School of Life Sciences

Department of Biological Sciences and Biotechnology

# Phytochemical and antioxidant analysis of C*itrullus ecirrhosus* seed: an in vitro and in silico evaluation towards atherosclerosis management

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## DEDICATION

I would like to dedicate this work to my father, who at the time of my birth envisioned everything that I am doing. He wants to see me always moving up the academic ladder.

**TABLE OF CONTENTS**


| | |
|---|---|
| DECLARATION AND COPYRIGHT | ii |
| CERTIFICATION BY SUPERVISOR | iii |
| ACKNOWLEDGEMENTS | iv |
| DEDICATION | v |
| LIST OF TABLES | ix |
| LIST OF FIGURES | x |
| LIST OF APPENDICES | xii |
| LIST OF ABBREVIATIONS | xiii |
| ABSTRACT | xiv |
| CHAPTER 1: INTRODUCTION | 1 |
| 1.1 Background | 1 |
| 1.2 Problem statement | 3 |
| 1.3 Hypothesis | 4 |
| 1.4 General Objective | 4 |
| 1.5 Specific Objectives | 4 |
| 1.6 Justification | 4 |
| CHAPTER 2: LITERATURE REVIEW | 5 |
| 2.1 Atherosclerosis, a global burden | 5 |
| 2.2 Development of Atherosclerosis | 5 |
| 2.3 Management of Atherosclerosis | 7 |
| 2.4 Citrullus ecirrhosus | 8 |
| 2.5 Phytochemical composition of *Citrullus spp* | 9 |
| 2.6 Antioxidant potential of *Citrullus ecirrhosus* seeds | 10 |
| 2.7 Role of phytochemicals on seed pigmentation/coloration | 10 |
| 2.8 Solvents for phytochemical extraction | 11 |
| 2.9 In silico docking | 11 |

CHAPTER 3: MATERIALS AND METHODS 13

3.1 Collection and preparation of plant extracts 13

3.1.1 Collection of plant samples 13

3.1.2 Preparation of seeds extracts 13

3.1.3 Plant confirmation 14

3.2 Quantitative Phytochemical Analysis 15

3.2.1 Total Phenolic Content 15

3.2.2 Total Flavonoids Content 15

3.2.3 Total Antioxidant Capacity 16

3.2.4 DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging capacity assay 16

3.2.5 ABTS (2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) Assay 17

3.3 Identification of phytochemicals - GC–MS (gas chromatography–mass spectroscopy) analysis 17

3.4 Molecular Docking 18

3.4.1 In-silico assessment of HMG CoA reductase enzyme inhibition and anti-atherosclerosis properties of *Citrullus ecirrhosus* phytochemicals. 18

3.4.2 Ligand and protein preparation 18

3.4.3 Site-specific molecular docking 19

3.4.4 Method validation 19

3.4.5. Visualization of binding position and interaction between HMG CoA reductase and bioactive compounds 19

3.4.6 Results analysis 19

3.5 Statistical Analysis 19

CHAPTER 4: RESULTS 21

4.2 Quantitative Phytochemical Analysis 22

4.2.1 Total Phenolic Content 22

4.2.2 Total Flavonoid Content 23

4.2.3 Total Antioxidant Capacity 24

4.2.4 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity assay 25

4.2.5 ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) Assay 27

4.3 Identification of phytochemicals - GC–MS (gas chromatography–mass spectroscopy) analysis 28

4.4 Molecular docking 40

4.4.1 Ligand and protein preparation 40

4.4.2 Site-specific molecular docking 41

4.4.3 Visualization of binding position and interaction between HMG-CoA reductase protein and bioactive compounds using Biovia Discovery Studio 2020 44

CHAPTER 5: DISCUSSION 47

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS 53

6.1 Conclusion 53

6.2 Study limitations and Recommendations 54

REFERENCES 55

APPENDICES 63

## LIST OF TABLES

Table 1.1: Phytochemical constituents identified in the methanol extracts of Citrullus ecirrhosus varieties using gas chromatography-mass spectrometry. 30

Table 1.2: Phytochemical constituents identified in the chloroform extracts of Citrullus ecirrhosus varieties using gas chromatography-mass spectrometry. 32

Table 1.3: Phytochemical constituents identified in the ethyl acetate extracts of Citrullus ecirrhosus varieties using gas chromatography-mass spectrometry. 35

Table 1.4: Phytochemical constituents identified in the hexane extracts of Citrullus ecirrhosus varieties using gas chromatography-mass spectrometry. 37

Table 2: Phytochemicals with documented antioxidant properties selected for docking. 39

Table 3: HMG CoA Reductase (PDB ID: 3CCZ) P2Rank predicted active site amino acid residues and vina search space X, Y, and Z coordinates. 43

Table 4: Molecular docking results of Citrullus ecirrhosus bioactive compounds against HMG CoA reductase enzyme. The controls are highlighted in red 43

## LIST OF FIGURES

Figure 1.1: A summary on how reactive oxygen species and oxidative stress play a role in the development of atherosclerosis. 8

Figure 1.2: The effect of statins on the mevalonate pathway in the liver inhibiting the conversion of HMG-CoA by the enzyme HMG-CoA reductase to mevalonic acid which ultimately hinders the formation of cholesterol. 10

Figure 1.3: Citrullus ecirrhosus plant .11

Figure 2: The three different varieties of the Citrullus ecirrhosus seeds with (A) Brown, (B) Red and (C) White. 15

Figure 3: Maximum Likelihood phylogenetic tree for Citrullus spp ITS2 region sequence to confirm the identity of the sample from Botswana. 23

Figure 4.1: Total phenolic content (mg ferulic acid equivalence/ g dry weight extract) of the Citrullus ecirrhosus seed extracts. 24

Figure 4.2: Total flavonoid content (mg quercetin equivalence (QE)/ g dry weight) of the Citrullus ecirrhosus seed extracts. 26

Figure 4.3: Total Antioxidant Capacity (mg Ascorbic Acid equivalence (AAE)/ g dry weight) of the Citrullus ecirrhosus seed extracts. 27

Figure 4.4: DPPH free radical scavenging capacity (mg Trolox equivalence (TE)/ g dry weight) of the Citrullus ecirrhosus seed extracts. 28

Figure 4.5: ABTS free radical scavenging capacity (mg Trolox equivalence (TE)/ g dry weight) of the Citrullus ecirrhosus seed extracts. 29

Figure 4.6: PrankWeb (P2Rank) predicted the active site of the HMG CoA Reductase protein PDB ID: 3CCZ. 42

Figure 4.7: Results of 3D (left) and 2D (right) molecular docking of HMG CoA reductase against vanillin lactoside. 46

Figure 4.8: Results of 3D (left) and 2D (right) molecular docking of HMG CoA reductase against 9, 10-secocholesta-5, 7, 10(19)-triene-3, 24, 25-triol. 46

Figure 4.9: Results of 3D (left) and 2D (right) molecular docking of HMG CoA reductase against stigmasterol. 47

Figure 4.10: Results of 3D (left) and 2D (right) molecular docking of HMG CoA reductase against gamma-Tocopherol. 47

Figure 4.11: Results of 3D left and 2D molecular docking of HMG CoA reductase against Phenol, 2, 4-bis (1, 1-dimethylethyl). 48

Figure 4.12: Results of 3D left and 2D molecular docking of HMG CoA reductase against Atorvastatin. 48

## LIST OF APPENDICES

Appendix 1: Gel Electrophoresis of DNA Extracted from Citrullus ecirrhosus 65

Appendix 2: ITS2 Multiple Sequence Alignment (MUSCLE) by MEGAX-64 66

Appendix 3: Chromatogram showing the phytochemical compounds with known biological activity with the one in black representing those with known antioxidant activity for methanolic extracts for brown seed variety. 67

Appendix 4: Chromatogram showing the phytochemical compounds with known biological activity with the one in black representing those with known antioxidant activity for ethyl acetate extracts for red seed variety. 67

Appendix 5: Chromatogram showing the phytochemical compounds with known biological activity with the one in black representing those with known antioxidant activity for hexane extracts for white seed variety. 68

Appendix 6: Active site predictions for HMG-CoA reductase (3CCZ) obtained using Prankweb with the pockets found ranked according to scores and probability. 68

Appendix 7: Excel results showing the binding affinity and rmsd values of the ligands and the HMG-CoA reductase post docking in PyRx. 69

Appendix 8: Excel results showing the types of bond interactions between the vanillin lactoside and the HMG-CoA reductase using Biodiscovery studio. 69

## LIST OF ABBREVIATIONS

NCD - Non-communicable diseases

CVD - cardiovascular diseases

HMG-CoA - 3-hydroxy-3-methylglutaryl coenzyme A

GC-MS - Gas chromatography with tandem mass spectrometry

DPPH - 2,2-diphenyl-1-picrylhydrazyl

ABTS - 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

SREBP - sterol regulatory element binding proteins

LDL - Low density lipoprotein

VLDL - Very low-density lipoprotein

ANOVA - Analysis of variance

SAS - Statistical Analysis System

CDC- Center for Disease Control

FDA- Food and Drug Administration

**ABSTRACT**

Atherosclerosis is an inflammatory condition of the arteries that contributes significantly to around 84.5% of all CVD mortality. It is distinguished by lipid accumulation in coronary arteries, which results in plaque development, reduced blood flow, and oxygen availability to various body tissues. This study therefore aimed at assessing phytochemicals of *Citrullus ecirrhosus* seed powder from three varieties, their antioxidant potential and predicting the binding mode of HMG-CoA reductase enzyme towards phyto-compounds using in silico studies as a drive towards atherosclerosis management. Secondary metabolites were extracted from *Citrullus ecirrhosus* seed powder using water, methanol, ethyl acetate, chloroform and hexane, and evaluated for total phenolic content (TPC) and total flavonoid content (TFC). The polar extracts (methanol and water) had higher phenolic and flavonoid content as compared to the moderate and non-polar seed powder extracts ($P < 0.05$). The methanolic and water extracts also showed higher ($P < 0.05$) free radical scavenging capacity as compared to the other moderate and non-polar solvent extracts in the DPPH and ABTS assays. This showed that the seed powder extract was rich in polar phytochemicals as compared to the non-polar. Identification of secondary metabolites of the extract was done using GC-MS. From a range of phytochemicals identified, 19 of those had reported antioxidant activities. These are Hexadecanoic acid; methyl ester (methyl palmitate); n-Hexadecanoic acid; Phenol, 2,4-bis(1,1-dimethylethyl); γ-Tocopherol; Hydroxylamine O-decyl; 13-Octadecenoic acid methyl ester; 5-Hydroxymethylfurfural; Vanillin lactoside; Octadecanoic acid; 9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3β,5Z,7E); cis-Vaccenic acid; Pentanoic acid, octyl ester; 3-Dodecene, (Z); 1-Heptatriacotano; Nonadecane; 2-Piperidinone, N-[4-bromo-n-butyl]; Heptacosane; Stigmasterol; 2-Methoxy-4-vinylphenol. The molecular interaction between HMG-CoA reductase enzyme, which is significant in cholesterol regulation, was studied against the 19 phytoconstituents from the seed powder extracts in silico. The phytoconstituents with high binding affinity within the range of that for the statins used were Vanillin lactoside, 9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol, stigmasterol, gamma-Tocopherol and Phenol, 2,4-bis(1,1-dimethylethyl)-. Their binding affinities ranged from -8.2 to -6.3 kcal/mol. Vanillin lactoside showed the highest binding affinity of -8.2 kcal/mol and the interactions between the compound and the protein were predicted to be through

conventional hydrogen bond, carbon hydrogen bond, Pi-Cation, Pi-Alkyl and unfavorable donor-donor interactions. This study thus showed that the *Citrullus eccirhosus* seeds have phytochemicals with antioxidant activity and some phytochemicals having a binding mode towards the HMG-CoA reductase enzyme in silico. However, there is also need to carry out wet lab analysis so as to confirm the ability of the seed powder in reducing the atherosclerotic lesion in atherosclerosis induced albino wistar rats.

# CHAPTER 1.0

# INTRODUCTION

## 1.1 Background

Non-communicable diseases (NCDs) are the leading cause of death globally with developing countries mostly affected (Minja *et al.*, 2022, Herrington *et al.*, 2016). The NCDs include cardiovascular diseases (CVDs; atherosclerosis and heart attack), cancer, diabetes and chronic lung disease. These diseases combined contribute to 74% of global mortality (WHO, 2022). The incidence of these NCDs is exacerbated by lack of physical exercise, use of tobacco and alcohol as well as unhealthy diets. According to a Chinese Global Television Network (CGTN) report (2022) on Botswana, 46% of deaths are because of NCDs, and approximately 90% of Batswana are believed to be eating unhealthy diets. The constant intake of diets containing high saturated fats and cholesterol from processed meat and fried foods leads to the accumulation of low-density lipoproteins (LDL) cholesterol in the blood. As a result, LDL cholesterol raises the risk of arterial plaque formation and death due to cardiovascular diseases (CVDs) such atherosclerosis (Shih *et al.*, 2000). Among the CVDs, atherosclerosis is the leading in morbidity and mortality (Herrington *et al.*, 2016). Atherosclerosis is defined by cholesterol accumulation in coronary arteries, which leads in plaque formation, decreased blood flow, and decreased oxygen availability to various body tissues (Singh *et al.*, 2002). As a result, atherosclerosis causes further health problems. Notably, among the factors that increase the risk of dying from atherosclerosis are a lack of adequate treatment and proper food intake.

Under oxidative stress, free radicals modify the LDL by oxidation to oxidized low density lipoprotein (ox-LDL). These ox-LDL then stimulate the migration of the monocytes to the sub endothelial space. The monocytes then engulf the ox-LDL forming foam cells which leads to the formation of an atherosclerotic plaque (Bonomini *et al.*, 2016; Bonomini *et al.*, 2008).

Medicinal reduction of atherosclerosis involves the lowering of LDL in the blood by taking in statins. These statins will then reduce the formation of the LDL in the liver. However, statins, on the other hand, produce muscle soreness and are reportedly as being carcinogenic and increasing the risk of diabetes (Guber *et al.*, 2021, Thompson *et al.*, 2016). Statins are extremely costly and have a low response rate in individuals who are simultaneously on other medicinal drugs (Naci *et al.*, 2013). Currently researchers are focusing on searching for natural sources for management of

NCDs. Accumulating evidence have been shown plants as a source of phytochemicals including steroids, terpenoids, flavonoids, alkaloids, phenols, tannins, and glycosides with potent biological effects (Edrah *et al.*, 2016, Usunobun *et al.*, 2015, Oktay *et al.*, 2003, Parr and Bolwell, 2000). Fruits including watermelon are pertinent as they are a source of antioxidants, dietary fiber, minerals, phytochemicals, and vitamins (Septembre-Malaterre *et al.*, 2018), responsible for reducing the risk associated with development of cancer, cardiovascular disease, obesity, and type 2 diabetes (Reyes-Munguía *et al.*, 2016). Watermelon is a fruit belonging to the family of Cucurbitaceae, a plant family of world's economic important crop with several genera and species including *Citrullus amarus*, *Citrullus lanatus*, *Citrullus rehmii*, *Citrullus mucusospermus*, *Citrullus colocynthis*, *Citrullus ecirrhosus* and *Citrullus naudinianus* (Levi *et al.*, 2017, Chomicki and Renner, 2015).

The phytochemicals present in *Citrullus lanatus* seeds which have been studied previously include saponins, flavonoids, tannins, alkaloids, and phenolic compounds (Opara *et al.*, 2018b). *C. lanatus* extract administered to LDL receptor knocked out mice decreased plasma cholesterol concentrations, which are ascribed to lower levels of low-density lipoprotein cholesterol and decreased the atherosclerotic lesion size (Poduri *et al.*, 2013, Kaur *et al.*, 2021b). *C. colocynthis* showed the presence of phytochemicals which further led to analysis of their antioxidant potential confirmed by chemical tests (Kumar *et al.*, 2008).

*C. ecirrhosus,* known as Namib tsamma, is commonly known as *sesoswane* in Botswana. *C. ecirrhosus* is a plant that is native to Southern Africa (Chomicki and Renner, 2015). The seeds of this plant have been traditionally used as a food source by local communities in Botswana and are known to possess medicinal properties. However, research on their biological effects is limited. Therefore, it is important to carry out studies on the health benefits of the seeds to increase awareness of consumers on their beneficial effects. In this study, we assess the phytochemical and antioxidant potential and in silico studies towards management of atherosclerosis, to predict the hypothetical binding mode of phyto compounds. Our recent preliminary study showed that *C. ecirrhosus* seed is a source of phytochemicals with antioxidant and antibacterial activities.

## 1.2 Problem statement

Atherosclerosis is a major underlying cause of CVDs and the leading cause of mortality and morbidity worldwide. Because of an increase in bad cholesterol (LDL) levels linked to lifestyle changes, the prevalence of atherosclerosis is predicted to rise significantly. Around 18.2 million adults (6.7%) who are 20 years of age or older have coronary artery disease, and over 365 914 people died in the United States alone from the disease in 2017, according to the CDC database report. According to the World Health Organization's report on the geriatric population as reported by (Mwaila and Yousif, 2022), by the year 2050, there will be almost 2 billion people in the geriatric population, up from 524 million in 2010. However, because of their weakened immune systems, this population is more vulnerable to cardiovascular illnesses, which raises the risk of atherosclerosis in this age group. According to a report by Transparency Market Research as reported by (Timmis *et al.*, 2024), these statistics have led to an increase in the estimation of the drug market demand for atherosclerosis management. A 3% CAGR (Compound Annual Growth Rate) increase is anticipated by 2027.

The synthetic drugs used to treat this illness have some downside effects in terms of cost implications and negative side effects. Conversely, it has been shown that plant-based sources are a good source of bioactive compounds with potential for use as agents for the management of atherosclerosis. Finding medicinal plant extracts or metabolites that can be further developed into anti-atherosclerosis medications is made easier with the help of bio-prospection of medicinal plants. Because these plant-derived extracts are more widely available, more efficacious, patient-favored, affordable, and without known side effects, they provide a comprehensive approach to the treatment of atherosclerosis. These will lessen the financial toll that atherosclerosis takes on the world economy. Local communities in Botswana have long utilized *C. ecirrhosus* seeds as a food source because of their known medical benefits. On the other hand, there is scarcity of scientific literature on their antioxidant activity.

### 1.3 Hypothesis

*Citrullus ecirrhosus* seed powder is a source of phytochemicals with significant ($P < 0.05$) antioxidant potential and binding mode towards HMG-CoA reductase enzyme.

### 1.4 General Objective

Assess phytochemicals of *Citrullus ecirrhosus* seed powder, their antioxidant potential and predict the binding mode of HMG-CoA reductase enzyme towards phyto-compounds using in silico studies.

### 1.5 Specific Objectives

- To extract and evaluate total phenolic content and total flavonoid content of crude extracts from *Citrullus ecirrhosus* seed powder
- To identify secondary metabolites of the extract using GC-MS/MS
- To evaluate biochemical antioxidant activities of the seed powder extracts using DPPH, ABTS and TAC assays
- To study the molecular interaction between HMG-CoA reductase enzyme and the phytoconstituents with antioxidant properties from the seed powder extracts by molecular docking.

### 1.6 Justification

In our preliminary research, we successfully extracted and qualitatively determined phytochemical constituents of *Citrullus ecirrhosus* which include anthocyanins, alkaloids, flavonoids, phenols, quinones, saponins, sterols and terpenoids. The radical scavenging capacity of these extracts against DPPH and ABTS were also established. However, till date, no detailed investigation has been conducted to identify the phytochemicals as well as quantifying them and exploring their antioxidant capacity in vitro as a step towards management of atherosclerosis.

