

**DETERMINING THE INFLUENCE OF THE EXTRACTION METHOD
ON THE YIELD AND CHEMICAL COMPOSITION OF
ELLAGITANNIN AND PUNICALAGIN FROM POMEGRANATE
FRUIT PEEL**

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DECLARATION OF INDEPENDENT WORK

I Reaotshepa Setlhodi, 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

This thesis is dedicated to the memory of my beloved mother, Selemogeng Juila Setlhodi. If only God could grant us a moment to hear your words of pride, I know you would say that I am your wildest dream come true. I pledge to honour your memory by dedicating myself to meaningful work and striving for excellence, just as you always encouraged me to do.

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ABSTRACT

Background

Pomegranate fruit has oxidative, metabolic and vascular health benefits. This has been attributed to its phenolic constituents, including bioactive ellagitannins. Although underutilized, the peel of pomegranate also contains these beneficial ellagitannins, which could be of medicinal relevance in managing oxidative stress. However, the extraction conditions for optimum recovery of antioxidant ellagitannins from pomegranate fruit peel remains elusive. To ascertain extraction the possible extraction conditions for optimum recovery of antioxidant ellagitannins from pomegranate peel, this study investigated the influence of aqueous extraction temperature or type of organic solvent extraction on the antioxidant and ellagitannins profile of pomegranate peel using advanced chemometrics models.

Methodology

Class 1 (processing grade) “Wonderful” pomegranate fruits were procured from a local fruit producing and packaging company. The fruits were washed, and peel was removed. First, 1 kg freshly removed peel was separately soaked in 2.5 L of distilled water at different temperatures (25 °C, 37 °C, 50 °C, 65 °C, 78 °C and 95 °C) for 1 hour. There after the soaked peel was blended with the water, filtered and the extracts were recovered by freeze drying. On the other hand, equivalent dry mass (250 g) of the dried peel was extracted with 1 L of different solvents (methanol, ethanol, acetone and water). The extracts were recovered using a rotary evaporator (for the organic solvent extracts) or by freeze drying (for the water extract). The extracts were evaluated for total phenol (TPC) and flavonoid (TFC) contents, 2,2-diphenyl-1-picrylhydrazyl and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radicals scavenging activity, Fe^{3+} reducing activity (FRAP), oxygen radical absorbance capacity (ORAC), as well as inhibitory capacity on FeSO_4 -induced linoleic acid peroxidation and lipid peroxidation in Chang liver cells. Tannins were recovered from the extracts and ellagitannins were quantified using liquid chromatography–mass spectrometry (LC-MS). Advanced chemometric models, including principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) were used to correlate the influence of the extraction methods on the ellagitannins and antioxidant profiles of the extracts.

Results and discussion

In the dry peel extraction, acetone extraction had the highest ($p < 0.05$) TFC and TFC and strongest ($p < 0.05$) Fe^{3+} reducing effect, while methanol extraction had the strongest ($p < 0.05$) radical scavenging activity. The ORAC and anti-lipid peroxidative activities of both solvent extractions outperformed that of the ethanol and water extractions. Tannins were highest for acetone and methanol extractions. LCMS-quantified ellagitannins in the tannins varied for the different extractions. OPLS bi-plot showed that the high ellagic acid constituents and total tannins yield of the methanol extraction influenced its potent radical scavenging activity, while the ellagitannin constituents including punicalagins, granatin A, geraniin and casuarinin influenced the high TFC, as well as the potent Fe^{3+} reducing, ORAC and anti-lipid peroxidative activities of acetone extraction.

On the other hand, extraction at 78 °C had the most potent radical scavenging potency, oxygen ORAC and anti-lipid peroxidative activity, which was consistent with its highest ellagitannins constituents. OPLS biplot showed distinct clustering pattern of the antioxidant and ellagitannins variables around 78 °C extraction temperature, suggesting the observable influence of the ellagitannins on the potent antioxidant profile of the 78 °C extraction. The variable contribution plots, as well as the obtained antioxidant data suggest punicalagins, granatin A and ellagic acid as notable influencing ellagitannins. Also, more ellagitannins were recovered from fresh peel extraction at 78 °C than dry peel organic solvent extraction.

