

**EVALUATING THE ANTIHYPERGLYCEMIC AND ANTIOXIDATIVE
EFFECTS OF ELLAGITANNIN AND PUNICALAGIN FROM
POMEGRANATE FRUIT PEEL**

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DECLARATION OF INDEPENDENT WORK

I Boitumelo Mashile, student number _____, hereby declare that this research project submitted to the Central University of Technology, Free State for the degree MASTER OF HEALTH SCIENCES IN BIOMEDICAL TECHNOLOGY is my independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification. As such, where external sources were utilized, due acknowledgment was given by means of a comprehensive list of references in accordance with departmental requirements. I therefore give copyright of this dissertation in favour of the Central University of Technology.

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DEDICATION

I humbly dedicate this master's dissertation to God, my lovely mother Cristina Mashile who prayed for me and my siblings Amo, Katli and Lesego for their support and love. To my friends who stood by me throughout the challenging times of undertaking this research.

FOR HE WHO BELIEVES IN GOD ALL THINGS ARE POSSIBLE.

ABSTRACT

Background

The vascular and metabolic health benefits of pomegranate fruit have been frequently mentioned. Despite being under-utilized, the peel of this fruit contains ellagitannins that can potentially increase the medicinal relevance of this fruit in managing metabolic diseases such as diabetes and oxidative complications. Unfortunately, the optimum recovery methods of these bioactive ellagitannins from pomegranate peel and their bioactivity profiling has been scarcely reported. Therefore, the aim of this study was to optimally recover ellagitannins and punicalagin with potent antioxidant and glycemic control properties from the fruit peel of “Wonderful” pomegranate.

Methodology

“Wonderful” pomegranate fruits (Class 1 or processing grade) were purchased from Sonlia Fruit Packhouse (Wellington, Western Cape Province, South Africa). The fresh peel was extracted using water at different temperatures (25, 37, 50, 65, 78 and 95 °C). The extracts were preliminary evaluated for phenol and flavonoid contents, as well as in vitro 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging, Fe^{3+} reducing and α -amylase and α -glucosidase inhibitory activities at a concentration of 45 $\mu\text{g/mL}$. An Amberlite® XAD16N resin was used to chromatographically recover tannins from the extracts. Ellagitannins in the tannins were quantified by Liquid chromatography – mass spectrometry (LC–MS). Punicalagin was purified from the tannin recovered from the 78 °C crude extract using Sephadex LH-20 gel. The dose dependent radical scavenging, oxygen radical absorbance capacity (ORAC), antiglycation, α -amylase inhibitory and anti-linoleic acid peroxidative potential of the purified punicalagin was evaluated in vitro and compared with that of the corresponding crude extract and tannin. In Chang liver cells induced with oxidative stress, the ability of the purified punicalagin and tannin, as well as the corresponding parent crude extract to inhibit lipid peroxidation and improve catalase and superoxide dismutase activity was measured. Also, their modulatory potential on glucose uptake/utilization was evaluated in L-6 myotubes.

Results

The extract obtained at 78 °C had the highest phenolic, flavonoid, tannin and punicalagin contents, which potentiated a stronger DPPH and ABTS radical scavenging and enzyme inhibitory action compared to the other extracts. Dose-dependently, the purified ellagitannins showed stronger DPPH (IC_{50} = 4.80 versus 25.0 and 32.7 μ g/mL, respectively) and ABTS (IC_{50} = 1.63 versus 27.5 and 37.5 μ g/mL, respectively) radical scavenging, ORAC (IC_{50} = 9.64 versus 15.6 and 25.6 μ g/mL, respectively), anti-linoleic acid peroxidative (IC_{50} = 9.97 versus 19.9 and 27.3 μ g/mL, respectively) and antiglycation (IC_{50} = 29.1 versus 34.2 and 79.1 μ g/mL, respectively) activities than the extract obtained at 78 °C and the purified punicalagin. The anti-lipid peroxidative effect in Chang liver cells, as well as the glucose uptake modulatory activity in L-6 myotubes also followed similar trend, with the recovered tannin (IC_{50} = 3.67 μ g/mL; EC_{50} = 14.8 μ g/mL) outperforming the extract (IC_{50} = 24.3 μ g/mL; EC_{50} = 55.0 μ g/mL) and the purified punicalagin (IC_{50} = 59.0 μ g/mL; EC_{50} = 112 μ g/mL), which suggests antioxidant and glycemic control potentials of the recovered tannin. The ORAC, anti-linoleic acid peroxidative and cellular anti-lipid peroxidative effects of the purified tannin was comparable to and/or more potent than that of ascorbic acid (IC_{50} = 8.10, 7.18, 22.4 μ g/mL, respectively), suggesting its potential relevance in managing oxidative stress. The potent antioxidant and glycemic control effect of the recovered tannin may be influenced by the presence of LC-MS identified ellagitannins and/or phenolics (punicalagin, ellagic acid, granatin A, corilagin, casuarinin, gallic acid and quercetin hexoside). The α -amylase inhibitory data however showed the purified punicalagin as the most potent, suggesting its potential in influencing postprandial glycemic control.

Conclusion

While punicalagin appears to be a major ellagitannin influencing the α -amylase inhibitory action of pomegranate peel, it works in concert with other ellagitannin and polyphenol constituents, including ellagic acid and gallic acid in exerting potent radical scavenging, anti-lipid peroxidative and glucose uptake modulatory activities. The data of the present suggest that tannin/ellagitannins purified from pomegranate peel aqueous extract obtained at 78 °C may be a promising dietary supplement for exerting glycemic control and mitigating oxidative stress.

Keywords: *Pomegranate peel, ellagitannins, chromatography, antioxidants, glycemic control*

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LIST OF ABBREVIATIONS

ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
Adm	Administration
ACE	Angiotensin converting enzyme
ALT	Alanine transaminase
AST	Aspartate aminotransferase
AGI	α -glucosidase inhibitors
BSA	Bovine serum albumin
BG	Blood glucose
BMI	Body-mass index
CRP	C-reactive protein
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPP-IV	Dipeptidyl peptidase
FBG	Fasting blood glucose
FRAP	Fe ³⁺ reducing activity
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GLP-1	Glucagon- like peptide –1
GIP	Glucose-dependent insulintropic polypeptide
GT	Glucose tolerance
HbA1c	Glycated haemoglobin
HFD	High-fat-diet
IDDM	Insulin-dependent mellitus

IDF	International Diabetes Federation
IL-6	Interleukin 6
LDH	Lactate dehydrogenase
LC-MS	Liquid chromatography–mass spectrometry
LPO	Lipid peroxidation
NIDDM	Non-insulin-dependent
PTP1B	Protein tyrosine phosphatase 1B
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SD	Sprague Dawley
SHR	Spontaneously hypertensive rats
SOD	Superoxide dismutase
SU	Sulphonylurea
SUR-1	Sulphonylurea receptor
SGLT2	Sodium glucose co-transporter 2
STZ	Streptozotocin
T2D	Type 2 diabetes
TZDs	Thiazolidinediones
WHO	World Health Organization

RESEARCH OUTPUTS

Published article from this thesis:

1. **Mashile B**, Setlhodi R, Izu GO, Erukainure OL, Mashele SS, Makhafola TJ, Eze KC, Chukwuma CI*. (2024). Temperature dependent extraction and chromatographic recovery and characterization of ellagitannins with potent antioxidant and glycemic control properties from “Wonderful” pomegranate peel. **International Journal of Food Science & Technology**, 59(1), 408 – 424. <http://doi.org/10.1111/ijfs.16823>.

Other published articles during master’s program:

1. Setlhodi R, **Mashile B**, Izu GO, Gbashi S, Mashele SS, Bonnet SL, Makhafola TJ, Chukwuma CI*. (2023). Modeling the Influence of Extraction Temperature on the Ellagitannin and Antioxidant Profiles of “Wonderful” Pomegranate Peel Using Advanced Chemometrics Analysis. *Food and Bioprocess Technology*. <https://doi.org/10.1007/s11947-023-03104-2>
2. Setlhodi R, **Mashile B**, Izu GO, Gbashi S, Mashele SS, Bonnet SL, Makhafola TJ, Chukwuma CI*. (2023). Effect of solvent extraction on the antioxidant and phytochemical profiles of ellagitannins from “wonderful” pomegranate peel: an advanced chemometrics analysis. *European Food Research and Technology*, 249: 1807–1820 (2023). <https://doi.org/10.1007/s00217-023-04255-4>
3. Akuru EA, Chukwuma CI*, Oyeagu CE, Erukainure OL, **Mashile B**, Setlhodi R, Mashele SS, Makhafola TJ, Unuofin JO, Abifarin TO, Mpendulo TC. (2022). Nutritional and phytochemical profile of pomegranate ("Wonderful variety") peel and its effects on hepatic oxidative stress and metabolic alterations. *Journal Food Biochemistry*, 46(4), e13913. <https://doi.org/10.1111/jfbc.13913>.

CHAPTER 1

INTRODUCTION

The global prevalence of diabetes and related chronic metabolic diseases is still increasing. South Africa is among the countries with socioeconomic burden caused by the non-communicable diseases (Gouda et al., 2019). Diabetes mellitus is a non-communicable disease that is characterized by persistent hyperglycemia due to loss of insulin secretion or action in target cells or tissues (Kahn et al., 2014). Diabetes is caused by several risk factors such as genetics, age, diet and lifestyle (Khan et al., 2019; IDF, 2021). If diabetes is not managed appropriately it can progress into many serious health complications, such as blindness, kidney failure, heart disease, as well as other macro and microvascular complications (Diabetes ,2023).

According to the International Diabetes Federation (IDF), diabetes has three types, namely type 1 diabetes, type 2 diabetes (T2D) and gestational diabetes. Type 1 diabetes results from insufficient insulin production due to loss of pancreatic beta cells. T2D is caused by non-responsiveness of target cells to insulin. Gestational diabetes occurs in women during pregnancy as a result of intolerance to blood glucose increase (IDF, 2021). Among these types of diabetes, the most prevalent is T2D, which accounts for over 90% of diabetes cases worldwide (Oyebode et al., 2018; IDF, 2021).

If not well managed, all types of diabetes have the potential to develop diabetic complications, which are the main contributors to the high mortalities and disabilities linked to diabetes. Diabetes may lead to several microvascular and macrovascular complications (Oyebode et al., 2018). Oxidative stress plays a key role in the progression and complication of diabetes due to increased production of prooxidants in the body during diabetes (Giacco and Brownlee, 2010). This is because overload of blood glucose can increase the production of prooxidants (Burgos-Moron et al., 2019). Prooxidants are reactive substances such as reactive oxygen species (ROS) and free radicals, which are detrimental to cellular functions since they can cause oxidative damage to proteins, lipids, cells, genetic material (DNA and RNA) and organs (Islam, 2016).

There are several commercial drugs used to manage diabetes, but most of these drugs have been linked to side effects such as diarrhoea , renal failure, weight gain, gastrointestinal discomforts, as well as the risk of cardiac failure and hypoglycemia (Marín-Peñalver et al., 2016). Their prolonged usage can cause several adverse effects as mentioned above,

emphasizing the need of utilizing natural therapeutic alternatives. Commonly known alternatives to commercial drugs for diabetes are plant derived medicines or traditional medicines, especially in developing and low-income African countries, where quality healthcare is difficult to afford. The health-promoting benefits of traditional medicine are mainly attributed due to the phenolic compounds present in many indigenous plants (Upadhyay & Dixit, 2015).

Among the sources of bioactive compounds or phytochemicals is pomegranate (*Punica granatum* L.), an ancient fruit that is widely consumed. The fruit belongs to the family Lythraceae. Pomegranate fruit is rich in antioxidants and its juice has been documented to have cardiovascular health benefits (Kandylis & Kokkinomagoulos, 2020; Giménez-Bastida et al., 2021). The fleshy and juicy arils of pomegranate fruit are usually consumed, while the peel being discarded as waste. The peel makes up around 50% of the fruit. Despite the abundance of phenolic compounds, including ellagitannins in the peel (Gullon et al., 2016), it continues to contribute to under-utilized agro-wastes, which is limiting the medicinal and bio-economic value of this fruit (Khan et al., 2015).

There is evidence that ellagitannin constituents of pomegranate peel could be relevant in the management of diabetes and oxidative stress (Sun et al., 2017; Chukwuma et al., 2020; Akuru et al., 2022; Marra et al., 2022; Mo et al., 2022). Numerous studies have described the beneficial effects of ellagitannins on obesity, oxidative stress, inflammation, glycemic control, lipid metabolism, cardiac and vascular health (Larrosa et al., 2010; Sun et al., 2017; Chukwuma et al., 2020; Akuru et al., 2022; Marra et al., 2022; Mo et al., 2022). Ellagitannins are a class of hydrolysable tannins primarily composed of punicalagins, punicalin, ellagic acid, gallagic acid and relative glycosides or derivatives (Altieri et al., 2019).

The radical scavenging, as well as the cellular anti-lipid peroxidative effects of pomegranate peel have been reported (Chukwuma et al., 2020; Akuru et al., 2022; Marra et al., 2022). The anti-inflammatory and cardiovascular protective attributes of pomegranate peel have been reported in both animal models and human subjects (Mo et al., 2022). In a previous study the acetone extract of pomegranate peel exhibited glycemic control potential by inhibiting α -amylase and glucose-induced protein glycation, as well as modulating glucose uptake/utilization in Chang liver cells (Chukwuma et al., 2020).

While ellagitannins are perceived as key bioactive principles in pomegranate peel, punicalagin remains the predominant ellagitannin of pomegranate peel and possibly a key bioactive

principle (Sun et al., 2017; Mo et al., 2022). Sun et al. (2017) reported the potent in vitro and in vivo antioxidant potential of commercial punicalagin, which outperformed commercial ellagic acid and punicalin (other key components of pomegranate peel ellagitannins). However, the antioxidant and antihyperglycemic effects of ellagitannins and punicalagin optimally purified from pomegranate peel have not been studied. In the present study temperature dependent extraction and chromatography techniques were employed in optimally recovering ellagitannins and punicalagin from pomegranate peel and evaluated for their antioxidant and antihyperglycemic potentials.

CHAPTER 2

LITERATURE REVIEW

2.1 Diabetes mellitus

Diabetes mellitus is a chronic metabolic disorder, characterized by persistent high levels of blood glucose (hyperglycemia), which causes long-term health complications such as retinopathy, loss of kidney function, neuropathy, heart attack and stroke overtime (Roglic, 2016). In fact, diabetes is associated with premature death caused mainly by coronary artery disease, stroke and renal dysfunction (Zheng et al., 2018). Hyperglycemia occurs because cells cannot metabolize blood glucose due to low or no insulin secretion by the pancreas or impaired action of insulin in target cells or tissues (Kahn et al., 2014). Diabetes is caused by several risk factors such as genetics, age, diet and some other lifestyle related factors (Khan et al., 2019). Symptoms of diabetes often include frequent urination (polyuria), constant hunger (polyphagia) and increased thirst (polydipsia) and weight loss (IDF, 2021).

2.2 Classification of diabetes

In 1980 and 1985, the World Health Organization (WHO) proposed two main types of diabetes, namely insulin-dependent diabetes mellitus (IDDM) or non-insulin-dependent diabetes mellitus (NIDDM). However, as more literature evolved, these terms (IDDM and NIDDM) were replaced by more precise and clinically applicable terminology. A new classification system was developed, which it identifies four types of DM. The include type 1 diabetes, type 2 diabetes, gestational diabetes and “other specific types” of diabetes (Mayfield, 1998).

2.2.1 Type 1 diabetes

Type 1 diabetes or IDDM is an autoimmune-related disease that can affect people of any age, but it usually occurs in children or young adults. The autoimmune disorder results in insufficient insulin production because of the destruction of pancreatic β -cells (IDF, 2021). The mechanisms behind the β -cells destructive process remain speculative. However, genetic susceptibility (class II HLA alleles) and environmental triggers such as viral infection, diet and toxins have been implicated in the initiation of the autoimmune reaction (Barnett, 2018).

2.2.2 Gestational diabetes

Gestational diabetes refers to a mild case of hyperglycemia that is initially detected during pregnancy (IDF, 2021). It increases the chance of T2D and obesity for both the mother and the offspring (McIntyre et al., 2019). Obesity, elevated inflammation and oxidative stress in the placenta and foetus are characteristics of this metabolic complication of pregnancy, which is accompanied by glucose intolerance and insulin resistance linked to hormone production from the placenta (WHO, 2013; Mrizak et al., 2014). It has been reported that oxidative stress may play a significant role in gestational diabetes by lowering the insulin sensitivity index (Mohsen et al., 2017).

2.2.3 Type 2 diabetes

The majority of diabetes patients have T2D or NIDDM, which accounts for a high percentage (about 90%) of all patients with diabetes (IDF, 2021). T2D is characterized by hyperglycemia due to deficient cellular response to insulin action, a condition known as insulin resistance (IDF, 2021). It has been reported that T2D is strongly associated with several factors, such as old age, obesity, ethnicity, diabetic family history and overweight (Khan et al., 2019; IDF, 2021).

2.3 Epidemiology of diabetes and type 2 diabetes

Diabetes is a pervasive disease that has increased significantly in recent decades and will continue to increase in the next decades, imposing a heavy burden on public health and socio-economic development of most developed and developing countries, thus poses additional challenges in effective treatment (IDF, 2021). In South Africa, the most affected are the economically disadvantaged families (Pillay et al., 2016), who have limited access to quality healthcare. Alarming statistics from epidemiological data indicate a worrying projected future for diabetes prevalence. IDF estimated that 537 million adults worldwide had diabetes in 2021 with a projected increase to 783 million by 2045 if effective measures are not adopted (IDF, 2021) (**Figure 2.1**). In Africa, about 306 thousand (8.6%) diabetic individuals died from diabetes before the age of 60 years (**Table 2.1**).

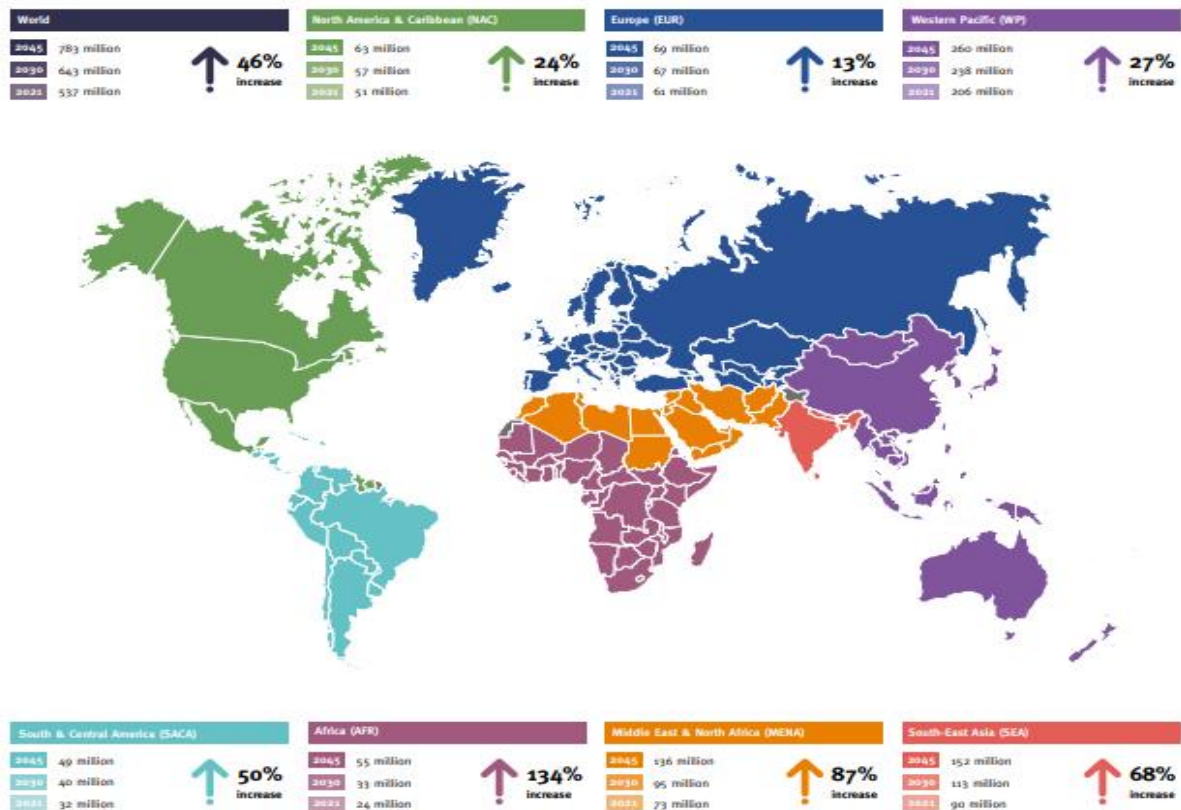


Figure 2.1: Number of people (20–79 years) with diabetes worldwide and per IDF Region in 2021–2045 (*adopted without permission from IDF, 2021*).

Table 2.1: Proportion and number of adults who died from diabetes before the age of 60 years in 2021

IDF region	Number of deaths due to diabetes before the age of 60 years (thousands)	Proportion of total deaths due to diabetes before the age of 60 years (%)
World	2,184.4	11.8
Africa	306.0	8.9
Europe	144.7	7.7
Middle East and North Africa	428.6	24.5
North America and Caribbean	199.9	18.4
South and Central America	86.7	8.0
South-East Asia	301.2	6.9
Western Pacific	717.4	15.0

Adopted without permission from IDF, 2021.

T2D incidence and prevalence differ by geographic area, which influences the prevalence of the associated morbidity and mortality outcomes. Cardiovascular diseases are the leading cause of morbidity and death associated with T2D, and patients with T2D have a 15% greater risk of mortality compared to people without diabetes (Matheus et al., 2013). Environmental and genetic factors both have an influence in T2D epidemiology. After being exposed to an environment that promotes sedentary behaviour and high calorie intake, genetic factors start to take influence. Genome-wide association studies have shown common glycemic genetic variations for T2D. However, these only account for 10% of the variance in all traits, indicating that uncommon variants are significant. Different ethnic groups may have different specific phenotypes that make them more susceptible to certain clusters of cardiovascular disease risk factors, such as insulin resistance, hypertension, and dyslipidaemia (Wong et al., 2016).

2.4 Type 2 diabetes risk factors

2.4.1 Ethnicity and genetic susceptibility

The prevalence and incidence of T2D varies significantly around the world depending on the ethnicity and region, with China being the global epicentre of the diabetes epidemic (Hu, 2011; IDF, 2021). In South Africa, T2D prevalence has been shown to be high among the coloured population compared to the Indian and Black populations (Pheiffer et al., 2021). Although no definite reasons have been identified, contributing factors such as genetic susceptibility and gene-environment interactions, socioeconomic development and modern lifestyle factors (that encourage obesity) have been hypothesized (Hu, 2011; Basu et al., 2013).

Genetic susceptibility has a significant role in the risk of developing T2D. Over the years, several genome-wide association studies for T2D have identified common genetic variants contributing to diabetes susceptibility (McCarthy, 2010). It has also been shown that most of these genes increase T2D risk through direct impacts on insulin secretion, while some act by inhibiting the action of insulin (Fuchsberger et al., 2016).

2.4.2 Unhealthy dieting, obesity and physical inactivity

The rise in T2D prevalence is driven by its main risk factors, namely unhealthy dieting obesity and physical inactivity, which are influenced by the rapid economic growth, and ageing

population (Basu et al., 2013; IDF, 2021). The global epidemics of T2D and obesity are largely caused by excessive calorie intake, but diet quality also has independent consequences. According to the Nurses' Health Study, the quality of lipids and carbohydrates had a significant impact on the development of T2D apart from BMI and other risk variables (Hu et al., 2001). A low-fiber and high-glycemic-index diet has been linked to an increased risk of T2D, while some dietary fats may contribute to insulin resistance (Wu et al., 2014). Additionally, sugar-sweetened beverages and soft drinks have been linked to a higher risk of metabolic syndrome, obesity, and T2D (Malik & Hu, 2019).

Obesity (body-mass index [BMI] ≥ 30 kg/m²) is a major risk factor for T2D (Bellou, et al., 2018; Ruze et al., 2023) and is connected to metabolic abnormalities that cause insulin resistance (Ruze et al., 2023). Increase in fat deposit, particular visceral and ectopic fat depots, has been linked to insulin resistance. Fat metabolism, including lipid oxidation is impaired due to insulin resistance (Wondmkun, 2020).

2.5 Pathophysiology of type 2 diabetes

In T2D, the body's regular regulation of glucose and fatty acids becomes impaired. It begins with a malfunction in the feedback loops between insulin action and insulin secretion, resulting in abnormal blood glucose levels (Galicía-García et al., 2020). The pancreatic hormone, insulin, is essential in regulating the metabolism of fatty acids and glucose. It enhances the uptake of glucose by skeletal muscle and adipose tissue, inhibits the liver's capacity to produce glucose, and inhibits the release of fatty acids from fat tissue. The factors that contribute to the pathogenesis of T2D impact both the secretion of insulin and its action as illustrated in the **Figure 2.2**. The amount of insulin that is available to target tissues is reduced by insufficient insulin production from pancreatic β -cells. Furthermore, the efficiency of insulin in its target tissues is impacted by pathways connected to insulin resistance.

Insulin resistance refers to impaired response of target tissues, particularly muscle, liver, and fat tissues to insulin action (Czech, 2017). In response to insulin resistance, pancreatic β -cells produce more insulin to compensate for insulin action, which results in hyperinsulinemia and eventually, pancreatic β -cells dysfunction. Normally, insulin prevents adipose tissue from releasing fatty acids. This anti-lipolytic action of insulin is impaired by insulin resistance, increasing the release of fatty acids into the bloodstream (Galicía-García et al., 2020). As a result, the level of circulating fatty acids is increased (Galicía-García et al., 2020).

Hyperglycemia and increased circulating fatty acids set off a negative feedback loop that further amplifies insulin resistance and insulin secretion.

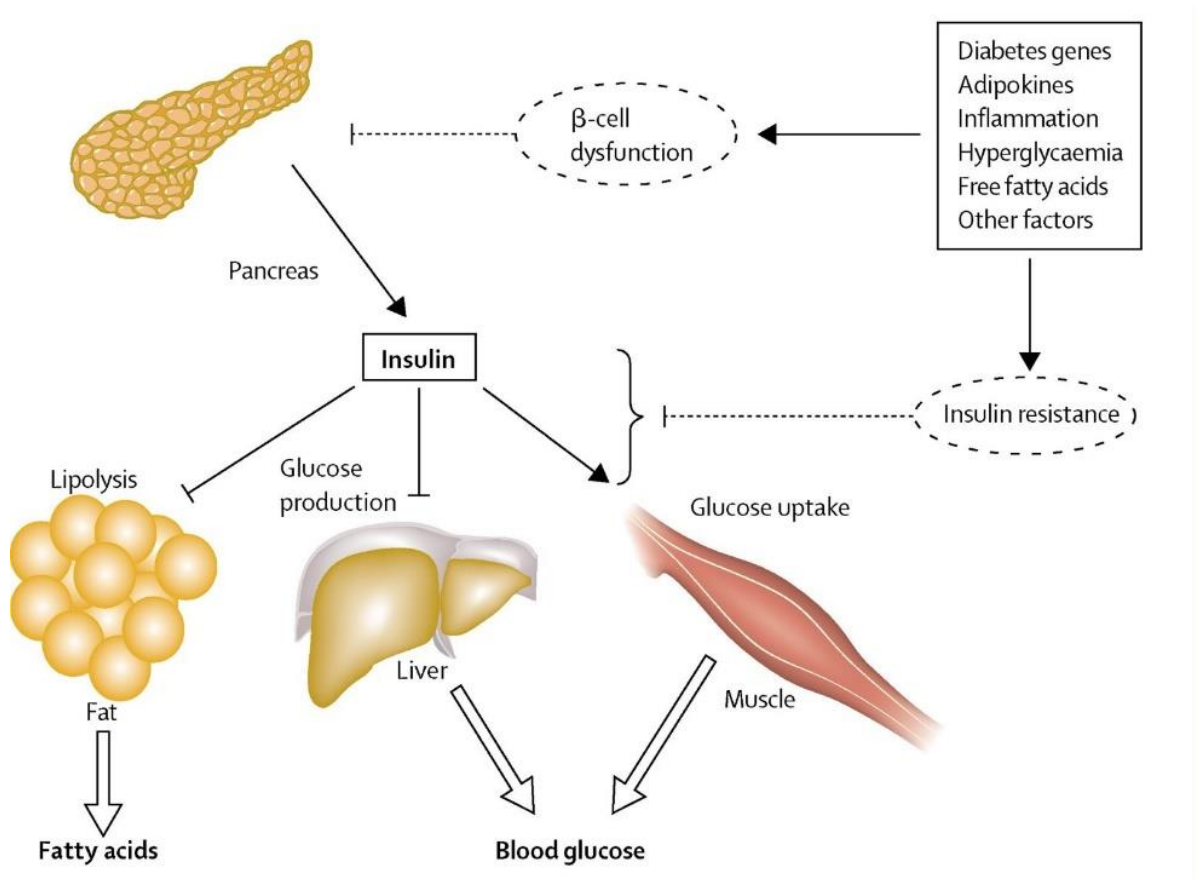


Figure 2.2: Pathophysiology of T2D (*adopted without permission from Stumvoll et al., 2005*).

The exact underlying mechanisms of the physiopathology of diabetes is not yet fully understood. However, it is well established that oxidative stress plays a significant role in all types of diabetes, notably T2D and development of diabetic complications. T2D is linked to increased oxidative stress caused by several disorders, such as hyperglycemia, inflammation, and dyslipidaemia (Giacco & Brownlee, 2010).

2.6 Oxidative stress in type 2 diabetes

Oxidative stress is known as an imbalance between the production and removal of reactive oxygen species (ROS) (Burgos-Morón et al., 2019). Chronic hyperglycemia induces ROS

overproduction resulting in detrimental cellular events such as formation of advanced glycation end-products formation (AGE), activation of protein kinase C, as well as polyol and hexosamine pathways (Giacco & Brownlee, 2010) (**Figure 2.3**). Under diabetic conditions, ROS overproduction exceeds the scavenging capacity of the cell's antioxidant defense causing damage to proteins, lipids, and DNAs. Moreover, ROS induced by diabetes trigger inflammation, which in turn worsens the oxidative stress.

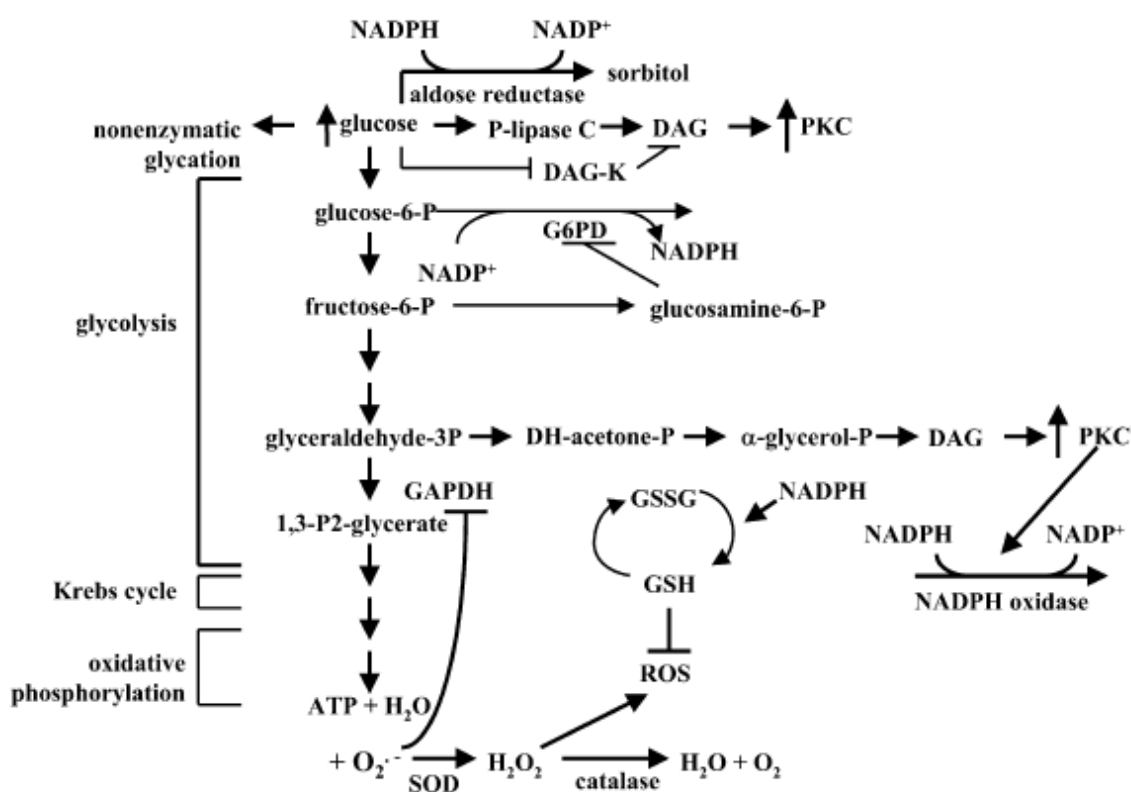


Figure 2.3: Schematic diagram of pathways that contribute to oxidative stress in response to increased glucose flux (*adopted without permission from King and Loeken, 2004*).

Examples of ROS that results in oxidative stress include nitric oxide, peroxynitrite, superoxide ion, hydrogen peroxide and singlet oxygen (Burgos-Morón et al., 2019). ROS are pro-oxidant oxygen species produced by normal cell metabolism and perform vital biological functions. But ROS can result in macromolecules damage, organelle injury and neuronal synaptic dysfunction due to their high chemical reactivity (Burgos-Morón et al., 2019). As a result,

protective pathways are activated to alleviate damage from ROS and regulate the production of ROS. Antioxidant molecules such as vitamins C, D, and K, as well as antioxidant enzymes, including superoxide dismutase (SOD), glutathione reductase (GSH-reductase) and glutathione peroxidase (GSH-Px), catalase, peroxiredoxins, thioredoxins are some key components of these protective pathway (Maiese et al., 2009; Burgos-Morón et al., 2019). **Figure 2.4** shows the biological or endogenous ROS and the action of a network of antioxidant defence systems. The progression of oxidative stress in diabetes has been linked to several diabetic complications.

