at 37°C in the dark for seven days (four weeks for BSA). The fluorescence of AGEs formed was measured at 335 nm (excitation) and 385 nm (emission). The percentage inhibition of AGEs was calculated using formula **2.5**.

4.1.4.2 Fructosamine content assay

The fructosamine content of the glycated protein samples was determined using the NBT assay (Ayyappan et al., 2015). The glycated protein sample (70 µL) was mixed

with 130 μL of 0.7 mM NBT. The reaction mixture was incubated at 37°C for 30 minutes, and the absorbance was measured at 540 nm. The fructosamine content was expressed as the nmol equivalence of 1-deoxymorpholino (DMF) per well (nmol/well).

4.1.4.3 Free amino group content assay

The TNBSA assay was used to determine the free amino group content of the glycated protein samples (Sattarahmady et al., 2007). The reaction mixture containing the glycated protein sample (70 μL) and 0.01% TNBSA (35 μL) was incubated at 37°C for 1 hour. The reaction was stopped by adding 10% SDS and 1 M HCl. The absorbance was read at 340 nm. The free amino group content was expressed as the nmol equivalence of L-arginine amino group content per well (nmol/well).

4.1.4.4 Congo red binding assay

The Congo red binding assay described by Klunk et al. (1989) was adapted to determine the content of protein aggregates in the glycated protein samples. A volume of 500 μL of glycated protein (BSA) was incubated with 100 μL of Congo red (100 μM) for 5 minutes at room temperature. The absorbance was measured at 530 nm. The higher the absorbance, the higher the content of protein aggregates.

4.1.4.5 Methylglyoxal-induced protein glycation assay

The antiprotein glycation activity of the plant extracts was also determined in MGO-induced BSA glycation (Bhatwadekar and Ghole, 2005). LDL could not be tested due to the reagent limitation. The reaction mixture was composed as in 4.4.1 except that MGO was used as a glycating agent, and the incubation period was two weeks. The antiglycation activity of the extracts was again confirmed by determining the presence of protein aggregates and free amino group contents of the glycated samples as described in 4.1.4.2 to 4.1.4.3.

4.2 Results

4.2.1 Inhibitory potential of the extracts on aldose reductase, sorbitol dehydrogenase and secretory phospholipase A₂ activities.

The inhibitory activity of the plant extracts was first looked into on ALR and SDH activity. The data showed that the extracts have inhibitory activity on the two enzymes. All three extracts exhibited a concentration dependent inhibition on both enzymes, however at varying degrees of inhibition (Figure 4.1 A and B). The maximum inhibitory activity of the three extracts was detected on ALR when compared to SDH. The hexane (HE) and methanol (ME) extracts exhibited the highest inhibitory activity on ALR with IC_{50} values of 75 and 90.8 $\mu\text{g/ml}$, respectively. Their IC_{50} values were lower than that of zopolrestat (127 $\mu\text{g/ml}$), a known standard inhibitor of ALR. The dichloromethane extract (DE) exhibited the lowest inhibitory activity with an IC_{50} of 139 $\mu\text{g/ml}$. The extracts further exhibited inhibitory activity on SDH, but their inhibitory activities were lower than that of quercetin (QCT), which was used as the reference inhibitor. DE and ME exhibited IC_{50} values of 186 and 185 $\mu\text{g/ml}$, respectively, when compared to 58 $\mu\text{g/ml}$ of QCT. HE (IC_{50} of >200 $\mu\text{g/ml}$) showed the lowest inhibitory activity on SDH.

Table 4.1. IC_{50} values of the extracts on aldose reductase and sorbitol dehydrogenase activities.

Extract	IC_{50} ($\mu\text{g/ml}$)	
	Aldose reductase	Sorbitol dehydrogenase
Hexane (HE)	75 ± 2.83	>200
DCM	139 ± 3.06	186 ± 3.06
Methanol (ME)	90.8 ± 2.71	185 ± 2.36
Zopolrestat (ZPT)	127 ± 2.45	-
Quercetin (QCT)	-	58 ± 1.22
Varespladib (VB)	-	-

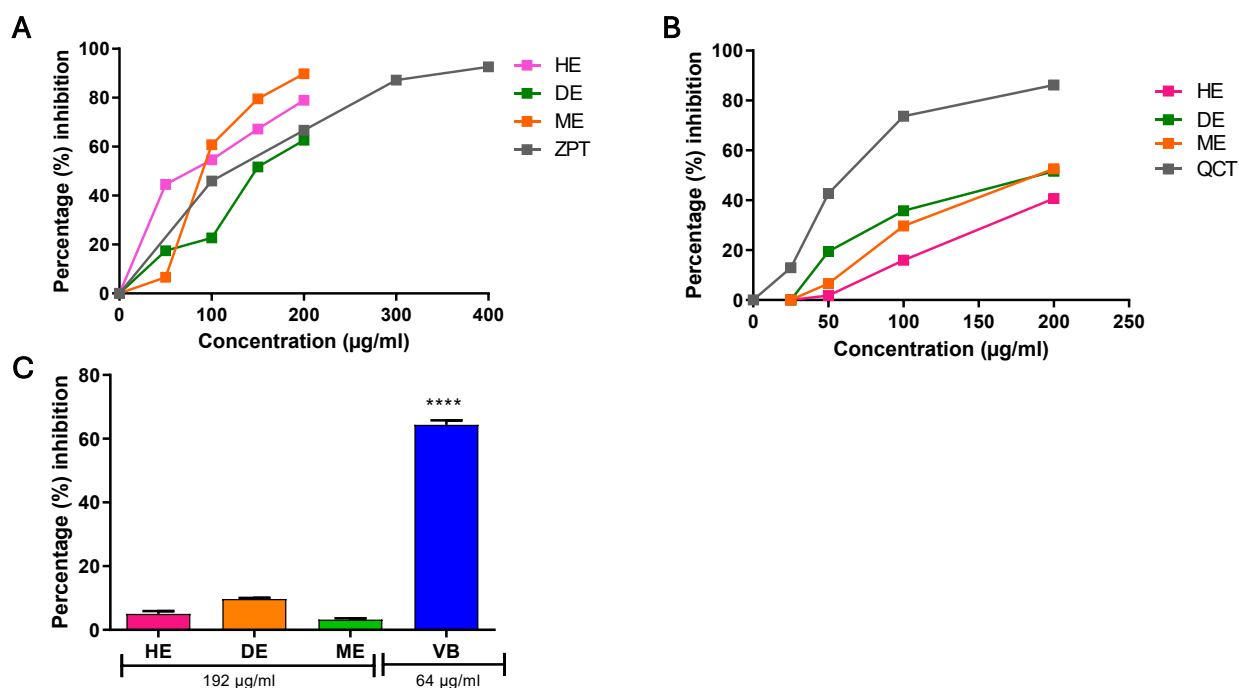


Figure 4.1. Inhibitory activity of the extracts on (A) Aldose reductase, (B) sorbitol dehydrogenase and secretory phospholipase A₂ (C) activity. QCT- quercetin, ZPT- zopolrestat and VB- varespladib. Data is depicted as mean $\pm$ SEM (n = 3), *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 as opposed to VB.

The crude extracts' inhibitory effect was further screened against secretory phospholipase A₂ activity and results are given in Figure 4.1C. The plant extracts (at 192 µg/ml) showed low inhibitory activity on the enzyme. The hexane, DCM and methanol extracts showed significant percentage inhibitions of 6%, 15% and 4%, respectively, when compared to 68% of Varespladib (at 64 µg/ml), which was used as a reference standard. Due to these lower percentage inhibitions of the extracts at 192 µg/ml, further tests at any higher concentrations were discouraged.

4.2.2 Effect of the extracts on fructose-induced protein glycation

The impact of *B. bowkeri* extracts on fructose-induced fluorescent AGEs generation was monitored over 4 weeks for BSA and 1 week for LDL as model proteins. The findings demonstrated that the *B. bowkeri* extracts inhibited the AGEs formation more as the concentration increased (Figure 4.3 A). The methanol extract (ME), at 500 µg/ml, showed the highest inhibitory activity (74.2%) on BSA glycation when compared to the DCM (DE) and hexane (HE) extracts with 67.2 and 53.4% inhibition,

respectively. The observed decreases in the fructosamine, amyloid contents (Figure 4.3C and F) and an increase in free amino group contents with the presence of the extracts (Figure 4.3E) further confirmed the antiprotein glycation activity of the extracts. The extracts (each at 500 µg/ml) also inhibited LDL glycation with a percentage inhibition of 59.6, 76.6 and 77.8% for HE, DE and ME, respectively. However, the standard inhibitor showed the highest percentage inhibition of 98%. This was confirmed by a decrease in fructosamine content (Figure 4.3D).

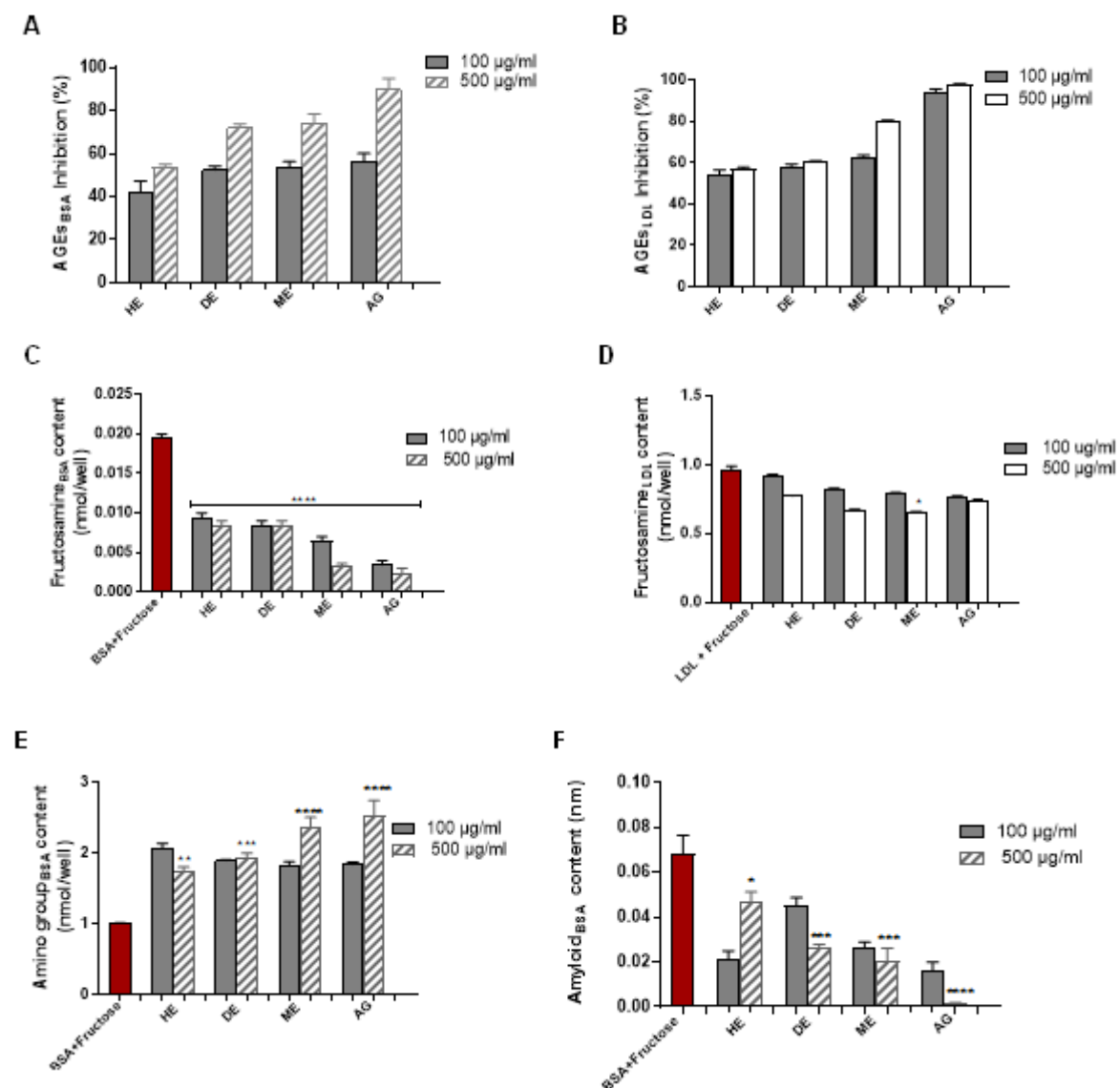


Figure 4.2. Inhibitory activity of the extracts on fructose-induced LDL and BSA glycation. (A) % inhibition of glycated BSA, (B) % inhibition of glycated LDL (C) Fructosamine content of glycated BSA, (D) Fructosamine content of glycated LDL, (E) Free amino group content of glycated BSA and (F) Amyloid content of glycated BSA. LDL and BSA were incubated with fructose at 37°C for 1 and 4 weeks, respectively. AG- aminoguanidine. Data is expressed as