# CHAPTER 2.0

# LITERATURE REVIEW

## 2.1 Atherosclerosis

Atherosclerosis is an inflammatory condition of the arteries that contributes significantly to CVDs morbidity and mortality. It is linked to lipids and other metabolic changes. Atherosclerotic cardiovascular disease (ACD) is a subset of two major conditions: ischemic heart disease (IHD) and cerebrovascular disease (mostly ischemic stroke). The IHD is the world's biggest cause of death, with cerebrovascular disease coming in third (Barquera *et al.*, 2015). These two together account for 84.5% of all CVDs. Morbidity and mortality from cardiovascular and other non-communicable chronic diseases (NCCDs) have recently increased, particularly in low to middle income nations. Traditionally, these countries were concerned with controlling undernutrition and infectious disease; however, they now face a growing threat from NCCDs if pre-transition health concerns are not fully addressed. This transition has been rapid, leaving countries and international health organizations with little time to respond (Moran *et al.*, 2014). IHD incidence, prevalence and mortality have been recorded to be on the rise between 2010 and 2019 with the trend projected to continue beyond 2019. Ischemic stroke also recorded an increase in the incidence and prevalence rates between the same periods of 2010 to 2019 (Chen *et al.*, 2023). These findings suggest the need to intensify efforts in the control and management of atherosclerosis globally.

## 2.2 Development of Atherosclerosis

The pathogenesis of atherosclerosis is associated with sequential events and a couple of factors. The process starts with damage to the endothelial cell of the inner wall of the arteries. These may be directly damaged by the free radicals or other factors like hypertension, hypercholesterolemia among others. The dysfunction of the endothelial leads to an increase in its permeability thus allowing the flow of substances such as low-density lipoprotein (LDL) cholesterol infiltrating the arterial wall (Sun *et al.*, 2020). The LDL then undergoes oxidative modification by the free radicals. The oxidized LDL (oxLDL) is a known potent trigger for inflammation. The oxLDL attracts monocytes to the endothelial site thus triggering the formation of foam cells within the

arterial wall. These foam cells trigger the formation of the fatty streaks which are the initial visible lesions in atherosclerosis (Berenji Ardestani *et al.*, 2020). Oxidative stress activates signaling pathways responsible for inflammation thus promoting the release of inflammatory cytokines and chemokines (Khosravi *et al.*, 2019). Also, recruited towards the atherosclerotic lesion are inflammatory cells which include macrophages and T lymphocytes. Smooth muscles in the arterial wall respond to the inflammatory signals by moving towards the lesion site thus leading to the formation of a fibrous cap over the atheromatous plaque (Badimon *et al.*, 2012, Badimon and Vilahur, 2012). Although this cap can stabilize the plaque, there's a chance it will become more brittle. Ruptures can occur in vulnerable plaques with thin fibrous caps. A blood clot, or thrombus, forms when a plaque bursts, exposing its contents to the bloodstream. Myocardial infarction, or heart attack, or stroke are examples of acute events that can result from a thrombus that blocks an artery either totally or partially (Jebari-Benslaiman *et al.*, 2022, Patterson *et al.*, 2023).

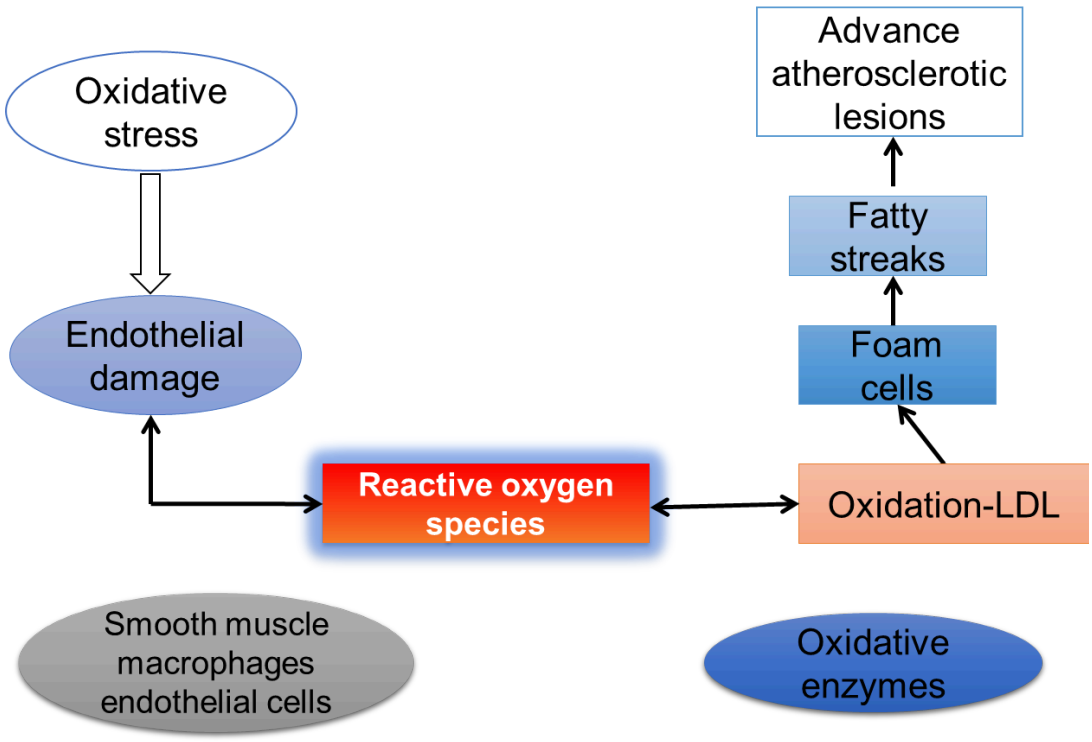



***Figure 1.1: A summary on how reactive oxygen species and oxidative stress play a role in the development of atherosclerosis (Vogiatzi et al., 2009; Rodella and Rezzani, 2012)***

**2.3 Management of Atherosclerosis**

The known key of management and prevention of CVDs is lipid lowering treatment. Currently, statin medication has been demonstrated to lower serum cholesterol as well as reducing the morbidity and mortality of CVDs (Reiner, 2013). These statins are the reversible competitive inhibitors of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase of the mevalonate pathway (du Souich *et al.*, 2017). Statins that were approved for use by the FDA include atorvastatin, rosuvastatin, simvastatin, pravastatin, fluvastatin, lovastatin and pitavastatin (O'Malley *et al.*, 2020). This inhibition causes a drop in cholesterol content in the endoplasmic reticulum thus triggering the movement of the sterol regulatory element binding proteins (SREBPs) to the Golgi from the endoplasmic reticulum. The SREPBs are then cleaved by the proteases and converted into active transcription factors. They then translocate to the nucleus and promote the expression of several genes including the HMG-CoA reductase and most critically, the LDL receptor. An increase in the HMG-CoA reductase expression restores hepatic cholesterol synthesis to normal, whereas increased LDL receptor expression results in an increase in the number of LDL receptors on the plasma membrane of hepatocytes. This will cause a fast removal of apolipoprotein B and E containing lipoproteins (LDL and VLDL). The reduction in plasma LDL-C and triglyceride levels can be attributed to enhanced LDL and VLDL clearance (Goldstein and Brown, 2015)(Feingold, 2016). Phytochemicals in plants can act as competitors of the statins in inhibition of the HMG-CoA reductase enzyme (Chouhan *et al.*, 2022). However, extensive research hasn’t been done on them with respect to this mechanism in lowering cholesterol levels in the management of atherosclerosis.

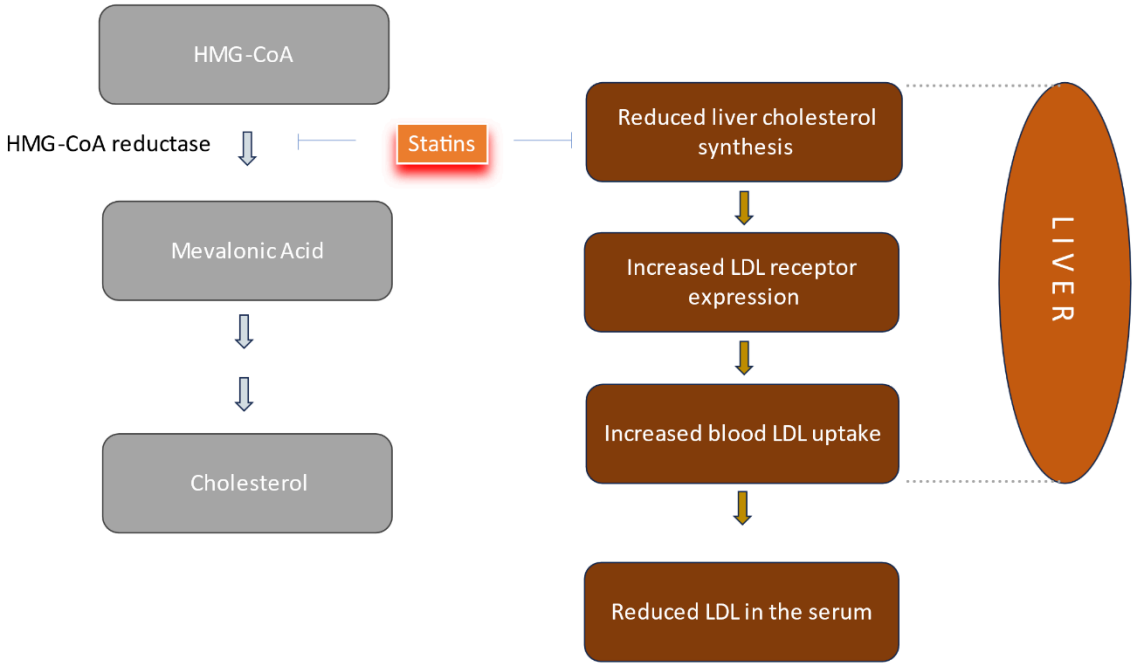



***Figure 1.2: The effect of statins on the mevalonate pathway in the liver inhibiting the conversion of HMG-CoA by the enzyme HMG-CoA reductase to mevalonic acid which ultimately hinders the formation of cholesterol. This ultimately increases the expression of the LDL thus causing an increase in the LDL uptake in the liver which will ultimately lower the serum LDL content. (Goldstein and Brown, 2015)***

### 2.4 Citrullus ecirrhosus

*Citrullus ecirrhosus* is commonly known as *sesoswane* in Botswana. It is a perennial desert vine that is mostly found in Botswana, Namibia, and South Africa with a bulk of it being in the desert areas such as Namib Desert. It is a *Cucurbitaceae* of the gourd family and a close relative of *Citrullus lanatus,* the popularly consumed watermelon. The plant can endure severe desert conditions with little water and high sunlight by relying on deep ground water and morning fogs. Its bitter-tasting fruit, known as *tsamma* melons, is a significant source of water for a variety of desert species. The plant has yellow flowers and annual stalks that die each year. It is a resilient species capable of crawling for up to two meters (Chomicki and Renner, 2015)

*Citrullus ecirrhosus* produces little seeds with a diameter of about 4-5 mm. The seeds have a dark brown or black outer covering that is firm, smooth, and shiny. The seeds inside the coat are white and have a slightly curved form. The seeds can be roasted and eaten by the local people as

a snack. (Umar *et al.*, 2013a), determined the nutritional content of the seeds. They found out that the seeds were rich in oil, protein and mineral content. These seeds have also been used in the treatment of various elements by the local people, such as stomach pains, constipation among others (Snijman, 2013).



***Figure 1.3: Citrullus ecirrhosus plant (Simmons et al., 2019)***

**2.5 Phytochemical composition of *Citrullus spp***

Phytochemicals are organic chemical molecules found in plants that are essential for their survival in an environment, according to (Choudhari *et al.*, 2020). These phytochemicals are also considered bioactive molecules due to their ability to regulate cholesterol synthesis, inhibit and reduce inflammatory reactions, and prevent DNA damage and organelle damage caused by oxidative stress, among other things (Carvalho *et al.*, 2020). Saponins, flavonoids, tannins, alkaloids, and phenolic compounds have previously been examined in *Citrullus lanatus* seeds, which is closely related to *Citrullus ecirrhosus* (Opara *et al.*, 2018a, Varghese *et al.*, 2013, Braide *et al.*, 2012). Flavonoids and tannins were also reported in *Citrullus colocynthis* extracts (Benariba *et al.*, 2013, Kumar *et al.*, 2008, Umar *et al.*, 2013b). Since the *Citrullus ecirrhosus* plant is known to thrive more in desert conditions, it is therefore expected to have an abundance of these phytochemicals (Ofir, 2020). Flavonoids are a group of secondary metabolites found in

foods of plant origin as well as medicinal plants which have been shown to exert potent antioxidant activity against the superoxide radical (Bombardelli and Gabetta, 2001). These flavonoids have been shown to be present in the most studied *Citrullus spp* which are the *C. lanatus* and the *C. colocynthis*. Saponins have also been reported to have antimicrobial and anticancer activities. Tannins also exhibit antioxidant activities which has led to the development of tannin based dietary supplements (Ofir, 2020).

### 2.6 Antioxidant potential of *Citrullus ecirrhosus* seeds

Antioxidants are compounds that inhibit oxidation which causes free radical formation. Free radicals are constantly being formed in the body. Studies have shown that reactive oxygen species (ROS) including free radicals can lead to lipid peroxidation and oxidative stress which damage biological structures such as proteins, lipids, and DNA (Goldstein and Brown, 2015). The human body has its own natural antioxidant system to stand against free radicals using enzymes, however, an intake of antioxidants as supplements reinforces the defense of the human antioxidant system (Edeoga *et al.*, 2005). When the generation of harmful chemicals known as free radicals exceeds the antioxidant defenses' ability to protect, oxidative stress ensues. While there is a vast of knowledge on the proximate and oil content of *Cucurbitaceae* seeds, particularly *Citrullus lanatus*, there is a scarcity of data on the seeds' phytochemicals and radical scavenging potential (Seidu and Otutu, 2016). The reported antioxidant activity of the *Cucurbitaceae* family was mostly on the *C. lanatus* and *C. colocynthis* (Tlili *et al.*, 2011, Kaur *et al.*, 2021a, Kumar *et al.*, 2008) leaving a dearth of information on the other family members. As a result, the current investigation concentrated on the phytochemical and antioxidant properties of *Citrullus ecirrhosus* seed contents.

### 2.7 Role of phytochemicals on seed pigmentation/coloration

Seed color may be influenced by many chemical components within their tissues. Various pigments such as anthocyanins, flavonoids and carotenoids play an integral role in seed coloration (Zhao *et al.*, 2022a). It is reported that flavonoids may contribute to the yellow or orange hues, carotenoids contributing to some yellow and red coloration and the anthocyanins resulting in the red, purple or sometimes blue tones of seeds (Khoo *et al.*, 2017b, Zhao *et al.*, 2022a). In other cases, polyphenolic compounds such as tannins and lignins may contribute to the brown coloration of seeds. Lignins add on to the hardness of the seed coat as well as adding some brown hues whilst the tannins contribute towards astringency and may also impart a

brownish color (Marles and Gruber, 2004). It is also reported that some flavonoids may contribute to the grey hues when in high concentration or being in some chemical forms. The other important aspect in the grey coloration might be an interplay amongst different pigments like flavonoids, anthocyanins and carotenoids in varying concentrations which then gives out the grey coloration (Marles and Gruber, 2004; Zhao *et al.*, 2022b).

**2.8 Solvents for phytochemical extraction**

The extraction process's selectivity and efficiency are greatly impacted by the solvent used to extract phytochemicals from plant materials. Because different phytochemicals are soluble in different solvents to varying degrees, choosing the right solvent or combination of solvents is essential. The chosen solvent should possess properties that are specifically designed to extract the desired phytochemicals. While non-polar solvents like hexane and chloroform are typically effective for non-polar compounds, polar solvents like water, methanol, and ethanol are typically used for the extraction of polar compounds (Pandey and Tripathi, 2014, Sasidharan *et al.*, 2011, Abubakar and Haque, 2020)

**2.9 In silico docking**

A computational technique useful in drug discovery for both predicting and analyzing the interaction between ligands and target proteins is called "in silico docking." Using this method, the binding affinity and orientation of the ligand within the binding pocket of the receptor are predicted by simulating the binding processes of the ligand to the active site of the protein of interest. This method is widely used in drug discovery for a variety of reasons. It provides researchers with the tools or capability to computationally screen vast libraries of various chemical compounds to find promising candidates for drugs (Kitchen *et al.*, 2004). These researchers can profile the best compounds that have the highest likelihood of binding for additional experimental validation and the ability to elicit therapeutic efficacy by using the binding affinity of ligands to the proteins of interest. Once promising drug candidates have been identified, in silico docking will be required to optimize their chemical structures for increased binding affinity and specificity. The design of chemical analogs and derivatives that might have improved pharmacological activities depends heavily on these computational simulations. By analyzing the interactions between the drugs and the targeted proteins, in silico docking provides some insights into the mechanism of action of pharmaceuticals. This aids in the development of more potent and highly selective medications as well as helps researchers better understand how

the drugs deliver their therapeutic effects. Researchers may expedite the drug discovery process, lower expenses, and find new treatments for a wider range of illnesses more effectively by utilizing computational techniques (Friesner *et al.*, 2004; Trott and Olson, 2010).

# CHAPTER 3.0

# MATERIALS AND METHODS

## 3.1 Collection and preparation of plant extracts

### 3.1.1 Collection of plant samples

The *Citrullus ecirrhosus* watermelon seeds were collected from Lemone farm, 30 Km west of Palapye, Botswana. The seeds were already air-dried and were of three distinctive varieties as shown in Figure 2. The air-dried seeds were ground to coarse powder using a coffee grinder and packed in an airtight Ziplock polythene bag. The samples were stored at -20 ℃ until analysis.

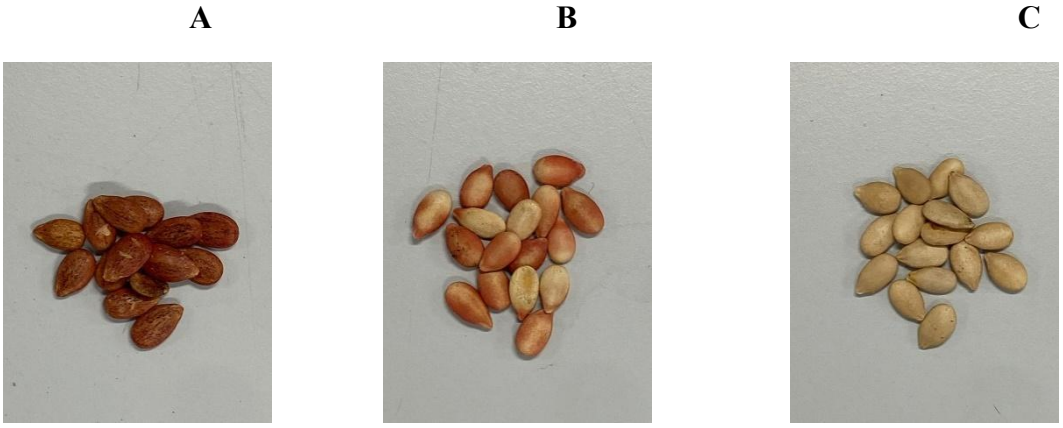



***Figure 2: The three different varieties of the Citrullus ecirrhosus seeds with (A) Brown, (B) Red and (C) White.***

### 3.1.2 Preparation of seeds extracts

The 2 g of each of the 3 grounded seed powder varieties were measured using an electronic balance. The measured powder was put in a conical flask. Each variety was measured five times to accommodate the solvents of extraction , water, methanol, chloroform, ethyl acetate and hexane. Maceration was then done with 20 mL of each respective solvent and left at room temperature with constant stirring for 24 hours. After soaking and stirring, the crude solutions were filtered through Whatman filter paper, Grade 1 to remove coarse impurities. The extracts were then concentrated in an oven at 40 °C. The resulting extracts were stored at 4–6 °C in the dark.