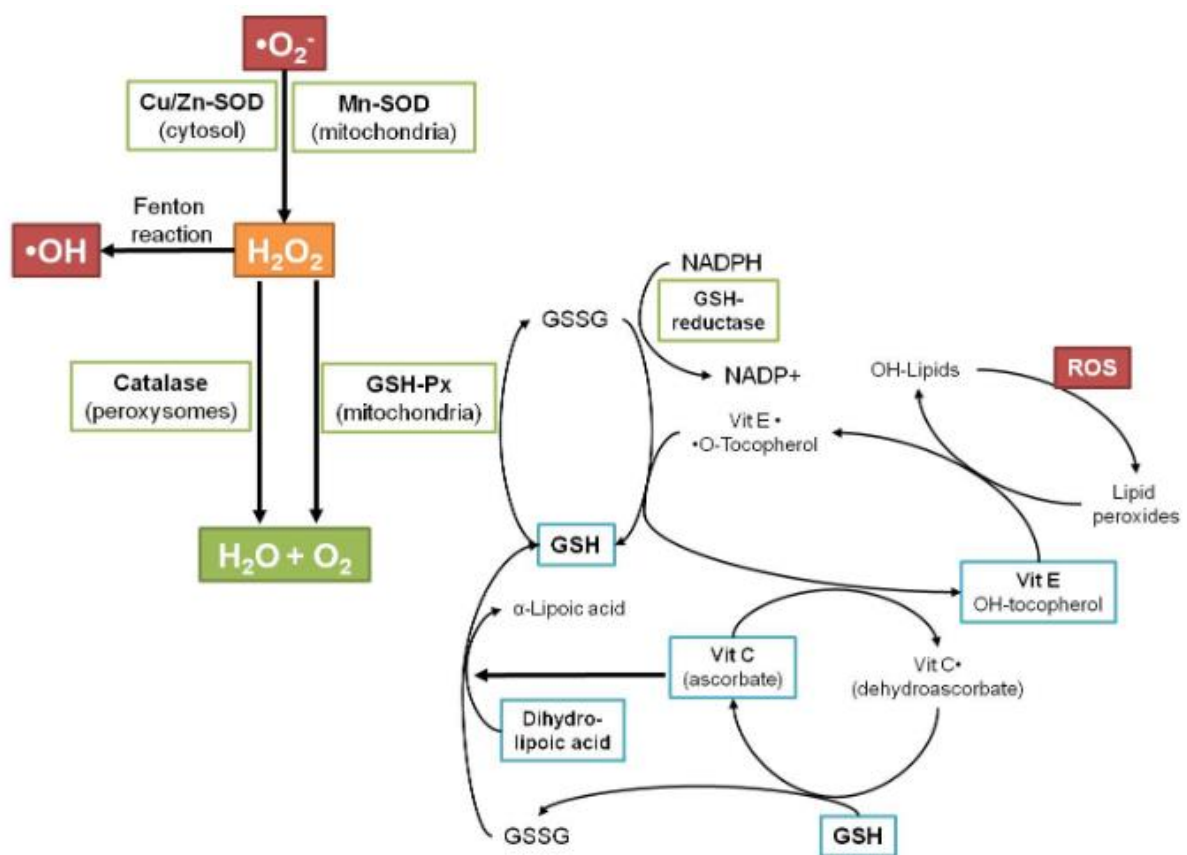


Figure 2.4: Biological antioxidant defence system (*Adopted without permission from Lazo-de-la-Vega-Monroy and Fernández-Mejía, 2013*)

2.7 Diabetes complications

2.7.1 Microvascular complications

Diabetic microvascular complications include damage of small vessels such as capillaries. The primary microvascular complications caused by chronic hyperglycemia include diabetic nephropathy, neuropathy, and retinopathy. These complications have several underlying mechanisms, including the generation AGEs, the development of proinflammatory microenvironment, and the induction of oxidative stress. Among individuals affected by diabetes, retinopathy accounts for 13% of microvascular complications, with neuropathy and nephropathy following at 4% and 3%, respectively (Ogurtsova et al., 2022).

Diabetic retinopathy

Diabetic retinopathy is one of the major primary causes of loss of vision (Leasher et al., 2016; Saigol, 2019). It is regarded as a neurovascular condition with retinal ganglion and glial cells damage (Rodríguez-Carrizalez et al., 2014), and it is classified as either non-proliferative or proliferative retinopathy. Patients with T2D have a higher chance of developing retinopathy, especially those with hypertension (Chawla et al., 2016). There are numerous kinds of pathogenic factors such as osmotic stress, aldose reductase modulation, AGEs accumulation and oxidative stress that may be involved in diabetic retinopathy (Fowler, 2008 ; Yamagishi et al., 2012).

Diabetic neuropathy

Diabetic neuropathy is a type of nerve damage that can happen to a person with diabetes. symptoms can vary from pain and numbness in the legs and feet to issues with the digestive tract, urinary tract, blood vessels and hearing (IDF, 2021). Up to 50% of diabetic people develop neuropathy with time (Andrei & Amorin, 2018). The duration and severity of hyperglycemia have a direct correlation with the risk of developing neuropathy. Additionally, some diabetic patients may have genes that make them susceptible to developing neuropathy (Fowler, 2008). The Pathophysiology of diabetic neuropathy includes both metabolic and vascular factors. Hyperglycemia leads to elevated levels of sorbitol and fructose, generation of AGEs (Giacco & Brownlee, 2010), overproduction of reactive oxygen species (ROS) and

inappropriate activation of protein kinase C. These factors have been collectively associated with nerve injury (Giacco & Brownlee, 2010).

Diabetic nephropathy

Diabetic nephropathy is one of the primary causes of significant renal failure (Faselis et al., 2020). Up to 40% of all diabetic patients develop this diabetic complication (Stavniichuk et al., 2014). Diabetic nephropathy is caused by glomerular damage resulting from hyperglycemia and hypertension. Other major risk factors are obesity, insulin resistance, ethnicity and dyslipidaemia (Raile et al., 2007). Diabetic nephropathy has a complicated pathophysiology that involves many interrelated mechanisms. Hyperglycemia causes glomerular hemodynamic alterations and activates the renin-angiotensin-aldosterone system, which are among the primary causes of diabetic nephropathy. The kidneys experience oxidative stress, inflammation, and extracellular matrix buildup, which results in renal fibrosis. The progression of the diabetic nephropathy has been linked to formation of AGEs, microvascular alterations, and kidney hypertrophy (Faselis et al., 2020).

2.7.2 Macrovascular complications

Diabetic macrovascular complications involve damage to large vessels including arteries and veins. The primary factor causing diabetes-related macrovascular complications is atherosclerosis. Atherosclerosis is a chronic inflammatory arterial disease, that comprises of peripheral vascular disease, cerebral vascular disease, and ischemic heart disease. Atherosclerosis develops in more than 50% of diabetic population, which results in morbidity and mortality (Herrington et al., 2016; Kobiyama and Ley, 2018). The primary causes of morbidity and mortality in diabetic patients are long-term macrovascular complications, including coronary artery disease, myocardial infarction and stroke (Huang et al., 2017; Cole & Florez, 2020). Diabetic patients are susceptible to diabetic macrovascular complications (Tan & de Haan, 2014), thus requires anti-diabetic medications that can suppress hyperglycemia, and prevent hyperglycemia-related oxidative complications.

2.8 Management of type 2 diabetes

The primary treatment strategy for managing T2D is lowering blood glucose levels to a healthy range, while avoiding excessively low levels (hypoglycemia). This is accomplished through a combination of therapeutic interventions, including antihyperglycemic medications, insulin administration, dietary guidance, and regular exercise.

The key element of T2D therapy is promoting a lifestyle that includes a balanced diet, regular exercise, and maintenance of a healthy body weight (IDF, 2021; WHO, 2023). The two main lifestyle changes include a balanced diet and physical activity (Marín-Peñalver et al., 2016). Evidently, engaging in regular physical activity is associated with a potential reduction in the susceptibility to T2D by approximately 30–50%, due to a decreased occurrence of obesity (Wu et al., 2014).

Exercise has two main advantages that can delay T2D, which are as follows. It improves the skeletal muscle cell's ability to induce blood flow into the muscle, which facilitates plasma glucose uptake/utilization, while reducing or eliminating inflammation and oxidative stress (Venkatasamy et al., 2013). Also, it lowers excessive intra-abdominal fat, a proven risk factor that encourages insulin resistance (Strasser, 2012). It has been demonstrated that exercising moderately increases glucose uptake by 40% (Ross, 2003). Additionally, it has been reported that T2D patients that exercise more notice an appreciable improvement in their ability to control blood sugar (Umpierre et al., 2011).

For diabetics, there is a strong advocacy for a balanced diet, which refers to a well-structured eating plan that enforces careful monitoring of an individual's intake of fats, sugars, carbohydrates, and alcohol. The advocacy also emphasizes on promoting the consumption of nutrient-rich foods, including vegetables, fruits, whole grains, and dairy products that possess minimal or reduced fat content (Ley et al., 2014; Maiorino et al., 2017; Wei et al., 2023). The WHO recommends 400g/day consumption of fruits and vegetables to reduce the risk of excessive weight gain and non-communicable diseases such as type 2 diabetes, cardiovascular disease and related metabolic health problems (WHO, 2013). Plant-based diets offer a wide range of antioxidants, such as polyphenols, which inhibit glucose absorption, stimulate insulin secretion, decrease hepatic glucose output, and promote glucose uptake.

However, in persistent hyperglycemic situations, blood glucose lowering medications are normally prescribed as mono/combination therapy.

2.8.1 Commercially available antihyperglycemic medications

Over the past decades, a wide range of orally administered and injectable medicines have been developed for the treatment of T2D, which are used as mono/combination therapy (Dahlén et al., 2022). The primary objective of these medications is to improve glycemic control, decrease body weight, and mitigate the risk of cardiovascular damage (Chaudhury et al., 2017).

Based on their mechanism of action, these medications can be divided into the following categories: "Sensitizers", which assist the action of the insulin (including biguanides and thiazolidinediones); "Secretagogues," which elevate the amount of insulin produced (including sulfonylurea, non-sulfonylurea, and insulin); alpha-glucosidase inhibitors; and incretin-based therapies (including glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors). Other groups include the sodium-glucose cotransporter 2 inhibitors, which reduces hyperglycemia by increasing urinary glucose excretion (van Baar et al., 2018); amylin agonists, which are analogues of the β -cell hormone amylin; and insulin, an endogenous blood glucose lowering hormone (Chaudhury et al., 2017).

2.8.1.1 Sensitisers

Biguanides

Metformin is one of the oldest biguanide medication for T2D treatment and is considered the first-line medication for the treatment of T2D if lifestyle changes are unsuccessful (Rena et al., 2017; Salvatore et al., 2020). Metformin has a variety of molecular mechanisms and sites of action (Rena et al., 2017). Its antihyperglycemic effects have been shown to take place through direct or indirect reduction in hepatic glucose production. Moreover, in both *in vitro* and *in vivo* experiments, the antioxidant activity of metformin has also been shown to reduce the production of ROS. For example, it inhibits mitochondrial respiration by diminishing mitochondrial complex I activity, thereby decreasing mitochondrial respiration (Cameron et al., 2018; Wu et al., 2017). In addition, it stimulates nitric oxide release and reduces nitric oxide stress (Sambe et al., 2018). The most common side effects of metformin, which are typically minor and temporary are gastrointestinal side effects, including anorexia, nausea, abdominal pain, and diarrhoea. Metformin also reduces vitamin B₁₂ intestinal absorption (DeMarsilis et al., 2022).

Thiazolidinediones

Thiazolidinediones (TZDs), also called glitazones, includes pioglitazone and rosiglitazone (Nanjan et al., 2018). Their primary mechanism of action involves binding to PPAR- γ receptors within adipocytes, thus maximising insulin sensitivity in the cells (Dowarah & Singh, 2020). TZDs also enhance the regulation of blood glucose levels by preserving the functionality of pancreatic β -cells (Nanjan et al., 2018). Additionally, the use of TZDs has been linked to a low risk of cardiovascular diseases (El-Atat et al., 2005; Nanjan et al., 2018), low levels of blood lipids, including free fatty acids (Nanjan et al., 2018) and anti-inflammatory properties (Davidson et al., 2018). TZDs also have some side effects. One of the most concerning side effects of TZDs is bone loss. Thiazolidinediones can influence the differentiation of bone marrow stem cells into adipocytes (fat cells) instead of osteoblasts (bone cells). This can lead to low bone density and an increased risk of bone fractures (Meier et al., 2008). Other potential side effects of TZDs include weight gain, fluid retention, edema, anaemia, cardiac failure and bladder cancer (Crunkhorn, 2007; Lebovitz, 2019).

2.8.1.2 Secretagogues

Sulfonylureas

Sulfonylureas (SUs) exhibit a distinctive affinity for the sulfonylurea receptors located on pancreatic β -cells. Through a direct mechanism, they stimulate the release of insulin and may function independent of glucose levels (Bösenberg & van Zyl, 2008). However, they are not effective in stimulating the release of insulin from a dysfunctional pancreas in the advanced stages of T2D. SUs are classified into three generations. The first comprises drugs like tolbutamide, tolazamide, chlorpropamide, and acetohexamide, while the second generation comprises glyburide (glibenclamide), glipizide, and gliclazide. The third generation includes glimepiride. Their mechanism of action centres around binding to the transmembrane sulfonylurea receptor (SUR-1), a regulatory component of ATP-sensitive K^+ channels. The binding triggers channel closure, influx of calcium ion and pre-formed insulin release. A notable consequence of this process is the risk of hypoglycemia (Lamos, 2013; Monami et al., 2014). This risk is particularly pronounced among elderly patients (Lamos, 2013), individuals on multiple medications, those with impaired renal function, and those grappling with liver disease. Alongside hypoglycemia, SUs may lead to side effects such as weight gain, skin reactions, acute porphyria and occasional hyponatremia (Bösenberg & van Zyl, 2008; Lim &

Chong, 2015). For patients with cardiovascular disease, SUs treatment holds critical significance and may increase risk of cardiovascular events (Bell, 2006). Additionally, concerns extending to potential bone fracture development have been linked to the use of SUs (Zhang et al., 2020). However, reports suggest the risk may be moderate (Zhang et al., 2020).

Meglitinides

Meglitinides, also called glinides, include nateglinide, repaglinide, and mitiglinide. Their mechanism of action involves binding to the SUR-1, which results in the closure of K-ATP channels and insulin secretion in a similar fashion as the SUs (Marín-Peñalver et al., 2016). Nonetheless, their binding affinity is comparatively weaker, thus they dissociate from the SUR-1 binding site faster compared to the SUs (Marín-Peñalver et al., 2016). Similar to SUs, meglitinides may lead to weight gain, yet their advantage lies in a reduced risk of hypoglycemia due to weaker receptor binding (Bösenberg & van Zyl, 2008). Meglitinides are generally well tolerated medications, with notable adverse effects including weight gain and episodes of hypoglycemia (DeMarsilis et al., 2022).

2.8.1.3 α -Glucosidase inhibitors

Alpha-glucosidase inhibitors (AGIs) have three common types namely, acarbose, miglitol, and voglibose. These inhibitors can be derived from medicinal plants, microbes (including bacteria and fungi), or synthesized chemically (Chougale et al., 2009). AGIs target the membrane-bound gastrointestinal enzyme α -glucosidase. This enzyme facilitates the breakdown of disaccharides and oligosaccharides and the absorption of glucose monomers in the small intestine (Kumar et al., 2011). The AGIs act as competitive inhibitors of α -glucosidases due to their strong affinity for the enzymes. The mechanism of action of AGIs involves binding to α -glucosidases, leading to a decrease in carbohydrate absorption in the small intestine, thus lowering postprandial blood glucose and insulin levels (Van De Laar et al., 2005). The most common side effects of AGIs are mostly gastrointestinal-related and include symptoms like vomiting, flatulence, diarrhoea, as well as headaches and insomnia (Marín-Peñalver et al., 2016; DeMarsilis et al., 2022).

2.8.1.4 Incretin-based therapy and dipeptidyl peptidase 4 inhibitors

Incretins are metabolic hormones secreted postprandially by L cells of small intestine to enhance insulin release from pancreatic β -cells (Hansen et al., 2010). These hormones are secreted after a meal intake. The two incretin hormones, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP), are responsible for 50 to 70% of the insulin release that occurs after oral glucose ingestion (Baggio & Drucker., 2007). Their secretion is similar to insulin secretion (Baggio & Drucker., 2007). However, these incretin hormones are subject to rapid degradation by the dipeptidyl peptidase 4 (DPP-IV) enzyme (Godinho et al., 2015). Patients with T2D have severely reduced or no incretin-mediated insulin production due to a drop in GLP-1 levels, which also reduces the ability of pancreatic β -cells to secrete insulin in response to glucose (Bösenberg & van Zyl, 2008). Incretin-based therapies refers to GLP-1 analogues, which mimic the action of native GLP-1 and are resistant to DPP-IV. GLP-1 analogues, due to their ability resist DDP-IV, can increase insulin secretion and inhibit glucagon release, leading to lowered blood glucose levels and reduced glycated haemoglobin (HbA1c) levels (Holst, 2019). However, these analogues can lead to side effects such as nausea, vomiting, headaches, indigestion, constipation, light-headedness, increased sweating and loss of appetite (Holst, 2019). Examples of GLP-1 analogues include Exenatide, Liraglutide, Exenatide, Albiglutide, Dulaglutide, Lixisenatide and Semaglutide (Gupta, 2013; Yu et al., 2018; Holst, 2019).

Dipeptidyl peptidase 4 (DPP-IV) inhibitors are another class of incretin-based medicines, which increase the availability and levels of endogenous incretin like GLP-1 (Godinho et al., 2015). They insulintropic effects that help to maintain normal blood glucose levels (Godinho et al., 2015). These inhibitors can increase glucose-stimulated insulin secretion, decrease the secretion of glucagon and support β -cell proliferation and survival (Pathak & Bridgeman, 2010). They work by inhibiting DPP-IV and thus prevents the deactivation of GLP-1 (Pathak & Bridgeman, 2010; Omar & Ahrén, 2014). Examples of available DPP- IV inhibitors include Alogliptin, Linagliptin, Gemigliptin, Anagliptin, Teneligliptin, Saxagliptin, Sitagliptin, Vildagliptin, Omarigliptin, and Linagliptin (Gallwitz, 2019). The use of IDD-IV inhibitors is associated with severe arthralgia (Men et al., 2017).

2.8.1.5 Sodium glucose co-transporter 2

Sodium glucose co-transporter 2 (SGLT-2) inhibitors are a class of blood glucose lowering medications that block the reabsorption of glucose in the kidneys (Nespoux & Vallon., 2018). They reduce hyperglycemia through enhanced urinary glucose excretion (van Baar et al., 2018; Nespoux & Vallon., 2018). SGLT-2 inhibitors can be derived from the flavonoid phlorizin, which is a natural compound extracted from the bark of apple trees (Abbas et al., 2019). Some phlorizin-based synthetic molecules are canagliflozin, dapagliflozin, luseogliflozin, tofogliflozin, empagliflozin and ipragliflozin (van Baar et al., 2018; Tentolouris et al., 2019). These inhibitors do not only exhibit glycemic control effects, but also improve cardiovascular function, lower blood pressure, cause weight loss and normalize lipid profiles (Tentolouris et al., 2019). However, SGLT2 inhibitors are associated with some adverse effects such as urinary tract infections, acute kidney failure, increased risk of bladder cancer, amputations and bone fractures (Choi., 2016; Tentolouris et al., 2019).

2.8.1.6 Amylin analogues

Human amylin is a hormone made up of a single chain of 37 amino acids that is secreted along with insulin by pancreatic β -cells (Bösenberg & van Zyl, 2008). Human amylin maintains fasting and postprandial blood glucose by reducing the rate of gastric emptying, inhibiting the secretion of glucagon and regulating food intake (Padhi et al., 2020). Amylin analogues are a class of chemical analogues developed to mimic the action of human amylin since the hormone is deficient in diabetic patients. The available medication in this class is pramlintide, which exerts its effects by maintaining blood glucose homeostasis in the same way as human amylin (Bösenberg & van Zyl, 2008). The most common side effects of pramlintide are nausea, vomiting, headache, anorexia, reduced appetite and hypoglycemia (Hieronymus & Griffin, 2015).

Due to the negative side effects of oral hypoglycemic medications used to treat diabetes, there has been an increased interest in herbal agents (El-Refaei et al., 2015). Several medicinal plants and their extracts have been reported to have potential relevance in the treatment of diabetes as they are abundant sources of polyphenols, flavonoids, gallotannins, amino acids, and other related phenolics with antidiabetic, anti-hyperlipidaemic, and antioxidant potentials (Hasan and Mohieldein, 2016; Vinayagam et al., 2016).

Natural antioxidants found in fruits can reduce oxidative stress and impede the development of metabolic and cardiovascular complications associated with diabetes (Wallace et al., 2019). The management of several chronic diseases, such as diabetes, hypertension, obesity, and cardiovascular complications, may benefit from the use of non-edible wastes, such as the peels of many fruits, which typically contain significantly more phenolics and other phytochemicals than their respective edible tissues (Wadhwa et al., 2016; Rafiq et al., 2018; Sagar et al., 2018).

2.9 Fruit wastes as potential sources of bioactive phytochemicals

Fruits and vegetables are among the most popular horticulture crops in the world, which is primarily attributed to their palatable quality, health benefits and high nutrient content (Sagar et al., 2018). Fruits are the potential source of a wide variety of nutrients, including micronutrients (vitamins, minerals, and bioactive substances) as well as macronutrients (carbohydrates, fats, and proteins). Around 909.64 million metric tons of fresh fruit were produced globally in 2021, which has doubled since 2000 (Statista, 2023). Nevertheless, up to 60% of the world's horticulture produce (fruits, vegetables, and root crops) is either wasted or lost early in the supply chain (Gupta et al., 2019).

Despite the enormous amounts of fruit produced and processed, there are still significant losses as a result of handling practices, inadequate facilities, and outdated technology that prevents the utilization of by-products from processing waste (Sagar et al., 2018). For instance, in South Africa, 10 million tons of food is wasted yearly with the wastes from fruits and vegetables contributing 44%, while 90% of this food waste end up in landfills (Oelofse & Nahman, 2012). Food waste disposal in landfills is detrimental to the environment, it has a negative impact on the environment through leachate, odours, pests, and atmospheric emissions, especially emissions of greenhouse gas like carbon dioxide and methane (Oelofse & Nahman, 2012). Methane is a potent greenhouse gas that affects climate change. For long, methane has had an environmental impact that is 28–36 times greater than that of carbon dioxide (Balcombe et al., 2021). Peels, seeds and pomace from fruits are among the most prevalent agro-wastes, including in South Africa (Khan et al., 2015). It is therefore imperative that fruit wastes are maximally utilized, including as sources of potential therapeutic agents.

Fruit wastes are a rich source of potent phytochemicals, such as phenolic compounds, flavonoids, tannins, pectin, vitamins, minerals, dietary fibres, and other bioactive compounds as shown in **Table 2.2**. Studies have shown that higher contents and composition of

phytochemicals and essential nutrients are available in the seeds, peels, and other parts of fruits and vegetables that are not commonly consumed than in the edible parts (Rudra et al, 2015; Wadhwa et al, 2016; Kumar et al, 2020). Many of these phytochemicals are bioactive compounds that affect metabolic functions and may have positive health effects.

Table 2.2: Fruit wastes as sources of bioactive compounds

Fruit waste	Bioactive phytochemicals compounds	Refences
Apple peel and pomace	p-coumaric acid, ferulic acid, and cafeic acid Flavonoids, proanthocyanidins, and flavan-3-ols (epicatechin, procyanidins)	(Leontowicz et al., 2007; Sudha et al., 2007)
Pomegranate peels	punicalin, punicalagin, punicalin, and ellagic acid	(Živković et al.,2018)
Grape pomace	Flavonol glycosides, anthocyanins, catechins, stilbenes	(Schieber et al.,2001)
Common grape seed	Procyanidin (isomers B1 – B8 and C1 - C4), gallic acid, (-)-epicatechin, galloyl(epi)catechin-(epi)catechin (isomers 1 – 6), Galloyl(epi)catechin-(epi)gallocatechin(+)-catechin, (+)-catechin, (epi)catechin gallate (isomers 1 and 2), quercetin (and its hexoside,, rhamnoside and glucuronide), ellagic acid, phloretin (and its glucoside and xyloglucoside) and amurenisin	Cádiz-Gurrea et al., 2017
Litchi seed	Procyanidin A1 and B2, epicatechin, quercetin, rutin, phlorizin, cinnamtannin B1 and B2, aesculitannin C and cyanidin-3-glucoside	Li et al., 2012; Jiang et al., 2013; Yao et al., 2021
Litchi peel	Catechin, epicatechin, epicatechin gallate, epigallocatechin gallate, procyanidin A1, A2, B1 and B2	Park et al., 2018

Fruit waste	Bioactive phytochemicals compounds	References
Banana leaves	Polyphenols (flavonoids and terpenoids)	(Marie-Magdeleine et al., 2010)
Guava peel and pulp	Melanin, polyphenolic substances, and dietary fiber	(Jiménez-Escrig et al., 2001)
Apricot seed	Gallic acid, 3,4-dihydrobenzoic acid, (+)-catechin, syringic acid, caffeic acid, rutin trihydrate, p-coumaric acid, trans-ferulic acid, resveratrol, apigenin-7-glycoside, quercetin, trans-cinnamic acid, naringenin, kaempferol and Isorhamnetin	Al Juhaimi et al., 2018
Mango peel	Carotenoids, vitamins, enzymes, dietary fibers, syringic acid, quercetin, mangiferin pentoside, and ellagic acid	(Ajila et al., 2007; Ajila et al., 2010)

2.10 Polyphenols

Polyphenols, also known as polyhydroxyphenols, are secondary plant metabolites that are utilised to defend plants from infections or ultraviolet radiation. These natural phytochemicals can be found in a variety of plant sources, including fruits and vegetables (Bahadoran et al., 2013). According to research, consuming a lot of polyphenols may reduce the risk of getting diabetes (Pandey & Rizvi, 2009). Remarkably, researchers have discovered over 8000 different polyphenol compounds (Pandey & Rizvi, 2009). Polyphenols can be categorized into many different classes depending on the strength of the phenolic ring, but the major categories of polyphenols are phenolic acids, flavonoids, stilbenes, tannins, and lignans, which sub-categorized into different groups as shown in **Figure 2.5** (Abbas et al., 2016; Tijjani et al., 2020).

Besides phenolic acid, flavonoids have a variety of biological properties, such as antioxidant, antibacterial, anticancer, and cardioprotective effects. Flavonoids are the predominant polyphenols in plant-based foods with more than 4000 different flavonoids that have been described (Bahadoran et al., 2013). In general, flavonoids are made up of a basic structure of diphenylpropanes (C6-C3-C6) with two aromatic rings connected by a three-carbon bridge.

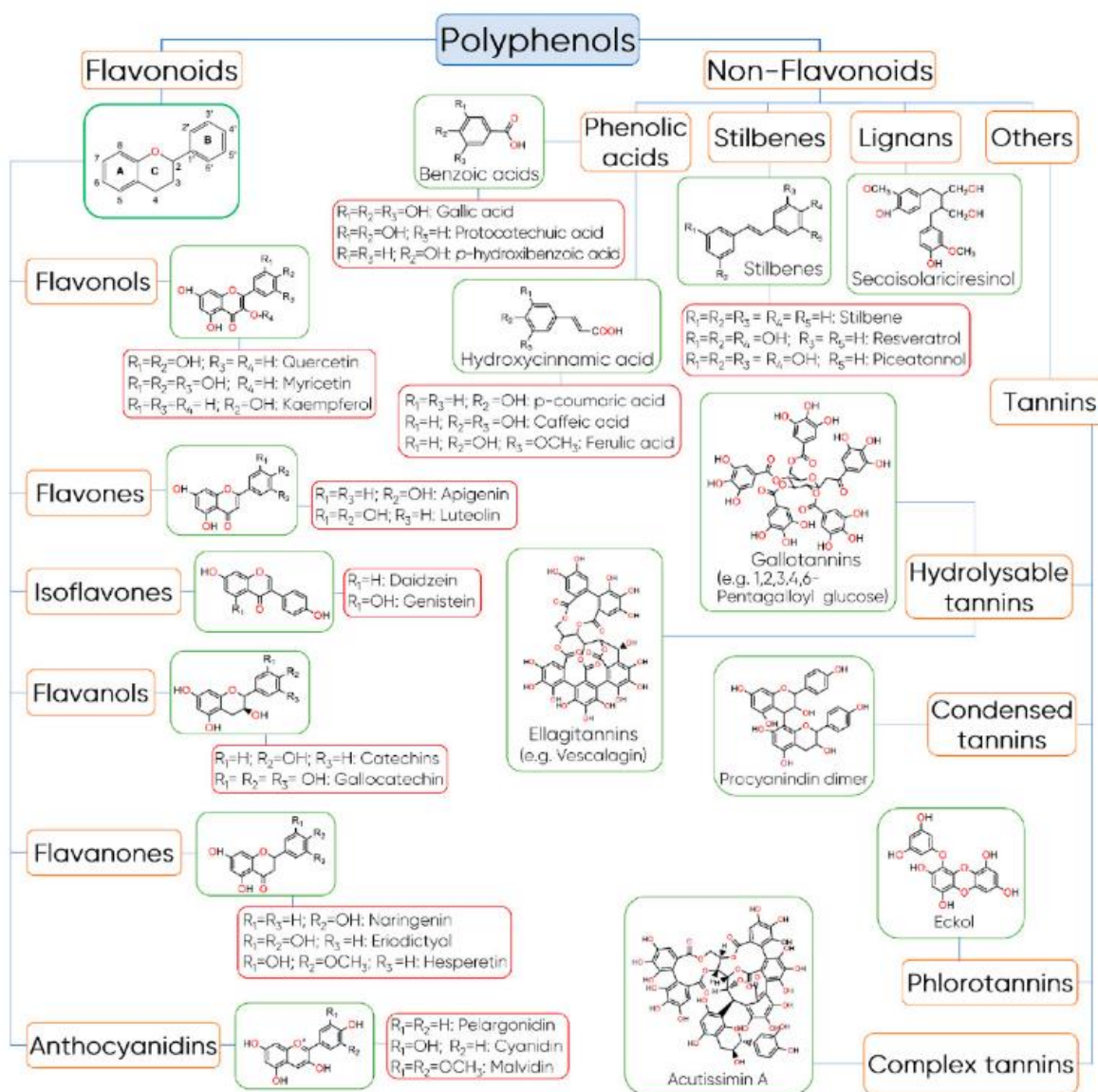


Figure 2.5: The major categories of plant-based polyphenols (*adopted without permission from Tijjani et al., 2020*).

Based on variations in the structure of the heterocyclic aromatic rings, flavonoids can be categorized into six major subclasses – flavonols, flavones, isoflavones, flavanols, anthocyanidins, and flavanones (Durazzo et al., 2019). The most abundant flavonoids present in a variety of food sources are flavonols, namely quercetin, kampferol, and myricetin. They are commonly found in plants like blueberries, leeks, broccoli, onions, and curly kale. Isoflavones, which include daidzein, genistein, and glycitein, are another important category. The main sources of these flavonoids with estrogen-like structures are soybeans and soy products (Abbas et al., 2016). *In vitro* and *in vivo* studies have provided evidence that

flavonoids have the ability to delay the onset of diabetes and its complications (Al-Dosari et al., 2017; Addepalli and Suryavanshi, 2018). They also inhibit the actions of hepatic enzymes and exhibit positive effects on both lipid and carbohydrate metabolism (Testa et al., 2016; Al-Ishaq et al., 2019).

Stilbenes are a group of polyphenols that contain two phenyl moieties connected by a two-carbon methylene bridge (Chong et al., 2009). Among stilbenes, resveratrol is one of the most well-known and extensively researched stilbenes due to its potent biological effects (Sirerol et al., 2016). Grapes, almonds, beans, blueberries, bilberries, peanuts, vines, cranberries, mulberries, plums, and wine are rich sources of stilbenes (Sirerol et al., 2016). The consumption of stilbenes has been associated with a reduction in overall mortality, as well as a lower risk of obesity, diabetes, and hypertension (Tresserra-Rimbau et al., 2014; Miranda et al., 2016; Grosso et al., 2017).

Lignans are organic, non-flavonoid phytoestrogens made up of 2,3-dibenzylbutane structure and are present in many kinds of plants, including flaxseed, wheat, tea, and fruits (Gorzkiewicz et al., 2021). Dietary lignans exist in aglycone and glycoside forms. The major dietary lignans include secoisolariciresinol, matairesinol, medioresinol, pinioresinol, lariciresinol, and syringaresinol (Pandey & Rizvi, 2009). Lignans have been shown to have anticancer, antihypertensive, antiviral and antioxidant effects (Deka et al., 2022; Mukhija et al., 2022; Xu et al., 2022; Jang et al., 2022).

Tannins are a large group of complex water-soluble polyphenols that are produced by a variety of plants (Al-Salih, 2010). Based on their chemical structure, tannins can be divided into four main groups, namely hydrolysable tannins (galotannins and ellagitannins), condensed tannins, phlorotannins, and complex tannins. Tannins can be found in a variety of foods, including cereals, beans, nuts, dried fruits, pomegranates, strawberries, barley, peaches, mint, basil, and oats. They can also be found in drinks including coffee, wine, fruit juice, beer, black tea, green tea, and coffee (Laddha & Kulkarni, 2019). These polyphenols comprise of the phenol hydroxyl groups which influences their antioxidant and antimicrobial properties (Akhtar et al., 2015).

Documented evidence has revealed that tannins are a key factor in the inhibition of α -amylase activity in some plants (Xiao et al., 2013; Ćorković et al., 2022). It has been proven that extracts rich in tannin from strawberries, raspberries, red grapes, red wine, and green tea are effective in inhibiting α -amylase (McDougall & Stewart, 2005). On the other hand, blueberry extracts lost some of their α -amylase inhibitory effects when the tannins were eliminated (McDougall

& Stewart, 2005). In fact, ellagitannins, a type of hydrolysable tannin, has been shown to be a key α -amylase inhibitory component of several berries (Xiao et al., 2013; Ćorković et al., 2022), suggesting the potential of fruit tannins to regulate blood glucose levels.