mean $\pm$ SEM (n = 3), *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 against BSA+Fructose and/or LDL+Fructose.

4.2.3 Effect of the extracts on methylglyoxal-induced protein glycation

The antiglycation potential of the plant extracts was further investigated using MGO. The BSA-MGO assay is commonly used to investigate the ability of antiglycation agents to inhibit the later stages of glycation. The results revealed that the extracts did not inhibit MGO-induced formation of fluorescent AGEs (Figure 4.4 A). There was a significantly high fluorescence of AGEs with the extracts present in contrast to the model control (BSA + MGO). A decline in AGEs formation was only detected with the known standard inhibitor, Aminoguanidine, present. These results were confirmed by free amino groups and amyloid contents shown in Figure 4.4 C and D, respectively. Low levels of amino groups and high levels of amyloid content were also observed in the protein incubated with MGO, with the extracts present as opposed to the glycated protein (BSA + MGO). A noticeable increase in amino groups and a decline in amyloid content were only observed in the standard inhibitor aminoguanidine.

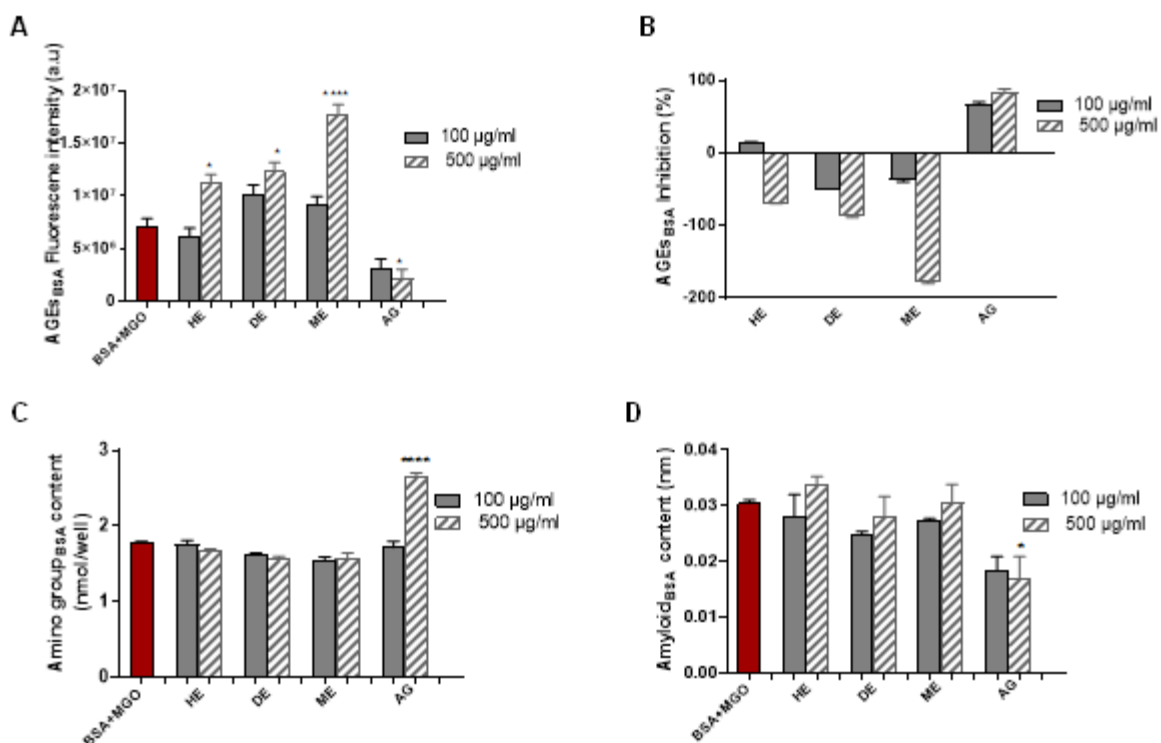


Figure 4.3. Inhibitory activity of the extracts on methylglyoxal-induced BSA glycation. (A) Fluorescence of AGEs, (B) % inhibition (C) Free amino group and (D) Amyloid content. BSA was incubated with MGO at 37°C for 1 week. Data is shown as mean $\pm$ SEM (n = 3), *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 in contrast to the BSA+MGO.

4.3 Discussion

The prevalence of people living with diabetes and dying from its micro- and macro-vascular complications is on the rise. The polyol pathway and AGEs formation are among the pathways that lead to diabetes complications, such as atherosclerotic cardiovascular diseases. These biochemical pathways have thus become therapeutic targets to reduce the risks of diabetes-related vascular complications (Putta and Kilani, 2020). This study investigated the inhibitory potential of *B. bowkeri* stem bark extracts on the polyol pathway and protein glycation.

The extracts' capability to inhibit the polyol pathway activity was determined on ALR and SDH, which are the two enzymes of the pathway. The polyol pathway rate-limiting step is catalyzed by aldose reductase, thus a critical therapeutic target towards inhibition of the pathway. The inhibitory activity exhibited by the extracts on ALR and SDH (Figure 4.1 A and B) indicates that the extracts could inhibit polyol pathway activity. The highest inhibitory activity of the extracts on ALR, when compared to SDH, suggests better inhibition of polyol pathway activity as the extracts exhibited better inhibitory activity on the crucial enzyme of the pathway.

The extracts' inhibitory activity could be a result of the plant extracts' TPC and TFC. According to the study reported by Kazeem et al. (2020), the inhibition of the two enzymes (ALR and SDH) by some extracts of the *Citrus* genus was attributed to their flavonoid content. The inhibitory potential of the *Euphorbia regis-jubae* extracts on ALR and SDH was also correlated with the phenolic and flavonoid components (Hmimid et al., 2021). Since polyol pathway activity results in both AGEs formation and production of ROS, the inhibitory activity of the *B. bowkeri* extracts on the polyol pathway enzymes could further prevent ROS production and protein glycosylation. This aligns with research carried out by Kazeem et al. (2021) in which inhibition of ALR and SDH by *V. amygdalina* extract ameliorated both oxidative stress and protein glycation.

The extracts' bioactivity was further investigated on secretory phospholipase A₂ (sPLA₂), an enzyme involved in the inflammatory response. Oxidative stress stimulates the expression and activity of sPLA₂, which catalyses the production of

arachidonic acid (AA) from phospholipids. AA produces pro-inflammatory cytokines which attract the oxidatively modified LDL (oxLDL) to the macrophage cells, which leads to foam cell formation (Bermúdez et al., 2021). The extracts showed little inhibition activity as compared to the known standard inhibitor. This indicates that the extracts do not have inhibitory activity on the AA inflammatory pathway. However, the antioxidant activity and extracts' inhibitory activity on the oxidative modification of LDL have been established (Chapter 3).

Since there is an established link between the polyol pathway and AGEs formation in mediating diabetic complications, the antiprotein glycation of the extracts was also investigated on BSA and LDL. While the BSA-fructose assay is commonly used to investigate the ability of antiglycation agents to inhibit the initial glycation stages, the BSA-MGO assay is employed for the later stages (Mohd Dom et al., 2020). The decreases in fluorescence of AGEs accompanied by lower fructosamine content and an increase in the free amino group content in fructose-induced protein glycation (Figure 4.3 C and E), indicated that the extracts possess antiprotein glycation properties. Inhibition of LDL glycation could be particularly crucial in the prevention of atherogenesis. LDL glycosylation and oxidation are critical steps in atherosclerosis development in diabetics (Poznyak et al., 2021). Failure of the extracts to inhibit the methylglyoxal-induced protein (BSA) glycation suggests that the plant extracts can only inhibit the early glycation stages but not the later stages.

Previous studies have indicated a strong connection between the phenolic contents of some plant extracts with their antiglycation activities (Favre et al., 2022; Sarmah et al., 2022). Since *B. bowkeri* extracts also showed high phenolic and flavonoid content (See Chapter 3), this phytochemical composition supports the observed antiglycation activity. The methanol extract (ME) indicated the highest antiglycation activity as opposed to the hexane extract (HE) and dichloromethane extract (DE). Methanol is a polar solvent that extracts polar phytochemicals. This implies that the highest phenolic content was observed with increasing polarity, thus the highest activity.

4.4 Conclusion

The findings of this investigative study demonstrated that the *B. bowkeri* stem bark extracts exhibited the potential to inhibit the polyol pathway activity and AGEs formation. Methanol extract, due to its high polarity and phenolic content, exhibited the highest activity among the hexane and dichloromethane extracts. The observed polyol pathway enzymes (ALR and SDH) inhibition and protein glycation may aid in the amelioration of diabetes-linked micro- and macro-vascular complications, including atherosclerosis. Further studies on the isolation of compound(s) that might be responsible for these bioactivities are recommended.

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CHAPTER 5

Phthalates from *Bauhinia bowkeri* extracts exhibit properties to inhibit the polyol pathway enzymes and low-density lipoprotein modification

Abstract

Bauhinia bowkeri extracts have shown promise in inhibiting the polyol pathway and protein glycation, which are critical to diabetes-associated macrovascular complications such as atherosclerosis. This study investigated the potential of selected *B. bowkeri* phytochemicals to inhibit the polyol pathway enzymes and LDL modification *in vitro*. The compounds were screened through *in silico* molecular docking, with the potential to inhibit ALR and SDH. The ALR and SDH *in vitro* bioassays were used to evaluate the enzyme inhibitory activity of the compounds. The antiprotein glycation activity of the compound(s) was investigated on LDL using fructose and methylglyoxal as glycating agents. The copper sulphate-mediated LDL oxidation assay was used to investigate the antioxidant activity of the compound(s). While all six selected compounds showed good ADMET properties, five showed good docking scores on ALR and SDH. Monoethylhexyl phthalate (BME) exhibited the highest docking scores of -7.01 and -4.75 on ALR and SDH, respectively. BME inhibited ALR and SDH with 0.33 and 0.30 mM IC₅₀ values, respectively. The compound also exhibited an inhibition that is dependent on concentration, LDL glycation and oxidation, with percentage inhibitions of 43.2 % and 54.13 %, respectively. These results indicate that the selected plant compounds have properties to inhibit the polyol pathway. BME can also inhibit the glycosylation and oxidative modification of LDL implicated in diabetes-associated risk of atherogenesis.