### 3.1.3 Plant confirmation

**DNA Extraction from *Citrullus ecirrhosus* and sequencing**

Leaf material of 0.1 g was harvested and crushed in mortar and pestle using liquid nitrogen. To homogenize the crushed leaf powder, 700 µL extraction buffer was used. About 0.5 mL of the homogenate was then transferred to a 1.5 mL microfuge tube and 33 µL of 20% sodium dodecyl sulfate (SDS) was added. Incubation was done for 10 minutes at 65ºC. Immediate addition of 160 µL (0.25 volume) of 5 M Potassium acetate was done. The mixture was vortexed thoroughly and incubated at 0ºC for 20 minutes followed by centrifuging at 13000 rpm for 10 minutes at room temperature and 450 µL of the clarified supernatant was carefully transferred to a new microfuge tube. About 0.5 µL of cold isopropanol was then added and the mixture vortexed and incubated at -20ºC for 30 minutes followed by centrifugation at 13000 rpm for 10 minutes to precipitate the DNA. The isopropanol was then decanted. The DNA pellet was then washed by centrifugation with 500 µL of 70% ethanol 13000 rpm at 4ºC for 5 minutes. The DNA pellet was then dried at room temperature for 15 minutes and suspended in 100 µL double distilled water. The extracted genomic DNA was amplified with universal primers, 5'-GCATCGATGAAGAACGCAGC-3' Forward primers and 5'- TCCTCCGCTTATTGATATGC-3' reverse primers, using Applied Biosystems ProFlex Thermal cycler (Thermo Fisher, Marsiling, Singapore). PCR product was detected by running agarose gel electrophoresis for the 1TS2 gene. The amplicons were sent to Inqaba Biotechnological Industries (Pty) Ltd sequencing using the Sanger Sequencing method. The sequence was then quality trimmed using BioEdit ver.7.2 (http://www.bioedit.com). Species identification was carried out by comparing with those in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (http:// www.ncbi.nlm.nih.gov/ blast). The sequence was then deposited in NCBI (accession number: PP860534).

**Phylogeny Analysis**

The evolutionary history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The phylogenetic tree with the highest log likelihood (-1414.82) was presented. The bootstrap support values were shown next to the branches, indicating the percentage of replicate trees in which the associated taxa clustered together during the bootstrap test (1000 replicates). The tree was drawn to scale, with branch

lengths representing the number of substitutions per site. The proportions of sites where at least one unambiguous base is present in at least one sequence for each descendant clade were shown next to each internal node. Codon positions included in the analysis were 1st+2nd+3rd+Noncoding, with a total of 492 positions in the final dataset. Evolutionary analyses were conducted using MEGA X software (Kumar *et al.*, 2018).

### 3.2 Quantitative Phytochemical Analysis

#### 3.2.1 Total Phenolic Content

Total phenolic content (TPC) of the extracts was determined by the Folin-Ciocalteu method (Singleton and Rossi, 1965) as modified by (Gao *et al.*, 2014). Stock solution of Ferulic acid used as a standard was prepared by mixing with methanol in a ratio 1 mg: 1 mL, and the standard solutions were made with dilutions over a concentration range of 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 mg/mL. The reaction mixture was prepared by mixing 0.2 mL of extracts or standard with 1.5 mL of 10% Folin-Ciocalteu's reagent (dissolved in distilled water) and incubated at room temperature for 15 minutes. After 15 minutes of equilibration, the mixtures were neutralized with 1.5 mL of sodium carbonate (60 g/L), of which post the set time, the reactions were incubated at room temperature in the dark for 90 minutes. The absorbance of the mixtures was measured at 725 nm using a Spec200E UV-Vis spectrophotometer (ThermoFisher Scientific, city, USA). The total phenolic content of the samples was calculated based on the ferulic acid standard curve equation and expressed as ferulic acid equivalence (FAE/g). Experimental measurements were carried out in triplicates.

#### 3.2.2 Total Flavonoids Content

The total flavonoid content of plant extracts was determined using the aluminum chloride colorimetric assay (Yadav and Agarwala, 2011). Briefly, 1 mL of each plant extract or 1 mL of quercetin solution (0 – 150 µg/mL) was mixed with 3 mL of methanol in test tubes. About 200 µL of 10% aluminum chloride was added to the mixture followed by 200 µL of 1 M potassium acetate. To make a total volume of 5 mL, 600 µL of distilled water was added. The reaction was then incubated for 30 minutes at room temperature and absorbance was read at 420 nm in a

Spec200E UV-Vis spectrophotometer (ThermoFisher Scientific, city, USA). By extrapolating from the quercetin (0 – 150 µg/mL) standard curve, the total flavonoid content was calculated, and the results were reported as quercetin equivalent/g (QE/g). Experimental measurements were carried out in triplicates.

### 3.2.3 Total Antioxidant Capacity

The phosphomolybdenum assay was used to assess total antioxidant capacity, with slight modifications to the method described by (Aliyu *et al.*, 2013). The 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate were combined to create the phosphomolybdenum reagent. About 3.3 mL of 99.9% sulfuric acid was diluted with 100 mL of distilled water to create 0.6 M sulfuric acid. To make 4 mM ammonium molybdate, 459 mg of sodium phosphate was reconstituted using 100 mL of distilled water, and 494 mg of powder was diluted using 100 mL of distilled water for 28 mM sodium phosphate. To make the working reagent, each of them was prepared separately first. The ascorbic acid standard that was utilized was 10 mM, and by diluting it with methanol, several standard concentrations were created, ranging from 1000 to 100 µg/mL. In addition, 3 mL of the working reagent was added to several labeled test tubes to create the reaction mixture. This was followed by the addition of 0.3 mL of standard solutions to correspondingly labeled concentration test tubes. In a similar manner, 0.3 mL of samples were added to test tubes with labels that held 3 mL of the working reagent for the sample reaction combination. Following their mixing and 90 minutes of incubation in a water bath at 90°C, they were allowed to cool to room temperature before the absorbance at 695 nm using Spec200E UV-Vis spectrophotometer (ThermoFisher Scientific, city, USA) was measured in comparison to a methanol blank. The ascorbic acid standard curve equation was used to express the results of each experiment, which were conducted in triplicates, as milligrams of ascorbic acid equivalents per gram (AAE/g). Experimental measurements were carried out in triplicates.

### 3.2.4 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay

The free radical scavenging activity was determined using DPPH (2, 2-diphenyl-1-picrylhydrazyl) as performed by (Blois, 1958) and modified by (Brand-Williams *et*

*al.*, 1995) with some minor modifications. The 10 mM Trolox stock solution was made by dissolving the powder in absolute ethanol. The standard working solutions were made in concentration ranges of 1.4, 1.2, 1.0, 0.8, 0.6, 0.4, 0.2 mM. The reaction solutions were made by taking 50 µL of the respective solution/ sample and mixing with 1900 µL of 100 µM methanolic DPPH. The reaction mixtures were then incubated at room temperature for 30 minutes in the dark. The absorbance was measured at 515 nm using Spec200E UV-Vis spectrophotometer (ThermoFisher Scientific, city, USA). The DPPH radical scavenging activity of the samples was calculated based on the Trolox standard curve equation and expressed as Trolox equivalence (TE/g). Experimental measurements were carried out in triplicates.

### 3.2.5 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) Assay

With some modifications, the 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay developed by (Re *et al.*, 1999) was used to measure the extracts' ability to scavenge free radicals. The 7 mM ABTS solution and the 2.45 mM potassium persulfate solution were made with distilled water to make the ABTS reagent stock solutions. The two solutions were combined and left at room temperature in the dark for the entire night. To prepare a working solution, the solution was then diluted with distilled water until an absorbance of 0.709 at 734 nm was achieved. The range in which the standard solutions were made was 1.4-0.2 mM, with 10 mM Trolox serving as the standard. About 1900 µL of a 7 mM methanolic ABTS solution was mixed with 50 µL of the corresponding standard solution or sample to create the reaction solutions. The reaction mixtures were then incubated at room temperature for 30 minutes in the dark. All the experiments were performed in triplicates. The absorbance was measured at 734 nm using Spec200E UV-Vis spectrophotometer (ThermoFisher Scientific, city, USA). The ABTS radical scavenging activity of the samples was calculated based on the Trolox standard curve equation and expressed as Trolox equivalent (TE/g). Experimental measurements were carried out in triplicates.

## 3.3 Identification of phytochemicals - GC–MS (gas chromatography–mass spectroscopy) analysis

GC–MS analysis of the extracts of methanol, chloroform, ethyl acetate and hexane of *Citrullus ecirrhosus* seeds was performed using Agilent 7890B GC, coupled to an Agilent 5977A mass

spectrometer detector. The HP-5 MS capillary column (Hewlett-Packard, CA, USA) which has a stationary phase of 95 % dimethyl silicone layered with 5% phenyl-methyl silicone of phases thickness of 0.25 mm was employed. The column dimensions were 30m x 320 µm x 0.25mm coated with 5% phenyl-methyl silicone, 95% dimethyl silicone. The GC parameters were set up as 250 °C inlet temperature, 90 °C oven temperature at 0 minutes raised to 200 °C for 2 minutes (3 °C/min) and then 280 °C for 2 minutes (15 °C/min). Helium gas was used as a carrier gas with a flow rate of 1.1 mL/min. The auxiliary temperature was 280 °C. The total retention time for the entire chromatographic analysis was 46 minutes. The MS parameters had a quad temperature of 150 °C, source temperature of 230 °C, scan mode and a mass range of 50–550 m/z. Compound identification and mass spectra analysis were performed using the "NIST-MS Library." The total ionic chromatogram's peak regions were used to calculate the relative percentage of separated substances. (Shajib *et al.*, 2023).

### 3.4 Molecular Docking

#### 3.4.1 In-silico assessment of HMG CoA reductase enzyme inhibition and anti-atherosclerosis properties of *Citrullus ecirrhosus* phytochemicals.

This evaluation implored the use of bioactive compounds from the *Citrullus ecirrhosus* seed extract that were determined by GC-MS analysis and demonstrated to possess antioxidant qualities; they also called for the retrieval of the bioactive compounds' Spatial Data Files (SDF) and the enzyme HMG CoA reductase; bioactive compound ligand and protein preparation; and site-specific molecular docking.

#### 3.4.2 Ligand and protein preparation

The Protein Data Bank (PDB) website (http://www.rscb.org/pdb/) provided the three-dimensional model of HMG CoA reductase (PDB ID: 3CCZ) (Sarver *et al.*, 2008). PyMOL Molecular Graphics (System Version 2.0 Schrodinger, LLC) was used to prepare the protein file by eliminating heteroatoms and water molecules (Ravi and Kannabiran, 2016). The three-dimensional models of the bioactive compounds were obtained as SDF from the online database PubChem (https://pubchem.ncbi.nlm.nih.gov/). The 2D compounds from PubChem were then converted to 3D compound SDF and Protein Data Bank, Partial Charge and Atom

Type (PDBQT) files using Open Babel. The PrankWeb (P2Rank) web-based front-end website (http://prankweb.cz/) was utilized to predict the protein-ligand binding sites for the prepared protein (Jendele *et al.*, 2019). The controls used were the FDA approved, on the market, statins (Atorvastatin, Simvastatin, Lovastatin, Pitavastatin, Rosuvastatin, Fluvastatin and Pravastatin).

### 3.4.3 Site-specific molecular docking

Using AutoDock 4.2.6 and AutoDock Vina in the PyRX 0.8 virtual screening tool, protein-ligand docking was carried out (Dallakyan and Olson, 2015). PDBQT files containing all the prepared compounds and proteins were loaded into AutoDock. The amino acid residues of the active site were labeled, and the vina search space was specified using the P2Rank predicted protein-ligand binding sites for pocket 1. For conformational search and docking simulation, the Lamarckian Genetic Algorithm (LGA) was chosen. Also, the other parameters were left at their default settings, and the number of evaluations was limited to 10.

### 3.4.4 Method validation

The docking process includes the conformation of the ligand, the prediction of the position of the ligand and its orientation within the sites/pockets (usually called poses) and the assessment of the binding affinity. The root mean square deviation (RMSD) calculation evaluates the accuracy of the pose prediction comparing the predicted pose against the experimental one (Sa'adah *et al.*, 2022)

### 3.4.5. Visualization of binding position and interaction between HMG CoA reductase and bioactive compounds

The 3-D crystal structure image of the HMG CoA reductase protein (PDB ID:3CCZ) and ligand with promising inhibitor properties were examined and visualized using PyMOL, and Discovery Studio Visualiser (Biovia, 2020).

### 3.4.6 Results analysis

Examining the receptor-ligand complex conformation and affinity energy value (kcal/mol) allowed for a thorough analysis of the molecular docking results. A higher degree of stability between the ligand and the receptor, which results in a stronger connection, is indicated by an affinity energy value that is more negative as compared to others.

### 3.5 Statistical Analysis

All analyses were performed in triplicates. Results of the analyses were analysed by one-way analysis of variance (ANOVA) followed by application of the Tukey test to assess the significance of specific intergroup differences using SAS software (JMP-SAS 17, USA, 2023) and results were considered significant at 95% confidence level ($P < 0.05$).

# CHAPTER 4.0

# RESULTS

## 4.1 Plant species confirmation

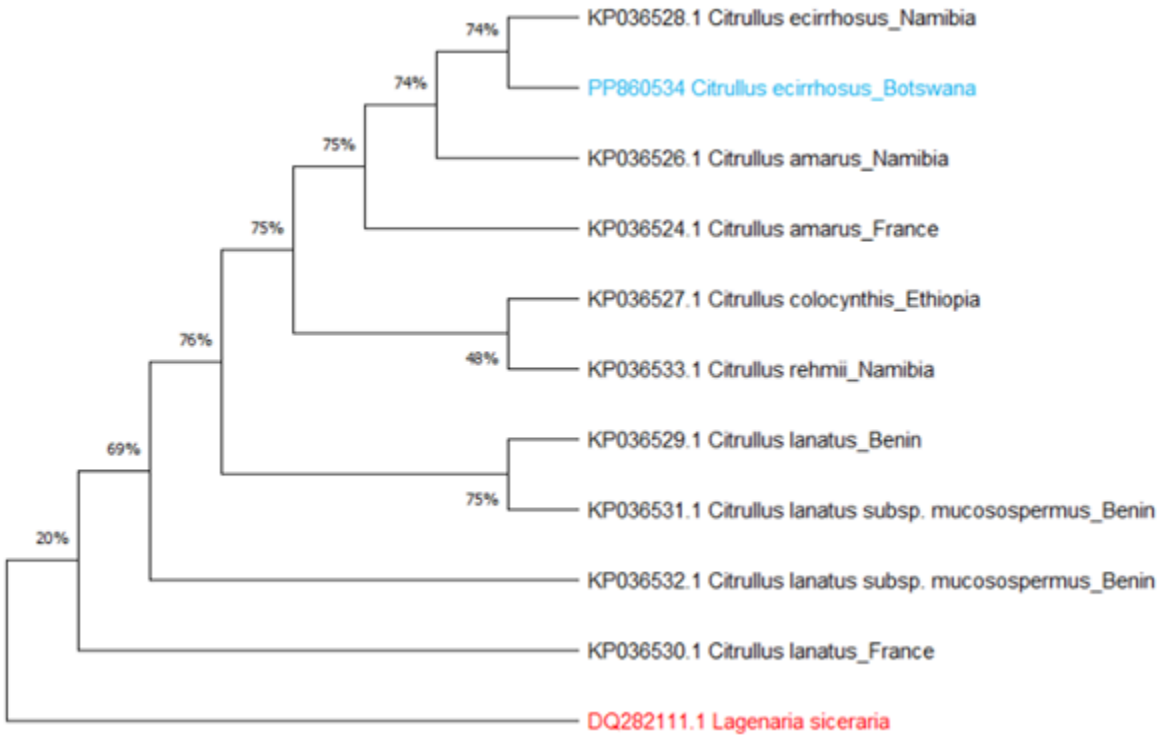



***Figure 3: Maximum Likelihood phylogenetic tree for Citrullus spp ITS2 region sequence to confirm the identity of the sample from Botswana. The sample is presented in sky blue and the outgroup is presented in red.***

With bootstrap values ranging from 74% to 76%, this species, which is found in Namibia (sample KP036528.1) and Botswana (sample PP860534), exhibits strong clustering support. This suggests a recent common ancestor between these samples and shows a close evolutionary link between them. The phylogenetic tree's high support values give rise to confidence in the validity of this grouping.

### 4.2 Quantitative Phytochemical Analysis

#### 4.2.1 Total Phenolic Content

The quantitative determination of the concentrations of phenols was based on the ferulic acid standard curve. The results found are presented as mg ferulic acid equivalence (FAE) /g dry weight of the extract. The highest concentration of phenols from the extracts was 0.0384 ± 0.0004 mg FAE/g dry weight found in the methanolic extracts of the brown variety followed by methanolic extracts of the white variety as shown in Figure 3.1. The hexane extracts of all the varieties were found to contain the lowest number of phenolics extracted. There was statistical difference ($P < 0.05$) amongst the solvents as they moved from one polarity to the other as shown in Figure 3.1. It can also be noted that the most phenolic compounds were recorded in the brown followed by white and lastly red varieties with the trend being maintained from the polar solvents to the non-polar.

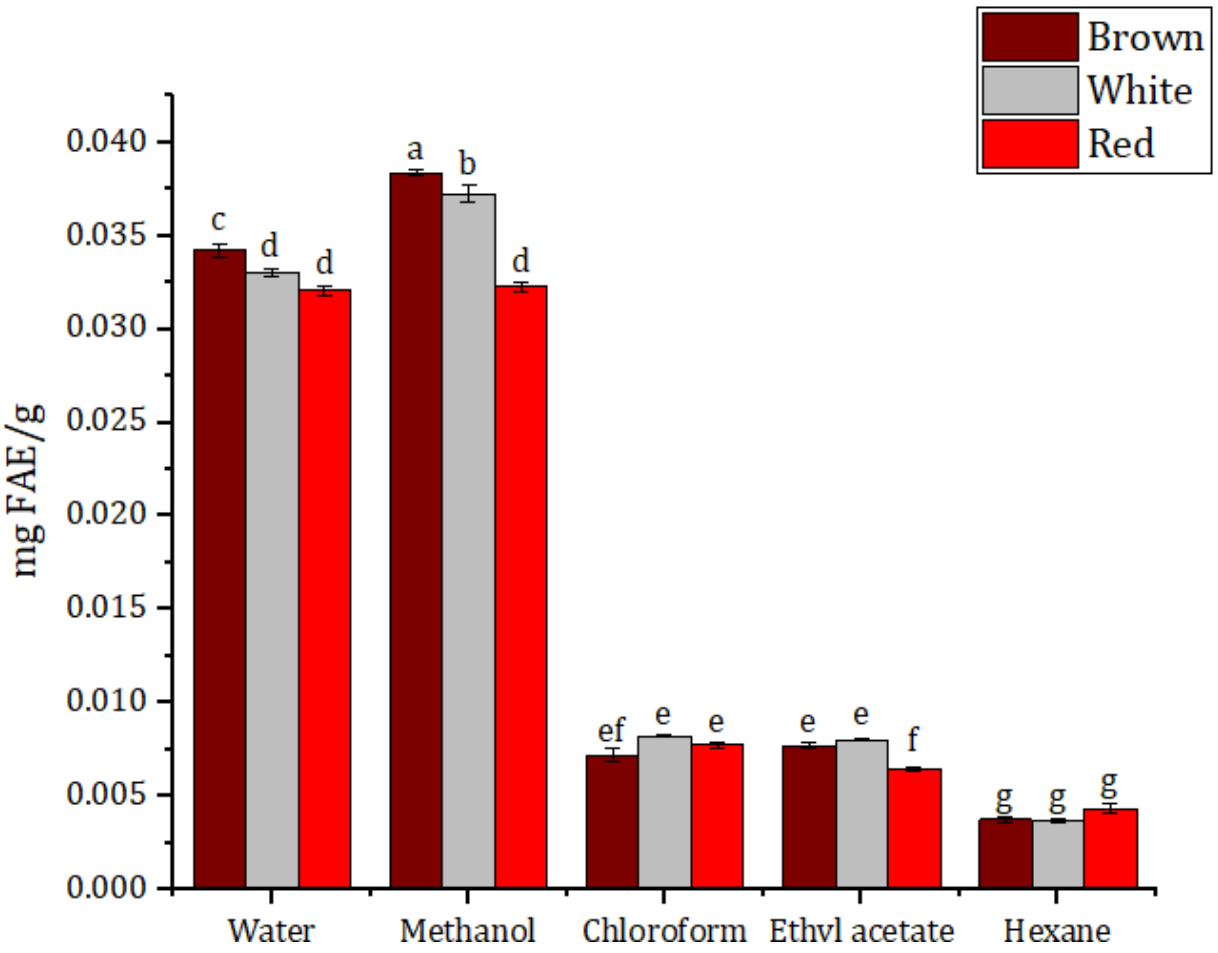


***Figure 4.1: Total phenolic content (mg ferulic acid equivalence/ g dry weight extract) of the Citrullus ecirrhosus seed extracts. Results are presented as mean (n = 3) ± standard deviation (SD). Bars with the same letters (a, b, c, d, e, f or g) are not significantly different (P < 0.05) while those with different letters are significantly different (P < 0.05).***

### 4.2.2 Total Flavonoid Content

Quantitative determination of flavonoids was estimated from the quercetin calibration curve and results were expressed as mg quercetin equivalence (QE)/g dry weight. Different concentrations of flavonoids were observed. Majority of the extracts were statistically different ($P < 0.05$) from each other. The highest concentration of flavonoids was 0.534 ± 0.003 mg QE/g dry weight for white methanolic extracts while the lowest recorded was 0.0036 ± 0.003 mg QE/g dry weight for red hexane extracts. Ethyl Acetate also extracted more flavanols as compared to the more polar chloroform. As shown in Figure 3.2, there is a higher total flavonoid content concentrations from polar solvent extracts as compared to the non-polar. Methanol had the highest extraction power followed by water. Chloroform and hexane extracts had the lowest concentration of flavonoids.