Pomegranate is one fruit that has a high tannin content, especially the water-soluble hydrolysable tannins known as ellagitannins. The fruit's health benefits have been linked to these phytochemical principles.

2.11 Pomegranate

Pomegranate (*Punica granatum* L.) is a fruit tree belonging to the family Lythraceae (**Table 2.3**), which grows between 5 to 10 meters tall (**Figure 2.6a**). It is planted in a variety of subtropical and tropical regions around the world including several countries in Asia, Europe, South and North America, Africa, and Australia (Holland et al., 2009). It is native to Central Asia but has a wide geographic range due to its ability to adapt to a variety of climatic conditions (Teixeira da Silva et al., 2013). Around 300, 000 hectares of pomegranate trees are thought to be grown worldwide, with a 3 million metric ton annual yield (Venkitasamy et al., 2019). South Africa competes with nations like Chile, Argentina, and Australia as one of the leading pomegranate growers in the Southern Hemisphere (Brodie, 2009).

Table 2.3: Taxonomic classification of Pomegranate

Classification	Name
Kingdom	Plantae
Sub-kingdom	Tracheobionta (vascular plants)
Super-division	Spermatophyta (seed plants)
Division	Magnoliophyta (flowering plants)
Class	Magnoliopsida (Dicotyledons)
Sub-class	Rosidae
Order	Myrtales
Family	Punicaceae, formerly, Lythraceae, currently
Genus	<i>Punica</i> L. (pomegranate)
Species	<i>Punica granatum</i> L. <i>Punica protopunica</i> Balf

Adopted without permission from USDA, 2014.

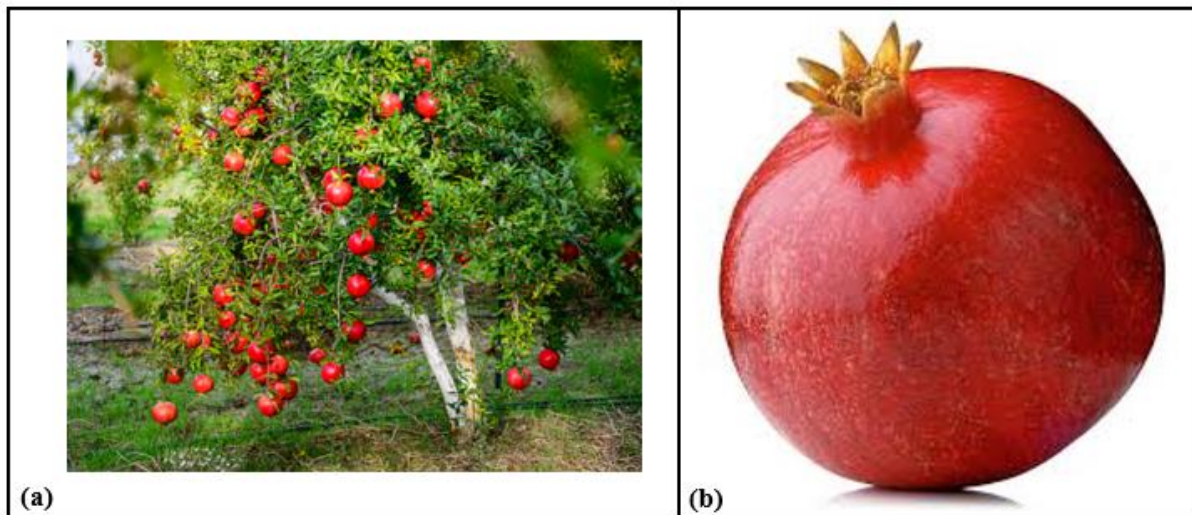


Figure 2.6: Image showing (a) pomegranate tree with fruits and (b) pomegranate fruit.

More than 500 varieties of *P. granatum* are known to exist (Stover & Mercure, 2007; Kahramanoğlu and Usanmaz, 2016). South Africa's most popular varieties/cultivars are “Wonderful”, “Herskovitz” and “Acco” (Kahramanoğlu, 2019), with the widely grown cultivar being the “Wonderful” cultivar. The pomegranate fruit husk is round with a reddish-purple colour (Figure 2.6b). As shown in Figure 2.7, the fruit is made up of 10% seeds, 40% arils and peels which account for roughly 50% of the fruit's weight (Jalal et al., 2018). The juice can range in colour from a light to a deep reddish colour (Holland et al., 2009).

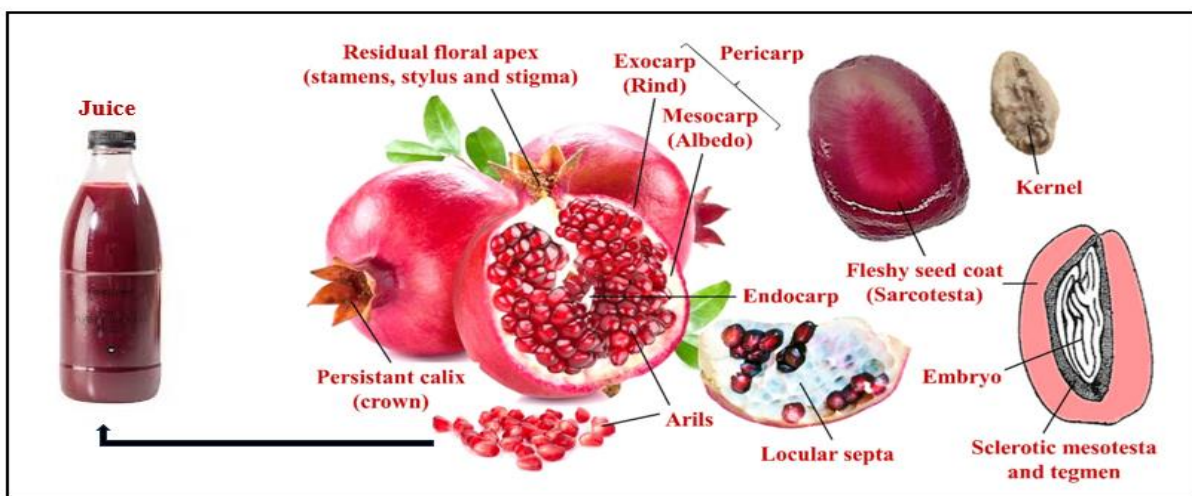


Figure 2.7: Composition of pomegranate fruit (adopted without permission from Montefusco et al., 2021).

Various pomegranate fruit components were used as traditional treatments for conditions such as stomach aches and bacterial infections (Holland and Bar-Ya'akov, 2014). The fruit also contains several potent therapeutic compounds that are advantageous to human health. Modern scientific research on health-beneficial metabolites, their therapeutic effects, and mechanisms of action on human and animal health supports the health benefits and traditional uses pomegranate.

2.11.1 Bioactivity potential of pomegranate plant in metabolic disorders

The potential bioactivity of pomegranate has been associated with its remarkable antioxidant activity (Glazer et al. 2012). In general, phenolic compounds are the main pomegranate bioactive compounds. Pomegranate plant has a wide variety of phytochemicals in all of its parts as indicated in **Table 2.4**, which are influential in their biological properties.

Table 2.4: Major phytochemicals in different part of pomegranate

Pomegranate parts	Phytochemicals
Peel	Phenolic acids, ellagitannis, flavonoids Several minerals
Juice	Tannins, ellagitannins (punicalin and punicalagin), flavonoids(anthocyanins flavanols and flavan-3-ols)
Bark and roots	Alkaloids (especially piperidine alkaloids), ellagitannins (punicalin and punicalagin)
Seed	Punicic acid, oleanolic, palmitic, linolenic acid
Leaves	Tannins, flavonoids, piperidine alkaloids, ellagic acids and esters ellagic acids with glucose
Flowers	Gallic and ursolic acid, also triterpenoids as asiatic acid and maslinic acid,

Adapted without permission from Magangana, et al., 2020.

The methanolic extract of pomegranate flower demonstrated antihyperglycemic action in Zucker diabetic fatty rats by modulating the expression of glucose transporter type 4 and insulin sensitivity (Huang et al., 2005). The ethanolic extract of the flower has also been proven to

reduce diabetes-induced hyperglycemia, insulin insensitivity, dyslipidaemia, and oxidative stress in STZ-induced (Manoharan et al., 2009) and high-fat-diet-induced (Tang et al., 2018) diabetic rats. Additionally, hydroalcoholic extracts (Adnyana, et al, 2014) and polyphenol-rich fraction (Yu et al., 2017) of the leaves exhibited anti-obesogenic effects by inhibiting pancreatic lipase activities and reducing fat intestinal absorption.

Pomegranate fruit has been shown to have the potential to ameliorate oxidative and metabolic diseases. Several studies have shown that extracts (water, methanol, and ethanol) or juice from the fruit's edible parts (arils) reduced blood glucose, increased liver glycogen content and insulin secretion, improved glucose tolerance, modulated insulin signalling factor expression, improved lipid profile, reduced lipid peroxidation and hepatic damage, and improved the antioxidant status of diabetic and oxidative stress-induced rats (Das et al., 2001; Chidambara et al., 2002; Das and Sama, 2009; Prasetyastuti et al., 2014; Salwe et al., 2015; Thanh et al., 2019; Gharib & Kouhsari, 2019).

In high-fat-diet-induced obese rats, the fruit juice reduced blood glucose and weight gain, improved satiety and insulin secretion and modulated the mRNA expression of several lipid and glucose metabolizing enzymes (Ahmed et al., 2015). *In vitro*, the hydro-ethanolic and methanolic extracts or isolates of the fruit, its peel and/or seeds showed radical-scavenging and ferric reducing antioxidant properties (Singh et al., 2002; Middha et al, 2013; Redha et al., 2018; Zeghad et al., 2019), as well as α -amylase (Jain et al., 2012; Redha et al., 2018) and lipase inhibitory activities (Shamsiya et al., 2016). Recent data on the fruit peel showed its ability to inhibit glucose-induced protein glycation and lipase activity, *in vitro* (Chukwuma et al., 2020). The study also showed the ability of the peel to inhibit lipid peroxidation and modulate glucose uptake in Chang liver cells (Chukwuma et al., 2020).

While the different parts of pomegranate fruit have been shown to have potent antioxidant and glycemic control bioactivity, attention has been drawn to its fruits due to its rich phytochemical profile, which influences its reported metabolic and antioxidant bioactivities.

2.11.2 The bioactive potential of pomegranate peel in metabolic and oxidative diseases

The metabolic and antioxidant bioactivities of pomegranate peel summarized in **Table 2.5**. *In vitro*, the peel of pomegranate has been shown to be an effective scavenger of radicals and ROS, as well as a potent iron chelating agent (Middha et al., 2013; Hernández-Corroto et al.,

2018; El-Hadary & Ramadan, 2019; Chukwuma et al., 2020), suggesting its ability to quench the deleterious outcome of radicals and ROS. The glycemic control, insulin modulatory, anti-glycation, anti-obesogenic, anti-atherogenic and anti-hypertensive potentials of pomegranate peel or its phytoconstituents have been demonstrated by its *in vitro* inhibitory effect on α -amylase, protein-tyrosine phosphatase 1B, aldose reductase, glucose-induced protein glycation, pancreatic lipase, cholesterol esterase and angiotensin converting enzyme (Jain et al., 2012; Hernández-Corroto et al., 2018; Chukwuma et al., 2020). At cellular level, the acetone extract of pomegranate peel inhibited lipid peroxidation and modulated glucose uptake in Chang liver cells (Chukwuma et al., 2020), suggesting its antioxidant and glycemic control potential.

Table 2.5: The bioactive potential of pomegranate peel in metabolic and oxidative diseases

Pharmacological action	Material, extract or isolate used	Remarks	References
25 days feeding of diet containing 1 and 5% peel powder protected against dextran sulfate sodium-induced intestinal and hepatic oxidative stress in rats by reducing small intestinal and hepatic LPO and increasing GSH content and GSH/GSSG ration	Peel powder		Al-Gubory et al., 2021
4 wks adm. (250 mg/kg bw, p.o.) of extract in female SHR reduced SBP and coronary artery ACE activity and improved the histology of the coronary artery.	Ethanol extract		Dos Santos et al., 2016
8 wks adm. (255 mg, p.o.) of extract in patients undergoing hemodialysis improved renal inflammatory status and endothelial functions by reducing serum CRP, IL-6, ICAM-1, VCAM-1 and TNF- α .	Peel extract (255 mg is equivalent to 90 mg of ellagic acid)		Jafari et al., 2020
28 days adm. (100 – 400 mg/kg bw, p.o.) of isolate in cyclophosphamide-induced immunosuppressed mice increased splenic LDH and acid phosphatase	Polysaccharide isolate		(Wu et al., 2019)

Pharmacological action	Material, extract or isolate used	Remarks	References
activity, lymphocytes proliferation, immunoglobins and cytokines secretion and antioxidant status.			
2 wks adm. (100mg/kg bw, p.o.) of extract protected against vancomycin-induced liver and kidney injury in male SD rats by reducing serum biomarkers of kidney and liver damage, tissue LPO, ROS production, inflammatory response and pro-apoptotic processes and improving renal and hepatic tissue histology, and antioxidant status.	Ethanol extract		El Bohi et al., 2021
• In vitro inhibition α-amylase, aldose reductase and PTP1B. • 21 days oral adm. of extract (200 na 400 mg/kg bw) and isolated compound (10 – 50 mg/kg bw) reduced FBG and improved GT and pancreatic histology	Compound (Valoneic acid dilactone) isolated from the ethyl acetate fraction of the peel methanol extract and the extract	The in vitro and in vivo activity of the compound was comparable and/or more to that of standards (Acarbose, quercetin and Glibenclamide).	Jain et al., 2012
4 wks adm. (500 and 1000 mg/kg bw, p.o.) of peel powder in HFD-fed albino rats reduced serum cholesterol, ALT, AST, CRP, IL-6, albumin and creatinine and protected against aortic histopathological degenerations.	Peel powder		(Salama et al., 2021)
• In vitro DPPH and NO radical scavenging activity • In vitro Fe^{3+} reducing activity • In vitro inhibition of glucose induced BSA glycation • In vitro α-amylase inhibitory activity • Pancreatic lase inhibitory activity • Increased GSH and reduced LPO in hepatocytes induced with oxidative stress	Acetone extract	• Up to 100 $\mu\text{g/mL}$ of extract did adversely affect the viability of kidney and liver cells. • The in vitro and cellular antioxidant	(Chukwuma et al., 2020)

Pharmacological action	Material, extract or isolate used	Remarks	References
Increased glucose uptake in hepatocytes		activity of the extract was comparable to that of Trolox, ascorbic acid and gallic acid.	
- In vitro ABTS and hydroxyl radicals scavenging activity - In vitro ACE inhibitory activity - In vitro cholesterol esterase inhibitory activity	Aqueous extract and protein isolates		(Hernández-Corroto et al. 2018)
- In vitro DPPH radical and H₂O₂ scavenging activity - In vitro Fe³⁺ reducing activity	Water and methanol extracts	The antioxidant activity of the extracts was comparable to that of ascorbic acid	(Middha, et al., 2013)
- In vitro DPPH and ABTS radicals scavenging activity 56 days adm. (200 mg/kg bw, p.o.) of extract reduced HbA1c and BG, improved lipid profile and serum biochemical markers of kidney and liver damage in alloxan-induced diabetic and HFD-fed male Wistar rats	75% methanol extract	The antidiabetic effect of the extract in the alloxan-induced diabetic rats was comparable to that of a 10 mg/kg bw Glibenclamide.	El-Hadary & Ramadan, 2019

Abbreviations: Adm., administration; ACE, angiotensin converting enzyme; BSA, bovine serum albumin; BG, blood glucose; CRP, C-reactive protein; FBG, fasting blood glucose; GSH, reduced glutathione; GSSG, oxidized glutathione; GT, glucose tolerance; HbA1c, glycated haemoglobin; HFD, high-fat-diet; LDH, lactate dehydrogenase; LPO, lipid peroxidation; PTP1B, Protein tyrosine phosphatase 1B; ROS, reactive oxygen species; SBP, systolic blood pressure; SD, Sprague Dawley; SHR, spontaneously hypertensive rats.

In vivo, diet containing 1 and 5% peel powder offered protection against dextran sulfate sodium-induced intestinal and hepatic oxidative stress in rats by reducing small intestinal and hepatic lipid peroxidation and increasing the ratio between oxidized and reduced glutathione

(Al-Gubory et al., 2021). In another study, the anti-hypertensive and cardioprotective potential of the peel's ethanol extract was demonstrated in spontaneously hypertensive rats by reducing systolic blood pressure and coronary artery angiotensin converting enzyme activity, as well as improving the histology of the coronary artery (dos Santos et al., 2016). The cardioprotective effect of pomegranate peel was also shown in high-fat-diet rats, where a 4-weeks oral administration (500 and 1000 mg/kg body weight) of the peel powder reduced serum cholesterol, ALT, AST, C-reactive protein, IL-6, albumin and creatinine and protected against aortic histopathological degenerations (Salama et al., 2021). The immunomodulatory potential of polysaccharides from the peel has been shown in immunosuppressed mice (Wu et al., 2019). A 28-days oral administration (100 – 400 mg/kg body weight) of the polysaccharide isolate increased splenic lactate dehydrogenase and acid phosphatase activity, lymphocytes proliferation, immunoglobins and cytokines secretion and antioxidant status (Wu, Zhu, Zhang, Li, & Sun, 2019). Furthermore, the blood glucose lowering effect of the peel ethanol extract has been demonstrated in alloxan-induced and high-fat-diet diabetic rat models (El-Hadary & Ramadan, 2019). The potency of the extract was comparable to that of glibenclamide (El-Hadary & Ramadan, 2019), suggesting the antidiabetic potential of pomegranate peel.

At both experimental and clinical levels, the following studies showed renal protective function of pomegranate peel. The peel ethanol extract protected against vancomycin-induced liver and kidney injury in male Sprague Dawley rats by reducing serum biomarkers of kidney and liver damage, tissue lipid peroxidation, ROS production, inflammatory response and pro-apoptotic processes, as well as improving renal and hepatic tissue histology, and antioxidant status (El Bohi et al., 2021). In patients undergoing haemodialysis, 8 weeks oral administration (255 mg per day.) of the peel extract improved renal inflammatory status and endothelial functions by reducing serum C-reactive protein, IL-6, ICAM-1, VCAM-1 and TNF- α (Jafari et al., 2020).

Pomegranate peel has a range of total phenolic content, from 18 to 510 mg/g dry matter. The majority of the phenolic components in pomegranate peel include tannins, flavonoids, and phenolic acids. (Babbar and Oberoi , 2013). The hydrolysable tannins (including gallotannins and ellagitannins) and flavonoids are the two most prevalent or notable subclasses. Pomegranate seeds, pericarp, and peel are rich in ellagitannins and gallotannins. (Chukwuma et al., 2020; Akuru et al., 2022). The main ellagitannin found in pomegranates is punicalagin. Ellagitannins can be hydrolysed into constituents such as ellagic acid (**Figure 2.8**), whereas gallotannins hydrolyses to release gallic acid.

Ellagitannins in pomegranate peel including punicalagin and ellagic acid have been shown to exert antioxidant and glycemic control action and are perceived to be some of the key phytoconstituents influencing the metabolic and oxidative health benefits of pomegranate. Moreover, dietary supplements rich in ellagitannins have been shown to exhibit inhibitory action on lipid peroxidation in overweight human subjects (Heber et al., 2007).

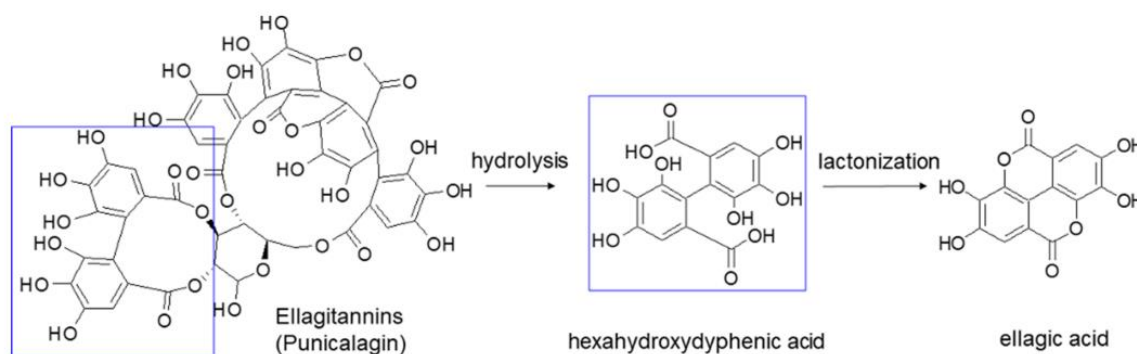


Figure 2.8: Chemical structure and hydrolysis of punicalagins (*adapted without permission from Zuccari et al., 2020*).

Sun et al. (2017) have also reported the potent *in vitro* and *in vivo* antioxidant potential of commercial punicalagin, which outperformed commercial ellagic acid and punicallin (other key components of pomegranate peel ellagitannins). Also, commercial ellagic acid has been shown to exert anti-lipid peroxidative activity in obese animals (Xu et al., 2021). Pomegranate punicalagin has also been shown to be an effective inhibitor of α -amylase and comparable to acarbose (Kerimi et al., 2017), suggesting the glycemic control potential of pomegranate ellagitannins. Additionally, insulin resistance and oxidative stress caused by high glucose treatment in HepG2 cells was improved following ellagic acid treatment.

2.12 Problem statement

While there are pharmacological evidence supporting the potential medicinal relevance of pomegranate peel ellagitannins in managing metabolic and oxidative health, the recovery of these bioactive principles for possible application, without sacrificing their biological properties proves difficult most times. Temperature dependent extraction has been shown to

improve recovery of plant phenolics, if conducted optimally. However, the optimum temperature and chromatographic conditions that will ensure the optimum recovery of pomegranate peel ellagitannins, as well as preserve their antioxidant and glycemic potential is still not certain. In fact, the antioxidant and antihyperglycemic effects of ellagitannins and punicalagin optimally purified from pomegranate peel have scarcely or not been studied.

2.13 Aim and objectives

The aim of this study was to optimally recover ellagitannins and punicalagin with potent antioxidant and glycemic control properties from the fruit peel of “Wonderful” pomegranate.

The objectives include the following:

- Use temperature dependent extraction and chromatography techniques to recover ellagitannins from pomegranate fruit peel.
- Characterize the extracts, fractions and isolates using LC-MS.
- Evaluate the extracts, fractions and isolates for their antioxidant and glycemic control potentials.

CHAPTER 3

MATERIALS AND METHODS

3.1 Collection fruit and preparation fruit peel aqueous extracts

Pomegranate fruits (variety: “Wonderful” variety; grade: Class 1 or processing grade) were purchased from Sonlia Fruit Packhouse (Wellington, Western Cape Province, South Africa) on 03 May 2021. The fruits were washed with clean tap water.

The peel of the fruits was removed. Six aliquots of 1 kg of the fresh fruit peel were separately soaked in distilled water at a mass (kg)/volume (L) ratio of 1:2.5, respectively, using six metallic bowls with fitted covers. The six metallic bowls were separately incubated at six different temperatures (25, 37, 50, 65, 78 and 95 °C) for 1 hour in a temperature-regulated oven. Exactly after 1 hour incubation, the fruit peel in each metallic bowl was blended with the incubation water to form a puree. The puree was first filtered with a cheese cloth and then with a filter paper, before recovering the crude extract by freeze drying (Martin Christ Alpha 1-2 LDplus Freeze Dryer supplied by Separations, Johannesburg, South Africa).

3.2 Total phenol and flavonoid measurement

The extracts were subjected to total phenol and total flavonoid estimation using methods that have been previously reported (Chukwuma et al., 2020).

For total phenol estimation, 125 µL of 10x diluted Folin–Ciocalteu reagent (Merck, South Africa) was aliquoted into the wells of a 96-well clear bottom plates containing 25 µL of the extracts (at 45 µg/mL in assay volume) or increasing concentration of gallic acid solution (3.75 to 120 µg/mL in assay volume). Thereafter, 100 µL of 0.7 M Na₂CO₃ was added and the plate was incubated for 30 min at room temperature. Absorbance was measured at 765 nm using a SpectraMax M2 microplate (Molecular Devices, California, USA).

For total flavonoid estimation, 80 µL of 98% ethanol was aliquoted into the wells of a 96-well clear bottom plates containing 20 µL of the extracts (at 45 µg/mL in assay volume) or increasing concentration of quercetin solution (3.75 to 120 µg/mL in assay volume). Thereafter, 100 µL of 2% AlCl₃ was added, and the plate was incubated for 1 hour at room temperature. Absorbance was measured at 430 nm.

The total phenol and flavonoid contents of the extracts were computed from the gallic acid and quercetin standard curves and expressed as mg/g GAE (gallic acid equivalent) and mg/g QE (quercetin equivalent), respectively, using the following formula:

$$\text{Total phenol (mg/g GAE) or Total flavonoid (mg/g QE) content} = \frac{C \times SV}{M}$$

Where, “C” is the concentration (mg/mL) extrapolated from gallic acid and quercetin standard curve; “SV” is the sample volume (mL), and “M” is the amount (g) of the sample in the “SV” (mL) of the sample solution; “GAE” and “QE” mean gallic acid equivalent and quercetin equivalent, respectively.

3.3 Preliminary measurement of *in vitro* antioxidant and enzyme inhibition activities of extracts

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS*+) scavenging, Fe³⁺ reducing and α-glucosidase and α-amylase inhibitory activities of the extracts or the positive controls were measured at a given concentration (45 µg/mL in assay mixture) using the methods described in previous reports (Chukwuma et al., 2020; Motlounng et al., 2020).

3.3.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The assay mixture contained 75 µl of the extracts or standards (ascorbic acid and Trolox) or their solvents (control) and 37.5 µl of a 0.3 mM DPPH solution in a 96-well plate. The mixture was incubated in the dark for 30 min, and absorbance was measured at 517 nm (SpectraMax M2 microplate reader, Molecular Devices, San Jose, CA, USA). Sample blank or sample solvent was used as the blank. The formula below was used to calculate the DPPH radical scavenging activity (%):

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

3.3.2 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging assay

The ABTS radical was generated by mixing and incubating equal volumes of 7 mM ABTS and 2.4 mM potassium persulfate solutions in the dark for 12 – 16 hours at room temperature. The mixture was, then, diluted 5 times with methanol. Assay was performed by mixing 50 μ L of the extracts or standards (ascorbic acid and Trolox) or their solvents (control) with 125 μ L of the diluted ABTS solution in a 96-well plate. Absorbance at 734 nm was measured after a 15 min incubation in the dark. The ABTS radical scavenging activity (%) was computed relative to the control using the following formula:

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

3.3.3 Measurement of Fe³⁺ reducing antioxidant power (FRAP)

The assay mixture was prepared by adding 25 μ L of the extracts or positive controls (Trolox and ascorbic acid) or increasing concentrations (4 - 80 μ M in assay mixture) of gallic acid to 0.2 M phosphate buffer (pH, 6.6) and 1% potassium ferricyanide solutions. The mixture was, then, incubated at 50 °C for 20 minutes, followed by the addition of 25 μ L of 10% trichloroacetic acid. Thereafter, 100 μ L of distilled water and 50 μ L of 0.1% FeCl₃ solution were added consecutively, before absorbance was measured at 700 nm. The FRAP computed from the gallic acid standard curves and expressed as mg/g GAE (gallic acid equivalent) using the following formula:

$$\text{FRAP (mg/g GAE) of} = \frac{C \times SV}{M}$$

Where, “C” is the concentration (mg/mL) extrapolated from gallic acid standard curve; “SV” is the sample volume (mL), and “M” is the amount (g) of the sample in the “SV” (mL) of the sample solution; “GAE” means gallic acid equivalent.

3.3.4 Measurement of α -glucosidase inhibition

To perform the assay, 25 μ L of the extracts or acarbose (positive control) or distilled water (control) were mixed in a 96-well plate with 50 μ L of 2 U/mL α -glucosidase enzyme solution (dissolved in 100 mM phosphate buffer, pH 6.8). The mixture was incubated for 10 minutes at 37 °C. Thereafter, 25 μ L of 5 mM para-nitrophenyl β -D-glucopyranoside solution (dissolved in 100 mM phosphate buffer, pH 6.8) was added and the mixture was, further, incubated for 20 - 30 minutes at 37°C. After incubation, 100 μ L of 100 mM Na₂CO₃ was used to end the enzyme reaction and the absorbance was measured at 405 nm. The enzyme activity inhibition (%) was calculated as follows:

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

3.3.5 Measurement of α -amylase inhibition

To perform the assay, 75 μ L of the extracts or acarbose (standard) was mixed with 75 μ L of 3 U/mL porcine pancreatic amylase solution (dissolved in 100 mM phosphate buffer, pH 6.8) in a vial. Equivalent volume of distilled water was used as the control. The mixture was incubated at 37 °C for 15 minutes, before the addition of an equivalent volume of 1% starch solution (dissolved in 100 mM phosphate buffer, pH 6.8). Thereafter, the mixture was incubated for another 30 min at 37 °C and 75 μ L of dinitrosalicylate color reagent was added. The mixture was put in a boiling water bath for 10 minutes. After cooling, the mixture was centrifuged (Hettich Mikro 200 microcentrifuge, Hettich Lab Technology, Tuttlingen, Germany) at 5000 x g for 5 min. An aliquot of 150 μ L of the supernatant was transferred into a 96-well plate and absorbance was measured at 540nm. The enzyme inhibition (%) was calculated as follows:

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

3.4 Total tannin (ellagitannins) recovery from extracts

A method modified from a previous report (Seeram et al., 2005) was adopted in recovering tannin (ellagitannins) from the extracts. An Amberlite® XAD16N resin (product code: XAD16; Merck, Johannesburg, South Africa) was used. First, 1.52 g of each extract was dissolved in 100 mL of distilled water, filtered with a Whatman filter paper, and stored in a clean screw-cap bottle (**Figure 3.1b**).

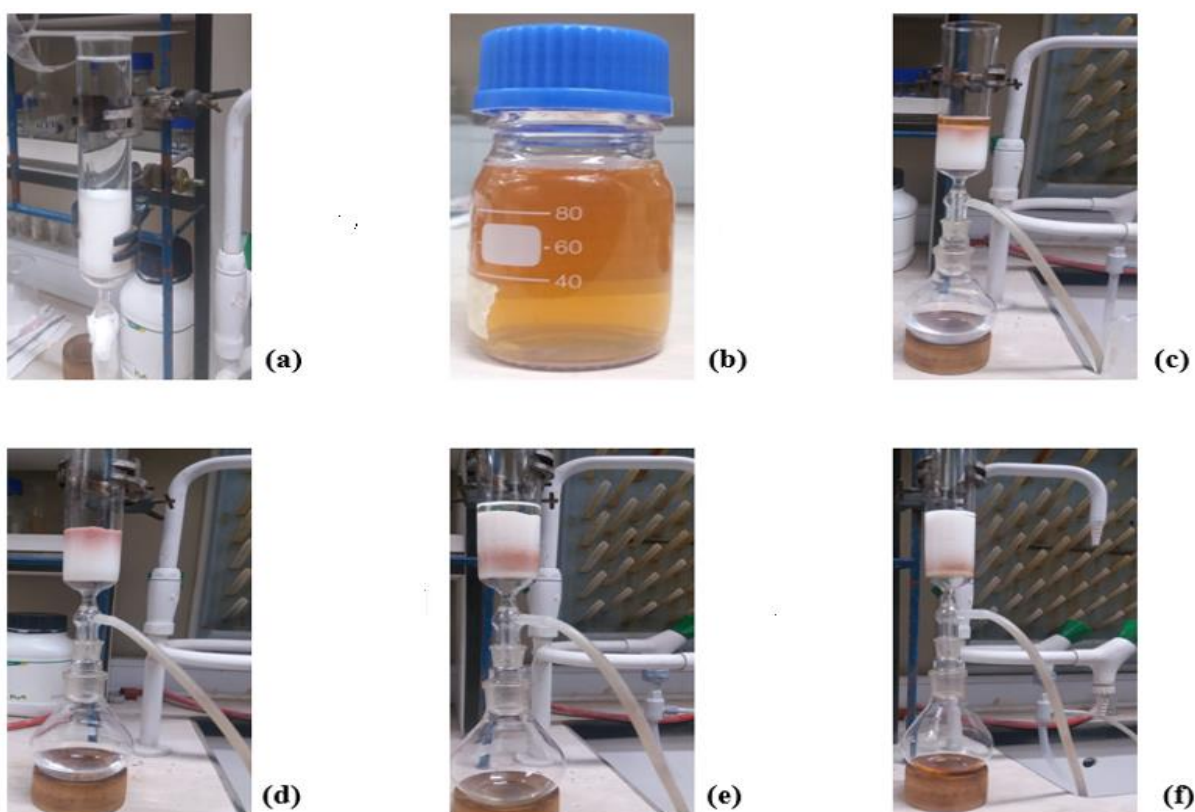


Figure 3.1: Illustration showing the recovery process of tannins from the pomegranate fruit peel extracts.

To prepare the XAD16N resin column, 35 g of the resin was washed twice with methanol, loaded into a column containing distilled water (**Figure 3.1a**) and allowed to equilibrate for 12 hours. After equilibrating, the water was completely drained using a water jet vacuum pump. Each of the dissolved extracts was separately poured into the column and gradually eluted with distilled water using the water jet vacuum pump. Elution continued until the tannin was completely adsorbed into the XAD resin and a consistent colourless eluent was observed

(Figures 3.1c and d). Any remaining water in the column was removed using the vacuum pump. The tannin trapped in the resin was eluted with methanol at a steady flow rate using the water jet vacuum pump (Figures 3.1e and f). The tannin eluent was air-dried under a fume hood and stored at -20 °C in clean screw-cap vials. The yield of the tannin recovered from each extract was calculated as follows:

$$\text{Yield (\%)} = \frac{\text{Mass (g) of tannin recovered from extract}}{1.52 \text{ g of extract}} \times 100$$

3.5 Purification of punicalagin from tannin

Punicalagin was purified from the tannin that was recovered from the peel extract prepared at 78 °C. The method previously reported by Seeram et al. (2005) was adopted with slight modifications for of isolation and purification of punicalagins. A Sephadex LH-20 gel column chromatography with methanol as the mobile phase was used for the isolation and purification. The tannin fraction (50 mg) obtained from the crude extract was dissolved in 2 mL of methanol and the resulting solution was subjected to a Sephadex LH-20 gel (Product code: 1003318800; Merck, Johannesburg, South Africa) column chromatography (45 cm x 18 mm filled at 80%) using the methanol as the mobile phase. The column flow rate was 0.8 mL/min.