5.0 Introduction

Atherosclerosis is a major underlying cause of diabetes-associated cardiovascular disease (CVD) mortalities (Poznyak et al., 2020). In a diabetic state, chronic hyperglycemia and hyperlipidemia directly or indirectly stimulate the atherogenic process that is clinically manifested as atherosclerosis. The increased polyol pathway activity, accelerated rate of protein glycation, and AGEs formation are considered among the underlying mechanisms (Rybarczyk et al., 2023). ALR catalyzes the first and rate-limiting step of the polyol pathway. The enzyme reduces glucose to sorbitol, which is then oxidized to fructose by the NAD⁺-dependent SDH. The produced fructose can be phosphorylated into a reactive dicarbonyl, 3-deoxyglucosone, which is a precursor in the formation of AGEs. AGEs bind to the RAGE and activate various cellular responses. These include pro-inflammatory cytokines and ROS production, which can oxidize proteins and lipids, including low-density lipoprotein. Activation of inflammatory reactions and LDL oxidation are critical steps in atherogenesis and atherosclerosis development (Xie et al., 2022).

Furthermore, the aldose reductase reaction depletes NADPH, which is required for the biosynthesis of reduced glutathione (GSH). Low cellular levels of GSH also lead to increased generation of ROS. The increased ROS production can further contribute to diabetes-related vascular complications through the oxidative modification of key proteins and LDL. Since the glycosylation and/or LDL oxidative modification is a critical step in atherogenesis (Poznyak et al., 2023), targeting the polyol pathway could also be ideal in mitigating diabetic vascular complications. Medicinal plants are highly recommended in the management of diabetes complications as the currently used drugs have therapeutic limitations and adverse effects (Frimpong et al., 2024). Traditionally used medicinal herbs are recognized for their wide availability and high efficacy with minimal side effects.

Bauhinia bowkeri is one of the recommended plants for Alzheimer's disease and diabetes treatment by some South African traditional healers. In the previous chapters, it was found that *B. bowkeri* extracts have properties to inhibit oxidative stress, the polyol pathway activity and AGEs formation. Since plant extracts have complex phytochemical composition, identification of specific phytochemicals responsible for

the observed bioactivities of the crude extracts is important in drug discovery and development. Thus, investigated the potential of selected phytochemicals from *B. bowkeri* extracts to inhibit the polyol pathway and LDL modification *in vitro*.

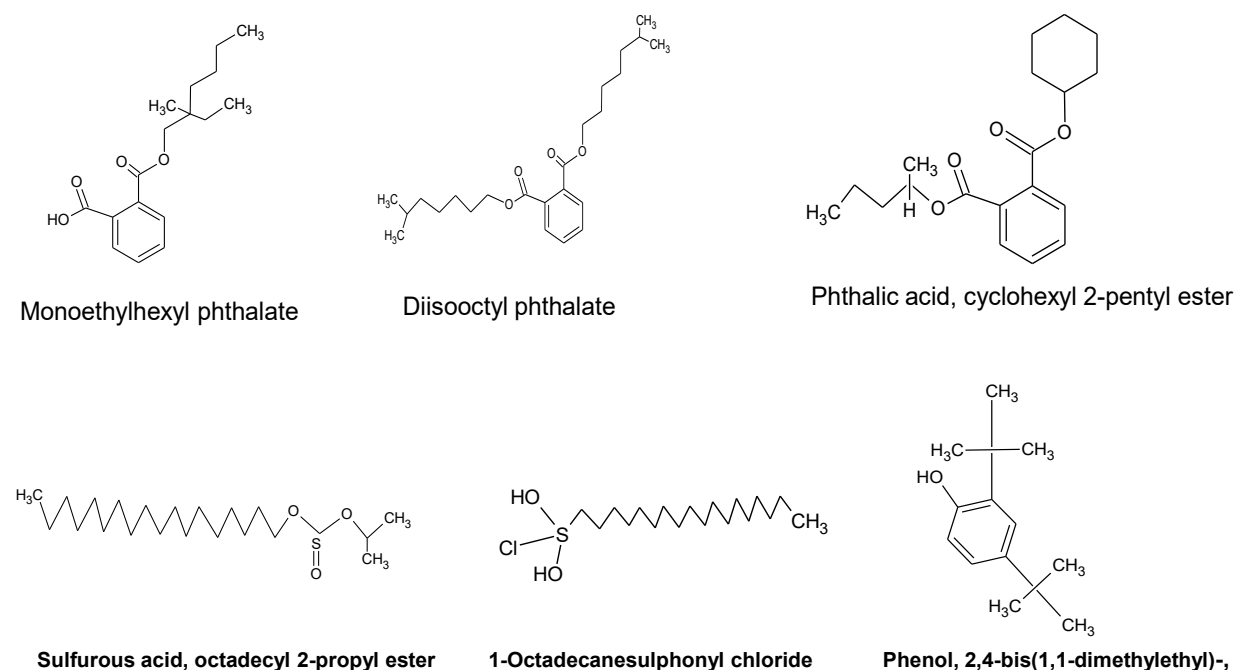
5.1 Materials and Methods

5.1.1 Reagents

The reagents employed in this chapter were of analytical grade, and they were the same as the reagents used in sections 3.1.1 and 4.1.1.

5.1.2 Selected compounds for *in silico* studies

The GC-MS analysis of *B. bowkeri* extracts revealed more than 50 bioactive compounds (Table 3.2). The compounds with a peak area of 90% and above were considered the major compounds and were selected for the *in silico* studies. These compounds are Monoethylhexyl phthalate (BME), 1,2-Benzenedicarboxylic acid, diisooctyl ester, Phthalic acid, cyclohexyl 2-pentyl ester, Sulfurous acid, octadecyl 2-propyl ester, 1-Octadecanesulphonyl chloride, and Phenol, 2,4-bis(1,1-dimethylethyl)-. Their chemical structures are shown in Figure 5.1 below.



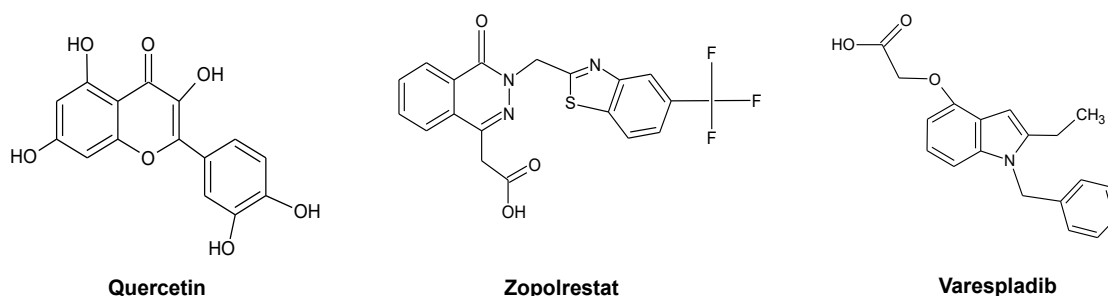


Figure 5.1. Chemical structures of the selected abundant phytochemicals in *B. bowkeri* extracts. The selected compounds had $\geq 90\%$ peak area in GC-MS data of the plant extracts. Zopolrestat, Varespladib and Quercetin were used as reference drugs (standard inhibitors).

5.1.2.1 Molecular docking

The molecular docking of the selected compounds was performed on Aldose reductase (3G5E), Sorbitol dehydrogenase (3QE3) and Secretory phospholipase A₂ (1TC8), using Maestro from Schrodinger (2022_1). The RCSB Protein Data Bank (PDB) was used to retrieve 3-dimensional protein structures. The Maestro software from Schrodinger was used to prepare ligands and proteins. The Maestro software was used in the macromolecular structure to calculate the binding free energy (Ugwu et al., 2018).

5.1.2.2 ADMET properties

The ADMET (absorption, distribution, metabolism, excretion, toxicity) properties of the selected compounds were determined by the pkCSM online tool (<https://biosig.lab.uq.edu.au/pkcsml/>). This pkCSM tool evaluates drug efficacy to predict the pharmacokinetic properties of small inhibitor molecules through graph-based signatures.

5.1.3 *In vitro* studies of the selected compound

Out of the six selected compounds, Monoethylhexyl phthalate (BME) was the only one considered for the *in vitro* studies. The selection was based on the most promising preliminary molecular docking results and commercial availability. BME was purchased from Sigma Aldrich. The test compound's stock solution (5 mM) made in ethanol was used to prepare 0.1-1 mM working solutions.

5.1.3.1 Aldose reductase and sorbitol dehydrogenase inhibition assays

The inhibitory activity of BME on ALR and SDH was investigated as described for the crude extracts (Refer to section 4.1.3 for a detailed description). The pure compound was tested at varying concentrations (0.1-0.5 mM). The compound's mode of enzyme inhibition was determined using different concentrations of DL-Glyceraldehyde (10-60 mM) and sorbitol (10-100 mM) as the substrates for ALR and SDH, respectively. The change in absorbance was converted into reaction velocities. To find the inhibition mode, $V_{\max}$ and K_m values, Lineweaver-Burk plots were plotted.

5.1.4 Anti-glycation activity of BME

The protein glycation inhibitory activity of BME was determined using the fructose and methylglyoxal (MGO) induced LDL glycation assays as described for the crude extracts (Refer to sections 4.1.4.1 and 4.1.4.5 for a detailed description). LDL was incubated with fructose or MGO, with and without the compound, for seven days at 37°C. The compound was tested at varying concentrations ranging from 0.1 to 0.5 mM.

5.1.4.1 Fructosamine content assay

The glycated protein samples' fructosamine content was measured using the procedure described in 4.1.4.2. A volume of 50 μ L of the glycated LDL sample was mixed with 110 μ L of 0.7 mM NBT and incubated at 37°C for 30 minutes. The absorbance was read at 540 nm. The fructosamine content of the samples was determined from the 1-deoxymorpholino (DMF) standard curve.

5.1.4.2 Free thiol group content assay

The free thiol group content of the glycated LDL samples was investigated by adapting the protocol of Ellman (1959) with minor modifications. A volume of 50 μ L of glycated LDL was combined with 110 μ L of 5 mM DTNB and incubated at 25°C for 15 minutes. The extracts' free thiol group content was extrapolated from the L-cysteine standard curve.

5.1.5 *In vitro* antioxidant assays

5.1.5.1 Free radical scavenging activity, ORAC and reducing power of the compound

The free radical scavenging activity, superoxide anion, reducing power and ORAC of the compound were determined following the method described for the crude extracts with minor modification (Refer to section 3.1.4 for a detailed description). The compound was tested at the concentration range of 0.1 to 0.5 mM.