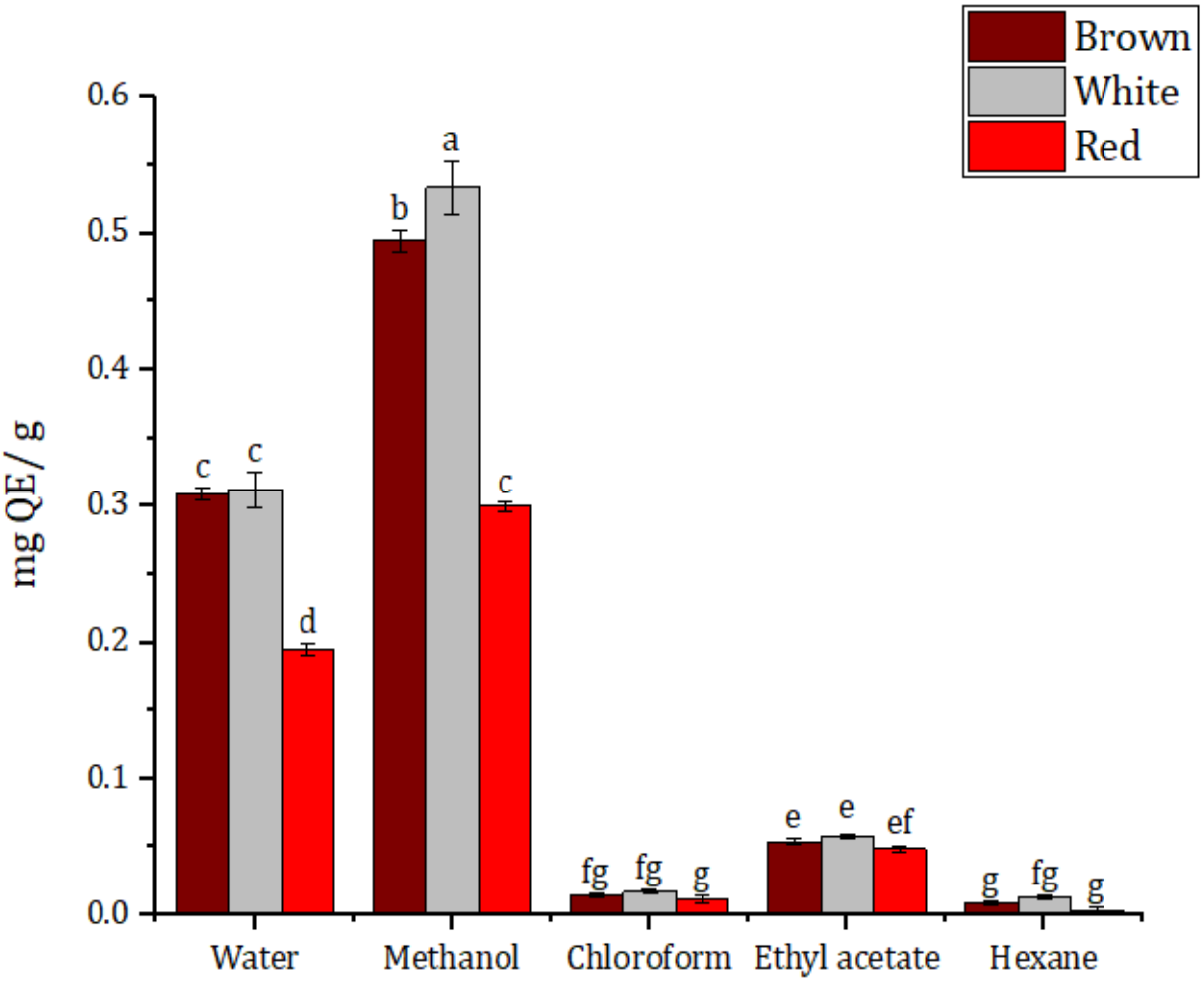



***Figure 4.2: Total flavonoid content (mg quercetin equivalence (QE)/ g dry weight) of the Citrullus ecirrhosus seed extracts. Results are presented as mean (n = 3) ± standard deviation (SD). Bars with the same letters (a, b, c, d, e, f or g) are not significantly different (P < 0.05), while those with different letters are significantly different (P < 0.05).***

**4.2.3 Total Antioxidant Capacity**

The total antioxidant capacity determination of the *Citrullus ecirrhosus* seed extracts was determined using the phosphomolybdenum method. This method is based on the reduction of Mo(VI) to Mo(V) by antioxidants which results in the formation of a stable green phosphate – Mo(V) complex. Ascorbic acid calibration curve was used to estimate the total antioxidant capacity with results expressed as mg ascorbic acid equivalence. The methanolic extracts from all varieties had the highest capacity with brown methanolic extracts having 24.5 ± 1.77 mg AAE/g dry weight, followed by white methanolic extraction having 20.7 ± 0.817 mg AAE/g dry weight and the red methanolic extraction with a concentration of 14.4 ± 0.328 mg AAE/g dry weight. There was an increased total antioxidant capacity with increasing polarity of extracting

solvent. There were statistical differences ($P < 0.05$) amongst these concentrations as shown in Figure 3.3. Hexane extracts had the lowest extraction capacity as they recorded the lowest concentrations.

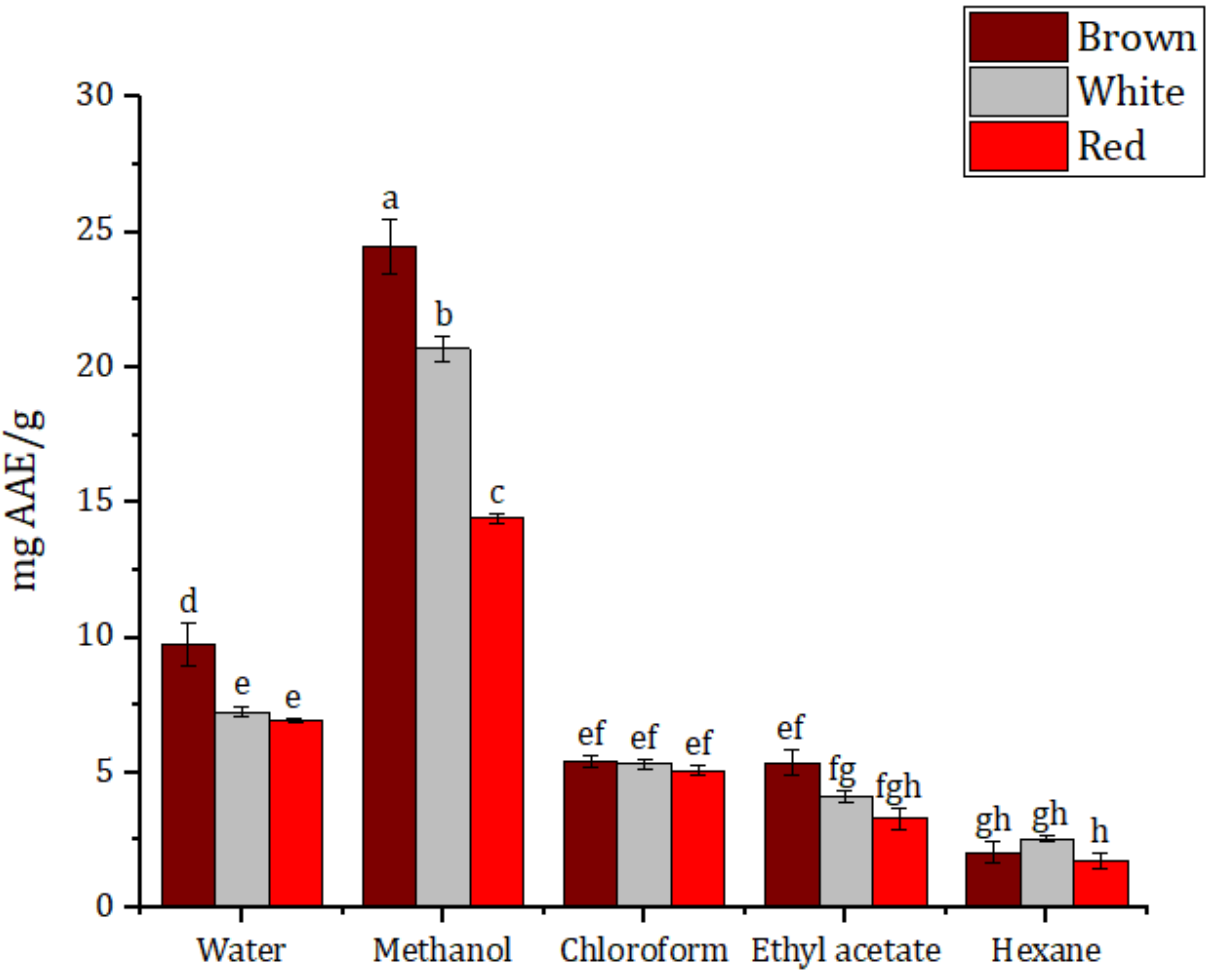



***Figure 4.3: Total Antioxidant Capacity (mg Ascorbic Acid equivalence (AAE)/ g dry weight) of the Citrullus ecirrhosus seed extracts. Results are presented as mean (n = 3) ± standard deviation (SD). Bars with the same letters (a, b, c, d, e, f or g) are not significantly different (P < 0.05), while those with different letters are significantly different (P < 0.05).***

**4.2.4 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging capacity assay**

The free radical scavenging capacity of the *Citrullus ecirrhosus* seed powder extracts quantitative determination was estimated from the Trolox calibration curve and results were expressed as mg Trolox equivalence (TE)/ g dry weight. An increasing DPPH radical scavenging capacity with increasing polarity of extracting solvent was observed. Figure 3.4 shows that the

methanolic extracts had the highest activity for the brown and white as there was no significant statistical difference ($P < 0.05$). The same trend is also seen across the rest of the solvents for the white and brown extracts. The water extracts for all the varieties showed no significant difference ($P < 0.05$). It can also be observed in Figure 3.4 that there is also no significant difference ($P < 0.05$) in the ethyl acetate and the hexane extracts.

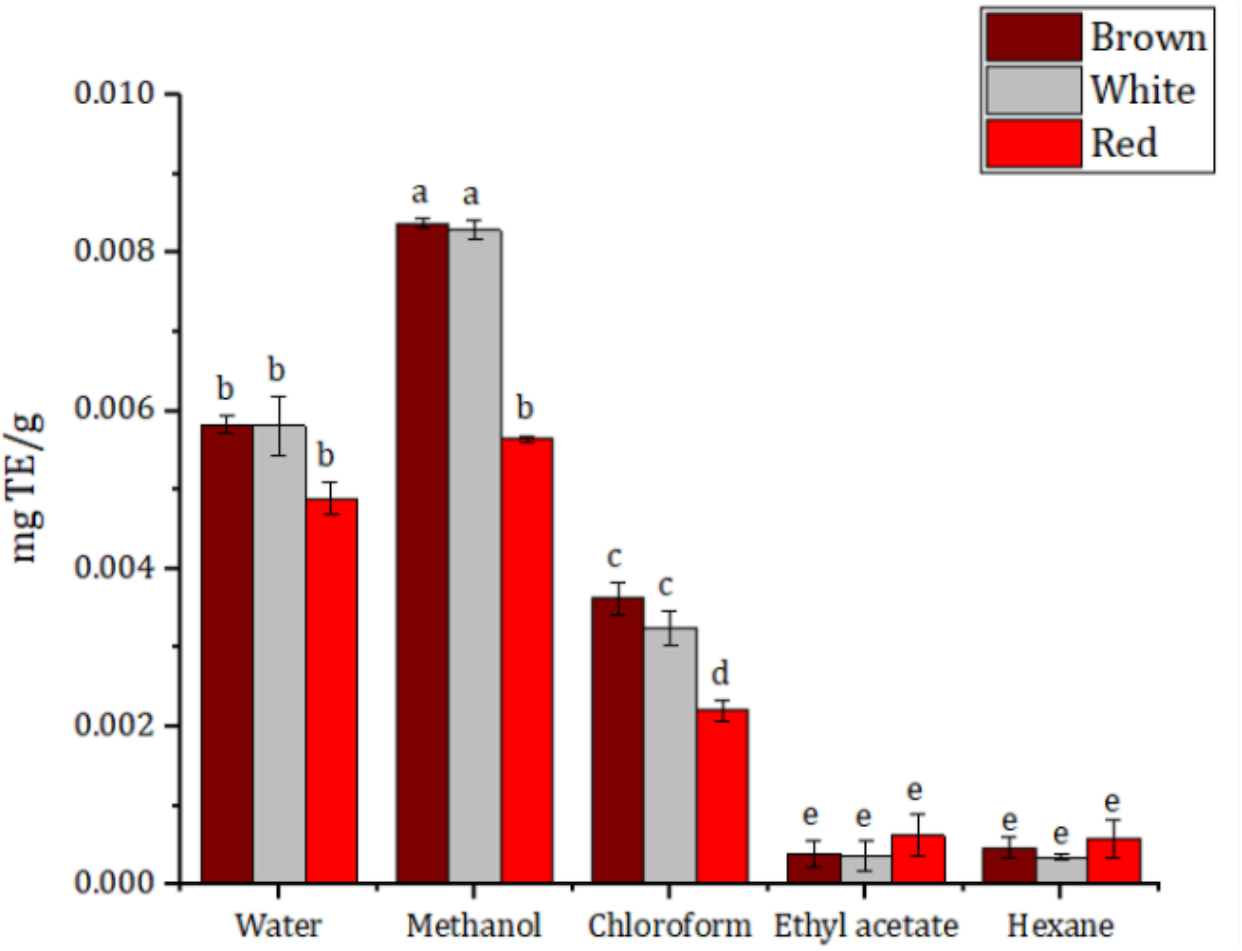



***Figure 4.4: DPPH free radical scavenging capacity (mg Trolox equivalence (TE)/ g dry weight) of the Citrullus ecirrhosus seed extracts. Results are presented as mean (n = 3) ± standard deviation (SD). Bars with the same letters (a, b, c, d, e, f or g) are not significantly (P < 0.05) different, while those with different letters are significantly (P < 0.05) different.***

### 4.2.5 ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) Assay

The ABTS free radical scavenging capacity of the *Citrullus ecirrhosus* was estimated from the Trolox calibration curve. The results were expressed as mg Trolox equivalence (TE)/ g dry weight. An increased ABTS free radical scavenging capacity with increasing polarity of extracting solvent was observed. Figure 3.5 shows that the methanolic extracts of the white and the brown extracts had the highest activity as no significant difference ($P < 0.05$) was noted. The water extracts of all the varieties also did not show significant differences ($P < 0.05$). There weren't much significant differences also from the ethyl acetate and the hexane extracts which had the lowest activities.

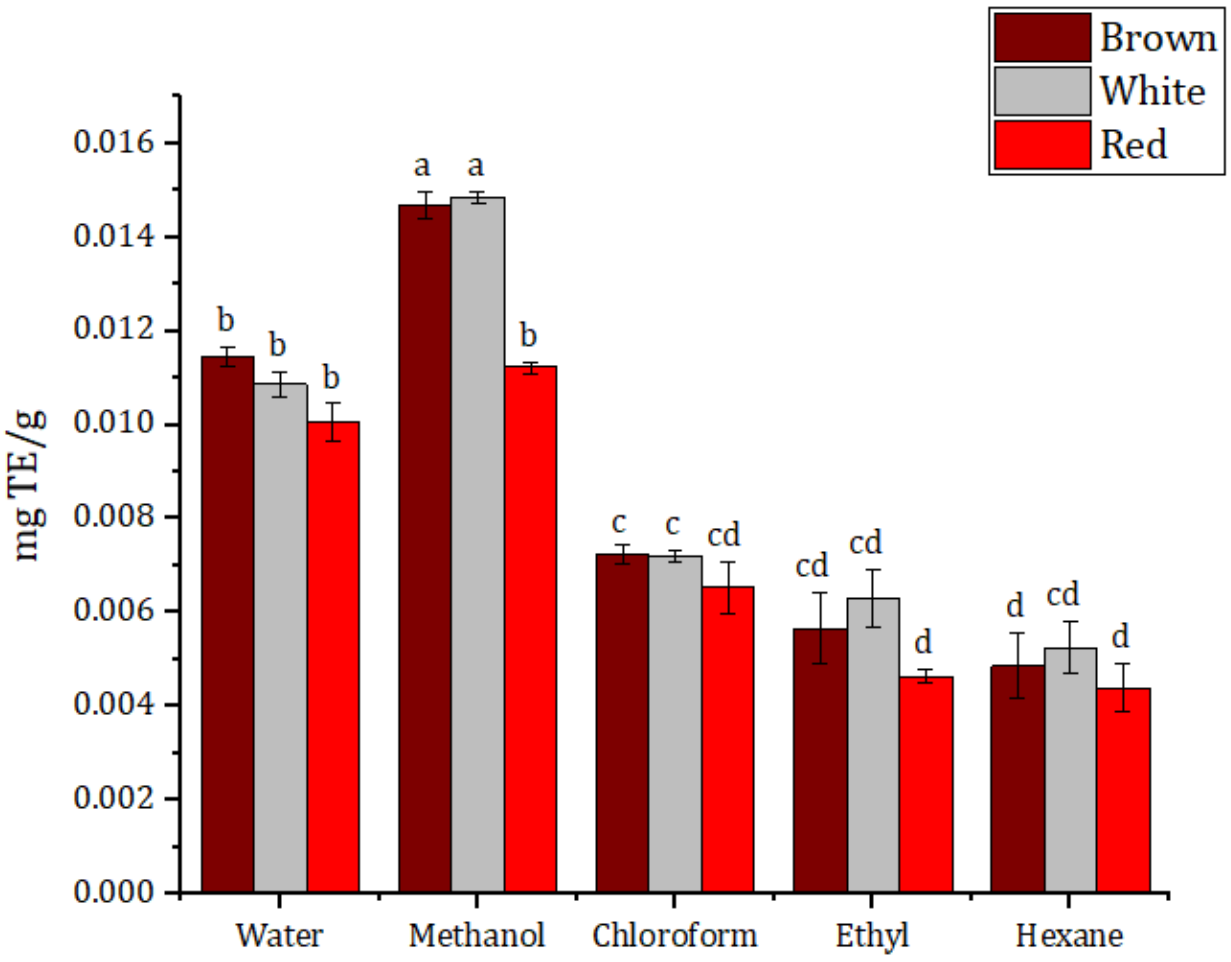



***Figure 4.5: ABTS free radical scavenging capacity (mg Trolox equivalence (TE)/ g dry weight) of the Citrullus ecirrhosus seed extracts. Results are presented as mean (n = 3) ±***

***standard deviation (SD). Bars with the same letters (a, b, c, d, e, f or g) are not significantly (P < 0.05) different, while those with different letters are significantly (P < 0.05) different***

**4.3 Identification of phytochemicals - GC–MS (gas chromatography–mass spectroscopy) analysis**

The GC-MS analysis of the seed powder extracts from the methanol, chloroform, ethyl acetate and hexane showed a range of phytochemicals as shown in Tables 1.1 throughout Table1.4. Some phytochemicals identified from the samples did not have any reported biological activity in literature. It may also be noted that the extraction power of the solvents across the seed varieties maintained a similar trend as the polar solvents had more as compared to the non-polar with hexane having fewer compounds as shown in Table 1.4.