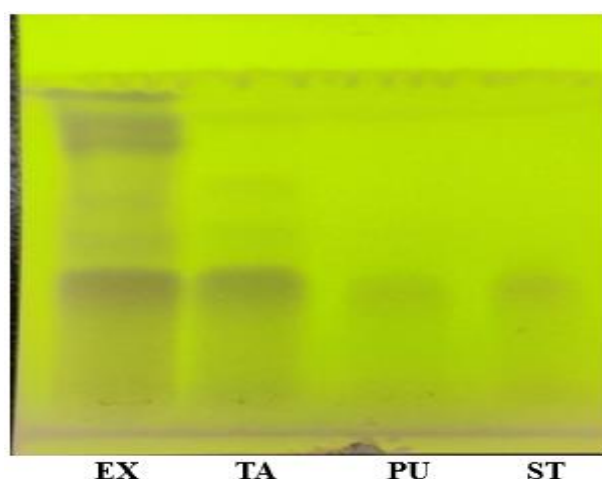


Figure 3.2: Thin layer chromatographic plate showing the 78 °C peel extract (EX), the tannin recovered from the extract (TA), the corresponding purified punicalagin (PU) and punicalagin standard (ST).

Thin layer chromatography (TLC) was developed with a solvent system of chloroform/ethyl acetate/ethanol/formic acid/distilled water (4: 3:3:2:1 v/v/v/v, respectively) and used to monitor the collected fractions (~1.6 mL) as shown in **Figure 3.2**.

3.6 Liquid chromatography - mass spectroscopic (LC-MS) quantification

The recovered tannins and the purified punicalagin were subjected to LC-MS. To perform the LC-MS protocol the following were used: A Waters Synapt G2 (Waters Corporation, MA, USA), ESI probe and ESI Pos, at a 15 V cone voltage. The operation of liquid chromatography was done using Acquity binary solvent manager. The chromatographic column used was a HSS T3. It had a dimension of 2.1 x 150mm and particle size of 1.8 μm (Waters Corporation, MA, USA). Two solvents systems (A and B) were used as the mobile phase. Solvent system A was water containing 0.1% methanoic acid, while solvent system B acetonitrile containing 0.1% methanoic acid. A 0.25 mL/min flow rate was applied for the gradient chromatographic separation at 30 °C. The gradient condition followed the following sequence: 100% (A):0% (B), 10 min; 72% (A):28% (B), 21 min; 60% (A):40% (B), 50 sec; 0% (A):100 % (B), 2 min. The samples were dissolved a mixture of acetonitrile and water (1:1 v/v), passed through a 0.22 μm filter and injected at a volume of 20 μL . The signals of the separated compounds were recorded at 254 nm. Punicalagin A and punicalagin B in the recovered tannin (ellagitannins) fractions and the purified punicalagin were identified and quantified from a Punicalagin A+B (product code: PHL80524, Sigma Aldrich, Johannesburg, South Africa) calibration curve. Using the accurate masses and elemental compositions, the other compounds in the tannin fraction recovered from the 78 °C peel extract were determined by semi-quantified relative to ellagic acid standard according to the extracted ion (**Appendix 1**). Quantified amounts were given in triplicates.

3.7 Measurement of dose-dependent antioxidant and glycemic control potential

The dose dependent bioactivity of the peel extract prepared at 78 °C was measured because of its stronger activity relative to the other extracts in the preliminary experiments. Its activity was compared to that of the tannin fraction and punicalagin recovered from it. Below are the performed dose dependent assays.

3.7.1 Radical scavenging assay

Three different *in vitro* experimental models were used to profile the radical quenching properties of the tested samples. These include DPPH and ABTS radical scavenging and oxygen radical absorbance capacity (ORAC) assays.

The DPPH and ABTS radical scavenging assays were done according to the methods described in **subsections 3.3.1 and 3.3.2** above. The assays were conducted using increasing sample concentrations (3.75 – 60 µg/mL in assay volume), while ascorbic acid and Trolox were used as positive controls at equivalent concentrations.

For the ORAC assay, a recently reported method (Setlhodi et al., 2023), which was modified from the method of Ou et al. (2013) was adopted. The assay was performed in a clear bottom opaque/black 96-well plate. A 0.112 µM fluorescein sodium salt solution and 152.66 mM 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) solution were prepared using a 75 mM potassium phosphate buffer (pH 7.3). Twenty-five microlitres of the samples or the positive control (ascorbic acid) at the tested concentration (7.5 – 60 µg/mL in assay volume) or their solvents (controls) was added into the wells of the plate. Next, 150 µL of the fluorescein sodium salt solution was added to the wells and the plate was incubated at 37 °C for 30 min. After incubation, 25 µL of the AAPH solution was added to the wells and the fluorescent reading (Excitation/Emission wavelength = 490 nm/515 nm) was immediately measured at 1 min interval for 35 min using a SpectraMax M2 microplate reader (Molecular Devices, San Jose, CA, USA) set at 37 °C. The time intervals (x-axis) were plotted against their corresponding fluorescent reading (y-axis) and the slope of the plot was computed from a linear regression. The positive version of the slopes was used to compute the ORAC of the tested samples (extracts, positive control and standards) using the following formula:

$$ORAC (\%) = \frac{Slope_{Control} - Slope_{Test}}{Slope_{Control}} \times 100$$

3.7.2 Measurement of the inhibition of linoleic acid peroxidation

The ability of the extracts, the recovered tannin and purified punicalagin to inhibit FeSO₄-induced linoleic acid peroxidation was performed using the method reported by Ramorobi et al. (2022), which was modified from the method reported by Choi et al. (2002). Into screw-cap

vials, 30 μL of the test samples (extract, recovered tannin, purified punicalagin and ascorbic acid) at the tested concentrations (5 – 80 $\mu\text{g/mL}$ in assay volume) or distilled water (in the vials assigned control and negative control) was dispensed. Next, 30 μL and 20 μL of 50 mM linoleic acid and Tris-HCl buffer (100 mM, pH 7.5) were respectively added. Twenty microliters of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (2 mM in incubation volume) was aliquoted into the vials containing the samples and the vials assigned negative controls to cause linoleic acid peroxidation, while an equivalent volume of distilled water was dispensed in the wells assigned controls. After 30 min incubation at 37 $^{\circ}\text{C}$, 5.5 % trichloroacetic acid and 0.25 % thiobarbituric acid were added to the vials at respective volumes of 80 and 50 μL . The vials were placed in a boiling water bath for 30 minutes. After cooling, the vials were centrifuged at 3500 x g for 5 min (Hettich Mikro 200 microcentrifuge, Hettich Lab Technology, Tuttlingen, Germany) and 150 μL of the clear layer was aliquoted into designated wells of a clear-bottom 96-well plate. The absorbance was read at 532 nm and the inhibitory potential of the tested samples on linoleic acid peroxidation was calculated as follows:

$$\text{Inhibition (\%)} = \frac{(A_{\text{Negative control}} - A_{\text{Normal control}}) - (A_{\text{Test}} - A_{\text{Normal control}})}{(A_{\text{Negative control}} - A_{\text{Normal control}})} \times \frac{100}{1}$$

Where, “A” means “Absorbance”.

3.7.3 α -Amylase inhibition assay

The samples were dose dependently (5 – 80 $\mu\text{g/mL}$ in assay volume) tested for their capacity to inhibit α -amylase activity and using the method reported in **subsection 3.3.5** above. Acarbose was used as the positive controls for the inhibition assay at equivalent concentrations as the tested samples.

3.7.4 Anti-glycation inhibition assay

The samples were dose dependently (5 – 80 $\mu\text{g/mL}$ in assay volume) tested for their capacity to inhibit glucose-induced bovine serum albumin (BSA) glycation using methods previously reported in a previous study (Motloun et al., 2020). Briefly, 50 μL volume of different concentrations (5 – 80 $\mu\text{g/mL}$ in assay volume) of the samples or standard (aminoguanidine)

or their solvents (control) was incubated with 50 μL of glucose (90 mg/mL) and 50 μL bovine serum albumin (10 mg/mL) in the dark for 3 weeks at 37°C using black 96-well plates. Thereafter, fluorescence was measured at Ex/Em = 360 nm/420 nm, and the glycation inhibition was computed using the following formula:

$$\text{Activity (\%)} = \frac{\text{Fluorescence of control} - \text{Fluorescence of test}}{\text{Fluorescence of control}} \times 100$$

3.7.5 Measurement of glucose uptake/utilization in L-6 myotubes

A reported method (Ramorobi et al., 2022) modified from a previous article (Oyedemi et al., 2013) was adopted for this assay. L-6 myoblast (ATCC CRL-1458, ATCC, VA, USA) cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum were trypsinized after confluence reached about 90%. The cells were seeded in designated wells of a 96-well plate at a 4000 cells/well density. After cells were confluent, the medium in each well was replaced with a differentiation medium (DMEM medium containing 2% horse serum) and incubated for 4 to 5 days. The spent medium was removed, and the differentiated cells were supplied with fresh medium that contained the tested samples (5, 37, and 50 $\mu\text{g/mL}$ in the incubation volume) or distilled water (control group). Wells containing medium without cells were used as blanks. Following a 48-hour incubation period, the sample-treated medium was removed, and the cells were washed with PBS. Next, RPMI medium containing 8 mM glucose and 0.1% BSA was dispensed into the wells. For the wells designated as the positive control, the RPMI medium contained insulin at a concentration of 1 μM . The 96-well plate was incubated for 2 hours, and glucose concentration was subsequently estimated in aliquots from each well using a Glucose-GO Assay Kit (Merck, Johannesburg, South Africa). The viability of the cells was monitored during the experiment using MTT assay. The experiment was conducted in three biological repeats. The formula below was used to compute the glucose uptake (%) of the test samples and control.

$$\text{Glucose uptake(\%)} = \frac{\Delta\text{GC of test or control} - \Delta\text{GC of blank}}{\Delta\text{GC of blank}} \times 100$$

Where, “ ΔGC ” is the change of glucose concentration in the incubation medium.

3.7.6 Measurement of antioxidant indices in Chang liver cells induced with oxidative stress

The effect of the tested samples on lipid peroxidation and the activity of key antioxidant enzymes was measured in Chang liver cells (ATCC® CCL-13™) induced with oxidative stress. Confluent cultured cells (DMEM high glucose with 10% FBS) were plated in a transparent 96-well cell culture plate at 10000 cells/well density. After 48 hours of incubation in a CO₂ incubator set at 5% CO₂ and 37 °C, the medium was replaced with fresh medium containing the tested samples or ascorbic acid (10 – 80 µg/mL in incubation medium) or distilled water (in the wells assigned control and negative control). After 30 min of incubation, FeSO₄.7H₂O (1 mM in incubation medium) was added to the wells of the tested samples/ascorbic acid and the wells assigned negative controls to induce oxidative stress. The incubation continued for 1 hours before lysing the cells with cold lysis buffer (50 mM sodium phosphate solution containing 0.5% v/v Triton X-100 and 1 mM EDTA, pH 7.5) and gentle agitation. Lipid peroxidation level, as well as the activity of catalase and superoxide dismutase (SOD) were measured in the supernatant (9500 x g for 10 min at 4 °C) of the lysed cells.

For lipid peroxidation a previous method (Ramorobi et al., 2022) was used with slight modifications. To perform the assay, 50 µL of supernatant or malondialdehyde (MDA) standards (0, 7.5, 15, 22.5, 30, and 45 µM in the assay volume) was mixed in designated vials already containing 250, 100 and 100 µL of 0.25 % w/v thiobarbituric acid, 20 % v/v acetic acid, and distilled water, respectively. The mixture was heated for 50 min in a boiling water bath, allowed to cool and centrifuged for 5 min at 3500 x g and ambient temperature. Aliquots of the supernatants were transferred into a transparent 96-well plate and the absorbance was measured at 532 nm. The lipid peroxidation levels were extrapolated from the MDA standard plot as thiobarbituric acid reactive substances. The inhibitory potential of the tested samples on lipid peroxidation was calculated as follows:

$$\text{Inhibition (\%)} = \frac{(C_{\text{Negative control}} - C_{\text{Normal control}}) - (C_{\text{Test}} - C_{\text{Normal control}})}{(C_{\text{Negative control}} - C_{\text{Normal control}})} \times \frac{100}{1}$$

Where, “C” is the concentration (µM) extrapolated from the MDA standard plot.

Catalase and SOD activities were measured in the cell lysates of cells treated with the highest concentration (80 µg/mL) of the tested samples. To measure the activity of the antioxidant enzymes, the protein concentration of the cell lysate was first measured using the bicinchoninic acid (BCA) method.

For catalase, the activity was measured by measuring the rate at which the enzyme decomposes H₂O₂ at room temperature using a Catalase Assay Kit (Sigma Aldrich, Johannesburg, South Africa; catalog number: CAT100). The activity was presented in mmol/mL/min. The activity was measured at a sample protein concentration of 1 mg/mL and the specific activity of the enzyme was expressed in mmol/min/mg protein. One unit of catalase will decompose 1.0 micromole of H₂O₂ to oxygen and water per minute at pH 7.0 at 25 °C at a substrate concentration of 50 mM H₂O₂. The specific activity of the enzyme was calculated as follows:

$$\text{Specific activity } (\mu\text{mol}/\text{min}/\text{mg protein}) = \frac{A \times DF}{RT \times SV \times PC}$$

Where, “A” = amount (µmole) of H₂O₂ that was used up during reaction time; “DF” = dilution factor of sample in reaction volume; “RT” = reaction time (min); “SV” = sample volume (mL); “PC” = sample protein concentration (mg/mL).

For SOD, activity was measured with an SOD Assay Kit (Sigma Aldrich, Johannesburg, South Africa; product number: 19160). The enzyme reduces superoxide ion to H₂O₂ and O₂. Thus, the samples’ SOD activity was determined monitoring how the samples inhibit the colorimetric reduction of a water-soluble tetrazolium by superoxide ion. Based on the sample protein concentration, the activity was normalized to reflect the specific activity (i.e., the enzyme activity per mg protein) using the following formula:

$$\text{Specific activity } (\% \text{ inhibition}/\text{mg protein}) = \frac{I \times DF}{SV \times PC}$$

Where, “I” = percentage inhibition (%) by enzyme on superoxide ion-mediated colorimetric reduction of a water-soluble tetrazolium salt; “DF” = dilution factor of sample in reaction volume; “SV” = sample volume (mL); “PC” = sample protein concentration (mg/mL).

3.7.7 IC₅₀ and EC₅₀ computation

The IC₅₀ and EC₅₀ values of the samples or standards are the sample concentrations that could cause 50% inhibitory (IC₅₀) or modulatory (EC₅₀) bioactivity. The values were computed on MS excel 2016 and GraphPad Prism 5 software using the linear and/or non-linear plot of transformed (log 10) sample concentrations (x-axis) versus the corresponding inhibitory or modulatory bioactivities.

3.8 Statistical analysis

Experimental data was presented as mean $\pm$ standard deviation of triplicate analysis ($n = 3$). The statistical analysis was done on the IBM SPSS Statistics (Windows version 29.0) software using the one-way ANOVA and Tukey post-hoc multiple comparative analysis. Statistical significance was set at $p < 0.05$.

CHAPTER 4

RESULTS

4.1 Phytochemical profiles of extracts and recovered tannins

4.1.1 Total phenol and flavonoid contents of extracts

The fruit peels contained polyphenols and flavonoids (**Figures 4.1**). As extraction temperature increased from 25 °C to 37 °C, the phenol and flavonoid contents appreciably decreased and significantly ($p < 0.05$) for flavonoid content. The phenol and flavonoid contents gradually increased as extraction temperature increased and peaked at 78 °C. Beyond 78 °C (at 95 °C), a significant drop in the levels of polyphenols and flavonoids was observed.

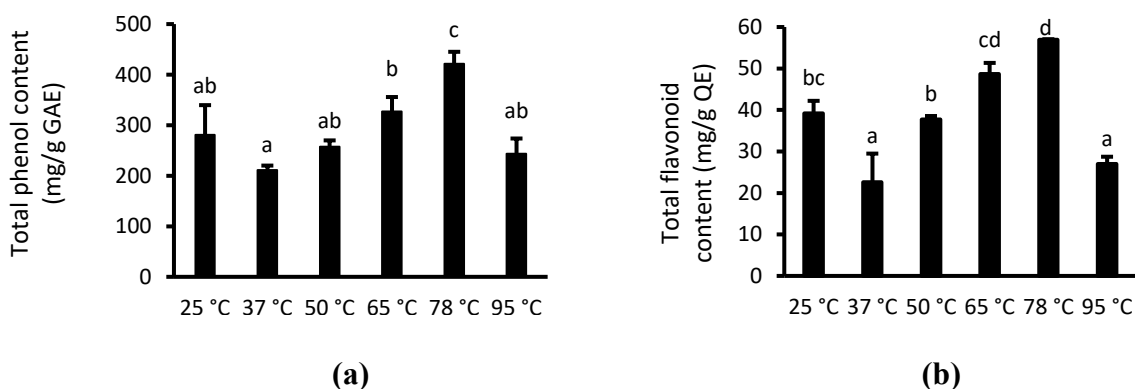


Figure 4.1: (a) Total phenol content and (b) total flavonoid content of the different pomegranate peel extracts at a tested concentration of 45 µg/mL. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, the letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

4.1.2 Ellagitannins profiles of extracts, recovered tannin and purified punicalagin

Phytochemical profiling of the peel extracts showed that the yield of the tannins recovered from the extracts was highest in the extract obtained at 78 °C (**Figure 4.2a**). Similarly, LC-MS profiling (**Figure 4.3 and Appendix 2**) revealed that punicalagin A and B were highest in the

tannin recovered from the extract obtained at 78 °C compared to the tannins recovered from the other extracts (**Figure 4.2b**). Other ellagitannins such as ellagic acid, granatin A, corilagin, casuarinin, and geraniin were also present in the tannin recovered from the extract obtained at 78 °C (**Figure 4.2c**)

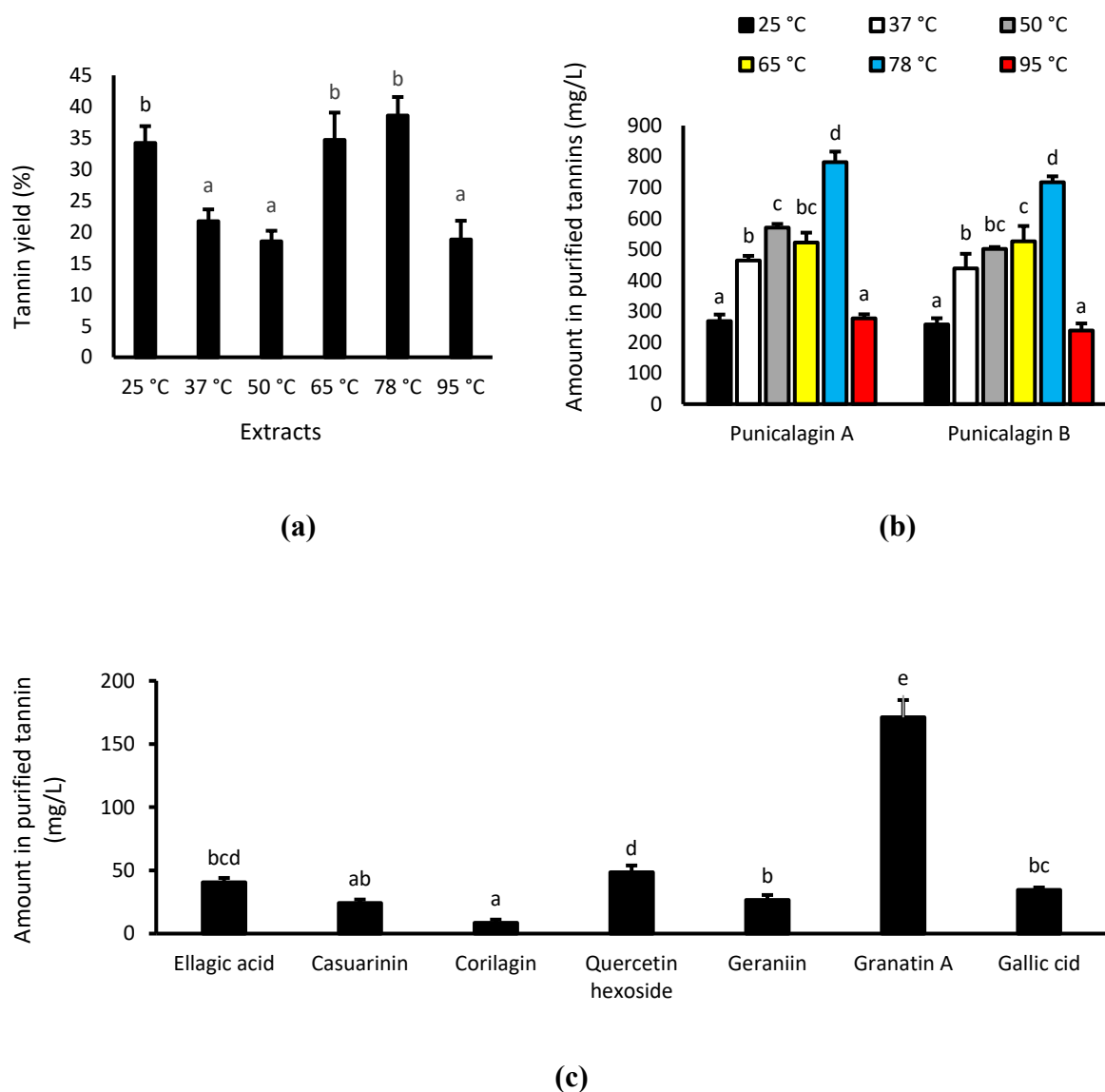


Figure 4.2: (a) The yield of the tannins recovered from the different peel extracts obtained at different temperatures (b) LC-MS quantified punicalagin A and B in the tannins recovered from the different extracts and (c) LC-MS quantified ellagitannins in the tannin recovered from the 78 °C peel extract. The replicate data are presented as mean $\pm$ SD of $n = 3$. For each quantification, the letters at the top of the bars represent significant differences ($p < 0.05$) between groups or between the quantified ellagitannins, when there are no similar letters.

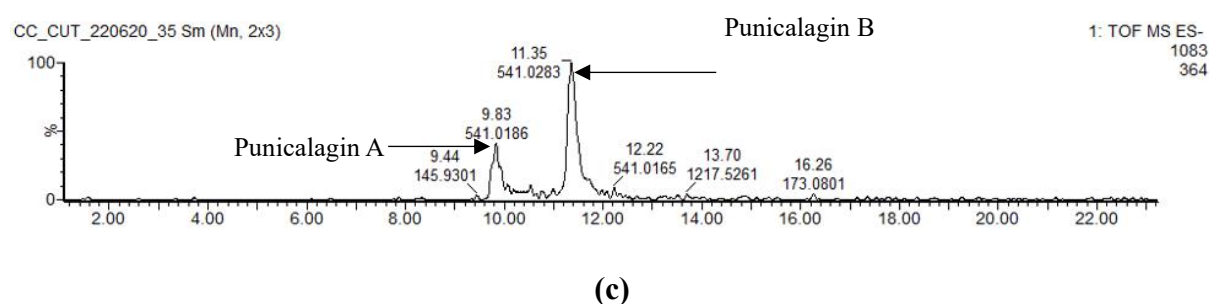
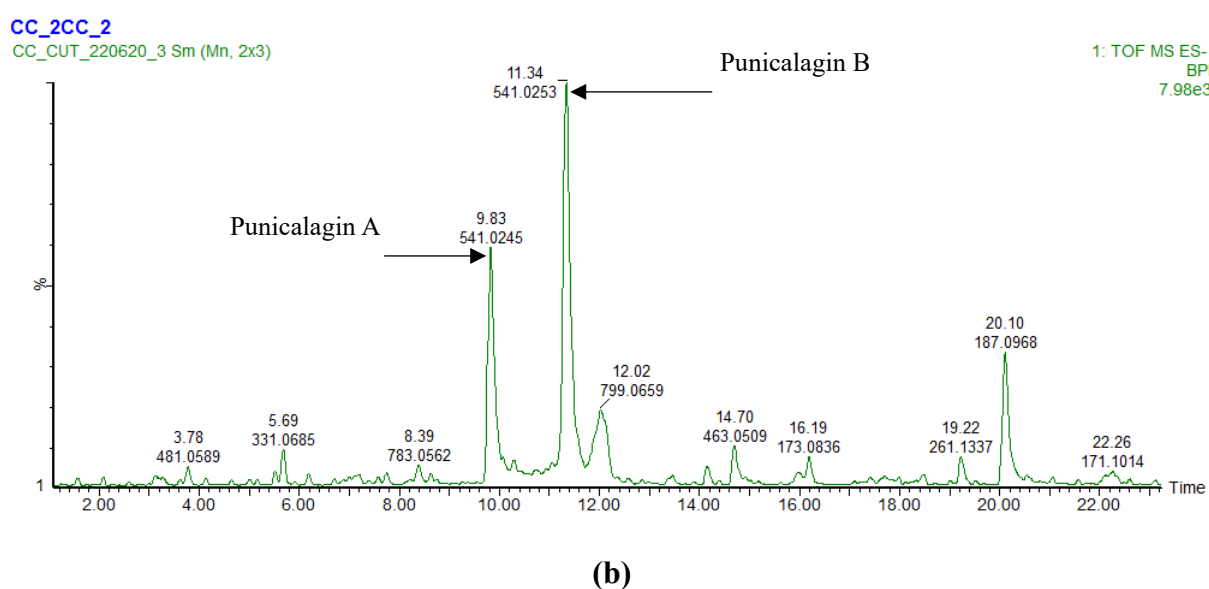
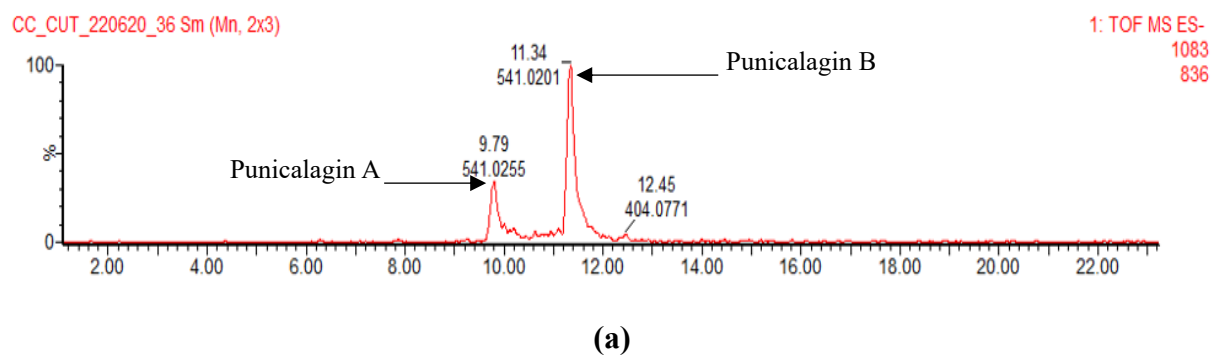


Figure 4.3: LC-MS chromatograms of (a) punicalagin A+B standard, (b) tannin recovered from pomegranate peel extract and (c) purified punicalagin.

4.2 Antioxidant and enzyme inhibitory properties of the different peel extracts

4.2.1 Radical scavenge and Fe^{3+} reducing capacity of extracts

The DPPH and ABTS radical scavenging and FRAP activity of the extract obtained at 78 °C was stronger than that of the other extracts and statistically comparable to that of ascorbic acid (Figures 4.4). In some instances, the activity of the extract obtained at 78 °C was significantly ($p < 0.05$) stronger than some of the other extracts.

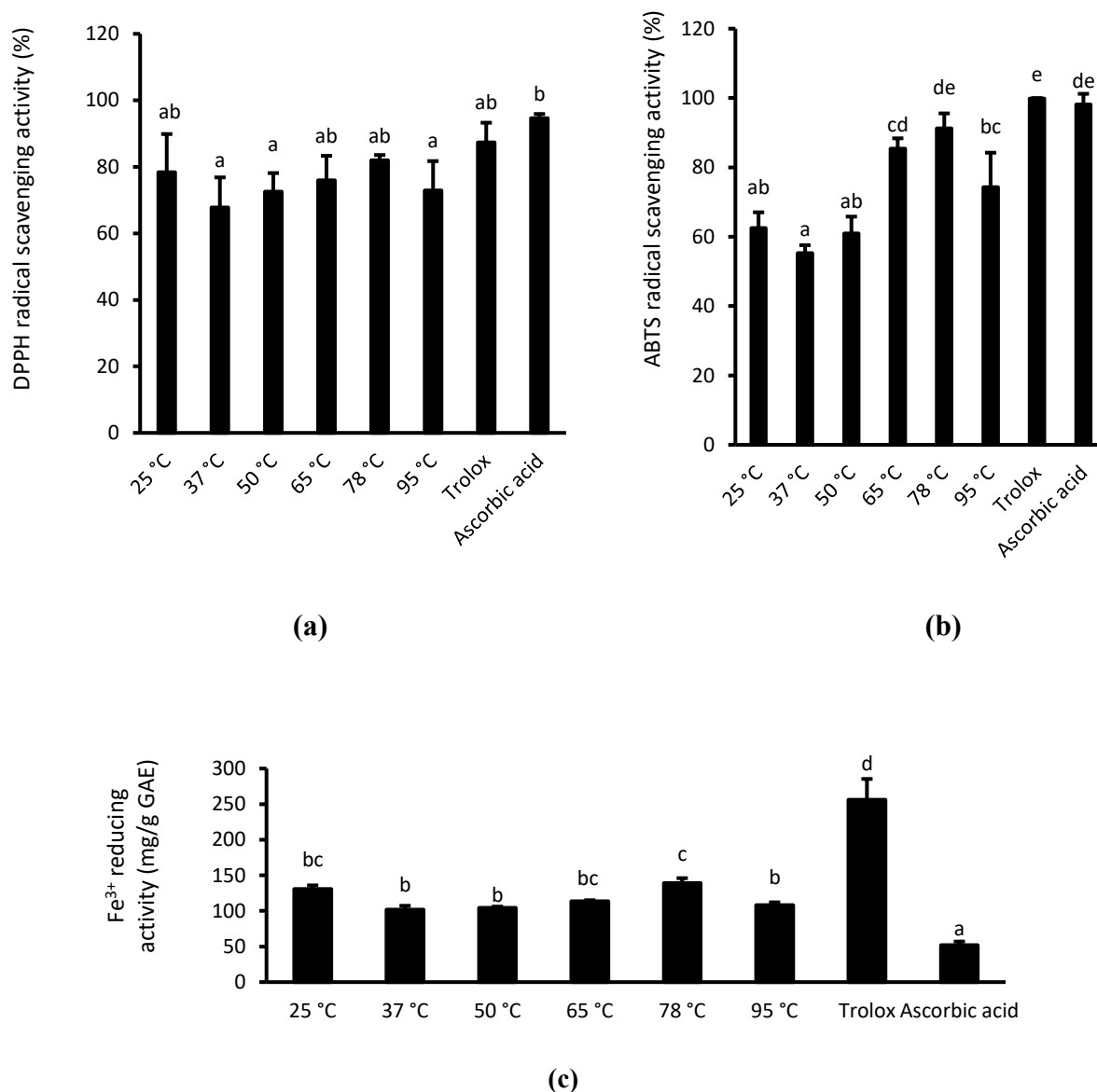


Figure 4.4: (a) DPPH radical scavenging, (b) ABTS radical scavenging and (c) FRAP activity of the different pomegranate peel extracts at a tested concentration of 45 $\mu\text{g/mL}$. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, the letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

4.2.2 α -Glucosidase and α -amylase inhibitory capacity of extracts

At the tested concentration of 45 $\mu\text{g/mL}$, all the peel extracts had a stronger inhibition of α -amylase than α -glucosidase (**Figures 4.5**). The α -amylase inhibitory action of the extract obtained at 78 $^{\circ}\text{C}$ was stronger than that of the other extracts (**Figure 4.5b**) and acarbose.