5.1.5.2 Oxidation of LDL

The inhibitory activity of the pure compound on LDL oxidation was investigated as described for the crude extracts in section 3.1.5. LDL was incubated with CuSO₄ at 37°C for 3 hours with and without the compound at different concentrations (0.1 to 0.5 mM), and the levels of conjugated dienes and MDA were determined.

5.2 Results

5.2.1 Molecular docking study

The selected compounds (6) were docked against aldose reductase, sorbitol dehydrogenase and secretory phospholipase A₂. Molecular docking predicts the ligand-receptor binding affinity. Docking scores with more negative values are considered good docking scores (Tshiyoyo et al., 2022). A value of -5 and above is considered a better docking score. The compounds showed (Table 5.1) good docking scores on the tested proteins except for Phthalic acid, Cyclohexyl 2-pentyl ester and Sulfurous acid, octadecyl 2-propyl ester. While Phthalic acid, cyclohexyl 2-pentyl ester showed the lowest docking score of -1.39 on aldose reductase, Sulfurous acid, octadecyl 2-propyl ester showed the lowest docking scores (-3.5 and below) on all the tested enzymes. Interestingly, BME exhibited the highest docking scores on all the tested proteins as compared to the other compounds.

Table 5.1. Docking scores and glide energies (kcal/mol) of the *B. bowkeri* compounds.

Compound	ALR		SDH		sPLA ₂	
	Docking score	Glide energy	Docking Score	Glide energy	Docking score	Glide energy
BME	-7.01	-45.01	-4.75	-30.99	-6.83	-31.45
Diisooctyl phthalate	-4.40	-33.34	-3.92	-33.25	-5.70	-36.30
Phthalic acid, cyclohexyl 2-pentyl ester	-1.39	-39.64	-5.13	-36.22	-6.05	-32.27
Sulfurous acid, octadecyl 2-propyl ester	-3.45	-46.22	-3.33	-32.10	-2.83	-33.44
1-Octadecanesulphonyl chloride	-2.56	-49.14	-3.43	-34.55	-4.76	-32.75
Phenol, 2,4-bis(1,1-dimethylethyl)-	-5.44	-19.85	-6.30	-23.89	-5.88	-27.02
*Zopolrestat	-4.24	-36.10	-	-	-	-
*Quercetin	-	-	-7.54	-41.13	-	-
*Varespladib	-	-	-	-	-7.10	-41.85

*- Reference inhibitor

5.2.2 ADMET properties of the compounds

The *in-silico* analysis of the compounds' ADMET properties was conducted, and the data is shown in Table 5.2. All the selected compounds showed better ADMET scores with the predicted high intestinal absorption and lack of hepatotoxicity. BME exhibited the highest intestinal absorption (97.04 %), comparable with other compounds. It was also predicted that all the compounds are not inhibitors of CYP3A4, and they all exhibited total clearance through excretion. While all the other compounds were further predicted to exhibit poor distribution through the blood-brain barrier, Phenol, 2,4-bis(1,1-dimethylethyl)- showed high potential (0.478) to readily cross the blood-brain barrier.

Table 5.2 ADMET properties of the major compounds of the *B. bowkeri* extracts

	Absorption	Distribution	Metabolism	Excretion	Toxicity
	Intestinal (%)	BBB permeability (logBB)	CYP3A4 inhibitors	Total clearance	Hepatotoxicity
BME	97.04	-0.085	0	1	0
Diisooctyl phthalate	91.448	-0.184	0	1	0
Phthalic acid, cyclohexyl 2-pentyl ester	94.009	0.124	0	1	0
Sulfurous acid, octadecyl 2-propyl ester	91.448	-0.184	0	1	0
1-Octadecanesulphonyl chloride	89.72	-0.23	0	1	0
Phenol, 2,4-bis(1,1-dimethylethyl)-	92.034	0.478	0	1	0

^a Absorption 89.72 (High), 91.448 (High), 92.034 (High), 94.009 (High), 97.04 (High); ^b BBB permeability -0.085 (poorly distributed), -0.184 (poorly distributed), -0.23 (poorly distributed), 0.124 (poorly distributed), 0.478 (readily cross blood brain barrier); ^cCYP3A4 substrate 0 (No), 1 (Yes); ^d Total clearance 0 (No), 1 (Yes); ^e Hepatotoxicity 0 (No), 1 (Yes).

Due to the most promising *in silico* predictions of BME, which include the highest docking scores and good ADMET properties, the predicted bioactivities of this compound were confirmed through *in vitro* biological assays.

5.2.3 Aldose reductase and sorbitol dehydrogenase inhibition

The *in vitro* inhibitory activity of BME on ALR and SDH was determined. The results of the IC₅₀ values of the compound, enzyme activity, mode of enzyme inhibition, K_m, and V_{max} values are presented in Tables 5.3 and 5.4. BME showed inhibitory activity on ALR and SDH activity. BME exhibited better activity than the standard inhibitors with IC₅₀ values of 0.33 mM and 0.30 mM on ALR and SDH, respectively. It was

observed that, similar to zopolrestat and quercetin (used as respective standard inhibitors), the compound inhibited both ALR and SDH in an uncompetitive mode of inhibition.

Table 5.3 The results of the inhibitory activity of the compound on aldose reductase.

Samples	IC ₅₀ (mM)	ALR activity (nmol/min/mg)	K _m (mM)	V _{max} (mM/min)	Mode of inhibition
No inhibitor		2.61	0.019	0.006	
BME (0.5 mM)	0.33	0.48	0.023	0.008	Uncompetitive inhibition
ZPT (0.5 mM)	0.50	0.39	0.071	0.007	Uncompetitive inhibition

BME- Monoethylhexyl phthalate, ZPT- Zopolrestat.

Table 5.4 The results of the inhibitory activity of the compound on sorbitol dehydrogenase

Samples	IC ₅₀ (mM)	SDH activity (nmol/min/ml)	K _m (mM)	V _{max} (mM/min)	Mode of inhibition
No inhibitor		0.03	0.0008	0.0001	
BME (0.5 mM)	0.30	0.01	0.0080	0.0006	Uncompetitive inhibition
QCT (0.5 mM)	0.41	0.01	0.0025	0.0002	Uncompetitive inhibition

BME- Monoethylhexyl phthalate, QCT- Quercetin.

5.2.4 Anti-glycation activity of BME

5.2.4.1 Inhibitory activity of BME against fructose-induced LDL glycation

The antiprotein glycation of the compound was first determined on LDL using fructose as a glycating agent. The findings of the compound's inhibitory effect on fluorescent AGEs formed following LDL exposed to a high concentration of fructose are shown in Figure 5.2. There was an observable increase in the fluorescence of AGEs formed in the LDL incubated with fructose only (gLDL) in contrast to the LDL incubated with fructose in the presence of the compound (BME) or aminoguanidine (Figure 5.2A).

BME inhibited the fluorescent AGEs formation in a manner that is concentration dependent. The observed decreases in fluorescence represented the highest inhibitory activity of 43.2% and 44.6% for BME and gallic acid, respectively. The observed inhibition of AGEs formation was accompanied by lower fructosamine and higher thiol group contents in the LDL incubated with fructose in the presence of BME (or aminoguanidine) when compared to the gLDL (Figure 5.2C).

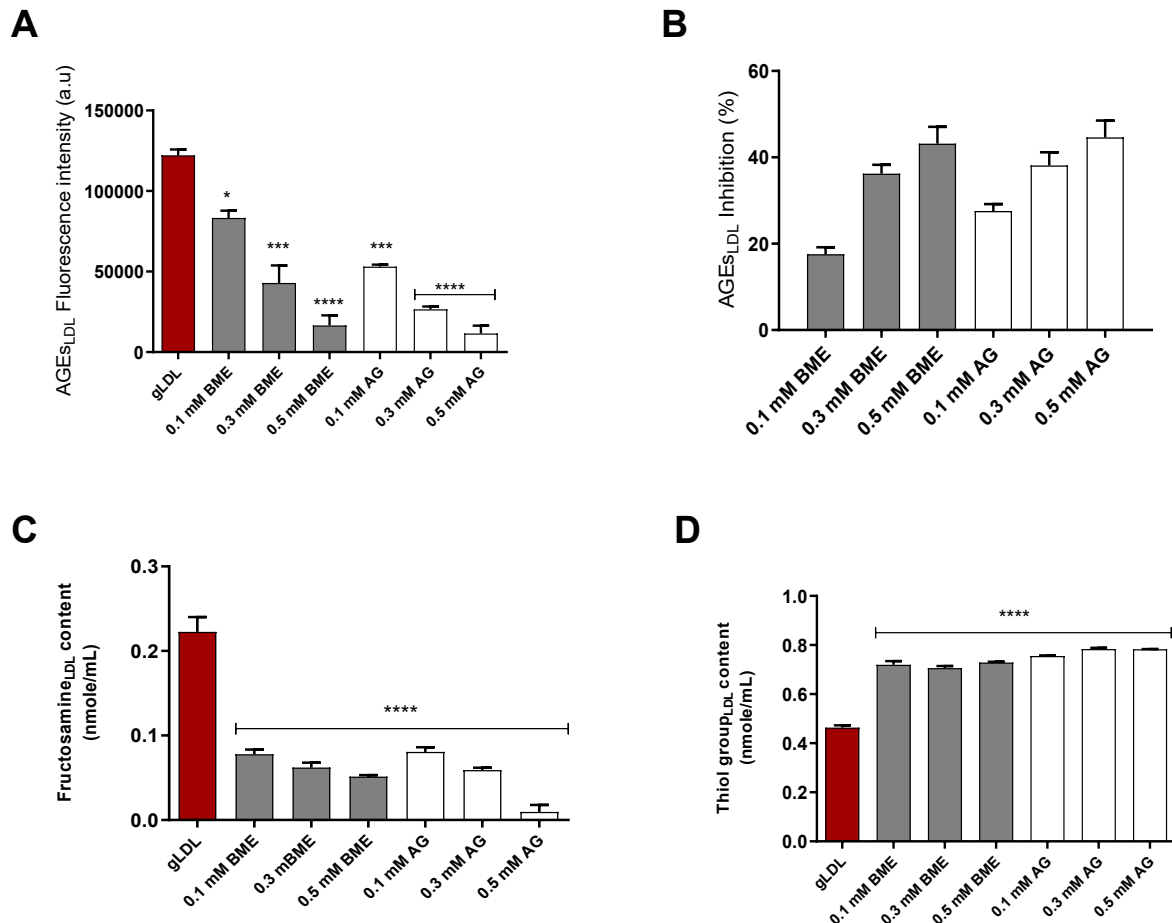


Figure 5.2. Inhibitory activity of BME on the fructose-induced glycation on LDL. LDL was incubated at 37°C with fructose with and without BME for 1 week, and levels of fluorescent AGEs formed (**A**), inhibition (%) of AGEs formation (**B**), fructosamine (**C**) and thiol content (**D**) were determined. Aminoguanidine (AG) functioned as a positive control. Data are depicted as mean $\pm$ SEM (n = 3), *p < 0.05, ***p < 0.001 and ****p < 0.0001 as compared to gLDL. gLDL-glycated LDL.