***Table 1.1: Phytochemical constituents identified in the methanol extracts of Citrullus ecirrhosus varieties using gas chromatography-mass spectrometry.***

| ***Brown seeds extract*** | | | | | |
|---|---|---|---|---|---|
| RT | Compound Name | Molecular Formula | Molecular Weight | Peak Area % | Probability % |
| 9.405 | 5-Hydroxymethylfurfural | $C_6H6O_3$ | 126.11 | 9.91 | 75 |
| 12.338 | 2-Methoxy-4-vinylpheno | $C_9H_{10}O_2$ | 150.1745 | 4.42 | 33 |
| 15.38 | Vanillin lactoside | $C_{20}H_{28}O_{13}$ | 476.4 | 2.01 | 54.3 |
| 16.868 | Sucrose | $C_{12}H_{22}O_{11}$ | 342.3 | 100 | 12.7 |
| 19.888 | 6-Amino-2-methyl-2H-indazole | $C_8H_9N_3$ | 147.18 | | 11 |
| 27.657 | 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxypheno | $C_{10}H_{12}O_3$ | 180.2005 | 6.74 | 81.5 |
| 34.157 | Hexadecanoic acid, methyl ester | $C_{17}H_{34}O_2$ | 270.45 | 5.42 | 70 |
| 35.23 | n-Hexadecanoic acid | $C_{16}H_{32}O_2$ | 256.4241 | 20.3 | 74.2 |
| 39.41 | 9,12-Octadecadienoic acid (Z,Z)-, methyl ester | $C_{19}H_{34}O_2$ | 294.4721 | 5.44 | 19.7 |

| | | | | | |
|---|---|---|---|---|---|
| 41.511 | Octadecanoic acid | $C_{18}H_{36}O_2$ | 284.48 | 15.79 | 61.1 |
| 44.203 | γ-Tocopherol | $C_{28}H_{48}O_2$ | 416.68 | 2.55 | 39.4 |
| 45.319 | 9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3β,5Z,7E) | $C_{27}H_{44}O_3$ | 416.637 | 2.61 | 10 |
| | Red seeds extract | | | | |
| RT | Compound Name | Molecular Formula | Molecular Weight | Peak Area % | Probability % |
| 5.866 | 1-Octanol, 2,7-dimethyl | $C_{10}H_{22}O$ | 158.2811 | 3.36 | 14.6 |
| 12.388 | 2,4-Decadienal | $C_{10}H_{16}O$ | 177.03 | 1.52 | 52.2 |
| 18.976 | Nonadecane | $C_{19}H_{40}$ | 268.518 | 1.55 | 14.3 |
| 25.716 | 2,6-Diisopropylnaphthalene | $C_{16}H_{20}$ | 212.336 | 2.42 | 42.1 |
| 26.876 | 2-Piperidinone, N-[4-bromo-n-butyl]- | $C_9H_{16}BrNO$ | 234.13 | 9.08 | 13.2 |
| 28.736 | 2-Dodecen-1-yl(-)succinic anhydride | $C_{16}H_{26}O_3$ | 266.3758 | 3.94 | 21.6 |
| 31.45 | E,E,Z-1,3,12-Nonadecatriene-5,14-diol | $C_{19}H_{34}O_2$ | 294.47206 | 2.91 | 15.3 |
| 34.12 | Cyclopropanebutanoic acid | $C_{25}H_{42}O_2$ | 374.4 | 3.74 | 44.3 |
| 36.331 | Hexadecanoic acid, ethyl ester | $C_{18}H_{36}O_2$ | 284.5 | 45.88 | 84.7 |
| 37.994 | E,E,Z-1,3,12-Nonadecatriene-5,14-diol | $C_{19}H_{34}O_2$ | 294.47 | 7.36 | 30 |
| 41.277 | 9-Octadecenamide | $C_{18}H_{35}NO$ | 281.477 | 74.97 | 64.5 |
| 45.304 | Oleic acid, | $C_{39}H_{76}O_3$ | 282.47 | 33.3 | 14.5 |
| 12.476 | 2,4-Decadienal | $C_{10}H_{16}O$ | 152.23 | 2.04 | 65.6 |
| | White seeds extract | | | | |
| RT | Compound Name | Molecular Formula | Molecular Weight | Peak Area % | Probability % |

| | | | | | |
|---|---|---|---|---|---|
| 9.427 | *5*-Hydroxymethylfurfural | $C_6H_6O_3$ | 126.11 | 23.4 | 86.9 |
| 12.338 | 2-Methoxy-4-vinylphenol | $C_9H_{10}O_2$ | 150.1745 | 4.51 | 15.8 |
| 15.38 | Vanillin lactoside | $C_{20}H_{28}O_{13}$ | 476.428 | 2.4 | 60.4 |
| 25.578 | 1-Octadecanesulphonyl chloride | $C_{18}H_{37}ClO_2S$ | 353.003 | 1.53 | 27.1 |
| 27.658 | 4-((1E)-3-Hydroxy-1-propenyl )-2-methoxyphenol | $C_{10}H_{12}O_3$ | 180.2005 | 5.68 | 82.7 |
| 34.157 | Hexadecanoic acid, methyl ester | $C_{17}H_{34}O_2$ | 270.45 | 5.5 | 69.4 |
| 35.252 | n-Hexadecanoic acid | $C_{16}H_{32}O_2$ | 256.4241 | 32.37 | 72.4 |
| 38.316 | 9,9-Dimethoxybicyclo [3.3.1] nona-2,4-dione | $C_{11}H_{16}O_4$ | 212.2423 | 16.6 | 27 |
| 41.555 | Octadecanoic acid | $C_{18}H_{36}O_2$ | 284.48 | 26.04 | 74.6 |
| 40.767 | 9,12-Octadecadienoic acid (Z,Z)- | $C_{18}H_{32}O_2$ | 280.4455 | 100 | 50.7 |

***Table 1.2: Phytochemical constituents identified in the chloroform extracts of Citrullus ecirrhosus varieties using gas chromatography-mass spectrometry.***

***Brown seeds extract***

| RT | Compound Name | Molecular Formula | Molecular Weight | Peak Area % | Probability % |
|---|---|---|---|---|---|
| 20.355 | Acenocoumarol | $C_{19}H_{15}NO_6$ | 353.326 | 1.16 | 65.1 |
| 29.984 | Nonadecane | $C_{19}H_{40}$ | 268.518 | 1.17 | 8.51 |
| 34.164 | Hexadecanoic acid, methyl ester (methyl palmitate) | $C_{17}H_{34}O_2$ | 270.45 | 11.62 | 74.7 |
| 35.237 | n-Hexadecanoic acid | $C_{16}H_{32}O_2$ | 256.4 | 16.83 | 81.9 |

| | | | | | |
|---|---|---|---|---|---|
| 39.351 | 9,12-Octadecadienoic acid, methyl ester | $C_{19}H_{34}O_2$ | 294.5 | 3.17 | 19.5 |
| 44.297 | 9,12,15-Octadecatrienoic acid, 2-(acetyloxy)-1-[(acetyloxy)methyl] ethyl ester | $C_{25}H_{40}O_6$ | 436.58 | 21.15 | 12.5 |
| 45.041 | Cholesta-22,24-dien-5-ol, 4,4-dimethyl | $C_{29}H_{48}O$ | 412.7 | 28.7 | 22.1 |

***Red seeds extract***

| RT | Compound Name | Molecular Formula | Molecular Weight | Peak Area % | Probability % |
|---|---|---|---|---|---|
| 8.318 | 2-Dodecene, | $C_{12}H_{24}$ | 168.319 | 9.41 | 4.6 |
| 11.185 | Nonadecane, 9-methyl | $C_{20}H_{42}$ | 282.5 | 1.54 | 34.7 |
| 12.738 | Heptanedioic acid, 2,4-dimethyl-, dimethyl ester | $C_{11}H_{20}O_4$ | 216.274 | 2.03 | 24.2 |
| 16.218 | 5-Methoxymethoxy-6-methyl-hept-6-enoic acid, methyl ester | $C_{11}H_{20}O_4$ | 216.27 | 2.85 | 38.3 |
| 18.429 | 3-Ethyl-3-phenyl-1-pentene | $C_{13}H_{18}$ | 174.282 | 3.2 | 16.4 |
| 20.595 | 1-Dodecanethiol | $C_{12}H_{26}S$ | 202.3998 | 3.5 | 39.5 |
| 24.163 | n-Dodecylmethyl sulfide | $C_{13}H_{28}S$ | 216.426 | 3.09 | 97.5 |
| 26.745 | 5,9-Tetradecadienedioic acid, 5,6,9,10-tetramethyl-, dimethyl ester | $C_{20}H_{34}O_4$ | 991.6 | 15.73 | 31 |
| 30.072 | 2,8-Decadienedioic acid, diethyl ester | $C_{14}H_{22}O_4$ | 258.3538 | 25.93 | 16.6 |
| 33.398 | Dodecane, 1,1'-thiobis | $C_{24}H_{50}S$ | 370.719 | 1.74 | 48 |
| 35.258 | Benzene, 1,3,5-tris(3-methyl-3-butenyl)- | $C_{21}H_{30}$ | 282.463 | 14.32 | 23.8 |
| 40.708 | 9,12-Octadecadienoic acid (Z,Z) | $C_{18}H_{32}O_2$ | 280.4455 | 18.703 | 24.2 |
| 40.905 | cis-Vaccenic acid | $C_{18}H_{34}O_2$ | 282.46 | 14.46 | 16.2 |

| | | | | | |
|---|---|---|---|---|---|
| 45.129 | Ethyl is-allocholate | $C_{26}H_{44}O_5$ | | 1.33 | 17.8 |

***White seeds extract***

| RT | Compound Name | Molecular Formula | Molecular Weight | Peak Area % | Probability % |
|---|---|---|---|---|---|
| 12.476 | 2,4-Decadienal | $C_{10}H_{16}O$ | 152.23 | 2.04 | 65.6 |
| | | | | | |
| 18.407 | 3-Ethyl-3-phenyl-1-pentene | $C_{13}H_{18}$ | 174.3 | 1.67 | 15.5 |
| 19.632 | Phenol, 2,4-bis(1,1-dimethylethyl)- | $C_{14}H_{22}O$ | 206.3 | 2.32 | 47.9 |
| 22.74 | 1,6-Heptadiene, 2-methyl-6-phenyl | $C_{14}H_{18}$ | 186.29 | 3.96 | 21.4 |
| 24.184 | n-Dodecylmethyl sulfide | $C_{13}H_{28}S$ | 216.426 | 1.63 | 93.3 |
| 25.651 | Cyclooctasiloxane, hexadecamethyl | $C_{16}H_{48}O_8Si_8$ | 593.23 | 2.38 | 91.2 |
| 31.188 | Cyclononasiloxane, octadecamethyl | $C_{18}H_{54}O_9Si_9$ | 667.3855 | 2.29 | 87.4 |
| 34.077 | Tetradecane, 2,6,10-trimethyl | $C_{17}H_{36}$ | 240.5 | 1.36 | 9.42 |
| 35.325 | n-Hexadecanoic acid | $C_{16}H_{32}O_2$ | 256.4241 | 39.51 | 72.5 |
| 36.287 | 5,8,11-Heptadecatriynoic acid, methyl ester | $C_{18}H_{24}O_2$ | 278.4296 | 68.4 | 41.5 |
| 39.613 | 9-Octadecenoic acid (Z)-, methyl ester | $C_{19}H_{36}O_2$ | 296.4879 | 1.76 | 18.5 |
| 40.817 | 9,12-Octadecadienoic acid (Z,Z)- | $C_{18}H_{32}O_2$ | 280.4455 | 73.63 | 37.1 |
| 45.238 | Cyclodecasiloxane, eicosamethyl | $C_{20}H_{60}O_{10}Si_{10}$ | 741.5394 | 10.82 | 75.4 |

***Table 1.3: Phytochemical constituents identified in the ethyl acetate extracts of Citrullus ecirrhosus varieties using gas chromatography-mass spectrometry.***

***Brown seeds extract***

| RT | Compound Name | Molecular Formula | Molecular Weight | Peak Area % | Probability % |
|---|---|---|---|---|---|
| 10.296 | Benzene, 1,3-bis(1,1-dimethylethyl)- | $C_{14}H_{22}$ | 190.3245 | 2.78 | 75.5 |
| 15.395 | Tetradecane | $C_{14}H_{30}$ | 193.39 | 11.68 | 36.7 |
| 19.619 | Phenol, 2,4-bis(1,1-dimethylethyl)- | $C_{14}H_{22}O$ | 474.61 | 72.65 | 63 |
| 22.639 | Cetene | $C_{16}H_{32}$ | 224.42 | 1.57 | 3.52 |
| 26.491 | Nonadecane | $C_{19}H_{40}$ | 268.518 | 7.27 | 20.2 |
| 29.993 | Tetradecane, 2,6,10-trimethyl | $C_{17}H_{36}$ | 240.4677 | 10.57 | 6.1 |
| 35.311 | n-Hexadecanoic acid | $C_{16}H_{32}O_2$ | 256.4241 | 50.32 | 72 |
| 36.295 | 5,8,11-Heptadecatriynoic acid, methyl ester | $C_{18}H_{24}O_2$ | 278.4296 | 35.796 | 42.3 |
| 40.738 | 9,12-Octadecadienoic acid | $C_{18}H_{32}O_2$ | 280.4455 | 65.41 | 29.8 |
| 41.548 | Oleic Acid | $C_{18}H_{34}O_2$ | 282.47 | 89.97 | 30.8 |
| 44.218 | γ-Tocopherol | $C_{28}H_{48}O_2$ | 416.68 | 22.53 | 39.3 |
| 45.159 | Ethyl iso-allocholate | $C_{26}H_{44}O_5$ | 436.6 | 2.58 | 7.71 |

***Red seeds extract***

| RT | Compound Name | Molecular Formula | Molecular Weight | Peak Area % | Probability % |
|---|---|---|---|---|---|
| 5.831 | Pentanoic acid, octyl ester | $C_{13}H_{26}O_2$ | 214.3443 | 2.55 | 9.48 |
| 7.341 | β-1,5-O-Dibenzoyl-ribofuranose | $C_{19}H_{18}O_7$ | 504.491 | 1.11 | 6.26 |
| 8.326 | 3-Dodecene, (Z)- | $C_{12}H_{24}$ | 168.319 | 10.02 | 9.08 |

| | | | | | |
|---|---|---|---|---|---|
| 10.58 | Nonanoic acid | $C_9H_{18}O_2$ | 158.23 | 1.61 | 53.1 |
| 11.806 | Tridecane | $C_{13}H_{28}$ | 184.4 | 3.81 | 46.7 |
| 19.575 | Phenol, 2,4-bis(1,1-dimethylethyl)- | $C_{14}H_{22}O$ | 278.505 | 26.39 | 62.6 |
| 28.658 | Benzyl Benzoate | $C_{14}H_{12}O_2$ | 212.2439 | 1 | 71.8 |
| 35.311 | n-Hexadecanoic acid | $C_{16}H_{32}O_2$ | 256.4241 | 40.95 | 73.6 |
| 36.252 | 5,8,11-Heptadecatriynoic acid, methyl ester | $C_{18}H_{24}O_2$ | 278.4296 | 20.14 | 51.1 |
| 40.804 | 9,12-Octadecadienoic acid (Z,Z)- | $C_{18}H_{32}O_2$ | 280.4455 | 100 | 45.7 |
| 41.57 | Oleic Acid | $C_{18}H_{34}O_2$ | 282.47 | 35.56 | 29.2 |
| 43.452 | 1-Heptatriacotano | $C_{37}H_{76}O$ | 537 | 2.31 | 53.9 |
| 45.115 | Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl | $C_{23}H_{32}O_2$ | 340.499 | 7.91 | 94.7 |

***White seeds extract***

| RT | Compound Name | Molecular Formula | Molecular Weight | Peak Area % | Probability % |
|---|---|---|---|---|---|
| 8.45 | Tetradecane | $C_{14}H_{30}$ | 198.39 | 18.35 | 13.9 |
| 9.989 | Undecanoic acid, 3-hydroxy-, methyl ester | $C_{12}H_{24}O_3$ | 202.2906 | 1.21 | 28.5 |
| 11.828 | Tridecane | $C_{13}H_{28}$ | 184.4 | 3.79 | 30.9 |
| 19.619 | Phenol, 2,4-bis(1,1-dimethylethyl)- | $C_{14}H_{22}O$ | 646.9216 | 27.05 | 62 |
| 35.332 | n-Hexadecanoic acid | $C_{16}H_{32}O_2$ | 256.4241 | 40.69 | 75.5 |
| 36.273 | 5,8,11-Heptadecatriynoic acid, methyl ester | $C_{18}H_{24}O_2$ | 278.4296 | 28.44 | 49.1 |
| 40.847 | 9,12-Octadecadienoic acid (Z,Z)- | $C_{18}H_{32}O_2$ | 280.4455 | 100 | 49.1 |
| 41.592 | Oleic Acid | $C_{18}H_{34}O_2$ | 282.47 | 33.92 | 30.1 |

***Table 1.4: Phytochemical constituents identified in the hexane extracts of Citrullus ecirrhosus varieties using gas chromatography-mass spectrometry.***

***Brown seeds extract***

| RT | Compound Name | Molecular Formula | Molecular Weight | Peak Area % | Probability % |
|---|---|---|---|---|---|
| 5.91 | Nonanal | $C_9H_{18}O$ | 142.2386 | 5.26 | 32.7 |
| 6.129 | D-Sphingosine | $C_{18}H_{37}NO_2$ | 299.5 | 1.55 | 23.6 |
| 29.918 | Hydroxylamine, O-decyl | $C_{10}H_{23}NO$ | 173.3 | 2.54 | 11.8 |
| 34.142 | Hexadecanoic acid, methyl ester | $C_{17}H_{34}O_2$ | 270.45 | 19.69 | 71.4 |
| 36.462 | Nonadecane | $C_{19}H_{40}$ | 268.518 | 4.89 | 19.4 |
| 39.417 | 9,12-Octadecadienoic acid (Z,Z)-, methyl ester | $C_{19}H_{34}O_2$ | 294.4721 | 61.33 | 22.2 |
| 39.657 | 13-Octadecenoic acid, methyl ester | $C_{19}H_{36}O_2$ | 296.4879 | 31.44 | 9.06 |
| 40.555 | Methyl stearate | $C_{19}H_{38}O_2$ | 298.5 | 37.52 | 58.2 |
| 42.349 | Tetradecane, 2,6,10-trimethyl | $C_{17}H_{36}$ | 240.4677 | 3.4 | 13.3 |
| 44.275 | γ-Tocopherol | $C_{28}H_{48}O_2$ | 416.68 | 100 | 50.8 |