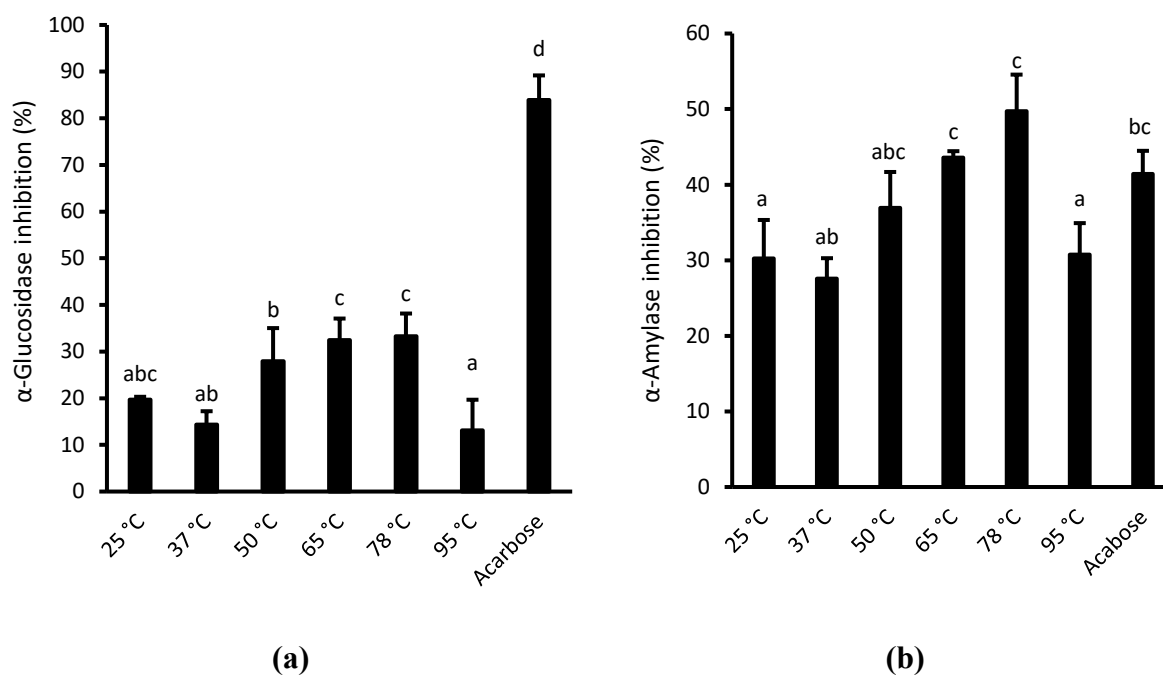
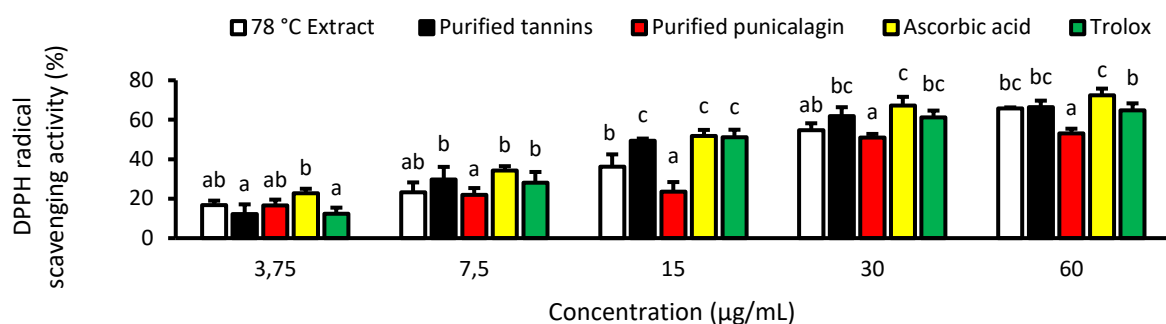


Figure 4.5: (a) α -glucosidase and (b) α -amylase inhibitory activity of the different pomegranate peel extracts at a tested concentration of 45 $\mu\text{g/mL}$. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, the letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

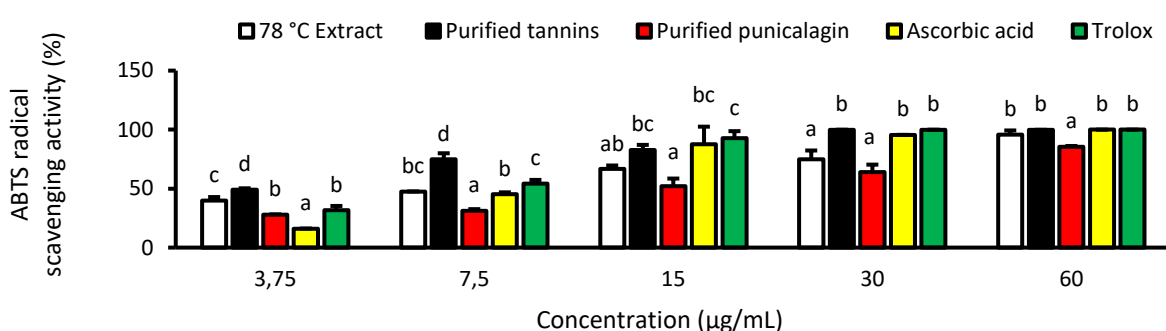
4.3 Dose-dependent antioxidant and glycemic control potential of punicalagin, tannin and crude extract

4.3.1 *In vitro* radical scavenging and ORAC activities

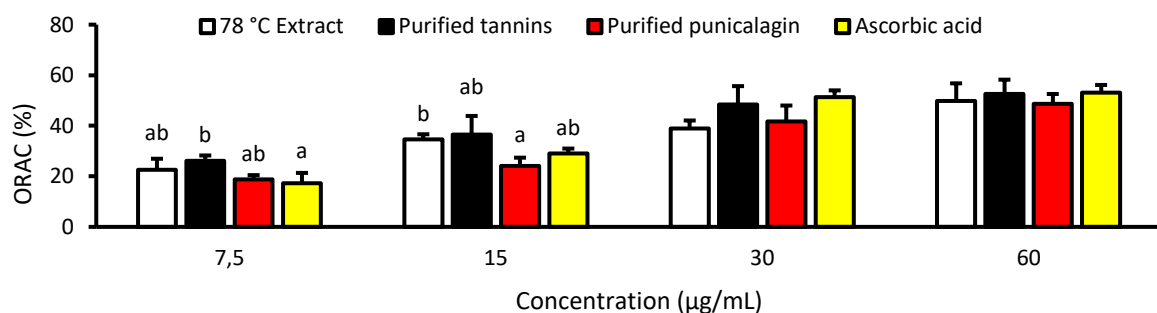
Based on the obtained data (**Table 4.1** and **Figure 4.6**), the extract obtained at 78 $^{\circ}\text{C}$, as well as the tannin and punicalagin recovered from it had appreciable radical scavenging and ORAC activities (**Figures 5a – c**). However, the IC_{50} values (**Table 4.1**) further suggest that the activity of the purified tannin was multiple times stronger than the activity of the extract and the purified punicalagin, but comparable to the scavenging strength of ascorbic acid and Trolox.



(a)



(b)



(c)

Figure 4.6: *In vitro* dose-dependent (a) DPPH radical scavenging, (b) ABTS radical scavenging and (c) ORAC activities of the 78 °C peel extract, the tannin recovered from the extract and the corresponding purified punicalagin. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, statistical comparison was done among the treatment groups at a given concentration. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

Table 4.1: IC₅₀ and EC₅₀ values for the dose dependent activities of the tested samples

Assays	78 °C Extract	TAN	PUN	Ascorbic acid	Trolox	Acarbose	AMG
IC ₅₀ or EC ₅₀ values (µg/mL)							
DPPH radical scavenging (IC ₅₀)	25.0 ± 3.94 ^a	4.80 ± 0.94 ^b	32.7 ± 8.61 ^a	9.30 ± 2.95 ^b	4.25 ± 0.95 ^b	ND	ND
ABTS radical scavenging (IC ₅₀)	27.5 ± 7.93 ^a	1.63 ± 0.52 ^b	37.5 ± 7.45 ^a	2.17 ± 0.68 ^b	2.18 ± 1.37 ^b	ND	ND
ORAC (IC ₅₀)	15.6 ± 4.35 ^b	9.64 ± 5.23 ^b	25.6 ± 4.68 ^a	8.10 ± 1.38 ^b	ND	ND	ND
Anti-linoleic acid peroxidation (IC ₅₀)	19.9 ± 5.70 ^{ab}	9.97 ± 3.50 ^{bc}	27.3 ± 9.42 ^a	7.18 ± 1.74 ^c	ND	ND	ND
Antiglycation (IC ₅₀)	34.2 ± 4.37 ^b	29.1 ± 8.32 ^{bc}	79.1 ± 9.48 ^a	ND	ND	ND	14.7 ± 5.55 ^c
α-Amylase inhibition (IC ₅₀)	59.8 ± 5.60 ^a	39.4 ± 8.43 ^b	31.9 ± 4.17 ^b	ND	ND	54.5 ± 7.57 ^a	ND
Anti-lipid peroxidation in Chang liver cells (IC ₅₀)	24.3 ± 8.98 ^b	3.67 ± 1.22 ^c	59.0 ± 14.3 ^a	22.4 ± 5.42 ^b	ND	ND	ND
Glucose uptake/utilization in L-6 myotubes (EC ₅₀)	55.0 ± 9.40 ^b	14.8 ± 4.96 ^c	112 ± 13.7 ^a	ND	ND	ND	ND

Abbreviations: “AMG”, aminoguanidine; “PUN”, purified punicalagin; “TAN”, recovered tannin. Data are presented as mean ± SD of triplicate analysis. For each assay, the superscript letters attached to each data represent significant differences ($p < 0.05$) between groups, when there are no similar letters

4.3.2 Inhibitory activity on FeSO₄-induced linoleic acid peroxidation

The *in vitro* ability of the purified tannin to inhibit linoleic acid peroxidation ($IC_{50} = 9.97 \mu\text{g/mL}$) was two and three times stronger than its corresponding extract ($IC_{50} = 15.6 \mu\text{g/mL}$) and purified punicalagin ($IC_{50} = 25.6 \mu\text{g/mL}$), respectively, and comparable to the activity of ascorbic acid ($IC_{50} = 7.18 \mu\text{g/mL}$) (Table 4.1 and Figure 4.7).

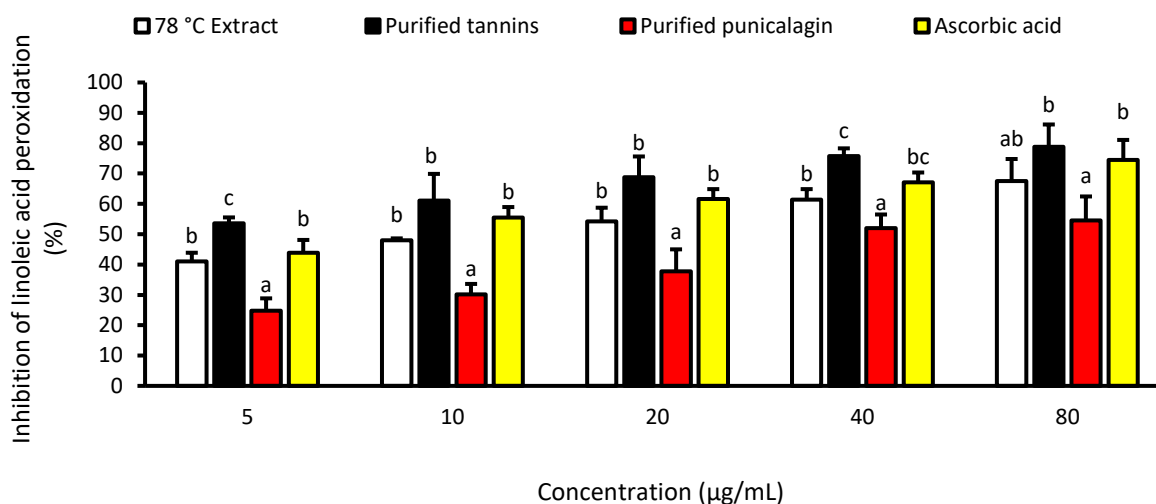
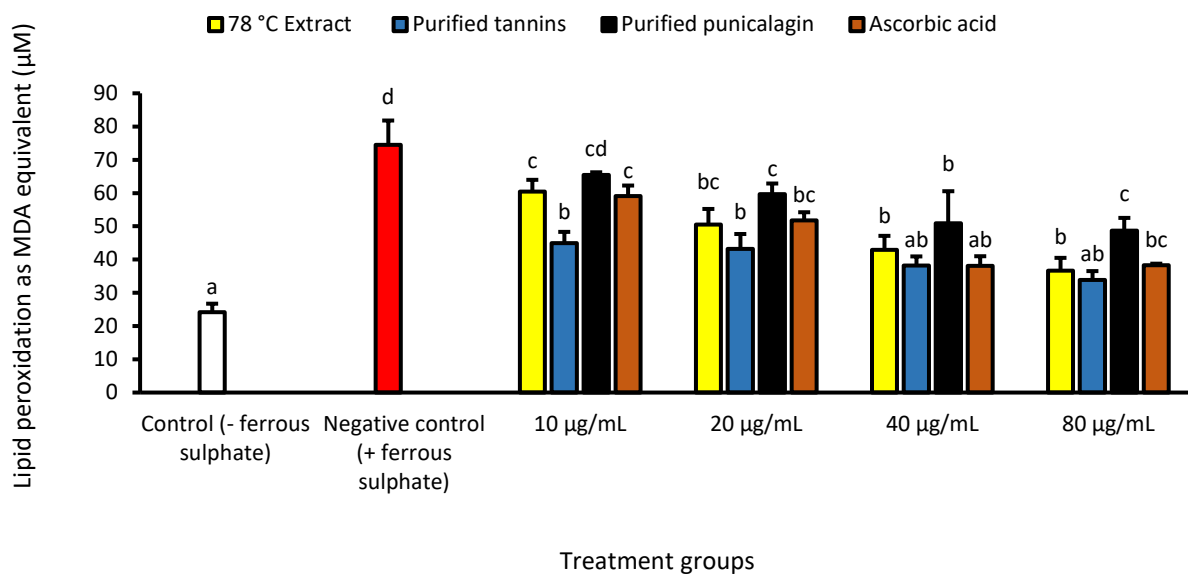


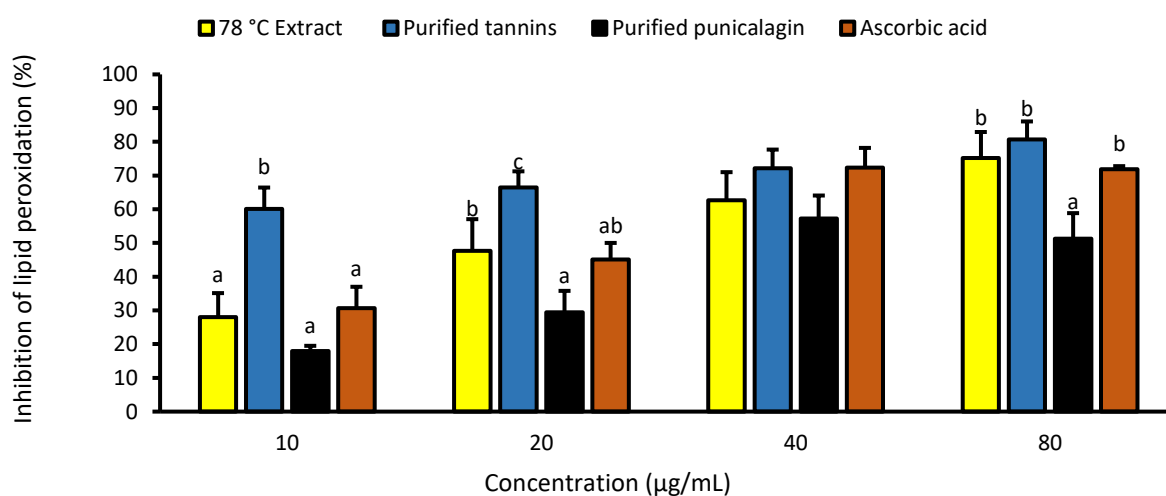
Figure 4.7: *In vitro* dose-dependent anti-linoleic acid peroxidative activity of the 78 °C peel extract, the tannin recovered from the extract and the corresponding purified punicalagin. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, statistical comparison was done among the treatment groups at a given concentration. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

4.3.3 Effect on lipid peroxidation and antioxidant enzyme status of Chang liver cells induced with oxidative stress

Like the *in vitro* anti-linoleic acid peroxidative activity, a similar trend of anti-lipid peroxidative activity was observed in Chang liver cells. The purified tannin ($IC_{50} = 3.67 \mu\text{g/mL}$) outperformed the corresponding crude extract ($IC_{50} = 24.3 \mu\text{g/mL}$) and purified punicalagin ($IC_{50} = 59.0 \mu\text{g/mL}$), as well as ascorbic acid ($IC_{50} = 22.4 \mu\text{g/mL}$) (Table 4.1 and Figure 4.8). The anti-lipid peroxidative activities of the crude extract and purified tannin in Chang liver cells were accompanied by a significant ($p < 0.05$) recovery of antioxidant enzymes (SOD and catalase) activities that were depleted by oxidative stress (Figure 4.9).

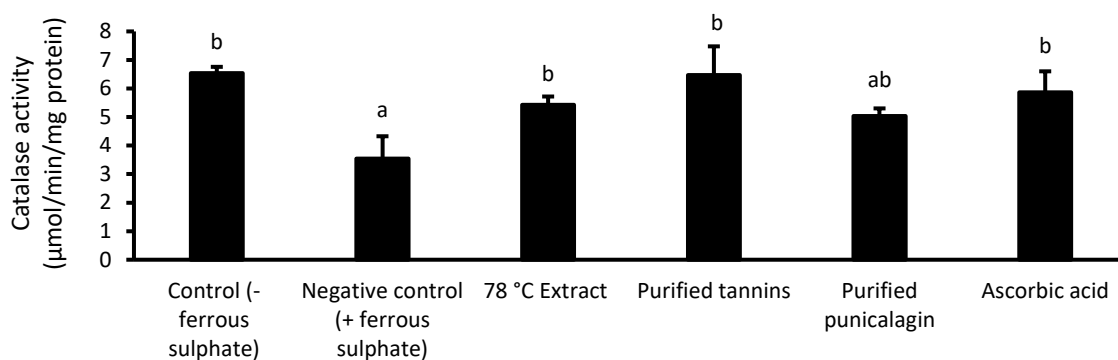


(a)

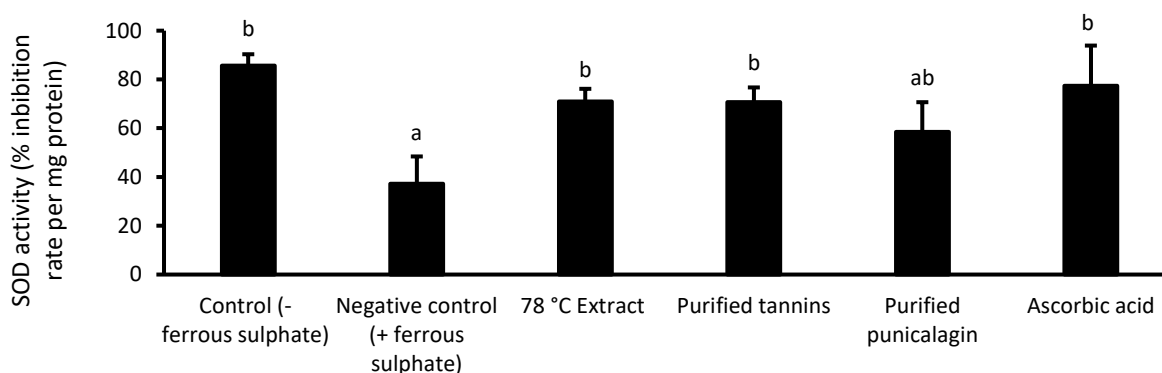


(b)

Figure 4.8: Dose-dependent (a) anti-lipid peroxidative effect and (b) percentage inhibitory activity of the 78 °C peel extract, the tannin recovered from the extract and the corresponding purified punicalagin on lipid peroxidation in Chang liver cells induced with oxidative stress. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, statistical comparison was done between the controls (control and negative control) and treatment groups at a given concentration or among the treatment groups at a given concentration. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.



(a)

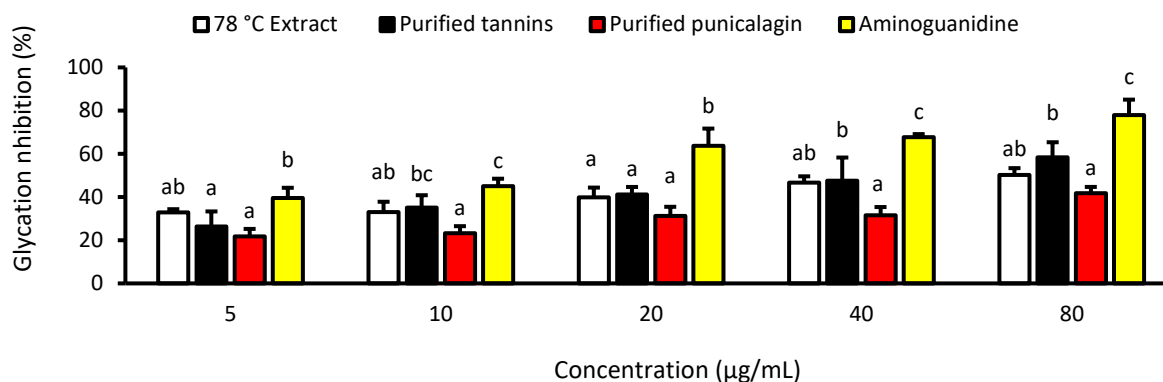


(b)

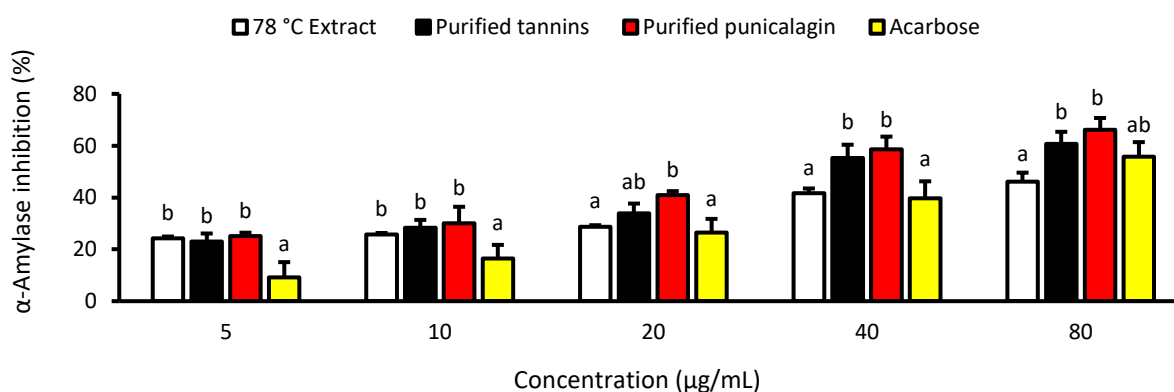
Figure 4.9: Effect of the 78 °C peel extract, the tannin recovered from the extract and the corresponding purified punicalagin (a) catalase and (b) SOD activities in Chang liver cells induced with oxidative stress. For each assay, statistical comparison was done between the controls (control and negative control) and treatment groups at a given concentration or among the treatment groups at a given concentration. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

4.3.4 *In vitro* inhibitory activity on glucose-induced BSA glycation and α -amylase

The anti-glycation activity of the crude peel extract and the recovered tannin was twice more potent than that of the purified punicalagin (Figure 4.10a and Table 4.1). The α -amylase inhibition data showed that purified punicalagin ($IC_{50} = 31.9 \mu\text{g/mL}$) outperformed the crude extract ($IC_{50} = 59.8 \mu\text{g/mL}$) and the tannin ($IC_{50} = 39.4 \mu\text{g/mL}$) recovered from the extract (Figure 4.10b and Table 4.1).



(a)



(b)

Figure 4.10: *In vitro* dose-dependent (a) α-amylase inhibitory and (b) anti-glycation activities of the 78 °C peel extract, the tannin recovered from the extract and the corresponding purified punicalagin. Data are presented as mean ± SD of triplicate analysis. For each assay, statistical comparison was done among the treatment groups at a given concentration. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

4.3.5 Effect on glucose uptake/utilization in L-6 myotubes

The 78 °C aqueous peel extract and its purified constituents dose dependently increased glucose uptake in muscle cells (L-6 myotubes), with the purified tannin ($EC_{50} = 14.8 \mu\text{g/mL}$) outperforming the extract ($EC_{50} = 55.0 \mu\text{g/mL}$) and purified punicalagin ($EC_{50} = 112 \mu\text{g/mL}$) (Table 4.1 and Figure 4.11).

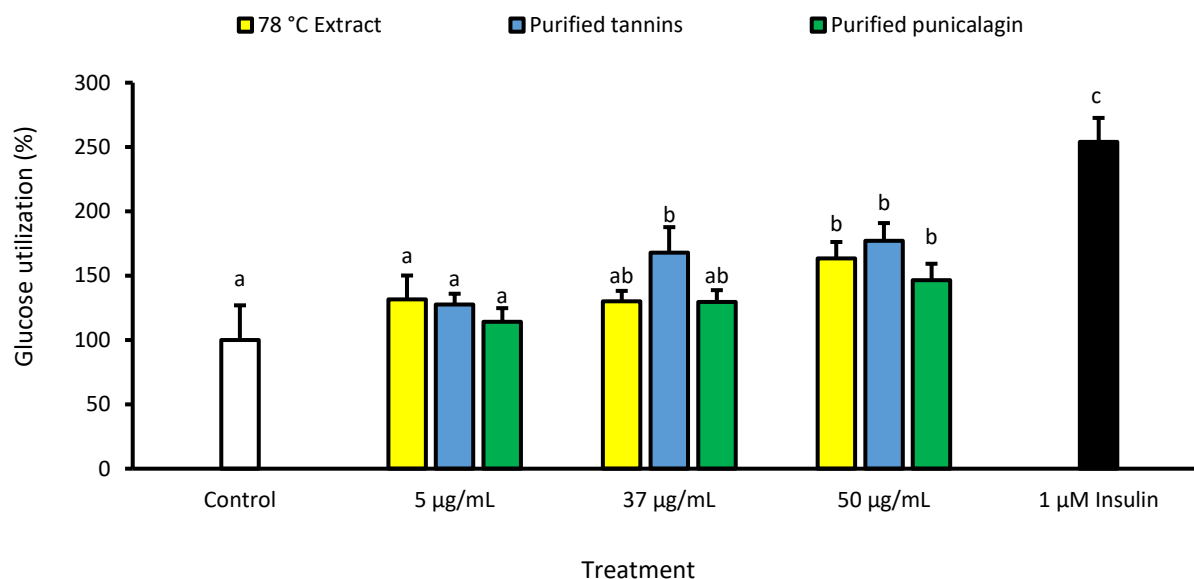


Figure 4.11: Dose dependent modulatory effect of the 78 °C peel extract, the tannin recovered from the extract and the corresponding purified punicalagin on glucose uptake/utilization in L-6 myotubes. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, statistical comparison was done between the controls (control and insulin) and treatment groups at a given concentration. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

The vascular and metabolic health benefits of pomegranate fruit have been frequently mentioned (Kandylis and Kokkinomagoulos, 2020; Giménez-Bastida et al., 2021). However, advances in research have shown that the peel of this fruit contains phytochemicals that can potentially increase the medicinal relevance of this fruit in managing metabolic diseases such as diabetes and oxidative complications. In the phytomedicinal context of the fruit and its peel, the ellagitannins have been considered as the major principles influencing the biological activity of pomegranate. Ellagitannins such as punicalagin and ellagic acid are considered key bioactive constituents (Sun et al., 2017; Chukwuma et al., 2020; Mo et al., 2022; Akuru et al., 2022; Marra et al., 2022). Nevertheless, the optimum recovery methods of these bioactive ellagitannins from pomegranate peel and their bioactivity profiling has been scarcely reported. In the present study, optimum extraction and purification methods were used to recover ellagitannins and punicalagin from pomegranate peel and comparatively evaluated for their glycemic control and antioxidant profiles.

As extraction temperature increased from 25 °C to 37 °C, the phenol and flavonoid contents appreciably decreased and significantly ($p < 0.05$) for flavonoid content (**Figure 4.1**). This decrease may be attributed to the action of polyphenol oxidase in the fresh fruit peels. Polyphenol oxidase degrades polyphenols and are optimally active at temperatures between 35 °C and 37 °C (Sikora et al., 2019). Thus, may have depleted phenol and flavonoid contents of the extracts obtained at 37 °C. The phenol and flavonoid contents gradually increased as extraction temperature increased and peaked at 78 °C (**Figure 4.1**). It has been documented that increasing extraction temperature facilitates the release of insoluble bound phenolic compounds and the breakdown lignin (Irakli et al., 2018), which may have contributed to the gradual temperature-dependent increase of phenol and flavonoid contents during extraction. Additionally, the increase in temperature beyond 37 °C may have caused inactivation of polyphenol oxidase, thus decreasing the rate of polyphenol degradation by the enzyme. Beyond 78 °C (at 95 °C), a significant drop in the levels of polyphenols and flavonoids was observed (**Figure 4.1**), which may be attributed to thermal degradation of polyphenols at high

temperatures. Moreover, it has been previously mentioned that temperatures beyond 80 °C could thermally degrade polyphenols, resulting in reduced phenol content, while extraction temperatures between 60 and 80 °C result in optimum recovery of polyphenols (Antony and Farid, 2022).

Plant polyphenols are known scavengers of free radicals due to their hydroxyl H-atom transfer abilities to form stable phenoxy radicals (Chen et al., 2020). Polyphenols also have iron chelating ability by binding to iron (Scarano et al., 2023). Thus, it is plausible that the high polyphenol and flavonoid contents of the peel extract obtained at 78 °C influenced its higher radical scavenging (**Figures 4.4a and b**) and Fe^{3+} reducing (**Figure 4.4c**) capacities at the tested concentration (45 µg/mL) relative to the capacities of the other extracts.

Despite a low α -glucosidase inhibitory action of the extracts (**Figure 4.5a**), the stronger α -amylase inhibitory action of the extract obtained at 78 °C relative to that of the other extracts (**Figure 4.5b**) and acarbose suggests its potential to promote glycemic control by delaying carbohydrate digestion (Ćorković et al., 2022). The α -amylase inhibitory action of many dietary polyphenols has been documented (Ćorković et al., 2022), which may be an important factor influencing the potent α -amylase inhibitory action of the extract obtained at 78 °C. This may be indicative of the higher tannin yield and punicalagin constituent of the 78 °C peel extract relative to the other extracts, as well as the presence of other ellagitannins (**Figures 4.2 and 4.3**), which may have worked synergistically with the constituent punicalagin to potentiate the promising α -amylase inhibitory action of this extract.

Documented evidence has revealed the notable contribution of tannins to the α -amylase inhibitory action of some plants (Xiao et al., 2013; Ćorković et al., 2022). Tannin rich extracts of strawberry, raspberry, red grape, red wine, and green tea have been shown to be effective in inhibiting α -amylase, while removing tannins from blue berry extracts weakened the α -amylase inhibitory action of the extracts (McDougall and Stewart, 2005). In fact, hydrolysable tannins, such as ellagitannins have been documented as major α -amylase inhibitory components of many berries (Xiao et al., 2013; Ćorković et al., 2022), which suggests a correlation between the high tannin yield (**Figure 4.2a**) and the potent α -amylase inhibitory activity (**Figure 4.5b**) of the extract obtained at 78 °C. Reports from previous studies (Kerimi et al., 2017) further suggest that the significantly ($p < 0.05$) higher amount punicalagin A and B observed in the tannin recovered from the extract obtained at 78 °C compared to the amounts in the tannins recovered from the other extracts (**Figure 4.2b**) may be notably influential in the potent α -

amylase inhibitory activity of the 78 °C extract. Moreover, pomegranate punicalagin has been shown to be an effective inhibitor of α -amylase and comparable to acarbose (Kerimi et al., 2017). It was, therefore, rationale to purify punicalagins from the tannin recovered from the 78 °C extract as shown in Figures 4d and e and comparatively examine the dose dependent glycemic control and antioxidant potential relative to the corresponding crude extract and tannin.

Furthermore, the dose-dependent radical scavenging (**Figure 4.6**) and anti-linoleic acid peroxidation (**Figure 4.7**) data suggest the ability of the extract obtained at 78 °C, as well as the tannin and punicalagin recovered from it to suppress lipid peroxidation and douse the deleterious action of free radicals on biological molecules during oxidative stress, with the purified tannin having stronger potential than the extract and the purified punicalagin (**Table 4.1**). The stronger anti-lipid peroxidative potential of the recovered tannin was replicated in Chang liver cells (**Figure 4.8** and **Table 4.1**). This was accompanied by a modulatory action on depleted antioxidant enzymes in the cells (**Figure 4.9** and **Table 4.1**), suggesting the potential of pomegranate peel extract and ellagitannins to improve in vivo antioxidant status and suppress detrimental oxidative stress.

The remarkable antioxidant potential of the purified tannin may be attributed to the presence of ellagitannins such as punicalagin, ellagic acid, granatin A, casuarinin, geraniin, etc and other phenolics such as gallic acid and quercetin hexoside (**Figure 4.2c**), which together, potentiated the potent antioxidant action of the purified tannin. Moreover, ellagitannins-rich dietary supplement from pomegranate has been shown to suppress lipid peroxidation in obese individuals (Heber et al., 2007), while commercial ellagic have been shown to exert anti-lipid peroxidative activity in obese animals (Xu et al., 2021). Also, the antioxidant properties gallic (Gao et al., 2019), as well as quercetin and its glucosides (Terao, 2023) have been well documented.

Besides antioxidant effects, the peel crude extract obtained at 78 °C, as well as the recovered tannin and punicalagin showed glycemic control and anti-glycation potential. The anti-glycation activity of the crude peel extract and the recovered tannin was twice more potent than that of the purified punicalagin (**Figure 4.10a** and **Table 4.1**), suggesting the bioactive contribution of other polyphenols present in the extract and tannin. Consistent anti-glycation activity of pomegranate peel acetone extract has been previously documented (Chukwuma et al., 2020), suggesting that potential of pomegranate peel to mitigate hyperglycemia induced

glycation and associated diabetic complications. The dose dependent α -amylase inhibition data (**Figure 4.10b**) suggests that pomegranate peel contains ellagitannins that may exert glycemic control by inhibiting α -amylase and delaying carbohydrate breakdown. The IC_{50} values (**Table 4.1**) further suggests that punicalagins may be the notable ellagitannins influencing the α -amylase inhibitory potential of pomegranate ellagitannins.

Another glycemic control potential exhibited by the pomegranate peel extract obtained at 78 °C and its purified constituents was a modulatory action on cellular glucose uptake/utilization. Glucose uptake/utilization in cells of peripheral tissue is a major mechanism by which insulin normalizes blood glucose levels (Ramorobi et al., 2022), which suggests the potential of pomegranate peel to improve insulin function and glucose tolerance. The glucose uptake effect of pomegranate peel has been previously demonstrated in Chang liver cells (Chukwuma et al., 2020). In the present study, the dose-dependent glucose uptake modulatory effect of the 78 °C aqueous peel extract in muscle cells (L-6 myotubes) was more potent than that of the extract and purified punicalagin (**Figure 4.11** and **Table 4.1**). Constituents such as ellagic acid and gallic acid present in the purified tannin (**Figure 4.2c**) may influence its potent glucose uptake. Moreover, the modulatory action of ellagic and gallic acid on insulin signalling, cellular glucose uptake and glycemic control has been documented (Prasad et al., 2010; Ding et al., 2019; Hidalgo-Lozada et al., 2022).