Conclusion

While acetone and/or methanol extraction may be promising for appreciable recovery of antioxidant ellagitannins from dry pomegranate peel, it is safe to speculate that water extraction at 78 °C optimally influenced the ellagitannins and antioxidant profiles of freshly removed pomegranate fruit peel than other extraction temperatures. Thus, may be promising for optimally recovering the ellagitannins from pomegranate peel, without sacrificing the antioxidant capacity.

Keywords: *Pomegranate peel, extraction, antioxidant, ellagitannins, advanced chemometrics analysis.*

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LIST OF ABBREVIATIONS

ABTS	:	3-ethylbenzothiazoline-6-sulfonic acid
ATP	:	Adenosine triphosphate
CAT	:	Catalase
DNA	:	Deoxyribonucleic acid
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
FRAP	:	Ferric reducing antioxidant power
G6PDH	:	Glucose 6 phosphatedehydrogenase
GAE	:	Gallic acid equivalent
GPx	:	Glutathione peroxidase
GR	:	Glutathione reductase
GSH	:	Reduced glutathione
GSSG	:	Glutathione di-sulphide
GST	:	Glutathione S transferase.
LC-MS	:	Liquid chromatography–mass spectrometry
NADH	:	Nicotinamide adenine dinucleotide
OPLS	:	Orthogonal partial least squares
ORAC	:	Oxygen radical absorbance capacity
PCA	:	Principal component analysis
QE	:	Quercetin equivalent
RMSECV	:	Root-mean- standard error for cross-validation
RMSEE	:	Root mean square error of estimation
RNS	:	Reactive Nitrogen Species
ROS	:	Reactive Oxygen Species
SOD	:	Superoxide dismutase
TFC	:	Total flavonoid content
TPC	:	Total phenol content

RESEARCH OUTPUTS

Published articles from this thesis:

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>

Other published articles and/or submitted manuscripts during master’s program:

1. 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>.
2. Mashile B, **Setlhodi R**, Izu GO, Erukainure OL, Mashele SS, Makhafola TJ, Eze KC, Chukwuma CI*. 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**. <http://doi.org/10.1111/ijfs.16823>.

CHAPTER 1

INTRODUCTION

Oxidative stress is defined as an oxidative imbalance caused by an inability to detoxify reactive products produced by reactive oxygen species (ROS) during cellular metabolism (Pizzino et al., 2017). The imbalance between radical species generation and antioxidant defence mechanisms causes damage to cellular macromolecules such as lipids, proteins, and DNA (Juan et al., 2021).

Antioxidant defence mechanisms defend biomolecules and the organism from the damaging effects of free radicals by reducing or repairing the damage done by ROS to the target molecule (Kran et al., 2023). Though the body has an inbuilt antioxidant defence mechanism to scavenge these free radicals that cause many diseases, there is a need for the utilization of additional antioxidants found in fruits and vegetables (Sridevi et al., 2018). Fruits, vegetables, and other plant-based diets are high in bioactive phytochemicals, which may give additional health advantages beyond basic nutrition by boosting the body's antioxidant status and lowering the risk of chronic disease development (Liu, 2004).

Pomegranate fruit is widely known for its cardiovascular and antioxidant health benefits (Singh et al., 2014). Additionally, it has antibacterial, anti-allergic, anti-diabetic, anti-atherosclerotic, and anti-inflammatory properties (Singh et al., 2014). The Pomegranate fruit has many secondary metabolites, particularly the ellagitannins (punicalagin and ellagic acid), as well as other polyphenols such as flavonoids, anthocyanidins, flavanones, catechins, and phenolic acids, which are responsible for its medicinal properties (Qua et al., 2021; Middha et al., 2013; Akuru et al., 2022). These phenolic compounds account for up to 92% of the fruits antioxidant property and are mostly concentrated in the fruit peel and juice compared to other parts of the fruit (Ismail et al., 2012; Yaritz et al., 2022). Pomegranate peel's capacity to scavenge free radicals has been proven and in certain instances, it was stronger than the pulp (Li et al., 2006).

The rising fruit production and commercialization of pomegranate causes accumulation of the fruit's wastes, particularly the peels (Bertolo et al., 2021). The fruit's peel has been researched as a potential source of antioxidants (Chidambara et al., 2002; Middha et al., 2013; Li et al., 2006; Akuru et al., 2022; Chukwuma et al., 2020). The findings provide support for the peel's

potential use in controlling oxidative stress and shocks, which may lead to the medicinal utilization of the fruit's peel.