***Red seeds extract***

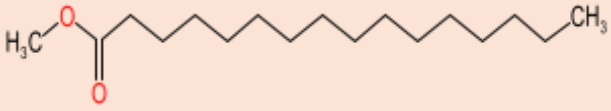
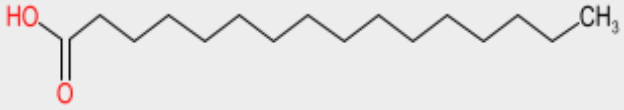
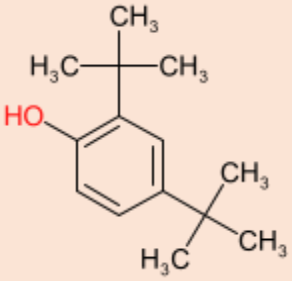
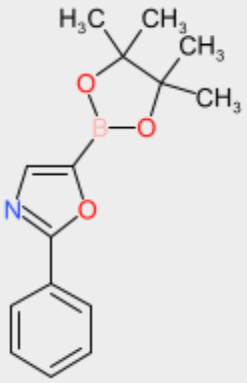
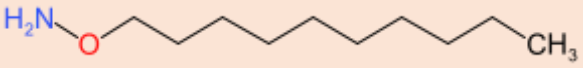
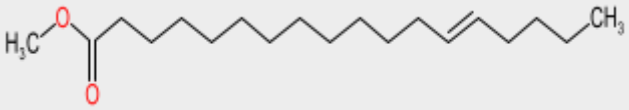
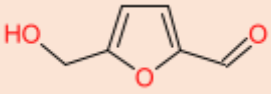
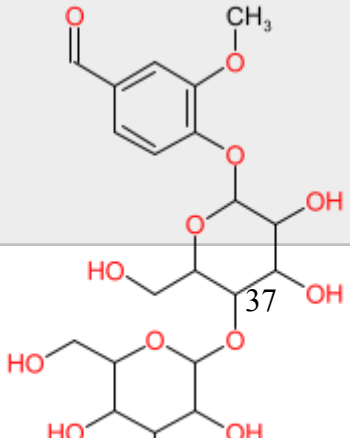
| RT | Compound Name | Molecular Formula | Molecular Weight | Peak Area % | Probability % |
|---|---|---|---|---|---|
| 5.057 | 1-Decanol, 2-octyl | $C_{18}H_{38}O$ | 298.5469 | 58.91 | 9.21 |
| 15.408 | Tetradecane | $C_{14}H_{30}$ | 198.39 | 1.05 | 47.1 |
| 22.828 | Hexadecane | $C_{16}H_{34}$ | 226.41 | 17.39 | 29.5 |
| 29.984 | Nonadecane | $C_{19}H_{40}$ | 268.518 | 1.61 | 7.83 |
| 34.142 | Hexadecanoic acid, methyl ester | $C_{17}H_{34}O_2$ | 270.45 | 2.02 | 70.9 |
| 39.417 | 9,12-Octadecadienoic acid, methyl ester | $C_{19}H_{34}O_2$ | 294.4721 | 2.16 | 21 |

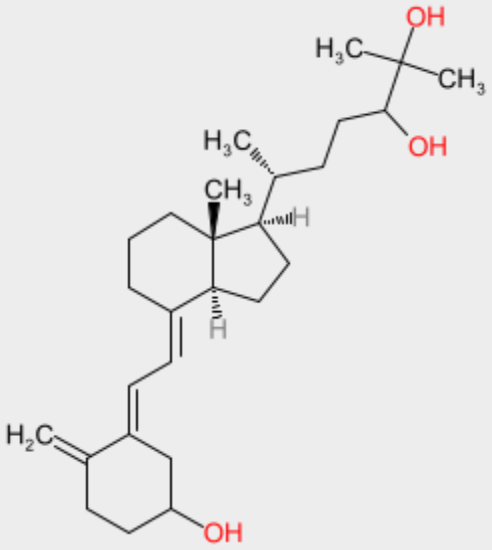
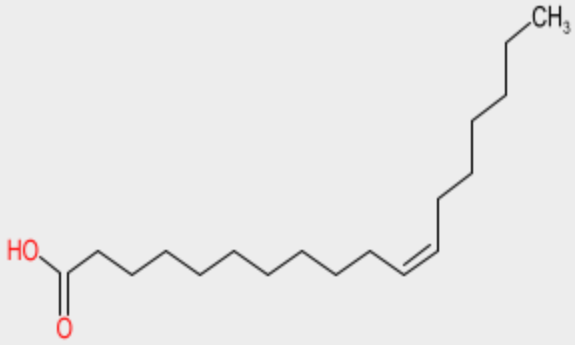
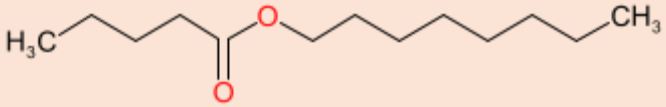
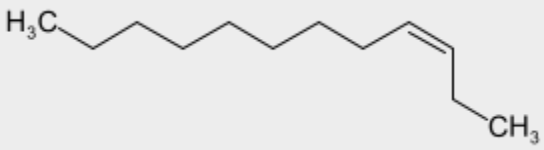
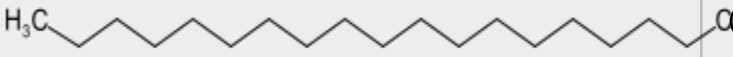
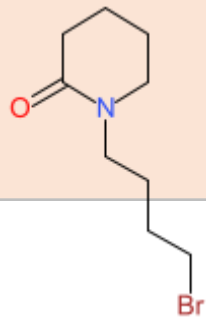
| | | | | | |
|---|---|---|---|---|---|
| 40.555 | Cyclopentaneundecanoic acid, methyl ester | $C_{17}H_{32}O_2$ | 200.3178 | 1.87 | 15.8 |
| 43.75 | 9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester | $C_{21}H_{40}O_4$ | 354.524 | 11.24 | 10.6 |
| 44.166 | γ-Tocopherol | $C_{28}H_{48}O_2$ | 416.68 | 66.7 | 44.6 |

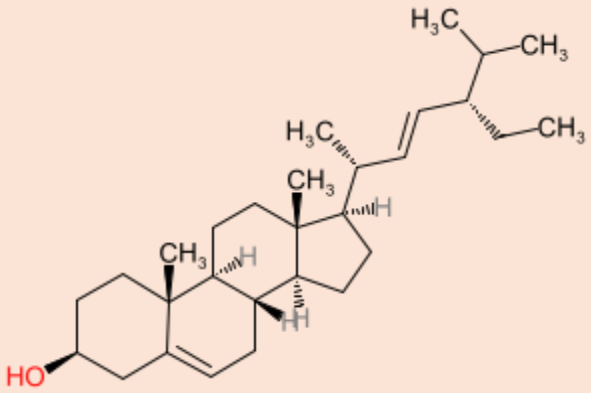
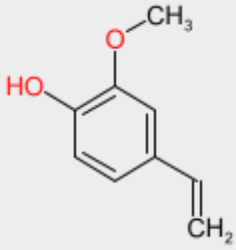
***White seeds extract***

| RT | Compound Name | Molecular Formula | Molecular Weight | Peak Area % | Probability % |
|---|---|---|---|---|---|
| 29.962 | Nonadecane | $C_{19}H_{40}$ | 268.518 | 1.43 | 9.47 |
| 34.164 | Hexadecanoic acid, methyl ester | $C_{17}H_{34}O_2$ | 270.45 | 7.54 | 51.2 |
| 35.236 | 9,9-Dimethoxybicyclo [3.3.1]nona-2,4-dione | $C_{11}H_{16}O_4$ | 212.2423 | 2.54 | 25.9 |
| 38.103 | Heptacosane | $C_{27}H_{56}$ | 380.7335 | 41.6 | 25.6 |
| 44.253 | γ-Tocopherol | $C_{28}H_{48}O_2$ | 416.68 | 47.6 | 49.7 |
| 45.019 | Stigmasterol | $C_{29}H_{48}O$ | 412.69 | 100 | 45 |

*Table 2: Phytochemicals with documented antioxidant properties selected for docking.*

| SN | Name | Structure | Activity Reference |
|---|---|---|---|
| 1 | Hexadecanoic acid, methyl ester (methyl palmitate) |  | (El-Agamy *et al.*, 2017) |
| 2 | n-Hexadecanoic acid |  | (Ganesan *et al.*, 2022) |
| 3 | Phenol, 2,4-bis(1,1-dimethylethyl)- |  | (El Agamy *et al.*, 2017) |
| 4 | γ-Tocopherol |  | (Gottstein *et al.*, 1990) |
| 5 | Hydroxylamine, O-decyl |  | (Mahalingam *et al.*, 2011) |
| 6 | 13-Octadecenoic acid, methyl ester |  | (Manonmani *et al.*, 2015) |
| 7 | 5-Hydroxymethylfurfural |  | (Turkut *et al.*, 2018) |
| 8 | Vanillin lactoside |  | (Charles *et al.*, 2009) |

| | | | |
|---|---|---|---|
| **9** | Octadecanoic acid |  | (Luo Y *et al.*, 2017) |
| **10** | 9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3β,5Z,7E) |  | |
| **11** | cis-Vaccenic acid |  | (Malik *et al.*, 2022) |
| **12** | Pentanoic acid, octyl ester |  | (Yakubu *et al.*, 2021) |
| **13** | 3-Dodecene, (Z)- |  | (Sumithra D., 2016) |
| **14** | 1-Heptatriacotano |  | (Elshaarawy *et al.*, 2023) |
| **15** | Nonadecane |  | (More K *et al.*, 2022) |
| **16** | 2-Piperidinone, N-[4-bromo-n-butyl]- |  | (Al-Salman *et al.*, 2019) |

| | | | |
|---|---|---|---|
| **17** | Heptacosane |  | (Bi, S.F *et al.*, 2016) |
| **18** | Stigmasterol |  | (Liang Q *et al.*, 2020) |
| **19** | 2-Methoxy-4-vinyl phenol |  | (Rubab *et al.*, 2020) |

### 4.4 Molecular docking

The molecular docking results are a depiction of the comparison of docking scores of a compound with other compounds. This also shows whether the compound in question has potential or not. A high binding affinity is represented by molecules with a lowest docking score (minus value). The more negative, the higher the binding affinity

#### 4.4.1 Ligand and protein preparation

The predicted binding sites (pockets) for the HMG CoA reductase enzyme (PDB ID: 3CCZ) generated using PrankWeb (P2Rank) are shown in Figure 3.6 and Table 3. Fifteen binding pockets were predicted with pocket 1 showing a score of 72.93 and the highest binding probability of 0.991. The active site (pocket 1) is within chain A and B of the protein and the predicted binding amino acid residues in this pocket are Ala478, Arg495, Leu498, Tyr514,

Arg515, Pro513, Tyr517, Thr557, Thr558, Glu559, Arg590, Ile638, His635, Lys691, Ala751, Asn755, Leu812, Ala856.

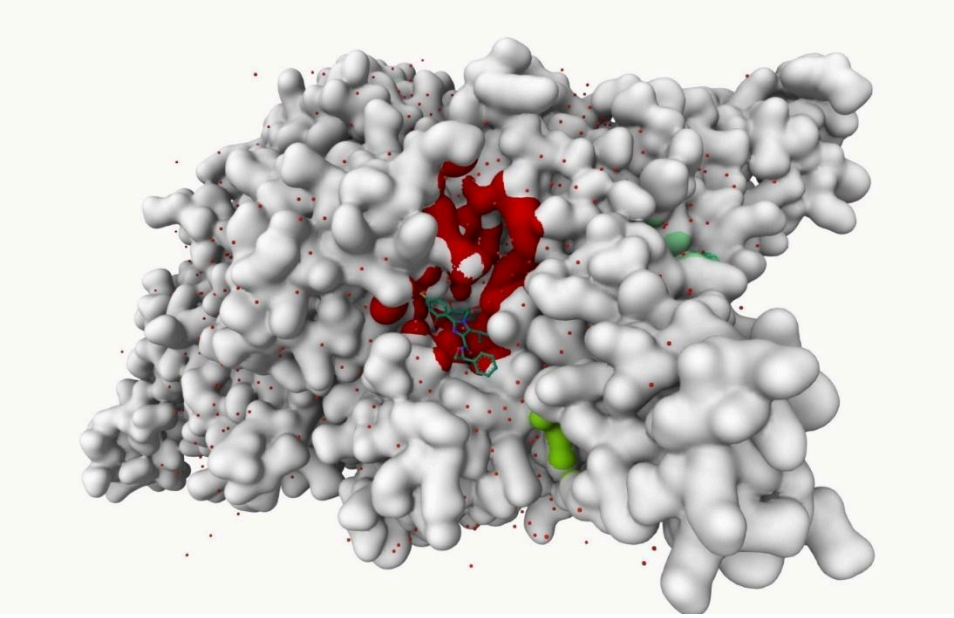


***Figure 4.6: PrankWeb (P2Rank) predicted the active site of the HMG CoA Reductase protein PDB ID: 3CCZ.The active site (pocket 1) is represented by the Red color. The green colors represent other pockets other than pocket 1 with less probability of being the active site.***

***Table 3: HMG CoA Reductase (PDB ID: 3CCZ) P2Rank predicted active site amino acid residues and vina search space X, Y, and Z coordinates.***

| Predicted Binding Pocket | Rank | Score | Probability | Centre x | Centre y | Centre z | Amino Acids residues IDs |
|---|---|---|---|---|---|---|---|
| **Pocket 1** | 1 | 72.93 | 0.991 | 81.5572 | 1.0687 | -5.8972 | Ala478, Arg 495, Leu498, Arg496, Arg 515, Tyr514, Pro535, Pro513, Tyr517, Tyr533, Arg590, Thr557, Thr558, Glu559, His635, Ile638, Ser661, Ser684, Asn686, Asp690, Lys691, Lys692, |

| | |
|---|---|
| | Ala751, His752, Asn755, Tyr761, Ala768, Leu812, Pro813, Leu853, Ala856 |

### 4.4.2 Site-specific molecular docking

Of the 19 bioactive compounds with antioxidant activities, the top five inhibitors according to their molecular docking binding affinity to the HMG CoA reductase protein were Vanillin lactoside, 9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol, stigmasterol, gamma-Tocopherol and Phenol, 2,4-bis(1,1-dimethylethyl)-. Their binding affinities ranged from -8.2 to -6.3 kcal/mol as shown in Table 3. Vanillin lactoside showed the highest binding affinity of -8.2 kcal/mol and the interactions between the compound and the protein were predicted to be through conventional hydrogen bond, carbon hydrogen bond, Pi-Cation, Pi-Alkyl and unfavorable donor-donor interactions.

***Table 4: Molecular docking results of Citrullus ecirrhosus bioactive compounds against HMG CoA reductase enzyme. The controls are highlighted in red***

| Ligand (Bioactive compound) | Binding affinity (kcal/mol) | Contact Receptor amino acid residues | Types of Interactions (Bonds) |
|---|---|---|---|
| **Vanillin lactoside** | -8.2 | Ala585:D, Lys606:A, Lys633:A, His635:A, Thr636:A, Ser637:A, Ile638:A, Glu700:D | Conventional hydrogen bond, carbon hydrogen bond, Pi-Cation, Pi-Alkyl, unfavorable donor-donor interactions |

| | | | |
|---|---|---|---|
| **9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol** | -8.0 | Arg496:B, Pro513:B, Arg515:B, Pro813:A | Conventional hydrogen bond, Alkyl |
| **stigmasterol** | -7.9 | Lys633:D, His635:D | Pi-sigma, Alkyl |
| **gamma-Tocopherol** | -7.6 | Tyr511:A, Tyro511:B,Pro513:B,Pro513:A,Tyr533:B,Leu812:B | Unfavorable acceptor-acceptor, Pi-sigma, Alkyl, Pi-Alkyl |
| **Phenol, 2,4-bis(1,1-dimethylethyl)** | -6.3 | Pro513:B , Tyr533:B | Conventional hydrogen bond, Alkyl, Pi-Alkyl |
| **Atorvastatin** | -8.8 | Cys561:B, Ala856:B, Leu853:B, Lys692:A, Lys735:B, Ser565:B, Ser661:A, Arg590:A, Asn686:A, Ser684:A, Asp690:A, Lys691:A, Glu559:B, Asn755:B | Conventional hydrogen bond, carbon hydrogen bond, unfavorable donor-donor, Pi-Cation, Pi-Alkyl, Pi-sigma |
| **Simvastatin** | -8.1 | Asp516:A, Arg515:A, Tyr533:A, Tyr533:B, Pro513:B, Pro535:B | Conventional hydrogen bond, Alkyl, Pi-Alkyl |
| **Lovastatin** | -8.0 | Arg515:B, Tyr514:B, Tyr511:B, Leu812:A, Pro813:A | Conventional hydrogen bond, Alkyl, Pi-Alkyl |
| **Pitavastatin** | -8.0 | Tyr514:B, Arg515:B, Tyr533:B, Leu812:A, Pro813:A, Pro513:B, Tyr533:A, Tyr517:A, Pro535:B | Conventional hydrogen bond, unfavorable donor-donor, Alkyl, Pi-Alkyl |
| **Rosuvastatin** | -7.9 | Glu559:A, Lys692:B, Ala856:A, Leu853:A, Ser565:A, Ala751:A, Arg590:B, Ser684:B | Conventional hydrogen bond, unfavorable donor-donor, Pi-Anion, |

| | | | |
|---|---|---|---|
| | | | Pi-Alkyl, Pi-sigma |
| **Fluvastatin** | -7.9 | Ala856:A, Leu853:A, Arg590:B, Ser661:B, Asp690:B, Ala751:A, Lys692:B, Lys691:B, Glu559:A, Asn755:A | Conventional hydrogen bond, carbon hydrogen bond, Unfavorable acceptor-acceptor, Pi-Alkyl, Pi-sigma |
| **Pravastatin** | -7.3 | His635: A, Ile638: A, Gln632: D, Glu700: D, Ser705: D | Conventional hydrogen bond, unfavorable donor-donor, Pi-Alkyl, Pi-sigma |

### 4.4.3 Visualization of binding position and interaction between HMG-CoA reductase protein and bioactive compounds using Biovia Discovery Studio 2020

The results of the 3D and 2D molecular docking of the HMG CoA reductase protein against the top five bioactive compounds and the control (Atorvastatin) with the highest binding capacity are shown in the following figures.