5.2 Conclusion

The present study demonstrated the antioxidant and glycemic control potential of pomegranate peel aqueous extract and its purified constituent ellagitannins. Extraction at 78 °C yielded optimum amounts of polyphenols, as well as ellagitannin constituents, which afforded the extract stronger antioxidant and α -amylase inhibition profiles, relative to the extracts obtained at other temperatures. While punicalagin appears to be a major ellagitannin influencing the α -amylase inhibitory action of pomegranate peel, it works in concert with other ellagitannin and polyphenol constituents, including ellagic acid and gallic acid in exerting potent anti-lipid peroxidative and glucose uptake modulatory activities. The data of the present suggest that tannin/ellagitannins purified from pomegranate peel aqueous extract obtained at 78 °C may be a promising dietary supplement for exerting glycemic control and mitigating oxidative stress.

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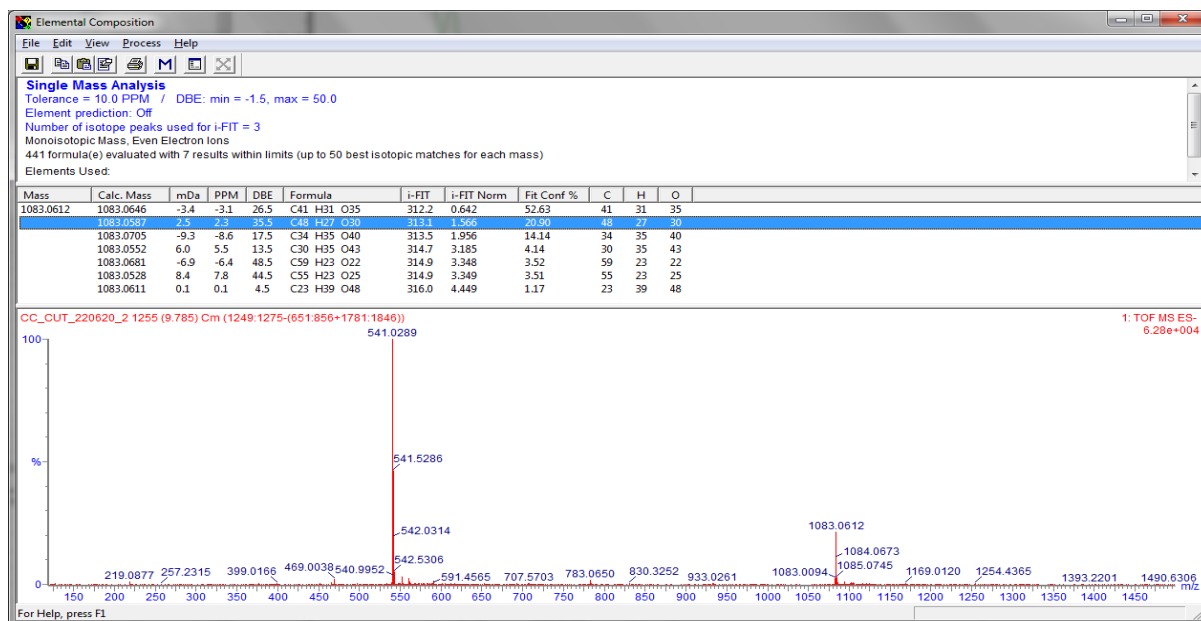
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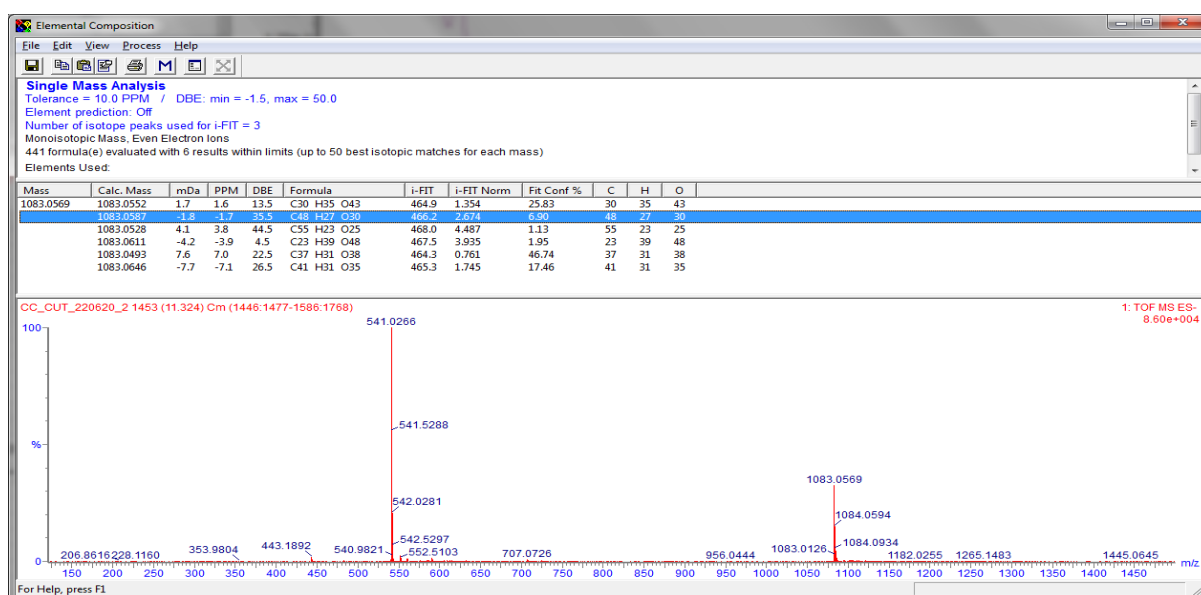
APPENDIX 1

SUPPLEMENTARY MATERIAL

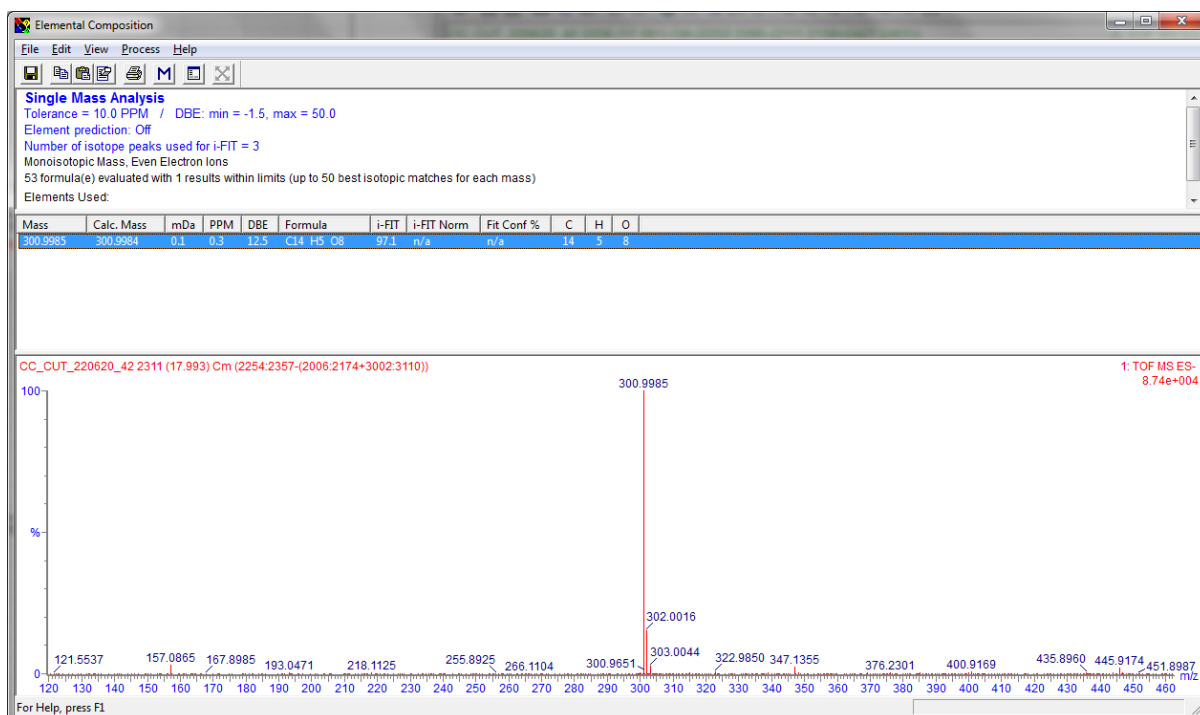
Single Mass Analysis of each of the analytes/compounds identified in the ellagitannins from the different solvent extracts.



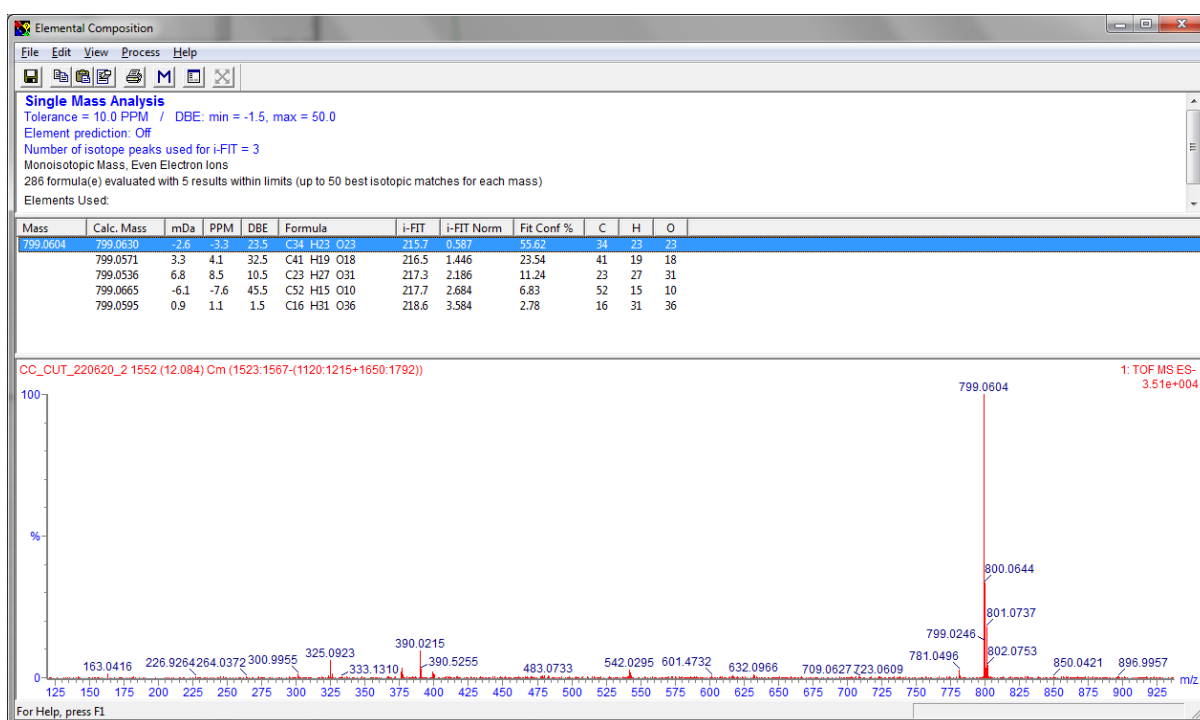
Punicalagin A



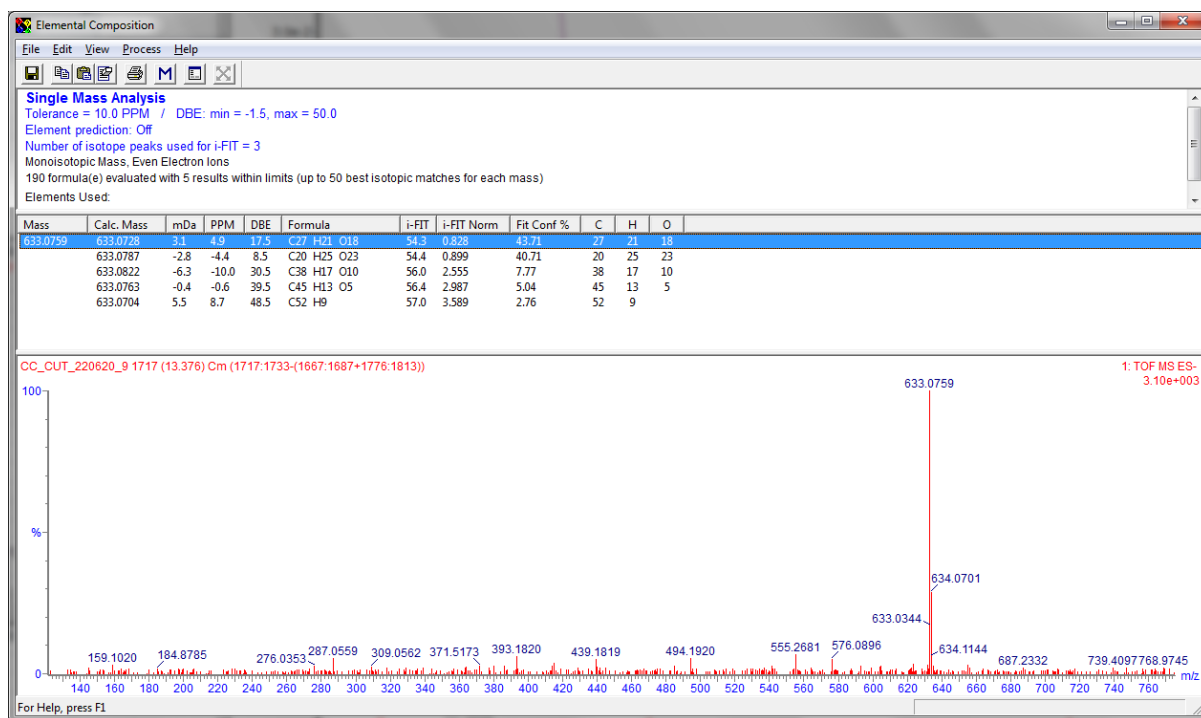
Punicalagin B



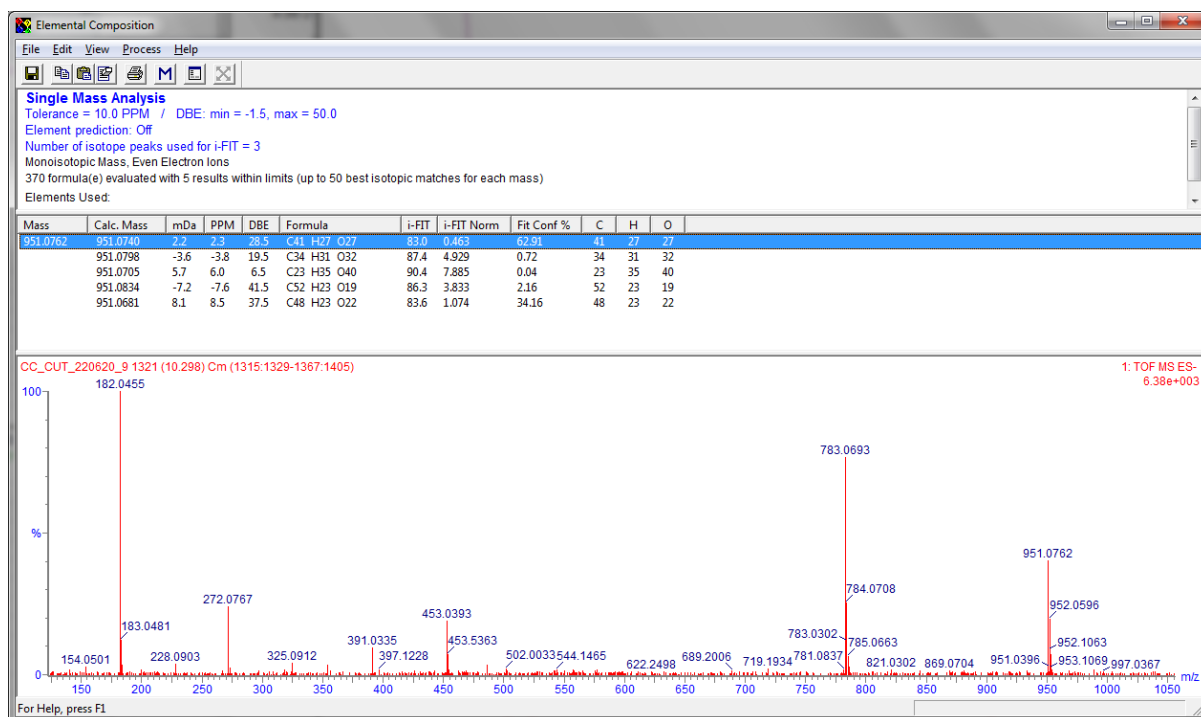
Ellagic acid



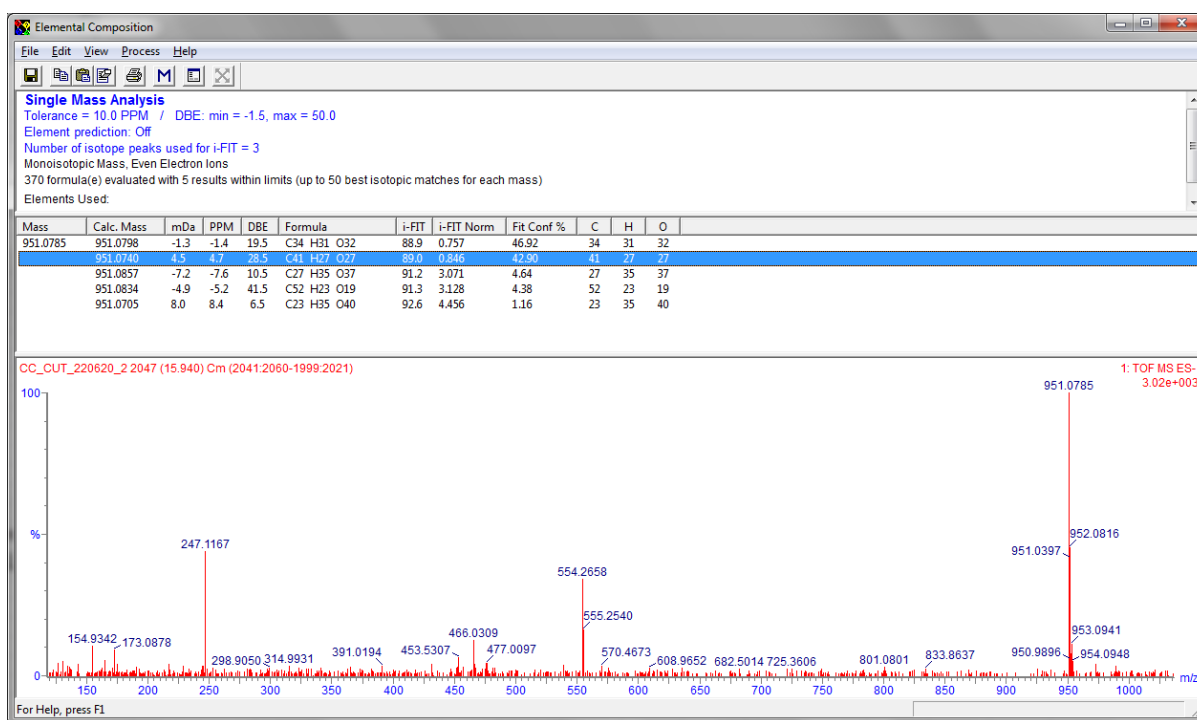
Granatin A



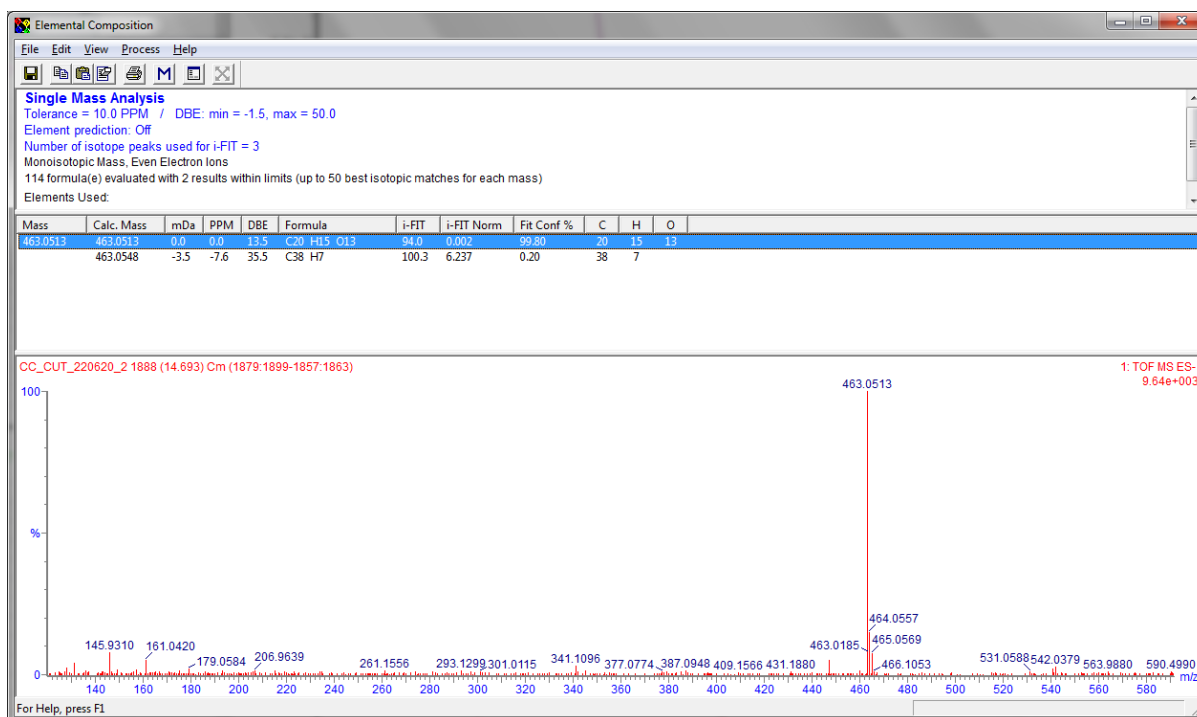
Corilagin



Casuarinin



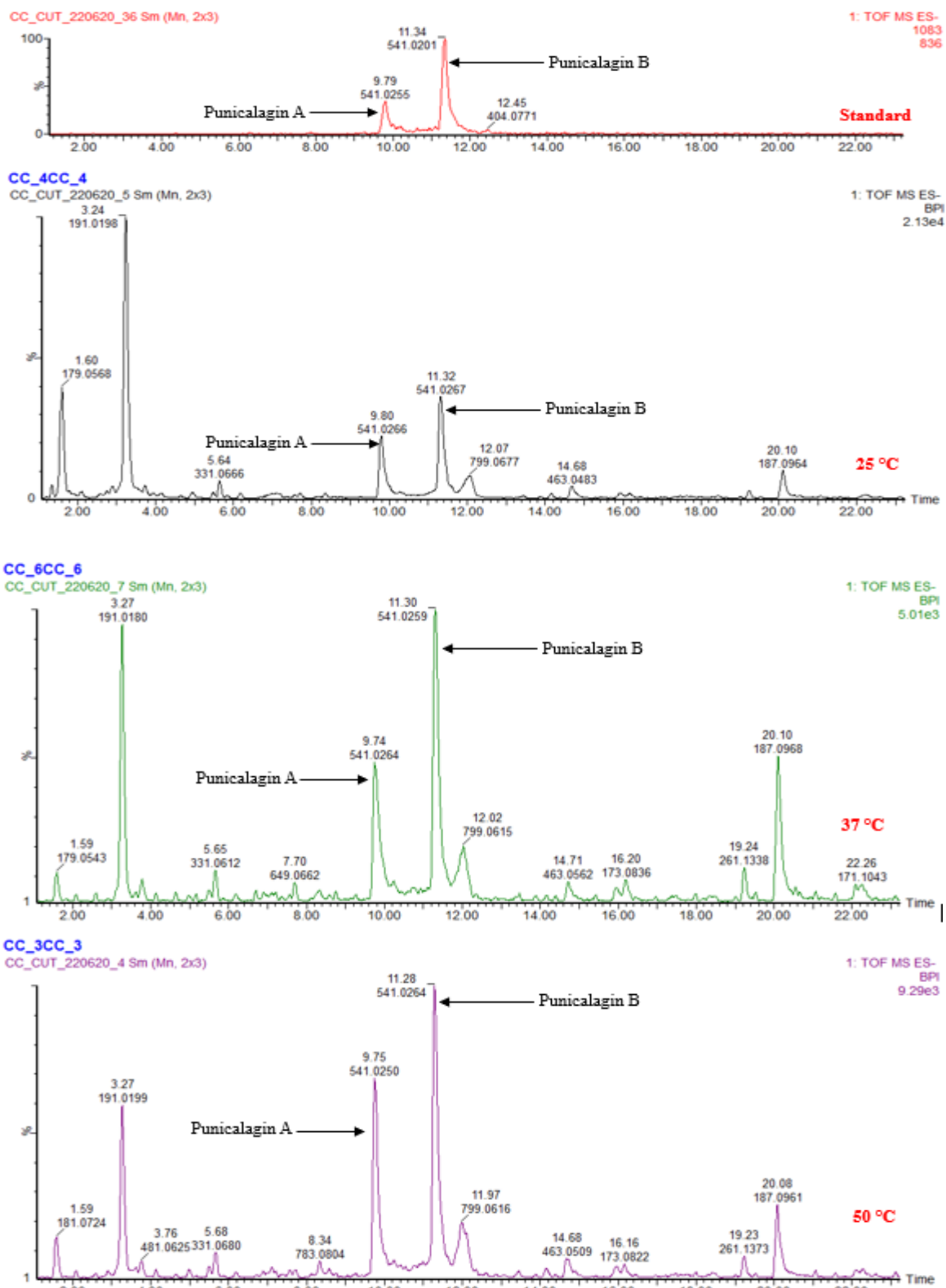
Geraniin



Quercetin hexoside

APPENDIX 2

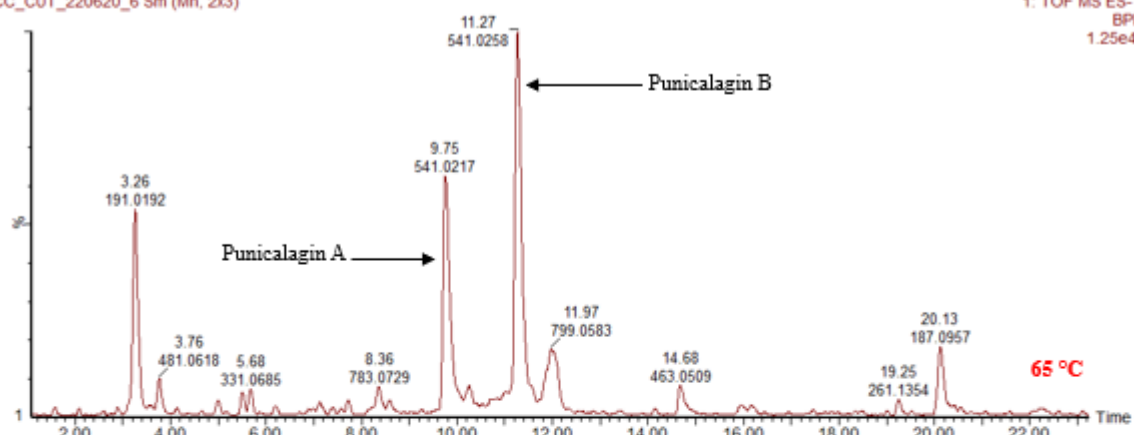
LC-MS chromatograms of punicalagin standard, as well as the tannins recovered from the different peel extracts.



CC_5CC_5

CC_CUT_220620_6 Sm (Mn, 2x3)

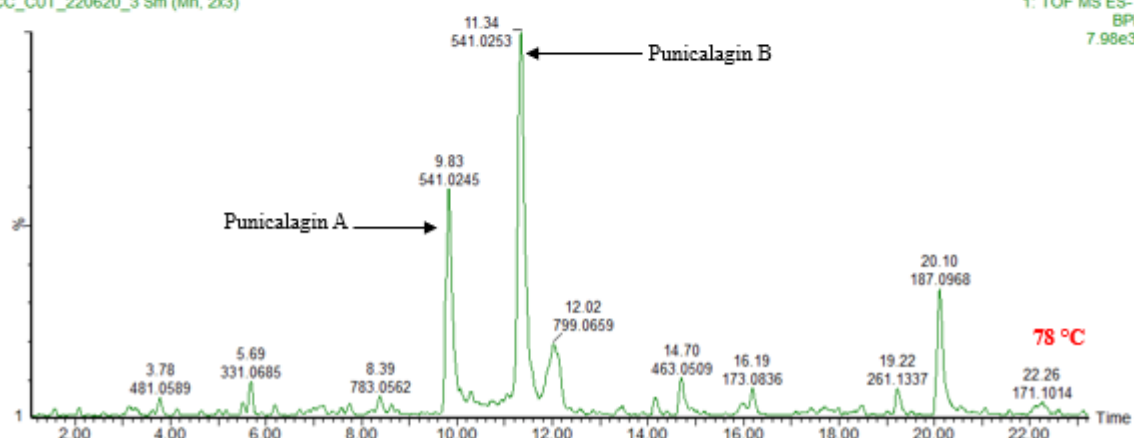
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BPI
1.25e4



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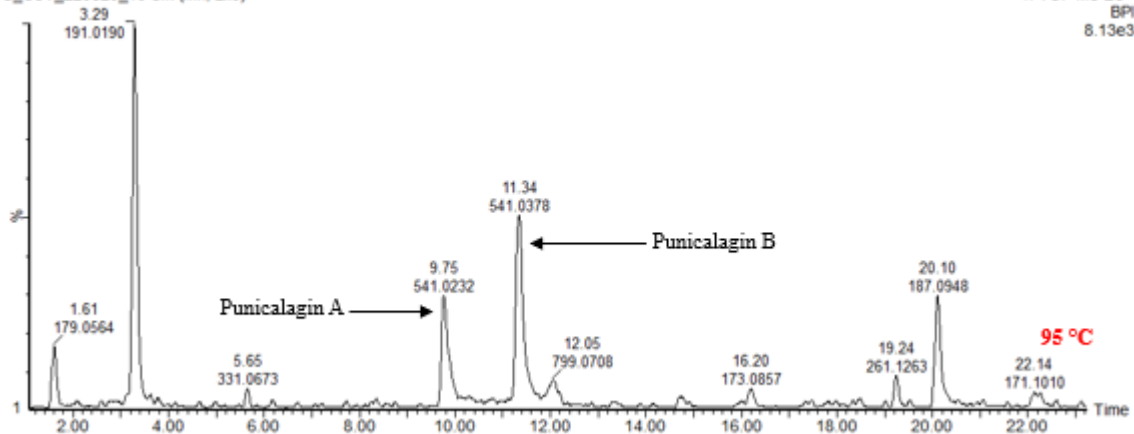
1: TOF MS ES-
BPI
7.98e3



CC_9CC_9

CC_CUT_220620_10 Sm (Mn, 2x3)

1: TOF MS ES-
BPI
8.13e3





Original article

Temperature-dependent extraction and chromatographic recovery and characterisation of ellagitannins with potent antioxidant and glycaemic control properties from 'Wonderful' pomegranate peel

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Summary Pomegranate peel contains bioactive ellagitannins including punicalagin, a major bioactive principle. However, studies have not optimally recovered and evaluated these ellagitannins for their antioxidant and glycaemic control potential. In the present study, a temperature-dependent extraction was employed to optimally recover bioactive ellagitannins and punicalagin from pomegranate (variety 'Wonderful') peel. The peel was extracted with distilled water at different temperatures (25, 37, 50, 65, 78 and 95 °C) and evaluated for phenol and flavonoid contents, *in vitro* antioxidant and enzyme inhibitory activities. XAD16N resin was used to recover tannins from the extracts and punicalagin was purified from the tannin recovered from the 78 °C crude extract. LC–MS was used to characterise the ellagitannins profile. The dose-dependent anti-lipid peroxidative and glucose uptake potential of the punicalagin and its parent crude extract and tannin were measured. The extract obtained at 78 °C had the highest phenolic, flavonoid, tannin and punicalagin contents, which potentiated stronger radical scavenging, ORAC (IC₅₀ = 9.64 vs. 15.6 and 25.6 µg/mL, respectively), anti-linoleic acid peroxidative (IC₅₀ = 9.97 vs. 19.9 and 27.3 µg/mL, respectively) and antiglycation (IC₅₀ = 29.1 vs. 34.2 and 79.1 µg/mL, respectively) activities than the extract and the purified punicalagin. Similarly, the cellular anti-lipid peroxidative and glucose modulatory effects of the recovered tannin (IC₅₀ = 3.67 µg/mL; EC₅₀ = 14.8 µg/mL) better than that of the extract (IC₅₀ = 24.3 µg/mL; EC₅₀ = 55.0 µg/mL) and the purified punicalagin (IC₅₀ = 59.0 µg/mL; EC₅₀ = 112 µg/mL), which may be attributed to the complementary action of the tannin's constituent compounds (punicalagin, ellagic acid, granatin A, corilagin, casuarinin, gallic acid and quercetin hexoside). The α-amylase inhibitory data, however, suggest the purified punicalagin influences enzyme inhibitory potential of pomegranate peel. The study suggests tannin/ellagitannin purified from pomegranate peel aqueous extract obtained at 78 °C may be a promising dietary supplement for exerting glycaemic control and mitigating oxidative stress.

Keywords Chromatography, diabetes, ellagitannins, oxidative stress, pomegranate peel.

Introduction

Recent data show that diabetes is a global health concern. Between 2019 and 2021, the number of adults living with diabetes increased from 463 to 537 million people, which has increased the mortality and

morbidity outcomes and health care expenditures associated with the disease (International Diabetes Federation, 2019, 2021). The disease is broadly classified into type 1 and type 2 (T2D) depending on the aetiology and pathophysiology. T2D is the most prevalent (International Diabetes Federation, 2021) and is characterised by insulin malfunction (Garcia-Garcia *et al.*, 2020). Cells involved in glucose metabolism are non-responsive to insulin signalling. Thus, metabolic

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processes (glucose uptake and utilisation and glycogen metabolism) that are key in glucose homeostasis are impaired, resulting in impaired glycaemic control and persistent hyperglycaemia (Galicia-Garcia *et al.*, 2020). Lifestyle-related factors including inactivity and unhealthy eating habits, as well as detrimental weight gain are some factors that may trigger insulin resistance and increase the risk of metabolic syndrome and T2D (Zhu *et al.*, 2021).