The antioxidant properties of its fruit peel have been majorly attributed to the presence of polyphenols, including the predominant ellagitannins constituents (Akuru et al., 2022; Yaritz et al., 2022; Marra et al., 2022; Mo et al., 2022). Ellagitannins are hydrolysed tannins common in pomegranate fruit, which include constituents like punicalagins, punicalin, gallagic acid, ellagic acid and relative glycosides (Altieri et al., 2019). Moreover, ellagitannins-rich dietary supplement from pomegranate, as well as commercial ellagic acid has been shown to have potent anti-lipid peroxidative activity in obese animals (Xu et al., 2021) and human subjects (Heber et al., 2007).

Several organic solvent extracts have been studied for the extraction of antioxidant phytoconstituents from pomegranate peel. This is because the kind of compounds extracted from plant material is greatly influenced by the solvent's polarity, which in turn affects the antioxidant and other bioactivity profiles of the resultant extracts (Singh et al., 2014). According to previous studies, the chemical and antioxidant profiles of pomegranate fruit peel extracts are highly influenced by the type of solvent extraction (Chidambara et al., 2002; Li et al., 2006; Middha et al., 2013; Akuru et al., 2022; Chukwuma et al., 2020).

On the other hand, water extraction remains the most widely used extraction method in folk medicine because it is perceived to be safe for consumption (Yang and Ross, 2010). A previous study has suggested that using fresh fruit peel for aqueous extraction may be promising for recovering ellagitannins from pomegranate fruit waste, perhaps to a commercial scale (Seeram et al., 2005). However, the temperature conditions that will optimally recover ellagitannins from the fresh peel without sacrificing the antioxidant profile remains elusive. Temperature is a very important factor in water phytochemical extraction. It is known that heat facilitates the infusion of phytochemicals present in plant materials and thus is commonly employed in Chinese traditional medicine (Yang and Ross, 2010). It is however important to understand the optimum extraction temperature to preserve the integrity and functional properties of the bioactive principles (Sójka et al., 2019).

A number of studies have investigated the effects of different extraction solvents, conditions and other extraction variables on the phytochemical and antioxidant profiles of phenolics from pomegranate peel (Calín-Sánchez et al., 2013; Alexandre et al., 2017; Chukwuma et al., 2020; Akuru et al., 2022). However, these studies lack a proper statistical correlation between the

extraction variables and the phytochemical and/or antioxidant profiles. Thus, the studies lack the capacity to ascertain the extraction method or variables for optimum recovery of antioxidant phenolics, including ellagitannins from pomegranate peel. Therefore, this study was done to investigate the influence of extraction solvent and temperature on the antioxidant and ellagitannins profile of pomegranate peel by employing advanced chemometrics models and analyses. The models include Pearson's bivariate correlation, principal component analysis (PCA) and orthogonal projections to latent structures (OPLS).

CHAPTER 2

LITERATURE REVIEW

2.1 Oxidative stress

The concept of oxidative stress was first used in 1985 by researchers in medicine. But there was limited knowledge around the topic (Abbas et al., 2021). Several studies are now being conducted on the subject worldwide, increasing knowledge about the role oxidative stress in normal physiology and the pathophysiology of many diseases. Oxidative stress is defined as an excess production of reactive oxygen species (ROS), which is due to an imbalance between the production and depletion of ROS. It results from increased free radical occurrence as well as decreased activity of the body's antioxidant defence mechanism (Poljsak et al., 2013). Chronic oxidative stress results in oxidative damage, which have been linked to a variety of human disorders (Asmat et al., 2016; Liguori et al., 2018)

2.2 Reactive oxygen species and reactive nitrogen species

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are unstable oxygen- and/or nitrogen-containing compounds that comprise both free radical and non-radical species (Ngo & Duennwald, 2022). Free radicals are tiny, diffuse molecules that have an unpaired electron. They are reactive and can cause chain reactions, in which a single free radical initiation event can harm multiple molecules (Jones, 2008). Examples of the free radicals include superoxide anion radical, hydroxyl radical, alkoxyl radicals, nitric oxide radical, and peroxy radicals. Non-free radicals include, among others, hydrogen peroxide, nitrogen dioxide, and peroxynitrites (Sies et al., 2020).