5.2.4.2 Inhibitory activity of BME against methylglyoxal-induced LDL glycation

The antiprotein glycation activity of BME was further investigated using the methylglyoxal-induced glycation assay. Methylglyoxal is a reactive dicarbonyl commonly used to investigate the later stages of glycation. It was observed that there was a decrease in fluorescence of AGEs formed in the presence of BME or AG as opposed to the LDL incubated with MGO (gLDL) only (Figure 5.3). However, BME showed poor inhibitory activity of AGEs when compared to AG (standard inhibitor). It inhibited the MGO-induced LDL glycation by up to 10% only. This was confirmed by the thiol group content, which is not statistically different from LDL incubated with MGO in Figure 5.3C. Furthermore, the inhibitory activity of the fluorescence of AGEs in the MGO-induced glycation is not comparable to the fructose-induced glycation (Figure 5.2) where a higher inhibitory activity was observed.

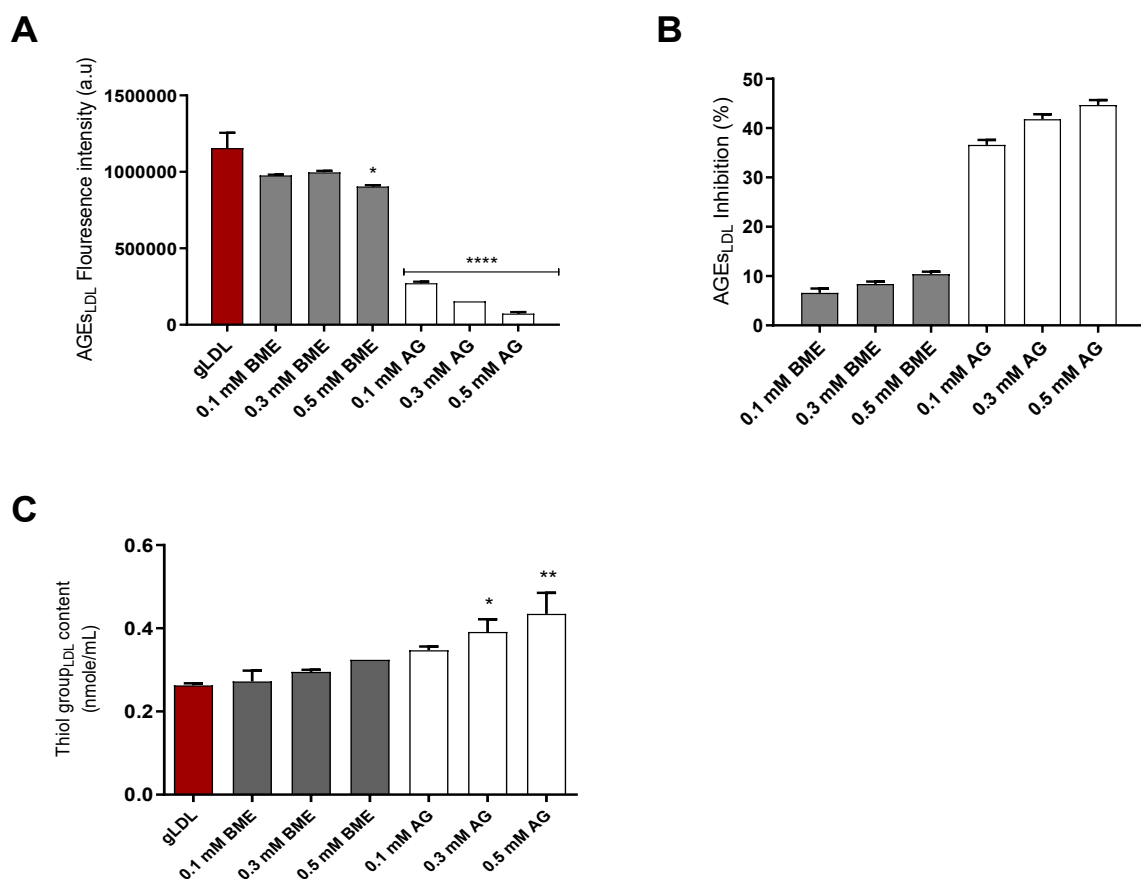


Figure 5.3. Inhibitory activity of BME on the methylglyoxal induced glycation on LDL. LDL was incubated at 37°C with MGO with and without BME for 1 week and levels of fluorescent AGEs formed (**A**), % inhibition of AGEs formation (**B**) and thiol content (**C**) were determined.

Aminoguanidine (AG) served as a standard. Statistical values are presented as mean $\pm$ SEM ($n = 3$), * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$ compared gLDL.

5.2.5 *In vitro* antioxidant activity of BME

The BME's antioxidant activity was analyzed by determining its free radical scavenging activity, reducing power and ORAC. The results are presented in Figure 5.4. The compound showed a concentration dependent weak free radical scavenging activity with high EC_{50} values (greater than the tested concentrations) on both DPPH and superoxide anion radicals (Figure 5.4 A&B). Low ORAC values and reducing power were also observed when compared with ascorbic acid and Trolox used as standard antioxidants (Figure 5.4 C&D).

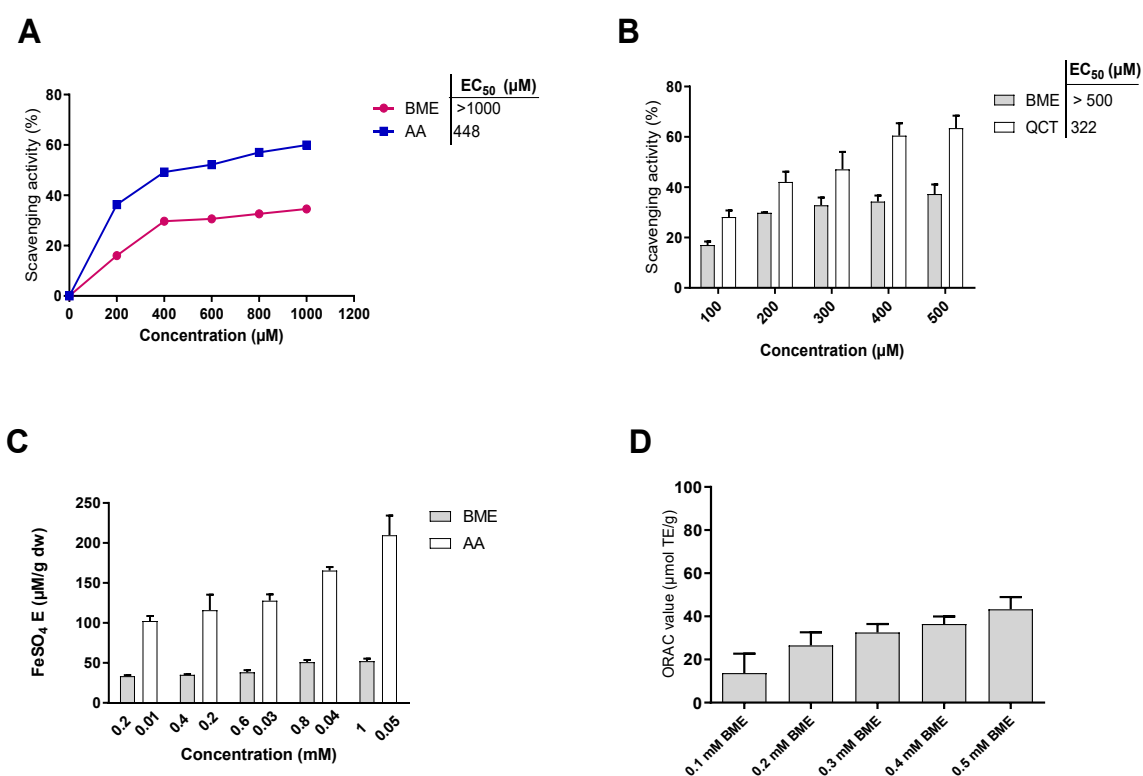


Figure 5.4. The antioxidant activity of BME. The activity of the compound was determined on DPPH (**A**) and superoxide anion radical (**B**) Ferric reducing power (**C**) and ORAC (**D**) of BME. The statistical values are stated as SEM ($n = 3$), *** $p < 0.001$ comparable to BME. BME- Monoethylhexyl phthalate, AA- ascorbic acid and QCT- quercetin.

5.2.6 Inhibition of LDL oxidation

The BME's antioxidant ability was additionally confirmed by evaluating its inhibitory activity on lipid peroxidation in the copper sulphate-induced LDL oxidation. The inhibitory activity on LDL oxidation was validated by measuring the conjugated dienes (CD) levels and MDA formed. The results are shown in Figure 5.5. A concentration dependent decrease of CD formation in LDL incubated with BME was observed when compared to the LDL incubated only with copper sulphate (Figure 5.5A). However, these results were not comparable to those of gallic acid (GA), used as a reference inhibitor. The reference inhibitor exhibited the highest inhibition of CD formation. A notable decrease in MDA concentration in the presence of BME or GA was observed (Figure 5.5B). The results displayed that the compound also inhibited the MDA formation in a manner that is concentration dependent, and the findings were less comparable to those of gallic acid. The percentage inhibitions at the highest concentrations of BME and GA were 53.3% and 66.3%, respectively.

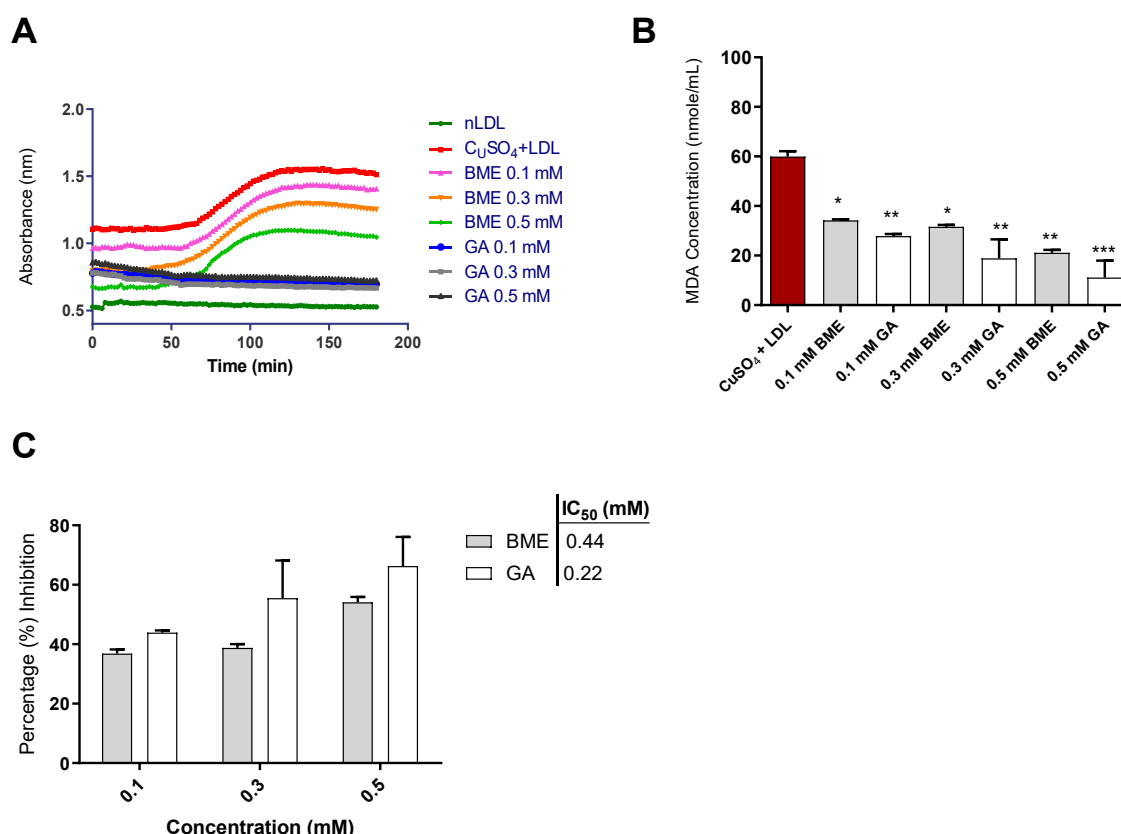


Figure 5.5. The BME inhibitory effect on LDL oxidation. **(A)** LDL-Conjugated diene formation inhibition, **(B)** LDL-MDA concentration, **(C)** LDL-MDA percentage inhibition. LDL was incubated with CuSO_4 at 37°C for 3 hours and the levels of conjugated dienes and MDA were

determined. The conjugated dienes formation was monitored at 2 min intervals for the 3 hours. The data was shown as mean $\pm$ SEM ($n = 3$), * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs LDL+CuSO₄.