While there are several synthetic antidiabetic medications, natural antioxidants are becoming relevant in managing oxidative stress-related damages in diabetic conditions (Kanwugu *et al.*, 2022). This is due to the strong link between diabetes and oxidative stress. In hyperglycaemic state, several processes trigger reactive oxygen species (ROS) production (Bhatti *et al.*, 2022). Processes involved in glycation are triggered more, which results in elevated production of ROS and other reactive radicals (Bhatti *et al.*, 2022). Glucose metabolism in hyperglycaemic state can lead to superoxide ion production, a deleterious ROS. Elevated lipid peroxidation has also been shown to trigger ROS production in diabetic state (Bhatti *et al.*, 2022). ROS can oxidatively damage biological molecules, which have been implicated in various diabetic complications (Bhatti *et al.*, 2022).

Pomegranate is rich in antioxidants and its juice has been documented to have cardiovascular health benefits (Kandylis & Kokkinomagoulos, 2020; Giménez-Bastida *et al.*, 2021), which has been linked to its phenolic constituents including the predominant ellagitannins (Giménez-Bastida *et al.*, 2021). Moreover, dietary supplements rich in ellagitannins have been shown to exhibit inhibitory action on lipid peroxidation in overweight human subjects (Heber *et al.*, 2007). Ellagitannins are hydrolysable tannins constituting mainly punicalagins, punicalin, ellagic acid gallagic acid and relative glycosides or derivatives (Altieri *et al.*, 2019).

Documented evidence has shown that the fruit peel of pomegranate is also rich in these ellagitannins and other phenolics, suggesting its therapeutic potential (Chukwuma *et al.*, 2020; Akuru *et al.*, 2022; Marra *et al.*, 2022; Mo *et al.*, 2022). The radical scavenging and the cellular anti-lipid peroxidative effects of pomegranate peel have been reported (Chukwuma *et al.*, 2020; Akuru *et al.*, 2022; Marra *et al.*, 2022). The anti-inflammatory and cardiovascular protective attributes of pomegranate peel have been reported in both animal models and human subjects (Mo *et al.*, 2022). In a previous study, the acetone extract of pomegranate peel exhibited glycaemic control potential by inhibiting α -amylase and glucose-induced protein glycation, as well as modulating glucose uptake/utilisation in Chang liver cells (Chukwuma *et al.*, 2020).

While ellagitannins are perceived as key bioactive principles in pomegranate peel, punicalagin remains

the predominant ellagitannin of pomegranate peel and possibly a key bioactive phytoconstituent (Sun *et al.*, 2017; Mo *et al.*, 2022). Sun *et al.* (2017) reported the potent *in vitro* and *in vivo* antioxidant potential of commercial punicalagin, which outperformed commercial ellagic acid and punicalin (other key components of pomegranate peel ellagitannins). However, the recovery of these bioactive pomegranate peel ellagitannins for possible medicinal application, without sacrificing their biological properties, proves difficult most times. Temperature-dependent extraction has been shown to improve recovery of plant phenolics, if conducted optimally. However, the optimum temperature and chromatographic conditions that will ensure the optimum recovery of pomegranate peel ellagitannins, as well as preserve their antioxidant and glycaemic potential, are still not certain. To the best of our knowledge, the antioxidant and antihyperglycemic effects of ellagitannins and punicalagin optimally purified from pomegranate peel have not been studied. Hence, in this study, a temperature-dependent extraction and chromatography techniques were employed in optimally recovering ellagitannins and punicalagin from pomegranate peel, which were evaluated for their antioxidant and anti-hyperglycaemic potentials.

Materials and methods

Collection fruit and preparation and extraction of fruit peel

Pomegranate fruits ('Wonderful' Var.; grade: Class 1 or processing grade) were purchased from Sonlia Fruit Packhouse (Wellington, Western Cape Province, South Africa) on 03 May 2021. The fruits were washed with clean tap water.

The peel of the fruits was removed. Six aliquots of 1 kg of the fresh fruit peel were separately soaked in 2.5 L of distilled water [*i.e.*, mass (kg)/volume (L) ratio of 1:2.5] using six metallic bowls with fitted covers. The six metallic bowls were separately incubated at six different temperatures (25, 37, 50, 65, 78 and 95 °C) for 1 h in a temperature-regulated oven. Exactly after 1 h incubation, the fruit peel in each metallic bowl was blended with the water in which it was incubated to form a puree. The puree was first filtered with a cheese cloth and then with a filter paper, before recovering the crude extract by freeze drying (Martin Christ Alpha 1–2 LDplus Freeze Dryer supplied by Separations, Johannesburg, South Africa).

Total phenol and flavonoid measurement

The total phenol and total flavonoid contents of the extracts were measured using methods that have been previously reported (Chukwuma *et al.*, 2020).

For total phenol estimation, 25 μL of the extracts (at 45 $\mu\text{g}/\text{mL}$ in assay volume) or increasing concentration of gallic acid solution (3.75–120 $\mu\text{g}/\text{mL}$ in assay volume) was aliquoted into designated wells of a clear-bottom 96-well plate. Next, 125 μL of 10 $\times$ diluted Folin–Ciocalteu reagent (Merck, South Africa) was added to the wells. Thereafter, 100 μL of 0.7 M Na_2CO_3 was added into the wells, and the plate was incubated for 30 min at room temperature. Absorbance was measured at 765 nm using a Spectra-Max M2 microplate (Molecular Devices, California, USA).

For total flavonoid estimation, 20 μL of the extracts (at 45 $\mu\text{g}/\text{mL}$ in assay volume) or increasing concentration of quercetin solution (3.75–120 $\mu\text{g}/\text{mL}$ in assay volume) was aliquoted into designated wells of a clear-bottom 96-well plate. Next, 80 μL of 98% ethanol was added into the wells. Thereafter, 100 μL of 2% AlCl_3 was added into the wells, and the plate was incubated for 1 h at room temperature. Absorbance was measured at 430 nm.

The total phenol and flavonoid contents of the extracts were computed from gallic acid ($y = 0.0447x + 0.0194$; $R^2 = 0.9988$) and quercetin ($y = 0.0345x + 0.0651$; $R^2 = 0.997$) standard curves and expressed as mg/g GAE (gallic acid equivalent) and mg/g QE (quercetin equivalent), respectively, using the following formula:

Total phenol(mg/g GAE)

or Total flavonoid(mg/g QE) content = $\frac{C \times SV}{M}$

where 'C' is the concentration (mg/mL) extrapolated from gallic acid and quercetin standard curves; 'SV' is the sample volume (mL), and 'M' is the amount (g) of the sample in the 'SV' (mL) of the sample solution; 'GAE' and 'QE' mean gallic acid equivalent and quercetin equivalent, respectively.

Preliminary measurement of *in vitro* antioxidant and enzyme inhibition activities of extracts

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS^{•+}) scavenging, Fe^{3+} reducing and α -glucosidase and α -amylase inhibitory activities of the extracts were measured at 45 $\mu\text{g}/\text{mL}$ using the methods described in previous reports (Chukwuma *et al.*, 2020; Motloung *et al.*, 2020). For DPPH and ABTS radical scavenging and Fe^{3+} reducing assays ascorbic acid and Trolox were used as the positive controls at an equivalent concentration (45 $\mu\text{g}/\text{mL}$), while acarbose was used as the positive control for the α -glucosidase and α -amylase inhibitory assays at an equivalent concentration (45 $\mu\text{g}/\text{mL}$).

Total tannin (ellagitannins) recovery from extracts

A method modified from a previous report (Seeram *et al.*, 2005) was adopted in recovering tannin (ellagitannins) from the extracts. An Amberlite[®] XAD16N resin (product code: XAD16; Merck, Johannesburg, South Africa) was used. First, 1.52 g of each extract was dissolved in 100 mL of distilled water, filtered with a Whatman filter paper and stored in a clean screw-cap bottle (Fig. 1b). To prepare the XAD16N resin column, 35 g of the resin was washed twice with methanol, loaded into a flash column containing distilled water (Fig. 1a) and allowed to equilibrate for 12 h. After equilibrating, the water was completely drained using a water jet vacuum pump. Each of the dissolved extracts was separately poured into the column and gradually eluted with distilled water using the water jet vacuum pump. Elution continued until the tannin was completely adsorbed into the XAD resin and a consistent colourless eluent was observed (Fig. 1c,d). Any remaining water in the column removed using the vacuum pump. The tannin trapped in the resin was eluted with methanol at a steady flow rate using the water jet vacuum pump (Fig. 1e,f). The tannin eluent was air-dried under a fume hood and stored at -20°C in clean screw-cap vials.

The yield of the tannin recovered from each extract was calculated as follows:

$$\text{Yield}(\%) = \frac{\text{Mass(g) of tannin recovered from extract}}{1.52 \text{ g of extract}} \times 100$$

Purification of punicalagin from tannin

Punicalagin was purified from the tannin that was recovered from the peel extract prepared at 78°C . The method previously reported by Seeram *et al.* (2005) was adopted with slight modifications for isolation and purification of punicalagins. A Sephadex LH-20 gel column chromatography with methanol as the mobile phase was used. Fifty milligrams of the tannin fraction obtained from the crude extract was dissolved in 2 mL of methanol, and the resulting solution was subjected to a Sephadex LH-20 gel (Product code: 1003318800; Merck, Johannesburg, South Africa) column chromatography (45 cm $\times$ 18 mm filled at 80%) using the methanol as the mobile phase. The column flow rate was 0.8 mL/min. The TLC was developed with a solvent system of chloroform/ethyl acetate/ethanol/formic acid/distilled water (volume ratio of 4: 3:3:2:1, respectively) and used to monitor the collected fractions (~ 1.6 mL).

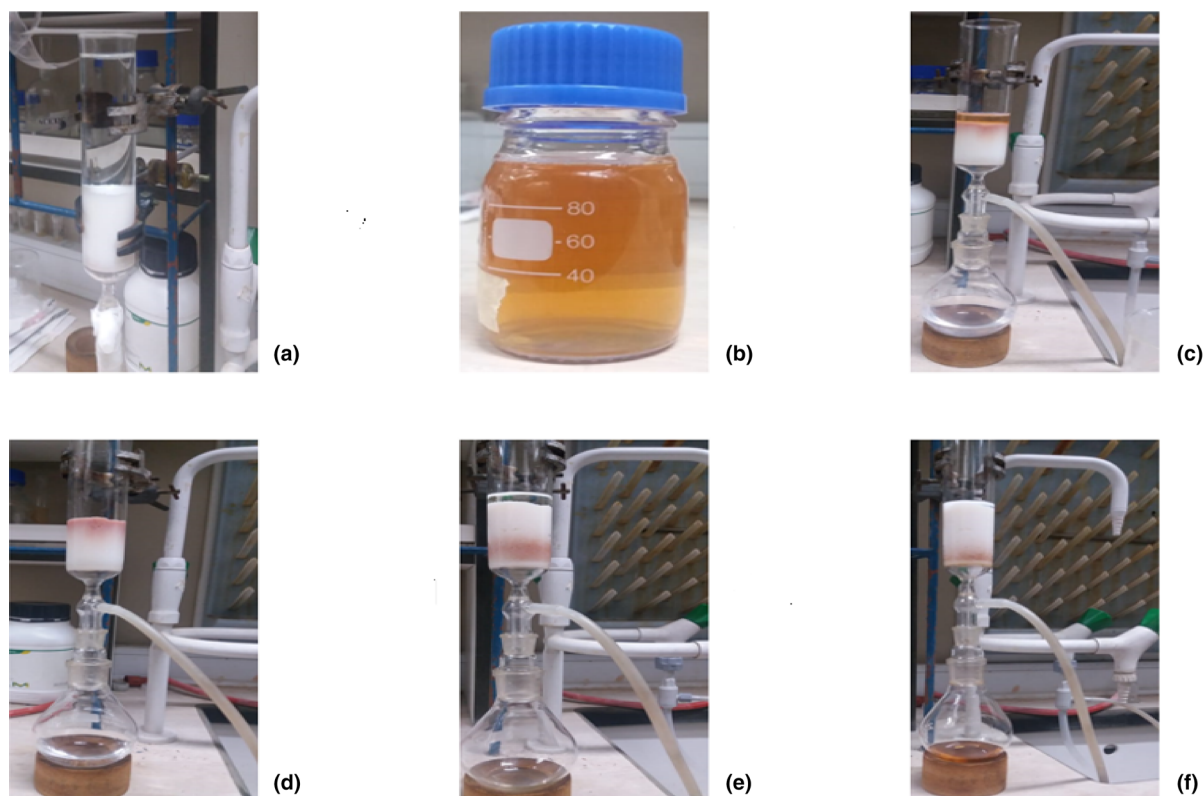


Figure 1 Illustration showing the recovery process of tannins from the pomegranate fruit peel extracts.

Liquid chromatography–mass spectroscopic (LC–MS) quantification

The recovered tannins and the purified punicalagin were subjected to LC–MS analysis. To perform the LC–MS protocol, the following were used: A Waters Synapt G2 (Waters Corporation, MA, USA), ESI probe and ESI Pos, at a 15 V cone voltage. The operation of liquid chromatography was done using Acquity binary solvent manager. The chromatographic column used was a HSS T3 column (Waters Corporation, MA, USA). It had a dimension of 2.1 × 150 mm and particle size of 1.8 µm. Two solvents systems (A and B) were used as the mobile phase. Solvent system A was water containing 0.1% methanoic acid, while solvent system B acetonitrile containing 0.1% methanoic acid. A 0.25 mL/min flow rate was applied for gradient chromatographic separation at 30 °C. The gradient condition followed the following sequence: 100% (A):0% (B), 10 min; 72% (A):28% (B), 21 min; 60% (A):40% (B), 50 s; and 0% (A):100% (B), 2 min. The samples were dissolved a mixture of acetonitrile and water (1:1 v/v), passed through a 0.22 µm filter and injected at a volume of 20 µL. The signals of the separated compounds were recorded at 254 nm. Punicalagin A and punicalagin B in the recovered tannin

(ellagitannins) fractions and the purified punicalagin were identified and quantified from a Punicalagin A + B (product code: PHL80524, Sigma Aldrich, Johannesburg, South Africa) calibration curve. Using the accurate masses and elemental compositions, the other compounds in the tannin fraction recovered from the 78 °C peel extract were determined and semi-quantified relative to ellagic acid standard according to accurate mass elemental composition and the extracted ion chromatography (Appendix S1). Quantified amounts were given in triplicates.

Measurement of dose-dependent antioxidant and glycaemic control potential

The dose-dependent bioactivity of the peel extract prepared at 78 °C was measured because of its stronger activity relative to the other extracts in the preliminary experiments. Its activity was compared with that of the tannin fraction and punicalagin recovered from it. Below are the performed dose-dependent assays.

Radical quenching activity

Three different *in vitro* experimental models were used to profile the radical quenching properties of the tested samples. These include DPPH and ABTS radical

scavenging and oxygen radical absorbance capacity (ORAC) assays.

The DPPH and ABTS radical scavenging assays were done according to the methods described in a previous report (Motloung *et al.*, 2020). The assays were conducted using increasing sample concentrations (3.75–60 µg/mL in assay volume), while ascorbic acid and Trolox were used as positive controls at equivalent concentrations.

For the ORAC assay, a recently reported method (Setlhodi *et al.*, 2023), which was modified from the method of Ou *et al.* (2013), was adopted. The assay was conducted using increasing sample concentrations (7.5–60 µg/mL in assay volume), while ascorbic acid was used as the positive control at equivalent concentrations.

Inhibition of linoleic acid peroxidation

The ability of the extracts, the recovered tannin and purified punicalagin to inhibit FeSO₄-induced linoleic acid peroxidation was performed using the method reported by Ramorobi *et al.* (2022), which was modified from the method reported by Choi *et al.* (2002). Into screw-cap vials, 30 µL of the test samples (extract, recovered tannin, purified punicalagin and ascorbic acid) at the tested concentrations (5–80 µg/mL in assay volume) or distilled water (in the vials assigned control and negative control) was dispensed. Next, 30 µL and 20 µL of 50 mM linoleic acid and Tris-HCl buffer (100 mM, pH 7.5) were respectively added. Twenty microliters of FeSO₄·7H₂O (2 mM in incubation volume) was aliquoted into the vials containing the samples, and the vials assigned negative control to cause linoleic acid peroxidation, while an equivalent volume of distilled water was dispensed in the wells assigned control. After 30 min incubation at 37 °C, 5.5% trichloroacetic acid and 0.25% thiobarbituric acid were added to the vials at respective volumes of 80 and 50 µL. The vials were placed in a boiling water bath for 30 min. After cooling, the vials were centrifuged at 3500 g for 5 min (Hettich Mikro 200 microcentrifuge, Hettich Lab Technology, Tuttlingen, Germany) and 150 µL of the clear layer was aliquoted into designated wells of a clear-bottom 96-well plate. The absorbance was read at 532 nm and the inhibitory potential of the tested samples on linoleic acid peroxidation was calculated as follows:

$$\text{Inhibition(\%)} = \frac{(A_{\text{Negative control}} - A_{\text{Normal control}}) - (A_{\text{Test}} - A_{\text{Normal control}})}{(A_{\text{Negative control}} - A_{\text{Normal control}})} \times \frac{100}{1}$$

where 'A' means 'Absorbance'.

α-amylase inhibition and antiglycation assays

The samples were dose dependently (5–80 µg/mL in assay volume) tested for their capacity to inhibit α-amylase activity and glucose-induced bovine serum albumin (BSA) glycation using the methods reported in a previous study (Motloung *et al.*, 2020). Acarbose and aminoguanidine were used as the positive controls for α-amylase and BSA glycation inhibition assays, respectively, at equivalent concentrations as the tested samples.

To perform the antiglycation assay, 50 µL of different concentrations (5–80 µg/mL in assay volume) of the samples or standard (aminoguanidine) or their solvents (control) was incubated with 50 µL of glucose (90 mg/mL) and 50 µL bovine serum albumin (10 mg/mL) in the dark for 1 week at 37 °C using black 96-well plates. Thereafter, fluorescence was measured at Ex/Em = 360 nm/420 nm, and the glycation inhibition was computed using the following formula:

$$\text{Activity(\%)} = \frac{\text{Fluorescence of control} - \text{Fluorescence of test}}{\text{Fluorescence of control}} \times 100$$

Measurement of glucose uptake/utilisation in L-6 myotubes

A reported method (Ramorobi *et al.*, 2022) modified from a previous article (Oyedemi *et al.*, 2013) was adopted for this assay. L-6 myoblast (ATCC CRL-1458, ATCC, VA, USA) cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum were trypsinised after confluence reached about 90%. The cells were seeded in designated wells of a 96-well plate at a 4000 cells/well density. After cells were confluent, the medium in each well was replaced with a differentiation medium (DMEM medium containing 2% horse serum) and incubated for 4–5 days. The spent medium was removed, and the differentiated cells were supplied with fresh medium that contained the tested samples (5, 37 and 50 µg/mL in the incubation volume) or distilled water (control group). Wells containing medium without cells were used as blanks. Following a 48-h incubation period, the sample-treated medium was removed, and the cells were washed with PBS. Next, RPMI medium containing 8 mM glucose and 0.1% BSA was dispensed into the wells. For the wells designated as the positive control, the RPMI medium contained insulin at a concentration of 1 µM. The 96-well plate was incubated for 2 h, and glucose concentration was subsequently estimated in aliquots from each well using a Glucose-GO Assay Kit (Merck,

Johannesburg, South Africa). The viability of the cells was monitored during the experiment using MTT assay. The experiment was conducted in three biological repeats. The formula below was used to compute the glucose uptake (%) of the test samples and control.

$$\begin{aligned} \text{Glucose uptake(\%)} &= \frac{\Delta\text{GC of test or control} - \Delta\text{GC of blank}}{\Delta\text{GC of blank}} \\ &\times 100 \end{aligned}$$

‘ΔGC’ is the difference between the initial and final glucose concentration in the incubation medium.

Measurement of antioxidant indices in Chang liver cells induced with oxidative stress

The effect of the tested samples on lipid peroxidation and the activity of key antioxidant enzymes was measured in Chang liver cells (ATCC® CCL-13™) induced with oxidative stress. Confluent cultured cells (DMEM high glucose with 10% FBS) were plated in a transparent 96-well cell culture plate at a density of 10 000 cells/well. After 48 h of incubation in a CO₂ incubator set at 5% CO₂ and 37 °C, the medium was replaced with fresh medium containing the tested samples or ascorbic acid (10–80 µg/mL in incubation medium) or distilled water (in the wells assigned control and negative control). After 30 min of incubation, FeSO₄·7H₂O (1 mM in incubation medium) was added to the wells of the tested samples/ascorbic acid and the wells assigned negative control to induce oxidative stress. The incubation continued for 1 h before lysing the cells with cold lysis buffer (50 mM sodium phosphate solution containing 0.5% v/v Triton X-100 and 1 mM EDTA, pH 7.5) and gentle agitation. Lipid peroxidation level as well as the activity of catalase and superoxide dismutase (SOD) were measured in the supernatant (9500 g for 10 min at 4 °C) of the lysed cells.

For lipid peroxidation a previous method (Ramorobi *et al.*, 2022) was used with slight modifications. To perform the assay, 50 µL of supernatant or malondialdehyde (MDA) standards (0, 7.5, 15, 22.5, 30 and 45 µM in the assay volume) was added to designated vials already containing 250, 100 and 100 µL of 0.25% w/v thiobarbituric acid, 20% v/v acetic acid and distilled water, respectively. The mixture was heated for 50 min in a boiling water bath, allowed to cool and centrifuged for 5 min at 3500 g and ambient temperature. Aliquots of the supernatants were transferred into a transparent 96-well plate, and the absorbance was measured at 532 nm. The lipid peroxidation levels

were extrapolated from an MDA standard plot as thiobarbituric acid reactive substances. The inhibitory potential of the tested samples on lipid peroxidation was calculated as follows:

$$\begin{aligned} \text{Inhibition(\%)} &= \frac{(C_{\text{Negative control}} - C_{\text{Normal control}}) - (C_{\text{Test}} - C_{\text{Normal control}})}{(C_{\text{Negative control}} - C_{\text{Normal control}})} \\ &\times \frac{100}{1} \end{aligned}$$

where ‘C’ is the concentration (µM) extrapolated from the MDA standard plot.

Catalase and SOD activities were measured in the cell lysates of cells treated with the highest concentration (80 µg/mL) of the tested samples. To measure the activity of the antioxidant enzymes, the protein concentration of the cell lysate was first measured using the bicinchoninic acid (BCA) method.

For catalase, the activity was measured by measuring the rate at which the enzyme decomposes H₂O₂ at room temperature using a Catalase Assay Kit (Sigma Aldrich, Johannesburg, South Africa; catalogue number: CAT100). The activity was presented in mmol/mL/min. The activity was measured at a sample protein concentration of 1 mg/mL, and the specific activity of the enzyme was expressed in mmol/min/mg protein. One unit of catalase will decompose 1.0 micromole of H₂O₂ to oxygen and water per minute at pH 7.0 at 25 °C at a substrate concentration of 50 mM H₂O₂. The specific activity of the enzyme was calculated as follows:

$$\begin{aligned} \text{Specific activity(}\mu\text{mol/min/mg protein)} &= \frac{A \times \text{DF}}{\text{RT} \times \text{SV} \times \text{PC}} \end{aligned}$$

where ‘A’ = amount (µmole) of H₂O₂ that was used up during reaction time; ‘DF’ = dilution factor of sample in reaction volume; ‘RT’ = reaction time (min); ‘SV’ = sample volume (mL); ‘PC’ = sample protein concentration (mg/mL).

For SOD, activity was measured with an SOD Assay Kit (Sigma Aldrich, Johannesburg, South Africa; product number: 19160). The enzyme reduces superoxide ion to H₂O₂ and O₂. Thus, the samples’ SOD activity was determined by monitoring how the samples inhibit the colorimetric reduction of a water-soluble tetrazolium by superoxide ion. Based on the sample protein concentration, the activity was normalised to reflect the specific activity (*i.e.*, the enzyme activity per mg protein) using the following formula:

$$\text{Specific activity (\% inhibition/mg protein)} = \frac{\text{I} \times \text{DF}}{\text{SV} \times \text{PC}}$$

where 'I' = percentage inhibition (%) by enzyme on superoxide ion-mediated colorimetric reduction of a water-soluble tetrazolium salt; 'DF' = dilution factor of sample in reaction volume; 'SV' = sample volume (mL); 'PC' = sample protein concentration (mg/mL).

IC₅₀ and EC₅₀ computation

The IC₅₀ and EC₅₀ values of the samples or standards are the respective concentrations that could cause 50% inhibitory (IC₅₀) or modulatory (EC₅₀) bioactivity. The values were computed on MS excel 2016 and GraphPad Prism 5 software using the linear and/or non-linear plot of transformed (log 10) sample concentrations (x-axis) vs. the corresponding inhibitory or modulatory bioactivities.

Statistical analysis

Experimental data were presented as mean $\pm$ standard deviation of triplicate analysis ($n = 3$). The statistical analysis was done on the IBM SPSS Statistics (Windows version 29.0) software using the one-way ANOVA and Tukey *post hoc* multiple comparative analysis. Statistical significance was set at $P < 0.05$.

Results and discussion

The vascular and metabolic health benefits of pomegranate fruit have been frequently mentioned (Kandylis & Kokkinomagoulos, 2020; Giménez-Bastida *et al.*, 2021). However, advances in research have shown that the peel of this fruit contains phytochemicals that can

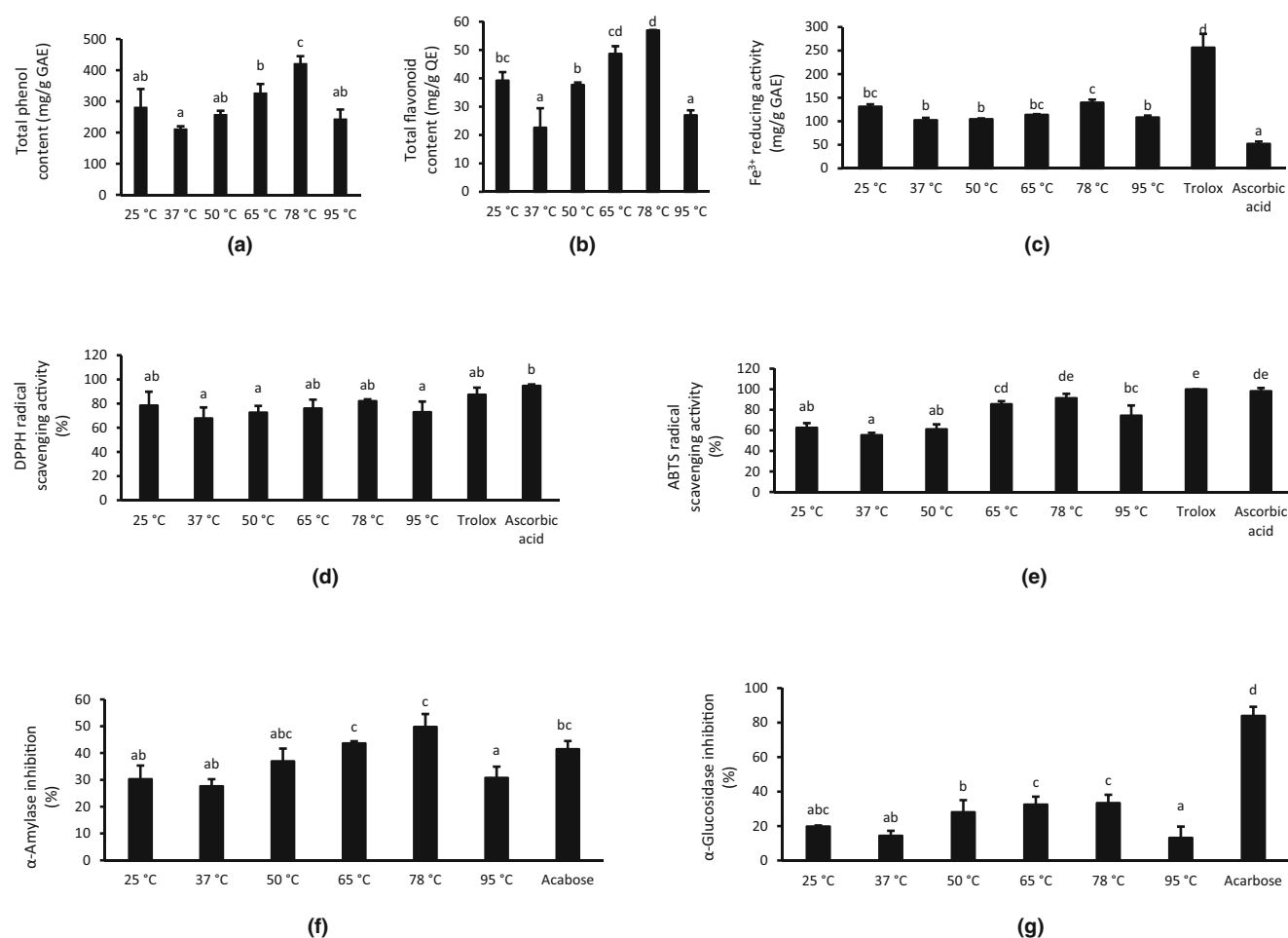


Figure 2 Preliminary data showing the (a) total phenol content (b) total flavonoid content (c) Fe³⁺ reducing capacity (d) DPPH radical scavenging activity (e) ABTS radical scavenging activity (f) α -amylase inhibitory activity and (g) α -glucosidase inhibitory activity of the different pomegranate peel extracts at a tested concentration of 45 μ g/mL. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, the letters at the top of the bars represent significant differences ($P < 0.05$) between groups, when there are no similar letters.

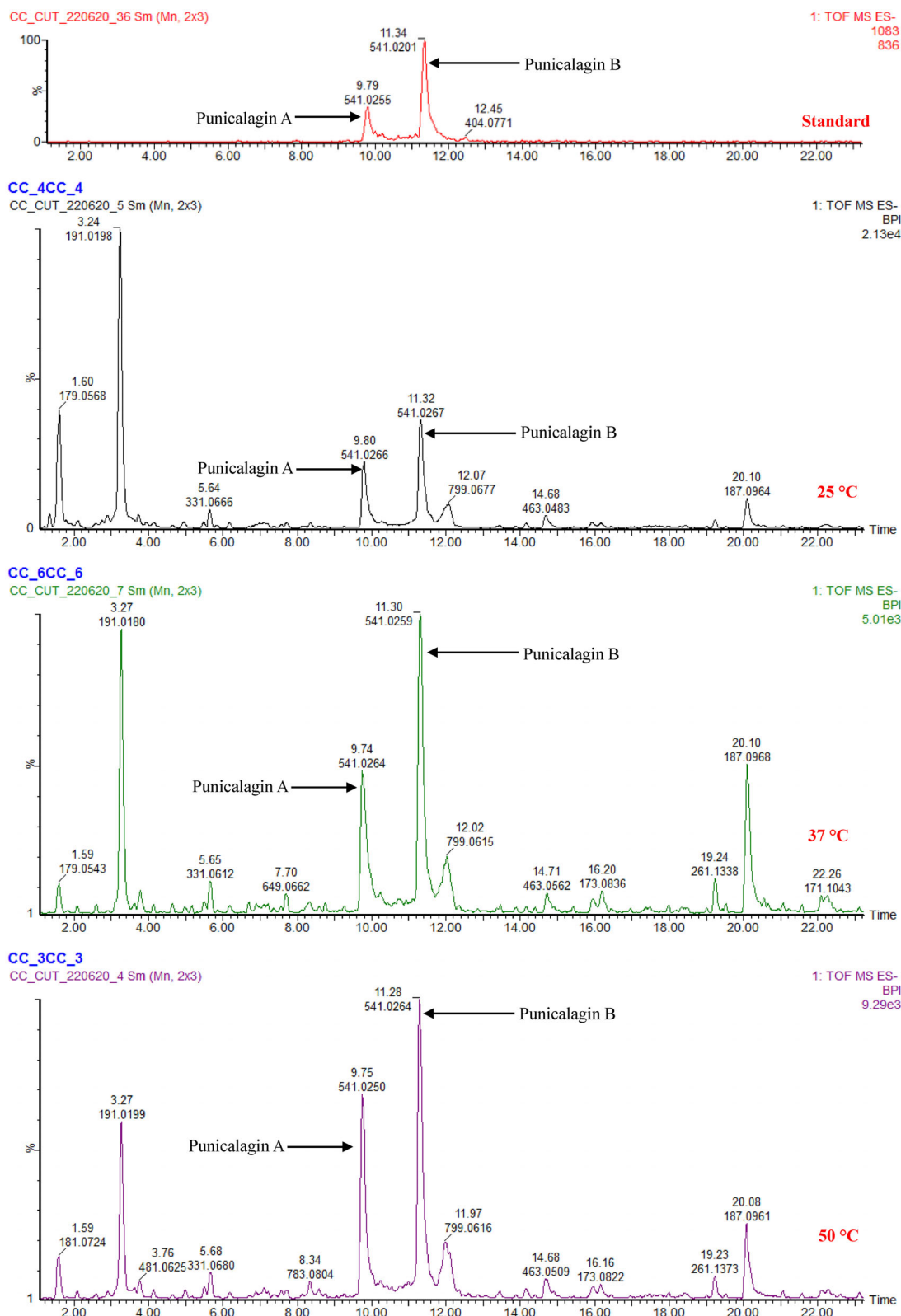


Figure 3 LC-MS chromatograms of punicalagin A + B standard and the different tannins recovered from the different pomegranate peel extracts.