2.3 Endogenous and exogenous production of ROS

In living organisms, ROS is produced and accumulated through cellular metabolic processes as shown in **Figure 2.1**. For endogenous ROS production, mitochondria are the main drivers. The body's mitochondria produce majority of ROS. Particularly, production of superoxide ion from oxygen reduction during cellular mitochondrial energy metabolism (Dröse and Brandt,

2012). During oxidative stress, mitochondria produce more ATPs to counteract the effects of oxidation on the body, but more ATPs also results in the production of more ROS.

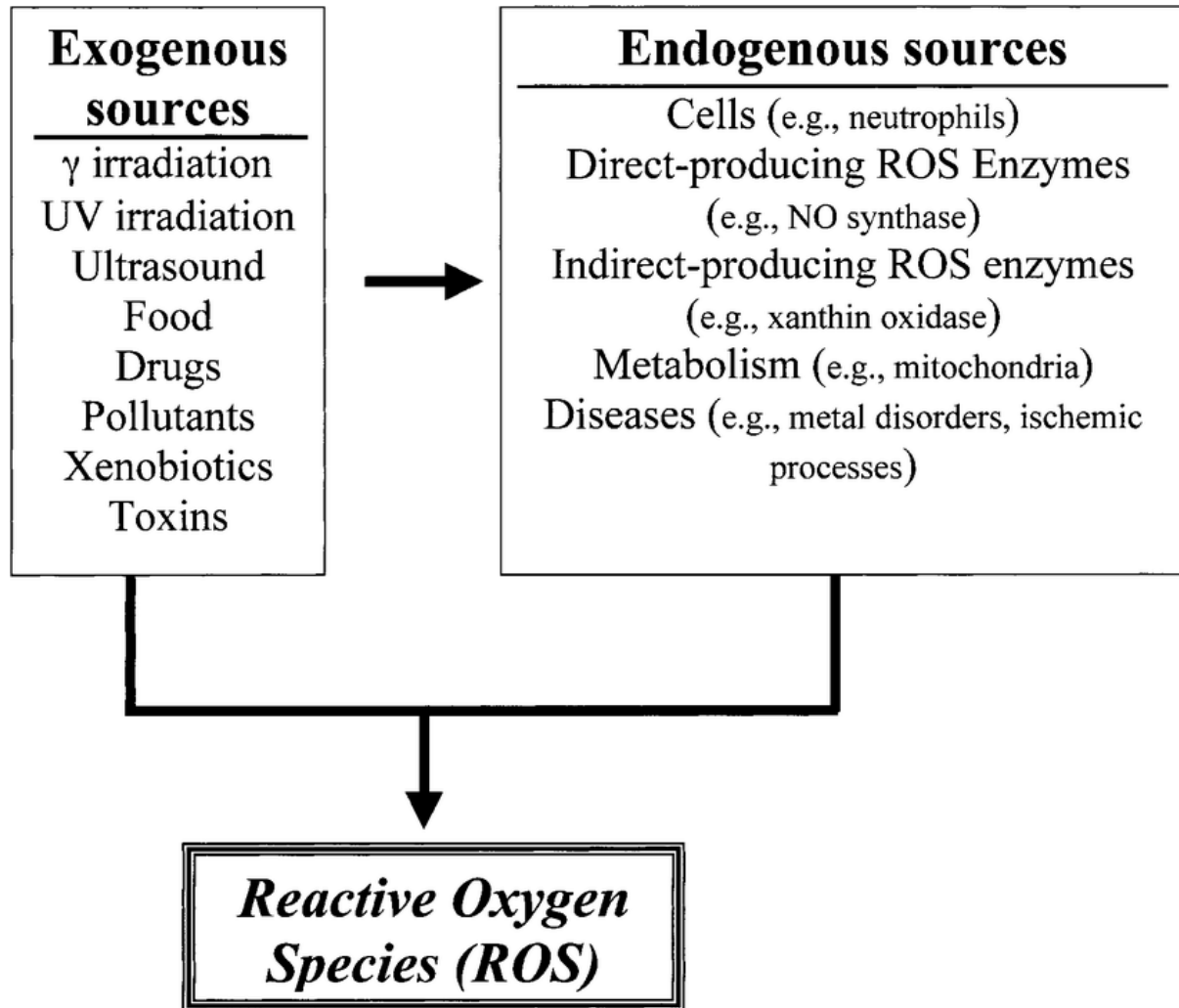


Figure 2.1: Endogenous and exogenous production of ROS (*adopted without permission from Kohen & Nyska, 2002*).