5.3 Discussion

The increased polyol pathway activity accelerated protein glycation and AGEs formation rate are considered among the underlying mechanisms of atherosclerotic cardiovascular complications of diabetes (Rybarczyk et al., 2023). In the previous chapter, it was observed that *B. bowkeri* extracts have antioxidant activity and can potentially inhibit the polyol pathway and formation of AGEs. This study investigated the potential of selected *B. bowkeri* compounds to inhibit the polyol pathway and LDL modification.

A total of six (6) *B. bowkeri* major compounds, as revealed by the GC-MS data (refer to Chapter 3), were selected for this research study. The potential bioactivities of the selected compounds were first investigated by *in silico* molecular docking and ADMET properties. The good docking scores exhibited by five (5) of the six (6) selected compounds on ALR, SDH and sPLA₂ predicted their potential inhibitory activity on the polyol pathway and inflammatory pathway activities. The tested compounds further exhibited good ADMET properties. Their predicted higher intestinal absorption suggests their higher bioavailability. CYP3A4 is an enzyme responsible for metabolizing many drugs, including statins and saxagliptin, in the liver (Bathaei et al., 2024; Li et al., 2021). Being predicted as non-inhibitors of CYP3A4 further suggests that these compounds would not interfere with the liver metabolism of other drugs. The predicted high total clearance and poor blood-brain barrier permeability suggest the compounds can be easily excreted from the body and minimize central nervous system-related side effects, respectively.

Due to the most promising *in silico* predictions of Monoethylhexyl phthalate (BME), which included the highest docking scores on the polyol pathway enzymes and good ADMET properties, its bioactivities were investigated by *in vitro* biological assays. BME reportedly has antioxidant, anti-inflammatory and anti-diabetic activities (Zayed and Sallam, 2019). The study's inhibitory activity on both ALR and SDH by BME

(Tables 5.3 and 5.4) indicates its potential to inhibit polyol pathway activity. BME could be one of the compounds contributing to the polyol's pathway enzyme inhibition by the *B. bowkeri* extracts. Polyol pathway activity results in the production of fructose, which further participates in protein glycation and AGEs formation. The inhibitory activity of BME on the polyol pathway could also imply its potential to inhibit protein glycation and the formation of AGEs.

To further investigate the potential bioactivities of BME, its antiprotein glycation activity was determined on LDL using fructose or MGO as glycating agents. While fructose is used to investigate the early stages of the glycation process, MGO is a reactive dicarbonyl commonly used to investigate the later stages of glycation (Mohd Dom et al., 2020). The compound's higher inhibitory activity against the fructose-induced LDL glycation, when compared to the MGO-induced protein glycation the compound showed to only inhibits the early stages and not the later stages of protein glycation. This further suggests that BME could be one of the major compounds contributing to antiprotein glycation activity of *B. bowkeri* extracts observed in the previous study. The obtained results imply that the compound (BME) may not only inhibit the polyol pathway activity but also the associated formation of AGEs. AGEs are among the major contributors to vascular disease development, including atherosclerosis.

The activity of the polyol pathway enzymes not only contributes to protein glycation but oxidative stress. Increased generation of ROS can further oxidize proteins, including LDL, which is implicated in several diseases, including atherosclerosis. Thus, the investigation of the BME antioxidant ability was of pivotal importance. The BME's antioxidant activity was first determined by ferric ion reducing power, ORAC, DPPH and superoxide anion scavenging assays. BME exhibited antioxidant activity but not comparable to the known standard antioxidants, ascorbic acid, quercetin and trolox (Figure 5.4). This implies that BME is not a good antioxidant that can protect against oxidative damage to lipids and proteins, which leads to various diseases, including atherosclerosis. These results suggest that BME is not one of the major compounds responsible for the observed antioxidant activity of *B. bowkeri* extracts (reported in Chapter 3).

The BME's antioxidant activity was also determined in the copper sulphate-induced LDL peroxidation. Lipid peroxidation occurs through initiation and propagation phases, which are characterized by the formation of conjugated dienes and malonaldehyde, respectively (Valgimigli, 2023). LDL peroxidation results indicated that BME has poor antioxidant activity or does not have high antioxidant activity as compared to the known standard inhibitor, gallic acid. Oxidative modification of LDL is one of the critical steps in atherogenesis and atherosclerosis development (Xie et al., 2022).

5.4 Conclusion

The findings obtained in this investigative study showed that the selected *B. bowkeri* bioactive compounds have properties to inhibit the polyol pathway. The plant compounds showed potential to inhibit the polyol pathway enzymes (ALR and SDH). BME can also inhibit the glycosylation and oxidative modification of LDL implicated in diabetes-associated risk of atherogenesis. This indicates that BME has the potential to be used as a structural template for the synthesis of active drugs against diabetes related macrovascular complications such as atherosclerosis.

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CHAPTER 6

General Discussion

6.0 Discussion

Chronic hyperglycemia, which characterizes diabetes, is implicated in the pathogenesis of inflammation and oxidative stress-mediated microvascular and macrovascular complications (Oguntibeju, 2019). The chronic hyperglycemia of diabetes directly or indirectly stimulates the atherogenic process that is clinically manifested as atherosclerosis. Atherosclerosis is the most prevalent microvascular complication of diabetes (Berbudi et al., 2020). The clinical limitations of the current drugs against diabetes associated atherosclerosis indicate the need to explore alternative therapeutics, preferably from natural sources, with improved efficacy and safety profiles.

This research investigated the therapeutic potential of *B. bowkeri* extracts against diabetes associated atherosclerosis. Since hyperglycemia is known to activate other glucose metabolising pathways like the polyol pathway and protein glycation pathway (Bardaş, 2024), the inhibitory potential of the plant extracts was first determined on the polyol pathway enzymes. The inhibitory activity of the extracts against ALR and SDH activities indicated the inhibition of the polyol pathway activity, thus preventing LDL glycation and oxidation. The hexane extract (HE) exhibited the highest ALR inhibition activity, and the methanol extract (ME) exhibited the highest inhibition activity of the SDH in contrast to the DCM extract (DE). The extracts' observed bioactivity could be caused by the extracts' TPC and TFC (Figure 3.3). Hmimid et al. (2021) reported that inhibition of ALR and SDH by *Euphorbia regis-jubae* extracts was due to their TPC and TFC. Inhibition of ALR and SDH also prevents the production of ROS from NADPH/NADP⁺, GSSG/GSH and NAD⁺/NADH systems, and the production of AGEs. ROS and AGEs can directly modify LDL, which stimulates foam cell formation and atherosclerotic plaque development. Thus, inhibition of ALR and SDH is important in the prevention of macrovascular complications.

To further determine the therapeutic potential of the extracts, their antiprotein glycation activity was determined on LDL and BSA using fructose and methylglyoxal (MGO) as

glycating agents. The observed inhibition of LDL glycation by the extracts implies that the extracts can inhibit the glycation of LDL under hyperglycemic conditions and prevent its susceptibility to oxidation, thus preventing foam cell formation, which is critical in atherosclerosis initiation and progression. ME exhibited highest inhibition activity of AGEs in comparison with other extracts which is correlated to its high TPC. AGEs can also bind to RAGE and produce ROS resulting in oxidative damage of cells and other biomolecules in addition to LDL (Zhou et al., 2024). It was, however, noted that the plant extracts have the potential to only inhibit the early stages of protein glycation but not the later stages. This was indicated by inhibitory activity only on the fructose-induced LDL glycation but not on the MGO-induced LDL glycation.

Increased production of ROS under chronic hyperglycemic conditions can also modify LDL. Oxidative modification of LDL stimulates atherosclerotic plaque development. The extracts' antioxidant activity was investigated on the copper sulphate-induced LDL peroxidation. The LDL lipid peroxidation inhibition showed that the extracts have antioxidant properties required to prevent oxidative modification of LDL. Their antioxidant capacity was also ascribed to the polyphenolic content of the extracts. The ME exhibited the highest inhibition of MDA as compared to HE and DE. Studies confirm that extracts antioxidant property highly correlates with TPC of the extracts (Ilie et al., 2024; Hari et al., 2024). In the three tested extracts (HE, DE and ME), HE only exhibited better inhibition of ALR than ME and DE, whereas ME exhibited good inhibition of SDH, antioxidant, and protein glycation activity. The reported HE and ME highest activities were attributed to TFC and TPC, respectively.

The inhibitory activities of the extracts on the polyol pathway enzymes, LDL glycation and oxidation prompted the need to identify some bioactive compound(s) that might be responsible for the extracts' activities. A total of six (6) bioactive compounds detected from the GC-MS analysis, which some have previously been said to possess antioxidant, anti-inflammatory activities (Kamel et al., 2024; Phillips et al., 2015) were selected for molecular docking and ADMET properties. The compounds showed good docking scores and ADMET properties. The observed *in silico* results suggest that the compounds can bind effectively to their target enzymes thus inhibiting them, and they can be effective and safe for drug design. BME, a compound selected from ME, showed the highest docking scores in all tested enzymes and good ADMET properties.

BME was selected for the *in vitro* inhibition assays on the polyol enzymes, AGEs formation and LDL oxidation. It was found that BME inhibited ALR and SDH and showed poor inhibition of the production of AGEs and LDL oxidation. This implies that BME is not one of the main compounds that have antioxidant activity and that *B. bowkeri* bioactive compounds may exhibit synergistic antioxidant activity. However, the inhibition of ALR and SDH consequently inhibits ROS and AGEs produced from the polyol pathway. These results indicate that the *B. bowkeri* extracts and their bioactive compound(s) have the potential to be used as therapeutic agents for the mitigation of diabetes associated complications.