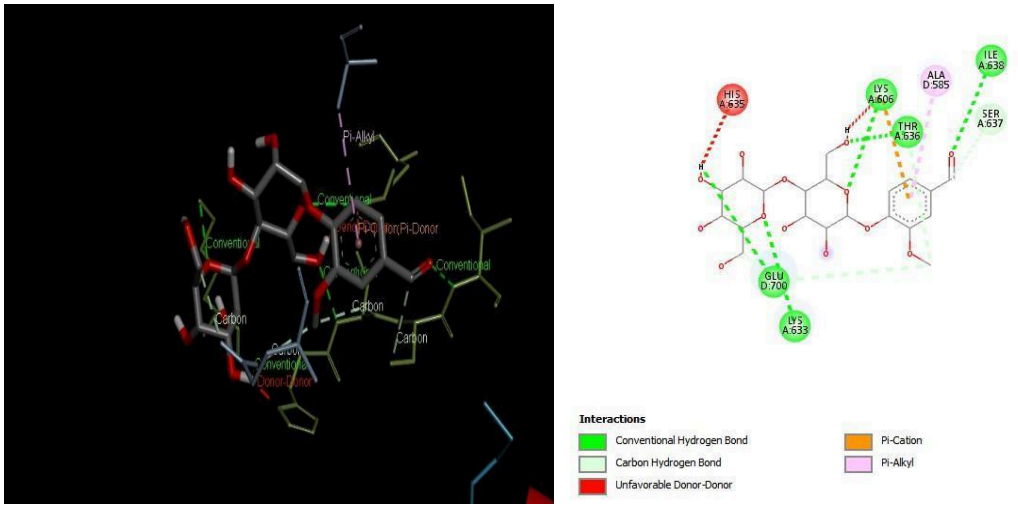



***Figure 4.7: Results of 3D (left) and 2D (right) molecular docking of HMG CoA reductase against vanillin lactoside.***

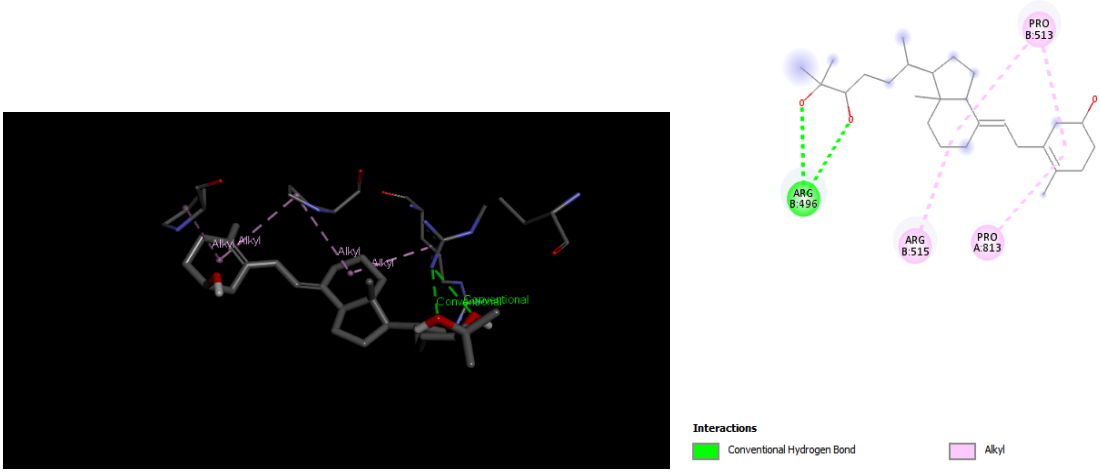



***Figure 4.8: Results of 3D (left) and 2D (right) molecular docking of HMG CoA reductase against 9, 10-secocholesta-5, 7, 10(19)-triene-3, 24, 25-triol.***

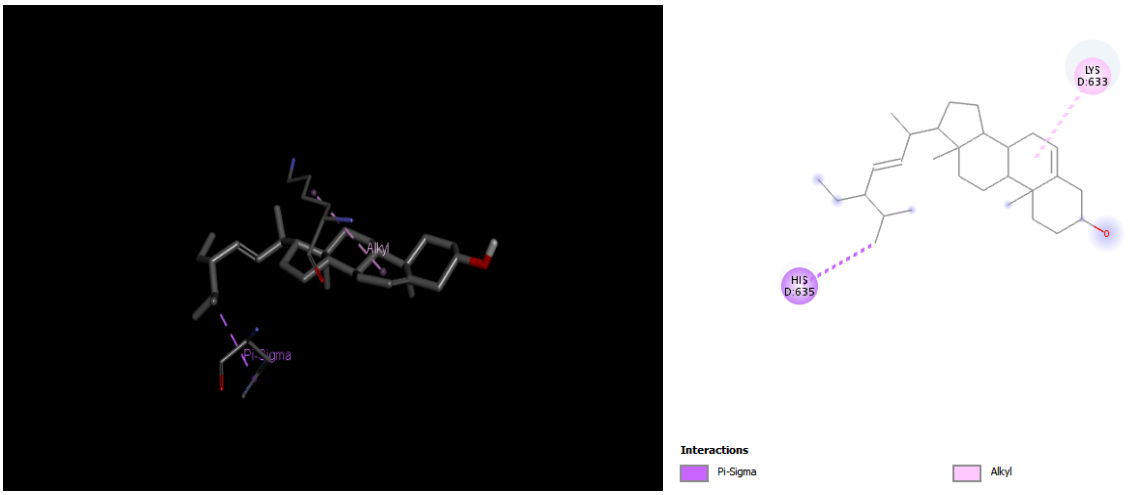



***Figure 4.9: Results of 3D (left) and 2D (right) molecular docking of HMG CoA reductase against stigmasterol.***

The docking result of stigmasterol shows the Pi-Sigma and Alkyl interactions however outside of the predicted binding site. This, however, was within the grid box when docking was done.

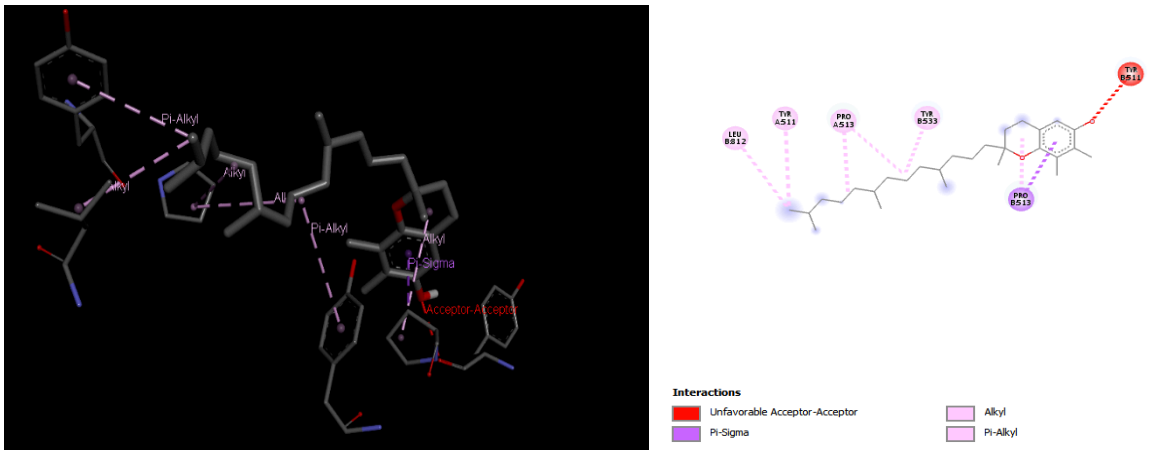



***Figure 4.10: Results of 3D (left) and 2D (right) molecular docking of HMG CoA reductase against gamma-Tocopherol.***

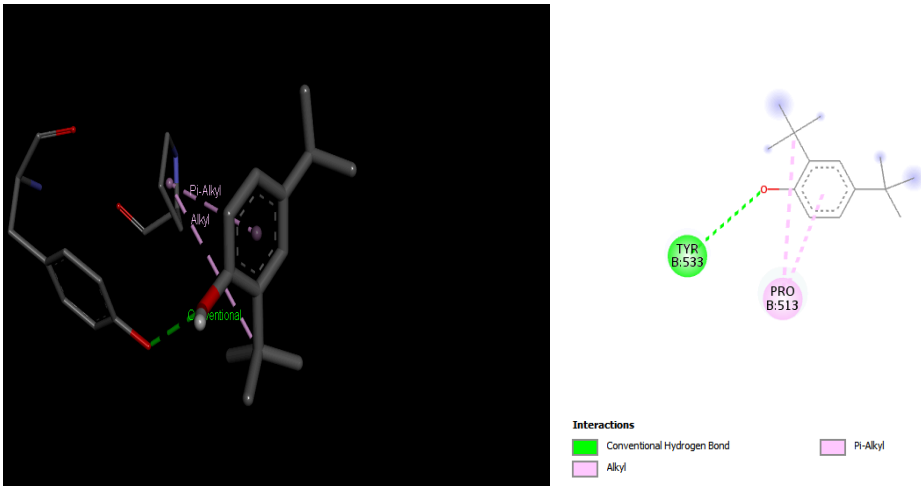



***Figure 4.11: Results of 3D left and 2D molecular docking of HMG CoA reductase against Phenol, 2, 4-bis (1, 1-dimethylethyl).***

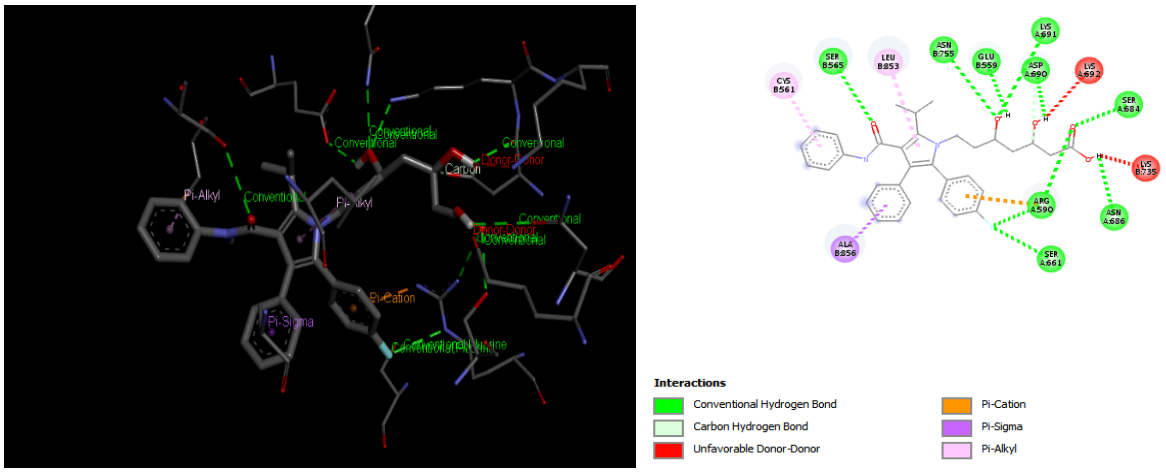



***Figure 4.12: Results of 3D left and 2D molecular docking of HMG CoA reductase against Atorvastatin.***

# CHAPTER 5.0

# DISCUSSION

Ethnomedicine has been using plants since the beginning of time. Plants can exhibit therapeutic effects on a range of illnesses because they are home to a diverse array of secondary metabolites responsible for antioxidant and anti-atherogenic activities. Certain plant parts must be harvested, including seeds, leaves, roots, and bark, and extraction must be carried out using a variety of solvents to obtain these phytochemicals (Kubi *et al.*, 2024, Odeh *et al.*, 2022, Quesada *et al.*, 2021). This study reported 37 phytocompounds from the plant extracts (Table 1.1-1.4). The major components identified from these extracts include vanillin lactoside, 9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol, stigmasterol, γ-Tocopherol, and phenol, 2,4-bis(1,1-dimethylethyl). It has been reported in literature that the *Citrullus Spp* seed powder contains a range of sterols and fatty acids as major constituents (Azhari *et al.*, 2014, Mariod and Matthaeus, 2008). It should be noted that hexane (a non-polar extract) had the least amount of bioactive chemicals extracted, whereas methanolic (polar) extracts had the most. Methanol breaks down non-polar cell walls and seeds, which may be why it is so effective (Masuku *et al.*, 2020, Handa, 2008, Daniel and Topo, 2012). The highest activity recorded and the trends seen from polar solvent extracts in comparison to non-polar solvent extracts can be attributed to this observation. The major compounds of interest identified across all varieties with their respective solvents are shown in Table 2. These compounds have some antioxidant activity reported on them. A similar trend amongst the seed varieties seems to be maintained as the brown seeds seem to generally have high activities followed by the white and lastly the red ones. This may be because of high tannins and lignin content in the brown seeds. The grey-white seeds pigmentation might be as a result of some flavonoids also responsible for its considerable activity with the red ones lastly depicting the presence of some anthocyanins (Marles and Gruber, 2004, Khoo *et al.*, 2017a, Zhao *et al.*, 2022b). The concentrations of these various phenolic compounds in these seed extracts explains their varying activities.

In this study, total phenolic and flavonoid contents were assessed and the results reported that the polar extracts had significantly higher phenolic compounds as compared to the non-polar

extracts. The concentration ranged from 0.01-0.0384 mg FAE/g (Fig 3.1). The findings are in line with those of (Saeed *et al.*, 2024), who reported that TPC ranged from 9.8±0.02 mg GAE/100 g to 11.1±0.03 mg GAE/100 g in various melon seed extracts. Similar findings were noted by (Santos *et al.*, 2018), who reported that TPC concentration ranged from 69.77 to 111.65 mg GAE/100 g in several melon seed extracts. Furthermore, Melon seed content was reported by (Neglo *et al.*, 2021) to be 0.042±0.003 mg GAE/g, whereas TPC was reported by (Mehra *et al.*, 2015) to be 4.222 µg GAE/mg. Phenolics are the largest group of the compounds known in plants and they include phenolic acids, simple and complex flavonoids, anthocyanins, tannins, lignans and stilbenes and have biological activities (Lin *et al.*, 2016, Isah, 2019). Flavonoids are a group of polyphenols that have the capacity to donate electrons, scavenge, and facilitate the formation of α-tocopherol as well as metal chelation. The concentration of total flavonoids in the present investigation ranged from 0.0036 to 0.534 mg QE/g (Fig 3.2) and increased with increasing polarity. This aligns with findings from (Saeed *et al.*, 2024) that documented TFC of several seed extracts with values ranging from 62.0 to 87.4 mg CE/100 g. Furthermore, TFC of 401 µg CE/mg was observed by (Mehra *et al.*, 2015), and TFC varying from 0.015 to 0.347 mg/g was demonstrated by (Mogotlane *et al.*, 2018) for various seed extract kinds.

As observed in this study, phenolic and flavonoid compounds found in these extracts suggest a high possibility of the *Citrullus eccirhosus* plant of having antioxidant properties hence substantiating the plant's ethnopharmacological use towards the management of oxidative linked diseases such as atherosclerosis (Chauke *et al.*, 2015). Flavonoids and polyphenols are abundant in a wide variety of plant species and studies have revealed their roles in anti-atherosclerosis. Studies have revealed the effects of flavonoids on the initiation and progression of atherosclerosis by conferring a protective effect (Grassi *et al.*, 2009b, Grassi *et al.*, 2009a, Ferri and Grassi, 2010). Various mechanisms with relation to the bioactivity of flavonoids and their related polyphenols have been reported, however, notably, the attention has been on their direct and indirect action as antioxidants. Working as antioxidants, polyphenols may protect the cell against oxidative damage thus limiting the risk of degenerative diseases associated with oxidative stress (Tedesco *et al.*, 2021). Notably, the consumption of these polyphenols seems to retard and limit the development of the atherosclerotic lesions thus impeding the low density lipoprotein from getting oxidized (Santangelo *et al.*, 2018). This is a very crucial and important aspect in the endothelial lesions which occur in atherosclerosis.

The body, in its fight against oxidative stress, employs its endogenous antioxidant system as well as leveraging on the exogenous antioxidants that come through the consumption of food (plants) or ingestion of medicines. This therefore shows the relevance of plants in their ability to scavenge free radicals in mitigating oxidative stress. In this study, *Citrullus ecirrhosus* seed extracts were assessed for their antioxidant capacity since their related counterparts *Citrullus lanatus* seeds who are reportedly as being antiatherogenic, showed the presence of phytocompounds like alkaloids, flavonoids, phenols and tannins (Varghese *et al.*, 2013). The extracts all showed free radical scavenging ability though with varying degrees from polar to non-polar solvents of free radicals from chemical oxidants like DPPH and ABTS as shown in Figure 3.4 and Figure 3.5. The methanolic extracts for all varieties had the highest activity followed by water and the hexane had the lowest activity for both DPPH and ABTS. This observation can correlate to the results already observed on the TPC and TFC for these extracts as the trend is similar. The presence of more phenolic compounds in the methanolic and water extracts contributed to their high free radical scavenging capacity as they have been reported to scavenge free radicals, chelate metal ions and donate hydrogen atoms (Santos-Sánchez *et al.*, 2019). Also, these high activities may have come as a result of these solvents having extracted a substantial number of the phenolic compounds with antioxidant activity (Gulcin, 2020). The impact of the polarity of the solvent can be seen as polar solvents exhibited much activity as compared to the non-polar ones.

The total antioxidant capacity of the extracts was analyzed based on the ability of the extracts to donate an electron to the unstable Mo (VI) to form a stable Mo (V) complex (Uddin *et al.*, 2016). In this study, the extracts had significant total antioxidant capacity expressed in ascorbic acid equivalence. The methanolic extracts from all varieties had the highest capacity with brown methanolic extracts having 24.5 ± 1.77 mg AAE/g dry weight, followed by white methanolic extraction having 20.7 ± 0.817 mg AAE/g dry weight and the red methanolic extraction with a concentration of 14.4 ± 0.328 mg AAE/g dry weight as shown in Figure 3.3. The high phenolic and flavonoid capacity also noted in the methanol and water extracts is responsible for the high overall antioxidant capacity. Hexane extracts showed the lowest, although a similar trend of a decrease from the polar to the nonpolar was also seen. In general, the extracts' capacity to scavenge free radicals plays a significant role in mitigating the consequences of oxidative stress, rendering them valuable as naturally occurring antioxidants.

For the molecular docking, the protein was prepared by removing ions, water molecules and ligands so as to avoid interaction and interference of these between the target molecules and the protein in AutoDock (Ravi and Kannabiran, 2016). The predicted binding sites for the HMG CoA reductase enzyme (PDB ID: 3CCZ) were generated using PrankWeb (P2Rank). The benefit of using PrankWeb is increased by the application of cutting-edge machine learning techniques, which are trained on large protein structure datasets. When compared to other conventional approaches, this alone gives it greater leverage in the prediction of active sites. Additionally, because of its computational efficiency, protein structures can be quickly analyzed, which makes it appropriate for high-throughput screening of potential active sites in large protein data sets (Jendele *et al.*, 2019).

The molecular docking results are a depiction of the comparison of docking scores of a compound with other compounds. This also shows whether the compound in question has potential or not. A high binding affinity is represented by molecules with a lowest docking score (minus value). The more negative, the higher the binding affinity. The ligand conformation, estimation of the ligand's orientation and location within the sites/pockets (sometimes referred to as poses), and evaluation of the binding affinity are all included in the docking process (Sa'adah *et al.*, 2022). By comparing the predicted pose with the experimental one, the root mean square deviation (RMSD) computation assesses how accurate the pose prediction was. To validate the method, an RMSD value of 0.00 Å for the ligands was obtained. RMSD helps in the description of possible changes in the crystal structure of the protein-ligand interaction before and after docking so as to determine how far the deviation was. The docking method is valid if the RMSD value is less than or equal to 2 Å. However, the smaller the RMSD value is the better is the predicted ligand of getting closer to the native ligand conformation thus the zero value would give the perfect value. Also, a negative value in the measure of the free bond energy further validates the method used. In this research, the RMSD value in mode one after docking had 0.00 Å thus the docking results will be considered a success because of the value that is less than or equal to 2 Å. There is a direct relation between the inhibition constant of a compound to its free bond energy on the docking score thus the free bond energy value can be used to predict the

inhibitory activity of compounds on the activity of proteins or enzymes (Dinata, 2024, Fernando *et al.*, 2019, Sa'adah *et al.*, 2022).