Production of RNS is activated in the circulation of ROS in the cell. In the presence of RNS and ROS, the integrity and functionality of DNA and mitochondrial enzymes is compromised, which leads to cellular and tissue damage (Abbas et al., 2021). ROS overproduction acts as a stimulant for mitochondrial toxicity. Other sources of ROS production include nicotinamide adenine dinucleotide phosphate (NADPH) oxidases pathways (Senoner & Dichtl, 2019). Other enzymes that contribute to ROS production are xanthine oxidase, nitric oxide synthase, cyclooxygenases, cytochrome P450 enzymes and lipoxygenases (Holmstrom & Finkel,

2014). Notable ROS production also occurs in cell organelles, like the peroxisome and endoplasmic reticulum, (Holmstrom & Finkel, 2014). Hydroxyl radical is produced in Fenton reaction and Haber–Weiss reaction pathway, while hypochlorous acid is produced from chloride ions and H_2O_2 through the myeloperoxidase reaction (Collin, 2019). Harmful exogenous sources of ROS include pollution, tobacco smoke, alcohol, ozone, environmental and industrial toxins, and radiation (Gutteridge & Halliwell, 2018).

A lot of important processes in the body can take place uninterrupted when the production of the ROS is minimal (Abbas et al., 2021). A moderately regulated production of ROS is needed in our body as it plays an important role in normal physiological functions. ROS act as secondary messengers in intracellular signalling cascades that control cell proliferation, autophagy, inflammatory and, and contribute to overall redox regulation (Bae et al., 2011).

ROS are strong oxidants and are harmful to the cell when there is an excessive production (Ngo & Duennwald, 2022). Since free radicals are reactive molecules, they react with other molecules in biological systems and damage cellular components including lipids, nucleic acids, proteins, and enzymes.

2.4 Oxidative stress and disease progression

Excessive production or accumulation of ROS and RNS results in oxidative stress. This is caused by pro-oxidant and antioxidant imbalance, which can lead to cellular damage in metabolism (Pizzino et al., 2017). According to Davalli et al. (2018), oxidative damage in cells can develop significantly during times of environmental stress and harm the structures of the cells. Lipid peroxidation of polyunsaturated fatty acids, such as membrane phospholipids (Ito et al., 2019), oxidation of proteins (Hawkins & Davies, 2019), and damage to DNA or RNA are among the negative effects of ROS in cells. Damages caused in the cell by oxidative damage includes cell inflammation, apoptosis, and necrosis. ROS can cause single-stranded and double-stranded DNA breakage, DNA and protein cross-links, lipid membrane damage, and mitochondrial dysfunction (Klran et al., 2023). These damages can progress to diseases such as metabolic, cardiovascular disease, chronic obstructive lung disease, chronic kidney disease, neurological disorders, and cancer (García-Sánchez et al., 2020; Klran et al., 2023) (**Figure 2.2**).