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CHAPTER 7

General Conclusion

7.1 Summary and Conclusions

The results obtained from this study indicated that the *B. bowkeri* extracts and its bioactive compound(s) inhibited the enzymes of the polyol pathway activity. Extracts with increasing polarity (Hexane, DCM and Methanol extracts) exhibited inhibition activity on polyol pathway enzymes (ALR and SDH), protein glycation and LDL oxidation. The extracts activity was strongly ascribed to the TPC, TFC and high polarity of the solvents. ME exhibited highest activity on SDH, protein glycation, LDL oxidation, and showed good antioxidant activity. ME exhibited high phenolic content as compared to HE and DE, and HE exhibited high flavonoid content in comparison with other extracts. HE and ME activity was due to high flavonoid and phenolic content, respectively. BME compound showed good ADMET and docking scores and inhibited ALR and SDH. BME further inhibited glycation of proteins and LDL oxidation. This indicates that the extracts and their bioactive compound(s) have the potential to inhibit the polyol pathway activity and the glycation and LDL oxidative modification, which is a major step in atherogenesis. This indicates that the *B. bowkeri* extracts and their bioactive compounds could be potential therapeutics in the search for drugs to manage diabetes associated complications such as macrovascular complications, including atherosclerosis.

7.2 Recommendations for future studies

Future studies should consider isolating the bioactive compounds of *B. bowkeri* from the ME as it demonstrated better activity than the other extracts and investigate the compounds' *in vitro* inhibition activities on the polyol pathway and LDL modification. It is also recommended that *in vivo* studies be done to further confirm the potential of plant extracts and the isolated compounds to ameliorate the development and progression of atherosclerosis in a diabetic state.

APPENDIX A

Additional data and Standard curves used

A1. Total phenolic and flavonoid contents

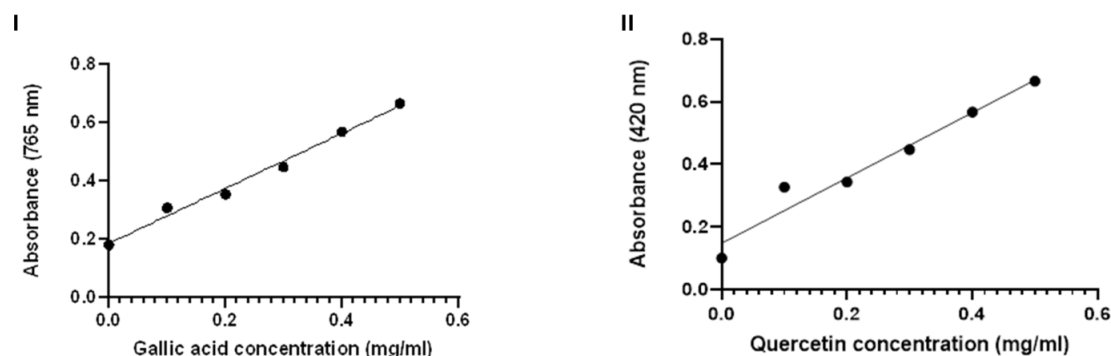


Figure A1: Standard curves of gallic acid (I) and quercetin (II). The gallic acid and quercetin curves were used to determine the total phenolic and flavonoid contents of the extracts, respectively.

A.1.1 Free radical scavenging activity of the extracts

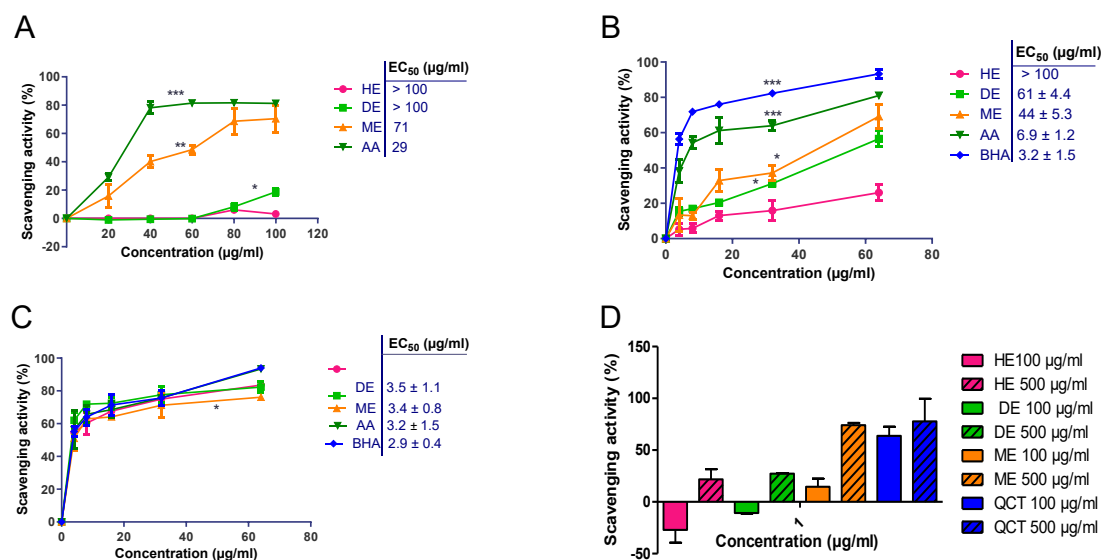


Figure A.1.1. Free radical scavenging activity of (A) DPPH, (B) ABTS, (C) Hydroxyl and (D) Superoxide anion radicals.

A2. LDL oxidation

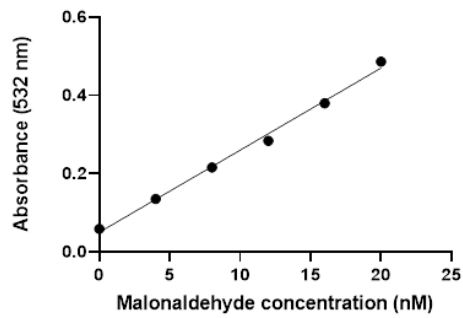


Figure A2: Standard curve of malonaldehyde. The standard curve was used to measure MDA concentration after oxidation.

A3. Ferric reducing power of the extracts

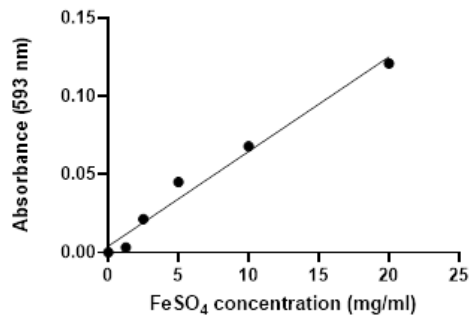


Figure A3: Standard of curve of ferrous sulphate. It was used to determine the ferric reducing power of the extracts.

A4. ORAC values of the extracts

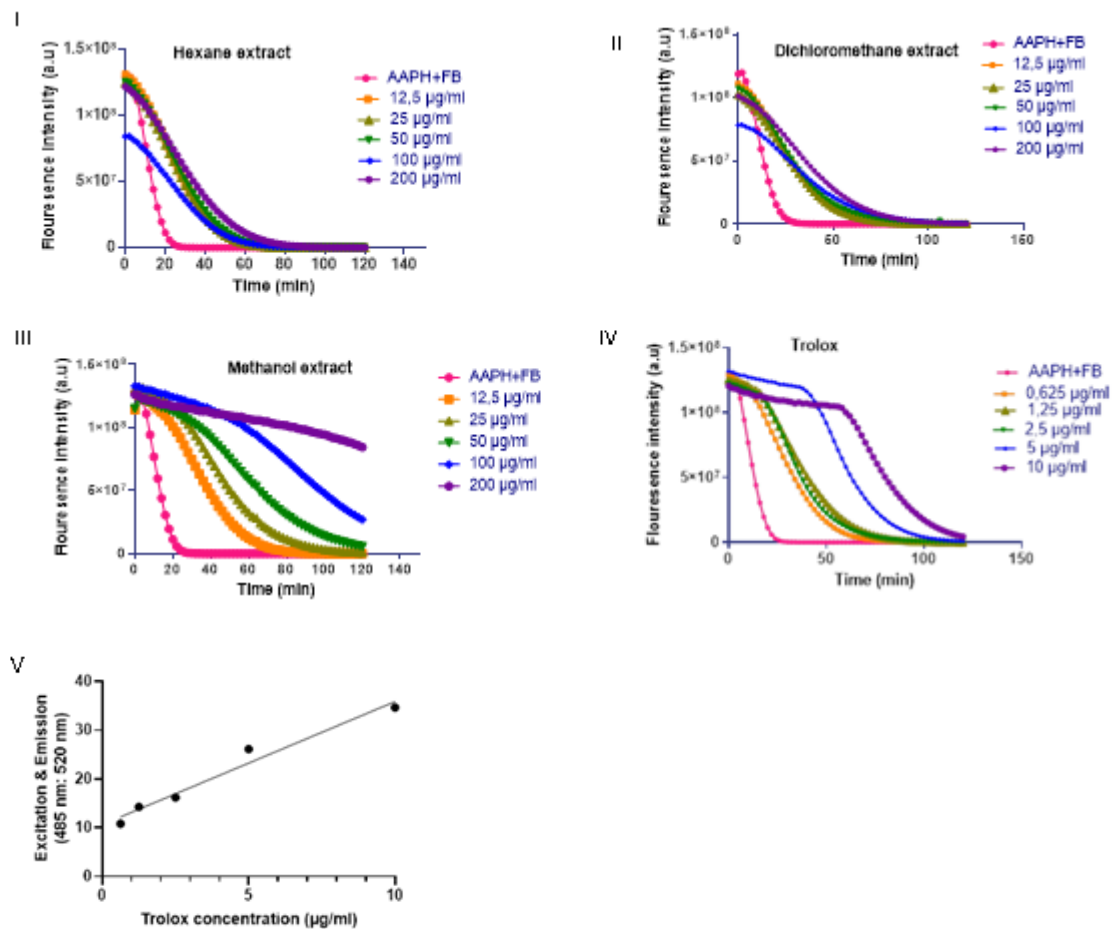


Figure A4: ORAC curves of hexane (I), DCM (II), Methanol (III), Trolox (IV) and Trolox standard curve (V). The Trolox standard curve was used to determine the ORAC values of the extracts.

A5. ALR and SDH inhibition

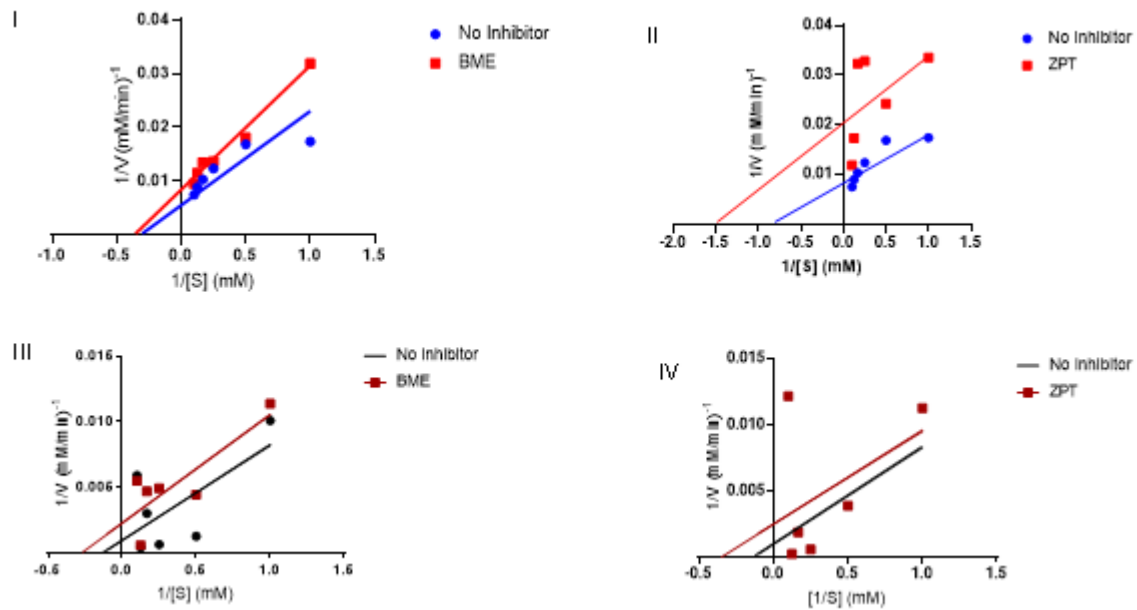


Figure A5: Lineweaver-burk plots of ALR (I & II) and SDH (III & IV), respectively. The K_m and V_{max} values were determined from the plots.