For maximum inhibition between the targeted protein and an inhibitor, site specific binding is integral. Site specific protein-ligand docking was performed using the predicted active sites coordinates from PrankWeb. The results obtained were presented as energies between the targeted protein molecule and the bioactive compound and their interaction position. From the 19 identified bioactive compounds with reported antioxidant activity after GC-MS analysis, the top 5 bioactive compounds according to their binding affinity to the HMG-CoA reductase were Vanillin lactoside, 9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol, stigmasterol, gamma-Tocopherol and Phenol, 2,4-bis(1,1-dimethylethyl). Their binding affinities ranged from -8.2 to -6.3 kcal/mol. Vanillin lactoside had the highest binding affinity amongst the bioactive compounds (-8.2 kcal/mol). Of the 7 FDA statin controls used, Atorvastatin was the only one which had a higher binding affinity (-8.8 kcal/mol) than vanillin lactoside. Several intermolecular interactions played pivotal roles in the protein-ligand interactions. These include hydrogen bonds, carbon-hydrogen bonds and the hydrophobic interactions like Pi-alkyl, alkyl as well as the Pi-cation and Pi-anion interactions. The number of these interactions were high in the Vanillin lactoside as compared to the other bioactive compounds. These forces of attraction are pivotal in drug design and development. The hydrogen bonds are known to provide structural ability, strength provision and are the strongest intermolecular forces to the protein-ligand complex. As for the other hydrophobic interactions, they are critical in holding the protein and ligand together and their intercalating maximizes the binding affinity of the ligands within the binding pocket of the targeted protein (Ashokbhai *et al.*, 2022, Padhi *et al.*, 2021). Therefore, the bioactive compound vanillin lactoside showing significant binding affinity and notable interactions at the binding sites of the HMG-CoA reductase may have a good potential in the inhibition of the HMG-CoA reductase enzyme against the inorganic statins. Vanillin lactoside is formed by the conjugation of the naturally phenolic compound vanillin and the disaccharide lactose. Vanillin on its own has been reported to scavenge for free radicals as a result of its phenolic hydroxyl groups. These groups donate hydrogen atoms to the free radicals thus neutralizing them in the process. Its role in the reduction of oxidative stress-related diseases as well as cytoprotection from oxidative damage (Makni *et al.*, 2011, Tai *et al.*, 2011, Xiong *et al.*, 2022). Lactose on its own doesn't have reported antioxidant activities, however, its conjugation

with phenolic compounds like vanillin enhance or modify their antioxidant properties by sometimes improving solubility, stability and bioavailability of these phenolics (Peinado *et al.*, 2010). Although the occurrence of vanillin might not be natural in plants as reported, there is still a possibility that it could be biosynthesized by certain plants under specific conditions, thus further research would be needed to confirm this possibility and also to have an understanding of the biosynthetic pathways involved.

9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol also showed high binding affinity of -8.0 kcal/mol which was the same as the FDA approved controls pitavastatin and lovastatin. The compound is structurally related to vitamin D derivatives given its nomenclature and structural components (Youssef *et al.*, 2023). The compound has been found as a major phytochemical in the methanolic extracts of *Ricinus communis* (Al-Gara *et al.*, 2019), *Citrullus colocynthis* (Idan *et al.*, 2015) and in the *Cnidoscolus aconitifolius* leaves (Chukwu *et al.*, 2020). Although the compound hasn't been widely recognized or being a standard derivative of vitamin D in mainstream scientific literature, its potential antioxidant capabilities may be approached through the examination of its structural and functional aspects typical of other related secosteroids and their derivatives. Its structural similarities to the vitamin D suggest possible mechanisms through which it could exhibit its antioxidant properties.

# CHAPTER 6.0

# CONCLUSION AND RECOMMENDATIONS

## 6.1 Conclusion

The present study has demonstrated that *Citrullus ecirrhosus* seed powder extracts have considerable phytochemicals which have antioxidative effects, associated with various mechanisms of action. The seed powder extract contained flavonoids as well as phenols and exhibited radical scavenging capabilities. The seed powder extract also had a high total antioxidant capacity. These are beneficial in combating oxidative stress which is key to the onset of cardiovascular diseases like atherosclerosis. Quite a number of phytochemicals were identified with some not having any reported biological activity yet thus giving room for future work on the isolated compounds to be carried out. It was also noted that the type of solvent used for extraction is significant to the quantity and quality of phytochemicals obtained thus precedence has to be given to the best solvent intended for the desired phytochemicals. In this study the polar solvents had higher radical scavenging activities as well as higher quantity of the flavonoids and phenols as compared to the non-polar solvents showing us that the seed powder extract had more polar phytochemicals as compared to the non-polar. Also, the seed coat colors had variations in terms of activity thus showing that the type of phytochemical within the seed contributes to its color and overall impact its activity as well. In conclusion, this study also showed that the seed powder extract has phytochemicals that can bind competitively to the HMG-CoA reductase enzyme in silico. Vanillin lactoside, 9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol, stigmasterol, gamma-Tocopherol and Phenol, 2,4-bis(1,1-dimethylethyl had binding affinity that was within the range of the FDA approved on market statins (Atorvastatin, Simvastatin, Lovastatin, Pitavastatin, Rosuvastatin, Fluvastatin and Pravastatin) thus making them potential phytochemicals that can be used towards the management of atherosclerosis.

## 6.2 Study limitations and Recommendations

- This study was conducted on a small budget resulting in shortage of resources and consumables especially for the total sterol content and cell culture assays as was proposed initially. It is therefore recommended for the study grant to be increased and made available on time so as to enable the purchasing of consumables for comprehensive studies.

- There is also need to carry out in vivo studies so as to confirm the ability of the seed powder in reducing the atherosclerotic lesion in atherosclerosis induced rats/mice.

- Vanillin lactoside and the other compounds which had high binding affinity to the HMG-CoA need to be extracted separately, purified and experimented individually on rats/mice to check if their effects on the management of atherosclerosis is consistent or maybe they have additive and synergistic effects.

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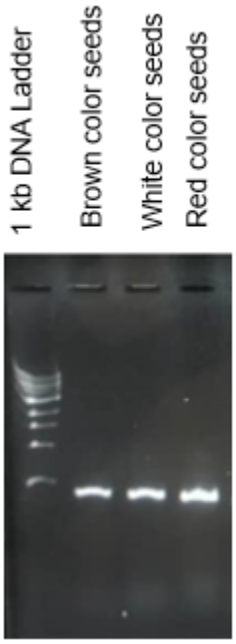
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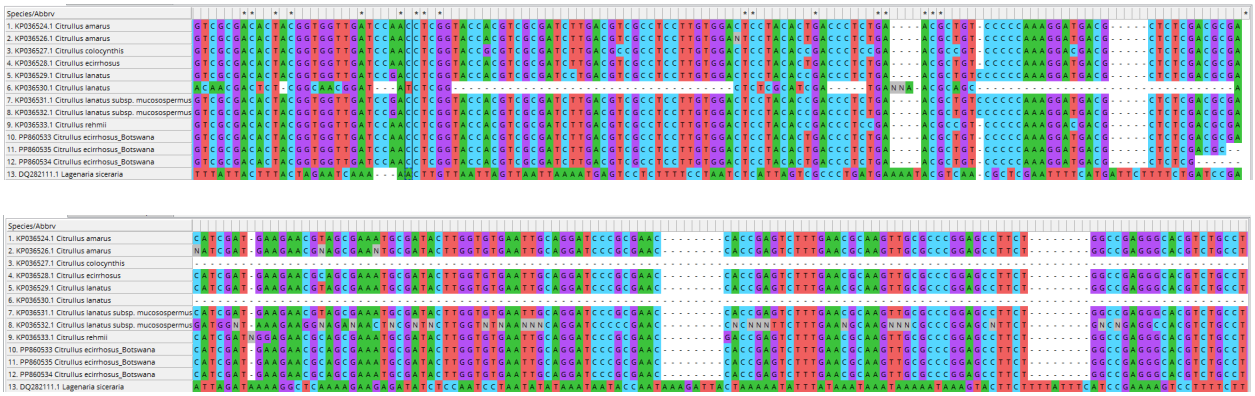
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## APPENDICES




***Appendix 1: Gel Electrophoresis of DNA Extracted from Citrullus ecirrhosus***



*Appendix 2: ITS2 Multiple Sequence Alignment (MUSCLE) by MEGAX-64*

a. 5-Hydroxymethylfurfural

b. 2-Methoxy-4-vinylpheno

c. Vanillin lactoside

d. 6-Amino-2-methyl-2H-indazole

e. 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxypheno

f. Hexadecanoic acid, methyl ester

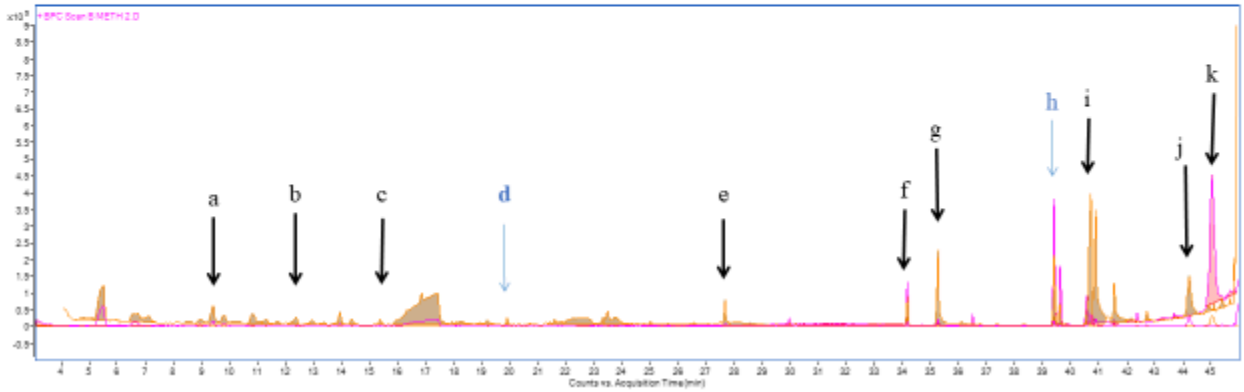
g. n-Hexadecanoic acid

h. 9,12-Octadecadienoic acid (Z,Z)-, methyl ester

i. Octadecanoic acid

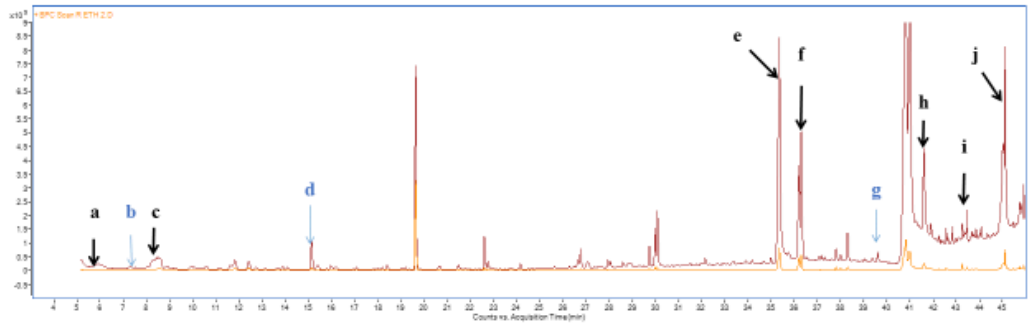
j. γ-Tocopherol

k. 9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3β,5Z,7E)



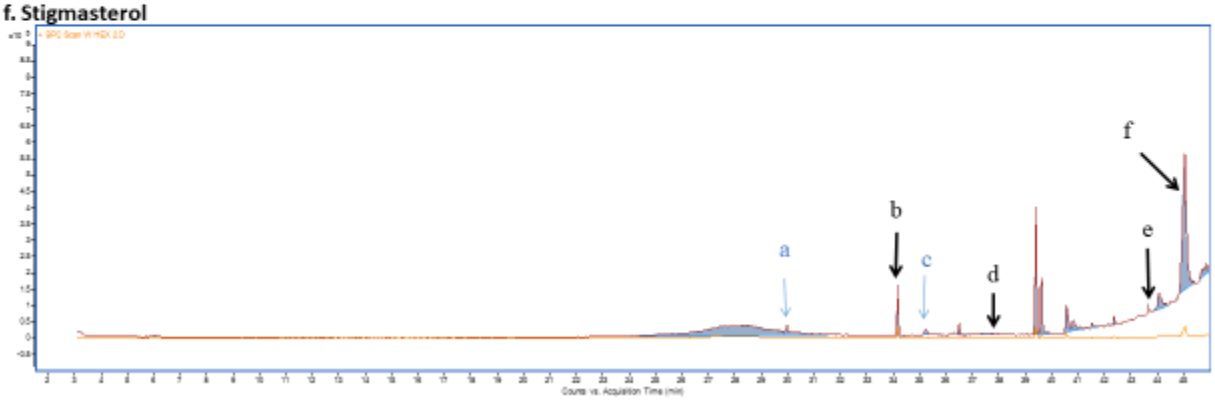

*Appendix 3: Chromatogram showing the phytochemical compounds with known biological activity with the one in black representing those with known antioxidant activity for methanolic extracts for brown seed variety.*

a. Pentanoic acid, octyl ester
b. β-1,5-O-Dibenzoyl-ribofuranose
c. 3-Dodecene
d. Tridecane
e. n-Hexadecanoic acid
f. 5,8,11-Heptadecatriynoic acid, methyl ester
g.9,12-Octadecadienoic acid (Z,Z)-
h.Oleic Acid
i. 1-Heptatriacotano
j. Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl




***Appendix 4: Chromatogram showing the phytochemical compounds with known biological activity with the one in black representing those with known antioxidant activity for ethyl acetate extracts for red seed variety.***

a. Nonadecane
b.Hexadecanoic acid, methyl ester
c. 9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione
d. Heptacosane
e. γ-Tocopherol
f. Stigmasterol




***Appendix 5: Chromatogram showing the phytochemical compounds with known biological activity with the one in black representing those with known antioxidant activity for hexane extracts for white seed variety.***

| A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|
| name | rank | score | probability | sas_point | surf_aton | center_x | center_y | center_z |
| pocket1 | 1 | 72.93 | 0.991 | 288 | 133 | 81.5572 | 1.0687 | -5.8972 |
| pocket2 | 2 | 64.89 | 0.989 | 261 | 123 | 52.5557 | -11.7301 | 30.6683 |
| pocket3 | 3 | 57.89 | 0.985 | 247 | 96 | 61.581 | 13.9467 | 39.9661 |
| pocket4 | 4 | 55.75 | 0.983 | 240 | 112 | 99.4073 | -1.3856 | 14.9213 |
| pocket5 | 5 | 6.52 | 0.358 | 22 | 22 | 54.5492 | 5.8751 | 38.0474 |
| pocket6 | 6 | 2.67 | 0.08 | 56 | 28 | 94.4963 | 19.7642 | -9.4877 |
| pocket7 | 7 | 2.1 | 0.047 | 39 | 16 | 64.8916 | 22.8023 | 10.0617 |
| pocket8 | 8 | 2.02 | 0.043 | 37 | 17 | 81.5601 | -22.3792 | 29.3967 |
| pocket9 | 9 | 1.59 | 0.024 | 3 | 7 | 96.5203 | 8.6008 | 19.9116 |
| pocket10 | 10 | 1.54 | 0.022 | 20 | 12 | 70.2802 | 19.8361 | 11.9481 |
| pocket11 | 11 | 1.43 | 0.019 | 42 | 18 | 57.7101 | -6.9927 | 10.1181 |
| pocket12 | 12 | 1.38 | 0.017 | 39 | 15 | 87.9377 | 10.9568 | 28.3619 |
| pocket13 | 13 | 1.36 | 0.016 | 22 | 18 | 57.6401 | 0.3841 | 12.5991 |
| pocket14 | 14 | 1.29 | 0.014 | 37 | 18 | 81.1203 | 7.917 | 37.2611 |
| pocket15 | 15 | 1.13 | 0.01 | 28 | 15 | 59.064 | -20.7112 | 38.6599 |

***Appendix 6: Active site predictions for HMG-CoA reductase (3CCZ) obtained using Prankweb with the pockets found ranked according to scores and probability.***

| A | B | C | D |
|---|---|---|---|
| Ligand | Binding Affinity | rmsd/ub | rmsd/lb |
| 3ccz_60823_uff_E=1011.75 | -8.8 | 0 | 0 |
| 3ccz_60823_uff_E=1011.75 | -8.7 | 0 | 0 |
| 3ccz_592475_uff_E=753.26 | -8.2 | 0 | 0 |
| 3ccz_54454_uff_E=352.22 | -8.1 | 0 | 0 |
| 3ccz_54454_uff_E=352.22 | -8 | 0 | 0 |
| 3ccz_53232_uff_E=334.51 | -8 | 0 | 0 |
| 3ccz_5282452_uff_E=1803.91 | -8 | 0 | 0 |
| 3ccz_91827817_uff_E=659.43 | -8 | 0 | 0 |
| 3ccz_53232_uff_E=334.51 | -7.9 | 0 | 0 |
| 3ccz_446155_uff_E=741.73 | -7.9 | 0 | 0 |
| 3ccz_446157_uff_E=975.12 | -7.9 | 0 | 0 |
| 3ccz_446155_uff_E=741.73 | -7.9 | 0 | 0 |
| 3ccz_5280794_uff_E=546.19 | -7.9 | 0 | 0 |
| 3ccz_92729_uff_E=258.14 | -7.6 | 0 | 0 |
| 3ccz_446157_uff_E=975.12 | -7.6 | 0 | 0 |
| 3ccz_592475_uff_E=753.26 | -7.4 | 0 | 0 |
| 3ccz_54687_uff_E=336.78 | -7.3 | 0 | 0 |
| 3ccz_54687_uff_E=336.78 | -7.3 | 0 | 0 |
| 3ccz_7311_uff_E=170.82 | -6.3 | 0 | 0 |
| 3ccz_7311_uff_E=170.82 | -6.3 | 0 | 0 |

***Appendix 7: Excel results showing the binding affinity and rmsd values of the ligands and the HMG-CoA reductase post docking in PyRx.***

| A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A:LYS606:I | Yes | 0 255 0 | Ligand No | 3.08232 | Hydrogen | Conventional Hydrogen Bond | A:LYS606:NZ | H-Donor | N:UNK1:O | H-Acceptor | 57.937 | 108.82 | | | |
| A:LYS633:I | Yes | 0 255 0 | Ligand No | 3.0189 | Hydrogen | Conventional Hydrogen Bond | A:LYS633:NZ | H-Donor | N:UNK1:O | H-Acceptor | 23.871 | 105.244 | | | |
| A:THR636: | Yes | 0 255 0 | Ligand No | 2.84854 | Hydrogen | Conventional Hydrogen Bond | A:THR636:N | H-Donor | N:UNK1:O | H-Acceptor | 16.785 | 122.24 | | | |
| A:ILE638:N | Yes | 0 255 0 | Ligand No | 3.0407 | Hydrogen | Conventional Hydrogen Bond | A:ILE638:N | H-Donor | N:UNK1:O | H-Acceptor | 19.849 | 131.876 | | | |
| N:UNK1:H | Yes | 0 255 0 | Ligand No | 2.62208 | Hydrogen | Conventional Hydrogen Bond | N:UNK1:H | H-Donor | D:GLU700: | H-Acceptor | | | 103.944 | 139.34 | |
| N:UNK1:C | Yes | 220 255 22 | Ligand No | 3.53083 | Hydrogen | Carbon Hydrogen Bond | N:UNK1:C | H-Donor | D:GLU700: | H-Acceptor | 105.472 | 132.324 | | | |
| N:UNK1:C | Yes | 220 255 22 | Ligand No | 3.2676 | Hydrogen | Carbon Hydrogen Bond | N:UNK1:C | H-Donor | A:THR636: | H-Acceptor | 101.062 | 147.734 | | | |
| N:UNK1:C | Yes | 220 255 22 | Ligand No | 3.54291 | Hydrogen | Carbon Hydrogen Bond | N:UNK1:C | H-Donor | D:GLU700: | H-Acceptor | 95.878 | 132.681 | | | |
| N:UNK1:C | Yes | 220 255 22 | Ligand No | 3.61404 | Hydrogen | Carbon Hydrogen Bond | N:UNK1:C | H-Donor | A:SER637: | H-Acceptor | 92.066 | 17.749 | | | |
| A:LYS606:I | Yes | 255 150 0 | Ligand No | 3.90511 | Hydrogen | Pi-Cation;Pi-Donor Hydrogen Bond | A:LYS606:NZ | Positive;H | N:UNK1 | Pi-Orbitals;Pi-Orbitals | | | | | 21.34 |
| N:UNK1 - I | Yes | 255 200 25 | Ligand No | 4.75858 | Hydropho | Pi-Alkyl | N:UNK1 | Pi-Orbital | D:ALA585 | Alkyl | | | | | |

***Appendix 8: Excel results showing the types of bond interactions between the vanillin lactoside and the HMG-CoA reductase using Biodiscovery studio.***