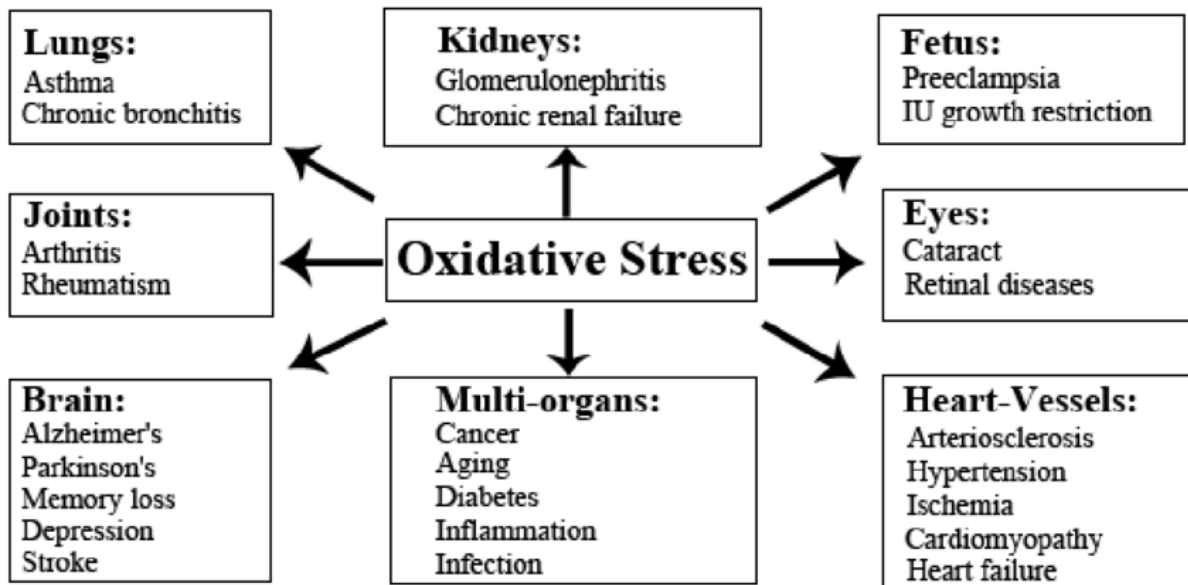


Figure 2.2: Diseases linked to oxidative stress (*adopted without permission from Pham-Huy et al., 2008*).

2.4.1 Reactive oxygen species damage on nucleic acids

Nucleic acids are prone to both endogenous and external damage, they are chemically unstable and susceptible to hydrolysis, nonenzymatic methylation, and oxidation (Araujo et al., 2016). According to Auboeuf (2020), DNA damage is the term for physical and chemical changes that can affect how genetic information is interpreted and transmitted. Various forms of molecular modification result from several exogenous and endogenous stresses that can cause damage to DNA (Juan et al., 2021). Deoxyribose and nitrogenous bases in DNA can react with ROS, leading to substantial oxidative reactions. This can result in mutations, carcinogenesis, apoptosis, necrosis, and genetic illnesses. The rupture of nucleosomes, which are essential for the organization of DNA within chromosomes, causes DNA fragmentation, which interferes with the compaction and coiling of DNA within chromatin (Juan et al., 2021). Because chromatin is crucial for the control of gene transcription (Bui et al., 2018), changes to its functional characteristics may result in mutagenesis. Among other reactions, hydroxyl radicals can be added to the C-8 position of guanine and generate a radical on the nitrogen at position 7 (**Figure 2.3**), which can be reduced by the addition of an electron and a proton to an unstable intermediate, and ultimately giving the ring-opening fragmentation product (Avendaño and Menendez, 2015; Juan et al., 2021). Alternatively, it can undergo oxidation to give the

derivative 8-hydroxyguanine. Hydroxylation of the C-8 position of the guanine derivative of DNA is the most well-studied DNA lesion induced by hydroxyl radical attack.