A6. Protein glycation inhibition

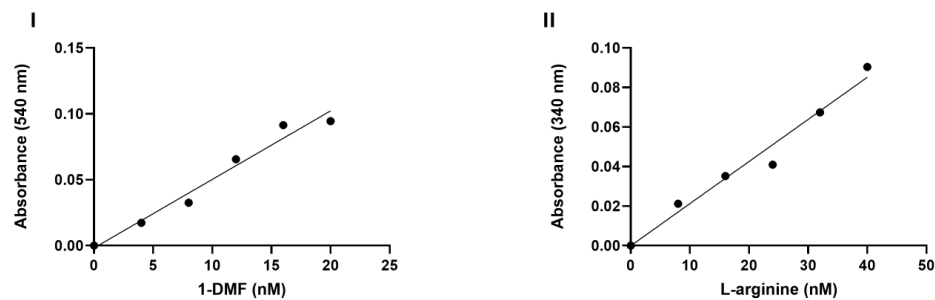


Figure A6: Standard of curve of 1-deoxymorpholino (1-DMF) (I) and L-arginine (II). Fructosamine and free amino group content were determined from the curves.

APPENDIX B

GC-MS spectra

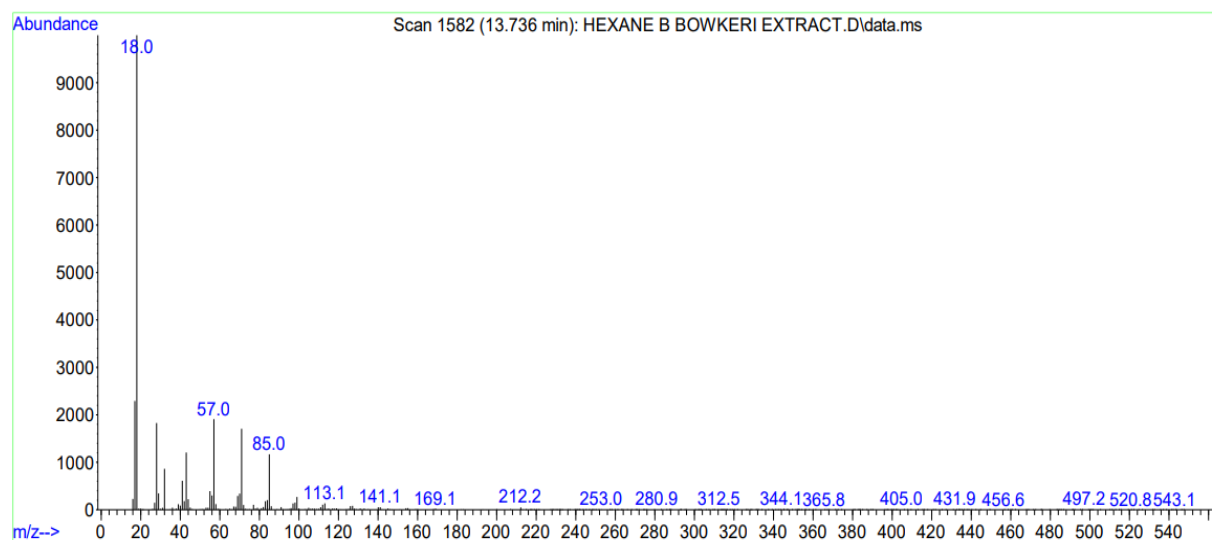


Figure B1: GC-MS spectrum of the hexane extract of *B. bowkeri*.

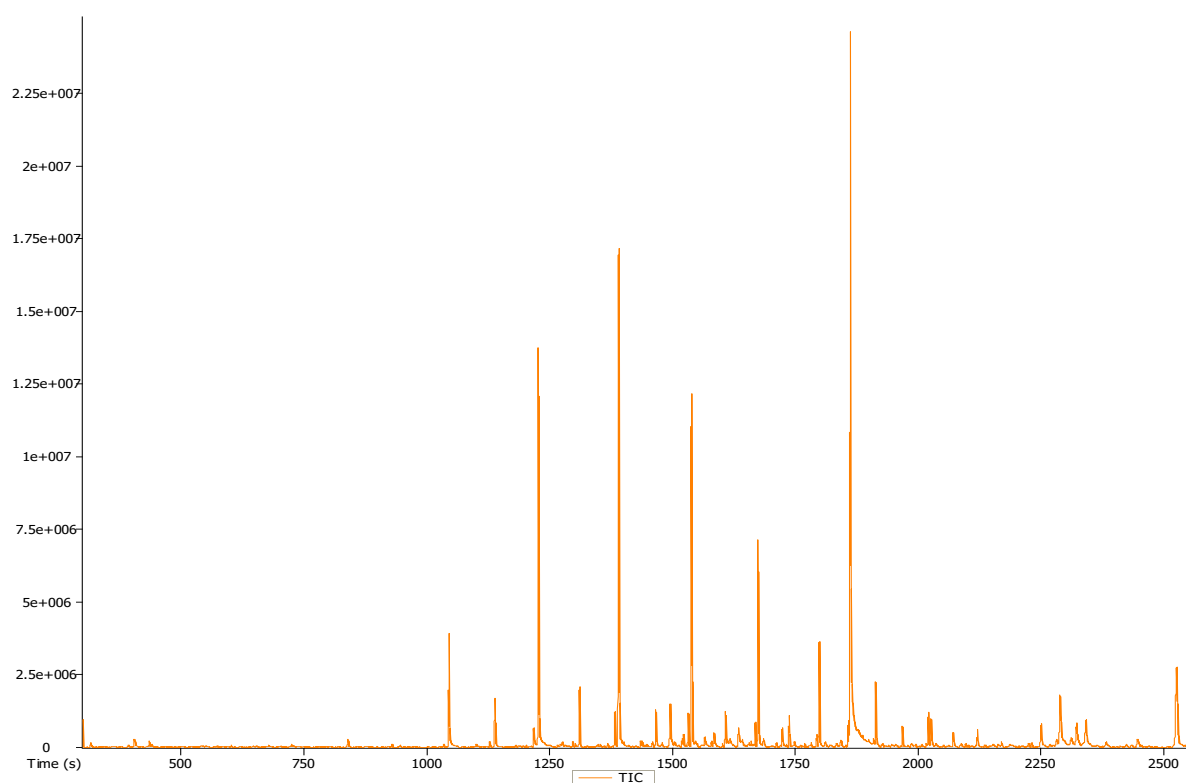


Figure B2: GC-MS spectrum of the DCM extract of *B. bowkeri*.

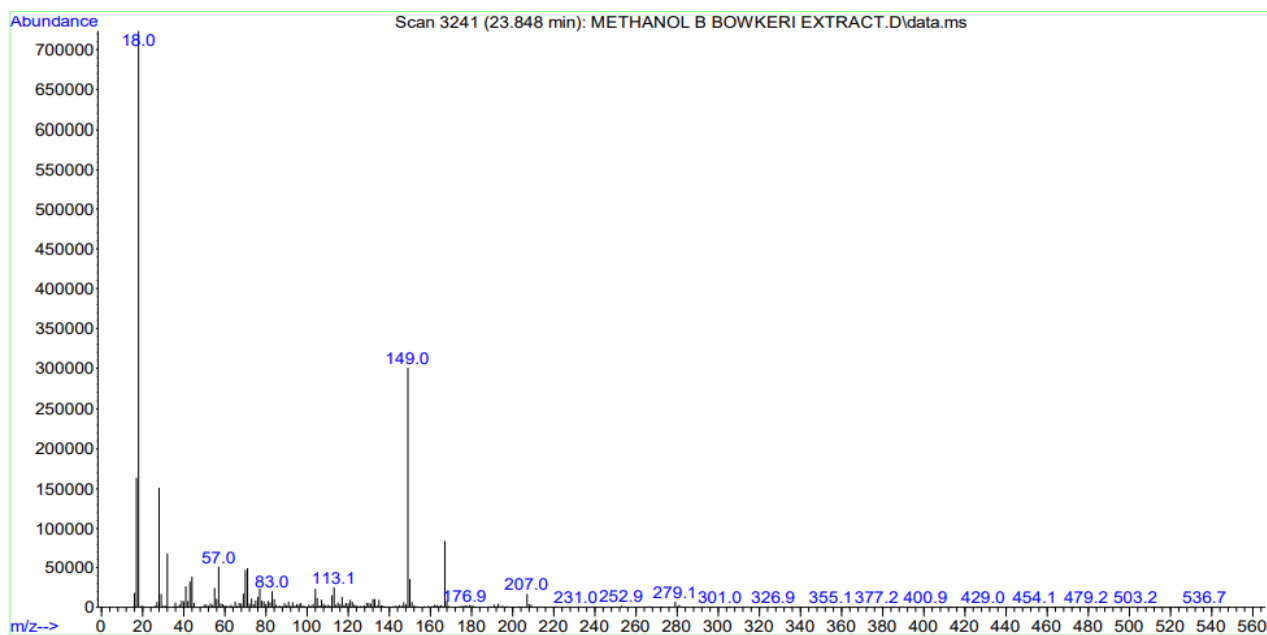


Figure B3: GC-MS spectrum of the methanol extract of *B. bowkeri*.

APPENDIX C

Ethical clearance certificate



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
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Faculty of Natural and Agricultural Sciences
Ethics Committee
E-mail: ethics.nas@up.ac.za

24 August 2022

ETHICS SUBMISSION: LETTER OF APPROVAL

Miss NN Ndlangamandla
Department of Biochemistry, Genetics and Microbiology
Faculty of Natural and Agricultural Science
University of Pretoria

Reference number: NAS066/2022

Project title: Targeting diabetes associated risks of atherogenesis with some bioactive compounds isolated from Bauhinia bowkeri extract

Dear Miss NN Ndlangamandla,

We are pleased to inform you that your submission conforms to the requirements of the Faculty of Natural and Agricultural Sciences Research Ethics Committee.

Please note the following about your ethics approval:

- Please use your reference number (NAS066/2022) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
- Please note that ethical approval is granted for the duration of the research (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
- The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
- **If Applications using GM permits:** If the GM permit expires before the end of the study, please make an amendment to the application with the new GM permit before the old one expires
- **If Applications using Animals:** NAS ethics recommendation does not imply that Animal Ethics Committee (AEC) approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

Post approval submissions including application for ethics extension and amendments to the approved application should be submitted online via the Ethics work centre.

We wish you the best with your research.

Yours sincerely,



Prof VJ Maharaj
Chairperson: NAS Ethics Committee

APPENDIX E

Turnitin Report

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