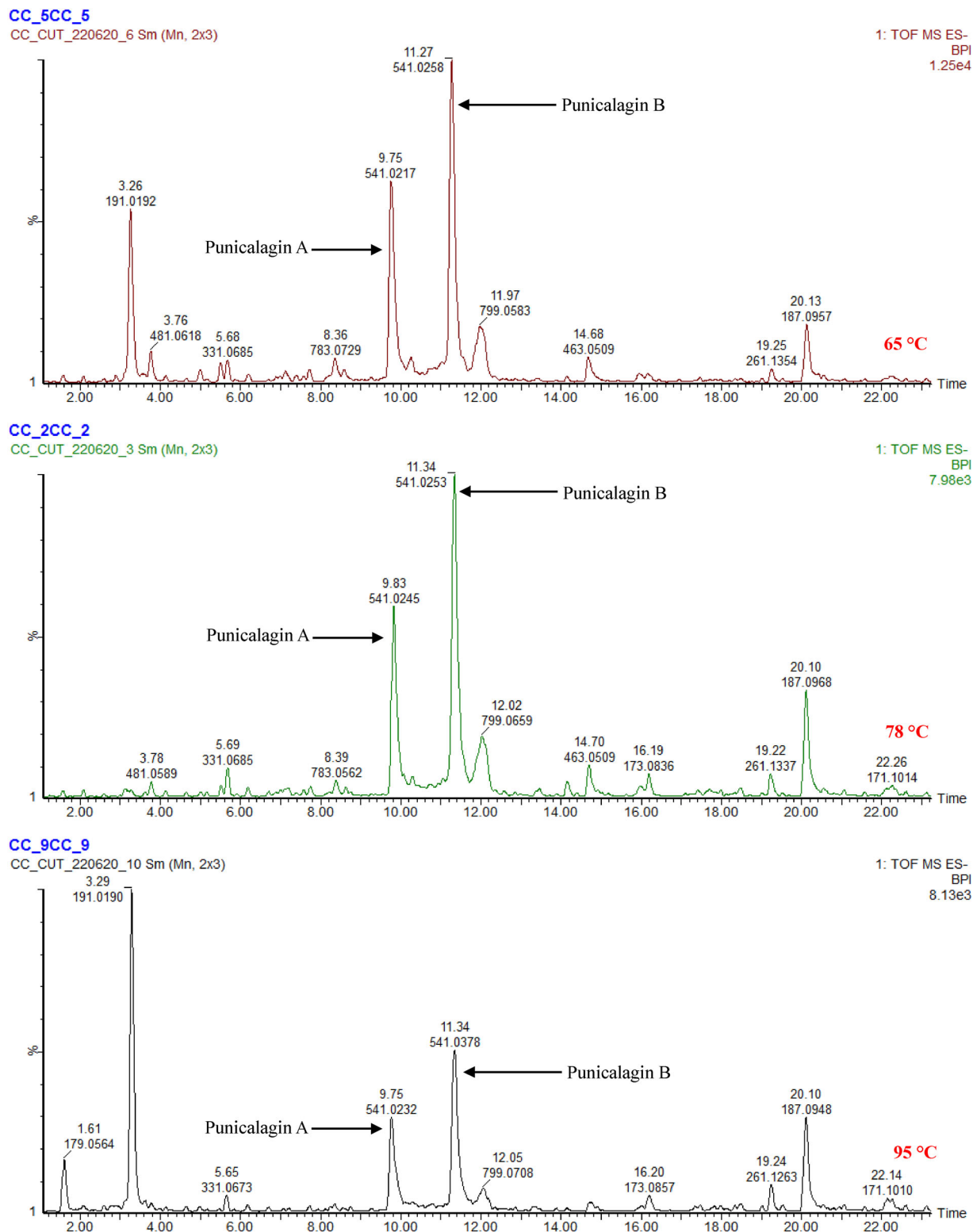


Figure 3 (Continued)

potentially increase the medicinal relevance of this fruit in managing metabolic diseases such as diabetes and oxidative complications. In the phytomedicinal context of the fruit and its peel, the ellagitannins have been considered as the major principles influencing the biological activity of pomegranate. Ellagitannins such as punicalagin and ellagic acid are considered key bioactive constituents (Sun *et al.*, 2017; Chukwuma *et al.*, 2020; Akuru *et al.*, 2022; Marra *et al.*, 2022; Mo *et al.*, 2022). Nevertheless, the optimum recovery methods of these bioactive ellagitannins from pomegranate peel and their bioactivity profiling have been scarcely reported. In the present study, optimum extraction and purification methods were used to recover ellagitannins and punicalagin from pomegranate peel and comparatively evaluated for their glycaemic control and antioxidant profiles.

Preliminary antioxidant, enzyme inhibitory and phytochemical profiles of the different peel extracts

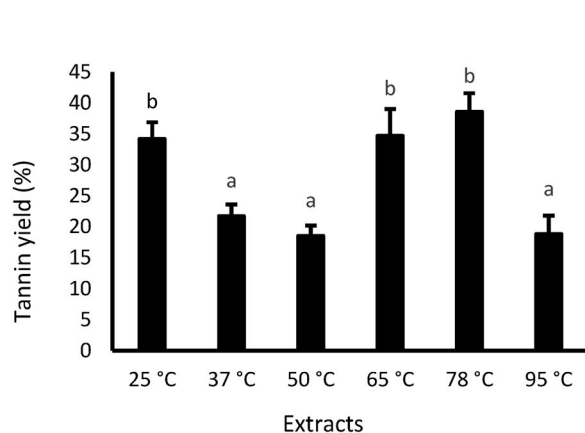
The peel extracts contained polyphenols and flavonoids (Fig. 2a,b). As extraction temperature increased from 25 to 37 °C, the phenol and flavonoid contents appreciably decreased, which was significant ($P < 0.05$) for flavonoid content. This decrease may be attributed to the action of polyphenol oxidase in the fresh fruit peels. Polyphenol oxidase degrades polyphenols and are optimally active at temperatures between 35 and 37 °C (Sikora *et al.*, 2019). Thus, may have depleted phenol and flavonoid contents of the extracts obtained at 37 °C. The phenol and flavonoid contents gradually increased as extraction temperature increased and peaked at 78 °C (Fig. 2a,b). It has been documented that increasing extraction temperature facilitates the release of insoluble bound phenolic compounds and the breakdown lignin (Irakli *et al.*, 2018), which may have contributed to the gradual temperature-dependent increase in phenol and flavonoid contents during extraction. Additionally, the temperature increase beyond 37 °C may have caused inactivation of polyphenol oxidase, thus decreasing the rate of polyphenol degradation by the enzyme. Beyond 78 °C (at 95 °C), a significant drop in the levels of polyphenols and flavonoids was observed, which may be attributed to thermal degradation of polyphenols at high temperatures. Moreover, it has been previously mentioned that temperatures beyond 80 °C could thermally degrade polyphenols, resulting in reduced phenol content, while extraction temperatures between 60 and 80 °C result in optimum recovery of polyphenols (Antony & Farid, 2022).

Plant polyphenols are known scavengers of free radicals due to their hydroxyl H-atom transfer abilities to form stable phenoxy radicals (Chen *et al.*, 2020). Polyphenols also have iron chelating ability by binding to

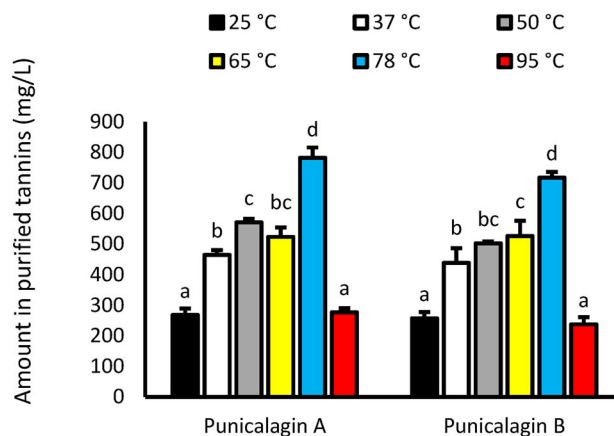
iron (Scarano *et al.*, 2023). Thus, it is plausible that the high polyphenol and flavonoid contents of the peel extract obtained at 78 °C influenced its higher radical scavenging (Fig. 2d,e) and Fe^{3+} reducing (Fig. 2c) capacities at the tested concentration (45 µg/mL) relative to the capacities of the other extracts.

At the same tested concentration of 45 µg/mL, all the peel extracts had a stronger inhibition of α -amylase than α -glucosidase (Fig. 2f,g). The α -amylase inhibitory action of the extract obtained at 78 °C was stronger than that of the other extracts (Fig. 2f) and acarbose, suggesting its potential to promote glycaemic control by delaying carbohydrate digestion (Čorković *et al.*, 2022). The α -amylase inhibitory action of many dietary polyphenols has been documented (Čorković *et al.*, 2022), which may be an important factor influencing the potent α -amylase inhibitory action of the extract obtained at 78 °C. Hence, the reduction in enzyme inhibitory activity as extraction temperature increased from 25 to 37 °C may be a reflection of the corresponding decrease in polyphenol content. Phytochemical profiling of the peel extracts showed that the yield of the tannins recovered from the extracts was highest in the extract obtained at 78 °C (Fig. 4a). Similarly, LC–MS profiling (Fig. 3) revealed that punicalagin A and B were highest in the tannin recovered from the extract obtained at 78 °C when compared to the tannins recovered from the other extracts (Fig. 4b). Other ellagitannins such as ellagic acid, granatin A, corilagin, casuarinin and geraniin were also present in the tannin recovered from the extract obtained at 78 °C (Fig. 4c), which may have worked synergistically with the constituent punicalagin to potentiate the promising α -amylase inhibitory action of this extract. This is, however, very speculative and is subject to experimental investigations, as the alpha amylase inhibitory effects of ellagitannins such as granatin A, corilagin and casuarinin have not been investigated, to the best of our knowledge. Nevertheless, the amylase inhibitory ellagitannins such as ellagic acid and geraniin has been documented, suggesting they may be influential in the inhibitory activity of the 78 °C peel extract. The potent amylase inhibitory activity of geraniin ($\text{IC}_{50} = 0.93$ µg/mL) was previously shown by Palanisamy *et al.* (2011). The amylase inhibitory effect of ellagic acid, however, remains controversial. While some studies have reported that it possesses both *in silico* (Bansode & Salalkar, 2016; Wang *et al.*, 2023) and *in vitro* (Wang *et al.*, 2023) amylase inhibitory activity and could potentiate hypoglycaemic action by inhibiting starch hydrolysis (Bellesia *et al.*, 2015), other studies have reported that it possesses little to no amylase inhibitory activity (McDougall *et al.*, 2005; Kam *et al.*, 2012).

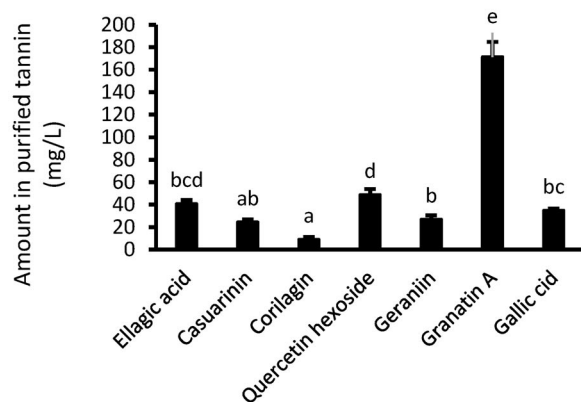
Documented evidence has revealed the notable contribution of tannins to the α -amylase inhibitory action



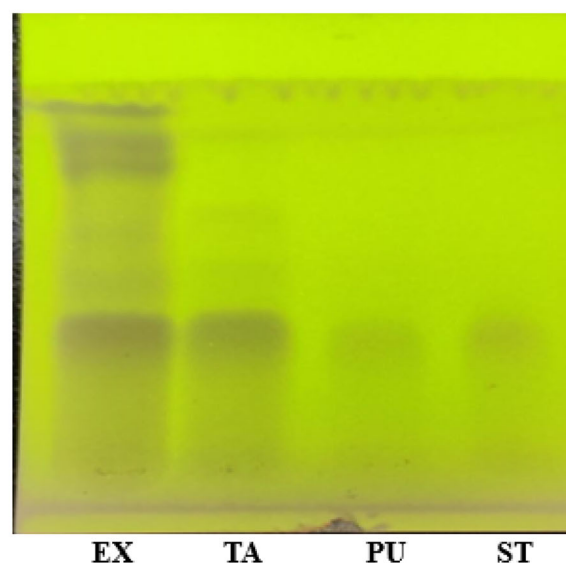
(a)



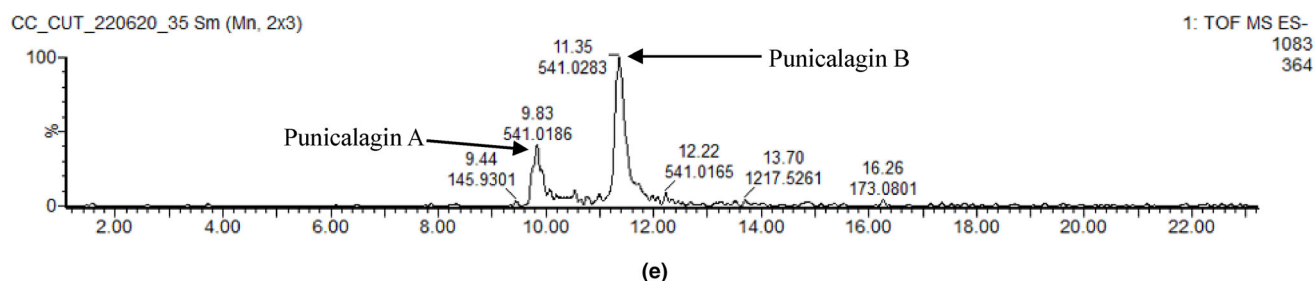
(b)



(c)



(d)



(e)

Figure 4 (a) Yield of the tannins recovered from the different peel extracts obtained at different temperatures (b) LC–MS quantified punicalagin A and B in the tannins recovered from the different extracts, (c) LC–MS quantified ellagitannins in the tannin recovered from the 78 °C peel extract, (d) thin layer chromatographic plate showing the 78 °C peel extract (EX), the tannin recovered from the extract (TA), the corresponding purified punicalagin (PU) and punicalagin standard (ST), (e) LC–MS chromatogram of the purified punicalagin. The replicate data are presented as mean $\pm$ SD of $n = 3$. For each quantification, the letters at the top of the bars represent significant differences ($P < 0.05$) between groups or between the quantified ellagitannins, when there are no similar letters.

Table 1 IC₅₀ and EC₅₀ values for the dose-dependent activities of the tested samples

Assays	IC ₅₀ or EC ₅₀ values (μg/mL)						
	78 °C extract	Purified tannins	Purified punicalagin	Ascorbic acid	Trolox	Acarbose	Aminoguanidine
DPPH radical scavenging (IC ₅₀)	25.0 $\pm$ 3.94 ^a	4.80 $\pm$ 0.94 ^b	32.7 $\pm$ 8.61 ^a	9.30 $\pm$ 2.95 ^b	4.25 $\pm$ 0.95 ^b	ND	ND
ABTS radical scavenging (IC ₅₀)	27.5 $\pm$ 7.93 ^a	1.63 $\pm$ 0.52 ^b	37.5 $\pm$ 7.45 ^a	2.17 $\pm$ 0.68 ^b	2.18 $\pm$ 1.37 ^b	ND	ND
ORAC (IC ₅₀)	15.6 $\pm$ 4.35 ^b	9.64 $\pm$ 5.23 ^b	25.6 $\pm$ 4.68 ^a	8.10 $\pm$ 1.38 ^b	ND	ND	ND
Anti-linoleic acid peroxidation (IC ₅₀)	19.9 $\pm$ 5.70 ^{ab}	9.97 $\pm$ 3.50 ^{bc}	27.3 $\pm$ 9.42 ^a	7.18 $\pm$ 1.74 ^c	ND	ND	ND
Antiglycation (IC ₅₀)	34.2 $\pm$ 4.37 ^b	29.1 $\pm$ 8.32 ^{bc}	79.1 $\pm$ 9.48 ^a	ND	ND	ND	14.7 $\pm$ 5.55 ^c
α-Amylase inhibition (IC ₅₀)	59.8 $\pm$ 5.60 ^a	39.4 $\pm$ 8.43 ^b	31.9 $\pm$ 4.17 ^b	ND	ND	54.5 $\pm$ 7.57 ^a	ND
Anti-lipid peroxidation in Chang liver cells (IC ₅₀)	24.3 $\pm$ 8.98 ^b	3.67 $\pm$ 1.22 ^c	59.0 $\pm$ 14.3 ^a	22.4 $\pm$ 5.42 ^b	ND	ND	ND
Glucose uptake/utilisation in L-6 myotubes (EC ₅₀)	55.0 $\pm$ 9.40 ^b	14.8 $\pm$ 4.96 ^c	112 $\pm$ 13.7 ^a	ND	ND	ND	ND

‘ND’ means ‘not determined’. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, the superscript letters attached to each data represent significant differences ($P < 0.05$) between groups, when there are no similar letters.

of some plants (Xiao *et al.*, 2013; Ćorković *et al.*, 2022). Tannin-rich extracts of strawberry, raspberry, red grape, red wine and green tea have been shown to be effective in inhibiting α-amylase, while removing tannins from blue berry extracts weakened the α-amylase inhibitory action of the extracts (McDougall & Stewart, 2005). In fact, hydrolysable tannins, such as ellagitannins have been documented as major α-amylase inhibitory components of many berries (Xiao *et al.*, 2013; Ćorković *et al.*, 2022), which suggests a correlation between the high tannin yield (Fig. 4a) and the potent α-amylase inhibitory activity (Fig. 2f) of the extract obtained at 78 °C. Moreover, the reduction in tannin yield as extraction temperature increased from 25 to 37 °C was consistent with a corresponding reduction in α-amylase inhibitory activity. Reports from previous studies (Kerimi *et al.*, 2017) further suggest that the significantly ($P < 0.05$) higher amount punicalagin A and B observed in the tannin recovered from the extract obtained at 78 °C compared with the amounts in the tannins recovered from the other extracts (Fig. 4b) may be notably influential in the potent α-amylase inhibitory activity of the 78 °C extract. Moreover, pomegranate punicalagin has been shown to be an effective inhibitor of α-amylase, which was comparable to acarbose (Kerimi *et al.*, 2017). It was, therefore, rationale to purify

punicalagins from the tannin recovered from the 78 °C extract as shown in Fig. 4d,e and comparatively examine the dose-dependent glycaemic control and antioxidant potential relative to the corresponding crude extract and tannin.

Dose-dependent antioxidant and glycaemic control potential of punicalagin, tannin and crude extract

Based on the IC₅₀ values (Table 1), the extract obtained at 78 °C, as well as the tannin and punicalagin recovered from it, had appreciable radical scavenging and ORAC activities (Fig. 5a–c), which suggest their ability to douse the deleterious action of free radicals on biological molecules during oxidative stress. However, the IC₅₀ values (Table 1) further suggest that the activity of the purified tannin was multiple times stronger than the activity of the extract and the purified punicalagin, but comparable to the scavenging strength of ascorbic acid and Trolox. Similarly, the *in vitro* ability of the purified tannin to inhibit linoleic acid peroxidation (IC₅₀ = 9.97 μg/mL) was two and three times stronger than its corresponding extract (IC₅₀ = 15.6 μg/mL) and purified punicalagin (IC₅₀ = 25.6 μg/mL), respectively, and comparable to the activity of ascorbic acid (IC₅₀ = 7.18 μg/mL) (Table 1 and Fig. 5f). A similar trend of anti-lipid peroxidative activity was observed

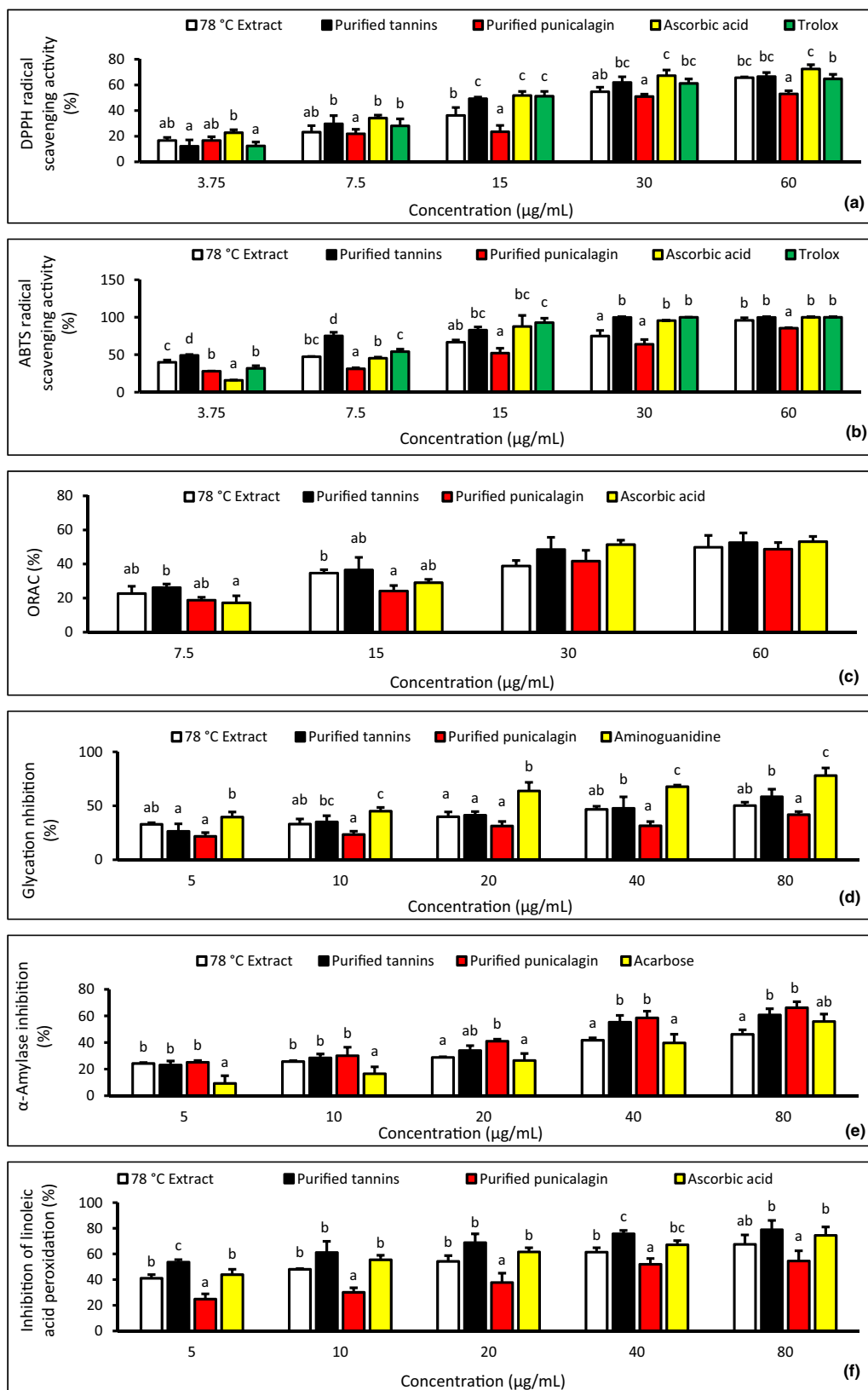


Figure 5 *In vitro* dose-dependent (a) DPPH radical scavenging, (b) ABTS radical scavenging, (c) ORAC, (d) antiglycation, (e) α -amylase inhibitory and (f) anti-linoleic acid peroxidation activities of the 78 °C peel extract, the tannin recovered from the extract and the corresponding purified punicalagin. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, statistical comparison was done among the treatment groups at a given concentration. The letters at the top of the bars represent significant differences ($P < 0.05$) between groups, when there are no similar letters.

in Chang liver cells, with the purified tannin ($IC_{50} = 3.67 \mu\text{g/mL}$) outperforming the corresponding crude extract ($IC_{50} = 24.3 \mu\text{g/mL}$) and purified punicalagin ($IC_{50} = 59.0 \mu\text{g/mL}$), as well as ascorbic acid ($IC_{50} = 22.4 \mu\text{g/mL}$) (Table 1 and Fig. 6a). The anti-lipid peroxidative activities of the crude extract and purified tannin in Chang liver cells were accompanied by a significant ($P < 0.05$) recovery of antioxidant enzymes (SOD and catalase) activities that were depleted by oxidative stress (Fig. 6b), suggesting the potential of pomegranate peel extract and ellagitannins to improve *in vivo* antioxidant status.

The remarkable antioxidant potential of the purified tannin may be attributed to the presence of ellagitannins such as punicalagin, ellagic acid, granatin A, casuarinin and geraniin, and other phenolics such as gallic acid and quercetin hexoside (Fig. 5c), which together, potentiated the potent antioxidant action of the purified tannin. Moreover, an ellagitannins-rich dietary supplement from pomegranate has been shown to suppress lipid peroxidation in obese individuals (Heber *et al.*, 2007), while commercial ellagic has been shown to exert anti-lipid peroxidative activity in obese animals (Xu *et al.*, 2021). Also, the antioxidant properties gallic (Gao *et al.*, 2019), as well as quercetin and its glucosides (Terao, 2023) have been documented.

Besides antioxidant effects, the peel crude extract obtained at 78 °C, as well as the recovered tannin and punicalagin, showed glycaemic control and antiglycation potential. The antiglycation activity of the crude peel extract and the recovered tannin was twice more potent than that of the purified punicalagin (Fig. 5d), suggesting the bioactive contribution of other polyphenols present in the extract and tannin. Consistent antiglycation activity of pomegranate peel acetone extract has been previously documented (Chukwuma *et al.*, 2020), suggesting that potential of pomegranate peel to mitigate hyperglycaemia induced glycation and associated diabetic complications.

The dose-dependent α -amylase inhibition data (Fig. 5e) suggests that pomegranate peel contains ellagitannins that may exert glycaemic control by delaying carbohydrate breakdown. The IC_{50} values (Table 1) showed that purified punicalagin ($IC_{50} = 31.9 \mu\text{g/mL}$) outperformed the crude extract ($IC_{50} = 59.8 \mu\text{g/mL}$) and the tannin ($IC_{50} = 39.4 \mu\text{g/mL}$) recovered from the extract, suggesting that punicalagins are the

notable ellagitannins influencing the α -amylase inhibitory potential of pomegranate tannins.

Another glycaemic control potential exhibited by the pomegranate peel extract obtained at 78 °C and its purified constituents was a modulatory action on cellular glucose uptake/utilisation. Glucose uptake/utilisation in cells of peripheral tissues is a major mechanism by which insulin normalises blood glucose levels (Ramorobi *et al.*, 2022), which suggests the potential of pomegranate peel to improve insulin function and glucose tolerance. The glucose uptake effect of pomegranate peel has been previously demonstrated in Chang liver cells (Chukwuma *et al.*, 2020). In the present study, the 78 °C aqueous peel extract and its purified constituents dose dependently increased glucose uptake in muscle cells (L-6 myotubes), with the purified tannin ($EC_{50} = 14.8 \mu\text{g/mL}$) outperforming the extract ($EC_{50} = 55.0 \mu\text{g/mL}$) and purified punicalagin ($EC_{50} = 112 \mu\text{g/mL}$) (Table 1 and Fig. 7). Constituents such as ellagic acid and gallic acid present in the purified tannin (Fig. 4c) may influence its potent glucose uptake effect. Moreover, the modulatory action of ellagic and gallic acid on insulin signalling, cellular glucose uptake and glycaemic control has been documented (Prasad *et al.*, 2010; Ding *et al.*, 2019; Hidalgo-Lozada *et al.*, 2022).

Conclusion

The present study demonstrated the antioxidant and glycaemic control potential of pomegranate peel aqueous extract and its purified constituent ellagitannins. Extraction at 78 °C yielded optimum amounts of polyphenols, as well as ellagitannin constituents, which afforded the extract stronger antioxidant and α -amylase inhibition profiles, relative to the extracts obtained at other temperatures. While punicalagin appears to be a major ellagitannin influencing the α -amylase inhibitory action of pomegranate peel, it works in concert with other ellagitannin and polyphenol constituents, including ellagic acid and gallic acid in exerting potent anti-lipid peroxidative and glucose uptake modulatory activities. The data of the present suggest that tannin/ellagitannins purified from pomegranate peel aqueous extract obtained at 78 °C may be a promising dietary supplement for exerting glycaemic control and mitigating oxidative stress.

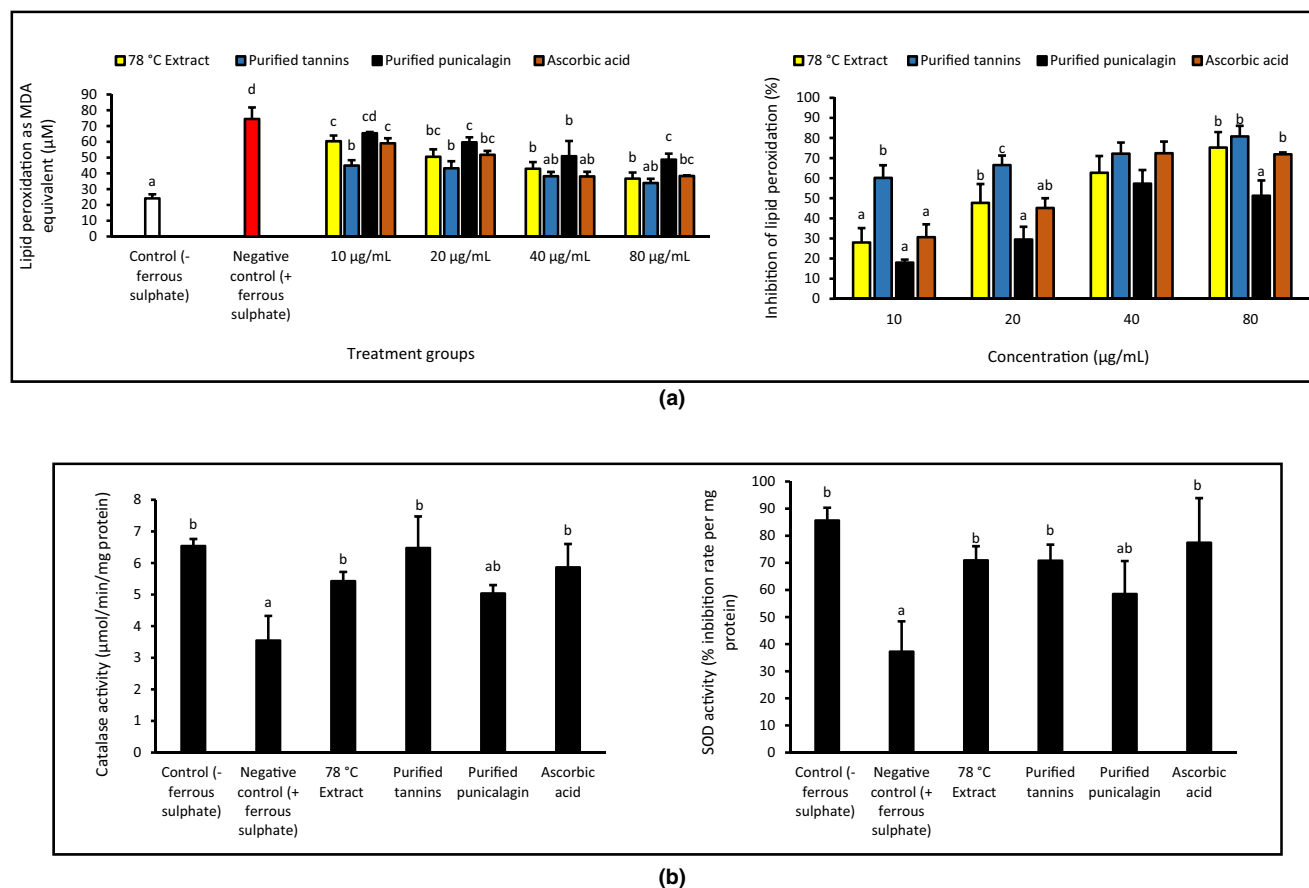


Figure 6 (a) Dose-dependent anti-lipid peroxidative and (b) antioxidant enzymes modulatory effects of the 78 °C peel extract, the tannin recovered from the extract and the corresponding purified punicalagin in Chang liver cells induced with oxidative stress. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, statistical comparison was done between the controls (control and negative control) and treatment groups at a given concentration or among the treatment groups at a given concentration. The letters at the top of the bars represent significant differences ($P < 0.05$) between groups, when there are no similar letters.

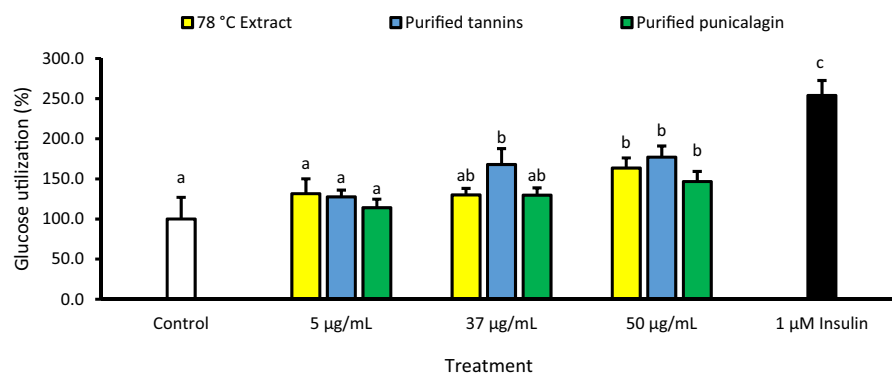


Figure 7 Dose-dependent modulatory effect of the 78 °C peel extract, the tannin recovered from the extract, and the corresponding purified punicalagin on glucose uptake/utilisation in L-6 myotubes. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, statistical comparison was done between the controls (control and insulin) and treatment groups at a given concentration. The letters at the top of the bars represent significant differences ($P < 0.05$) between groups, when there are no similar letters.

Conflict of interest

The authors declare that there is no conflict of interest with this work.

Ethical approval

Ethics approval was not required for this research.

Author contributions

Boitumelo Mashile: Data curation; methodology; formal analysis; writing – original draft. **Reaotshepa Setlhodi:** Methodology; data curation; formal analysis; writing – review and editing. **Gloria O. Izu:** Methodology; data curation; formal analysis; writing – review and editing. **Ochuko L. Erukainure:** Software; formal analysis; methodology; writing – review and editing. **Samson S. Mashele:** Methodology; resources; writing – review and editing. **Tshepiso J. Makhafola:** Methodology; resources; supervision; writing – review and editing. **Kenneth C. Eze:** Methodology; writing – review and editing. **Chika I. Chukwuma:** Conceptualization; methodology; software; formal analysis; supervision; funding acquisition; resources; writing – review and editing.

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Peer review

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ijfs.16823>.

Data availability statement

The data of this study are available from the corresponding author upon request.

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The study reported by Akuru *et al.* (2022) is one of the most recent studies reporting the antioxidant and glycaemic control potential of pomegranate peel extracts. Thus, it was rational to comment on this study to provide insights into the recent trends on the subject matter and provide a platform conducting the present study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Single Mass Analysis of each of the analytes/compounds identified in the ellagitannins from the different solvent extracts.