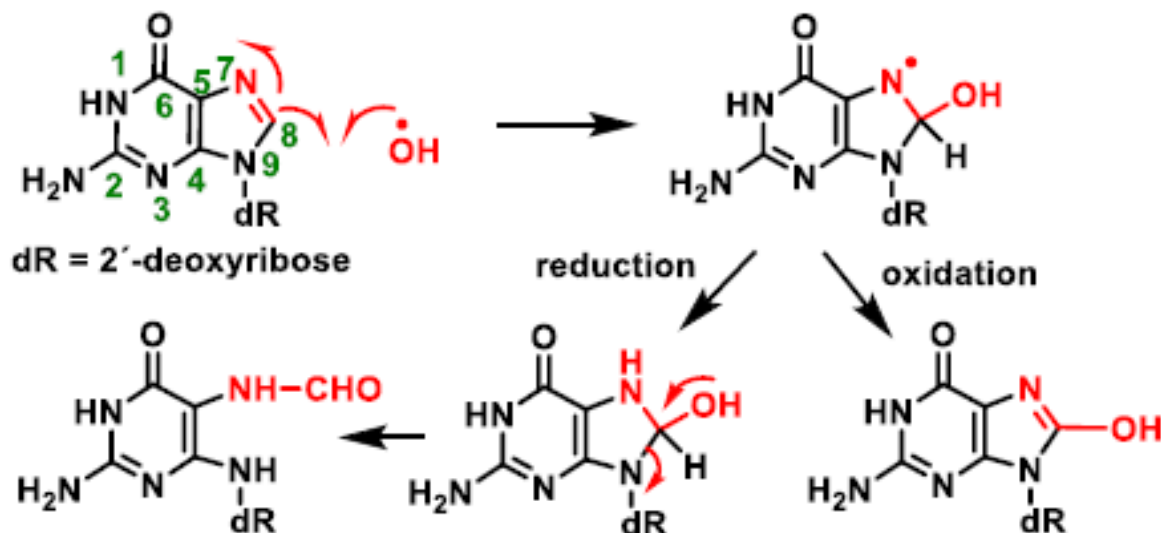


Figure 2.3: Mechanisms of oxidative damage to DNA-deoxyribose (*adopted without permission from Avendaño and Menendez, 2015*).

2.4.2 Lipid peroxidation

The oxidative degradation of polyunsaturated fatty acids is caused by lipid peroxidation. It is a chain reaction started by the abstraction of hydrogen or the addition of an oxygen radical (Repetto et al., 2012). The active methylene (RH) bridge represents a crucial target site, thus polyunsaturated fatty acids are more susceptible than the saturated ones (Repetto et al., 2012). A hydro-peroxidized lipid and an alkyl radical are produced because of the free radical's oxidation of an unsaturated lipid chain in this reaction. This affects the membrane structure, and thus the fluidity and integrity of the membrane are compromised (Yadav et al., 2019). This process is initiated by the attack of a hydroxyl radical on one of the bis-allylic sites in the fatty acid side chains, resulting in the production of an alkyl radical (Juan et al., 2021).

In the initial stage of lipid peroxidation, a radical species is formed (**Figure 2.4**). Free radical attacks a carbon that is bordered by double bonds in the aliphatic chain of a polyunsaturated fatty acid and hydrogenates a methylene group ($-\text{CH}_2$) that is connected to the carbon (Repetto et al., 2012; Juan et al., 2021). The radicals produced are stabilized by resonance with the

double bond (**Figure 2.4**). The radical reaction is extended during the propagation phase, and additional radical species such as peroxy radical are created as a result. Peroxyl radicals can combine with other surrounding polyunsaturated fatty acids to create a hydroperoxide and an alkyl radical, which further propagated the radical reaction with fatty acids (Repetto et al., 2012; Juan et al., 2021).

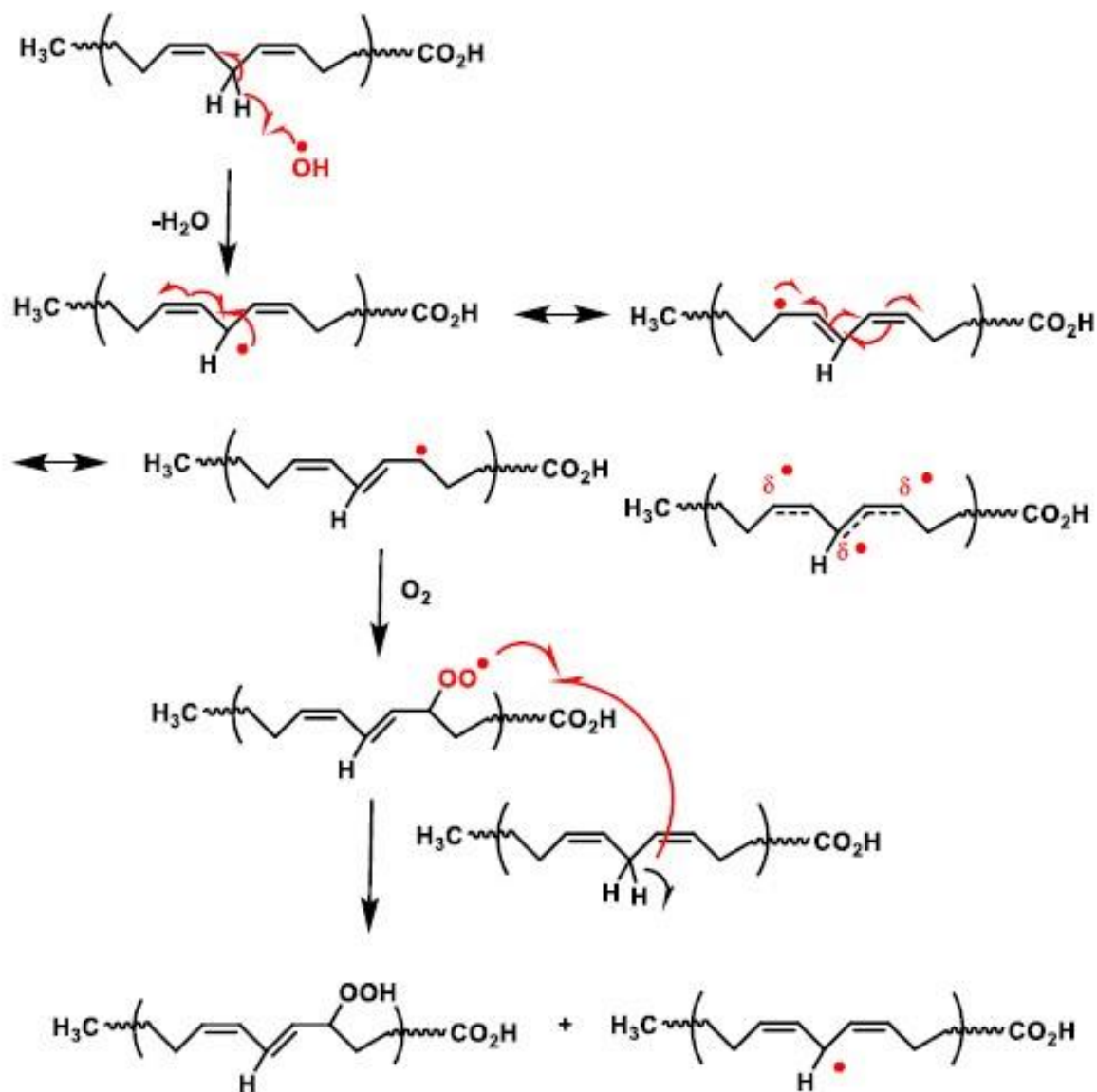


Figure 2.4: Mechanism of lipid peroxidation (*adopted without permission from Juan et al., 2021*).

Toxic breakdown products from lipid peroxidation, such as lipid hydroperoxides, isoprostanes and aldehydes like malondialdehyde, 4-hydroxynonenal can contribute to increased cellular toxicity by reacting with the tissue and cellular proteins to form advanced glycation end-products (AGEs) and advanced lipid peroxidation end-products (Araujo et al., 2016; Dal & Sigrist, 2016; Juan et al., 2021). This scenario causes protein dysfunctions, such as loss of activity and increased sensitivity to proteases and compromise cellular inflammatory responses, leading to apoptosis (Dal & Sigrist, 2016).

Lipid peroxidation generally changes membrane fluidity, permeability, and transport (Morita et al., 2016). The etiology of many chronic diseases, including atherosclerosis, diabetes, obesity, neurodegenerative disorders and cancer is influenced by the severity of lipid peroxidation (Lobo et al., 2010).

Oxidative stress has been linked to severe disorders such as cardiovascular disease, chronic obstructive lung disease, chronic kidney disease, neurological disorders and cancer (Klran et al., 2023) (**Figure 2.2**). Oxidative stress is reported to be involved in vitiligo, irritant contact and allergic contact dermatitis, adipose tissue dysfunction, asthma, atherosclerosis, hypertension, myocardial infarction, ischemia, metabolic syndrome, obesity, diabetes, dyslipidemia and impaired glucose tolerance (Chattopadhyay et al., 2015; Baek & Lee, 2016; Zhou et al., 2021; Sahiner et al., 2018; Dubois-deruy et al., 2020). Oxidative stress has also been associated with neurological disorders such as Parkinson's disease, Alzheimer's disease, Amyotrophic lateral sclerosis, and Huntington's disease (Klran et al., 2023). Oxidative stress has also been linked to an increase in autism and rheumatoid arthritis (Mateen et al., 2016; Bjørklund et al., 2020).

2.5 Antioxidant defence system

Antioxidants are chemicals that can delay or prevent the oxidation of biological molecules, such as proteins, lipids, carbohydrates and nucleic acids in biological systems, while antioxidants defence systems are mechanisms that protects biomolecules and the organism from the damaging effects of free radicals by reducing or repairing the damage done by ROS to the target molecule (Martn-Sierra et al., 2019). Overall, antioxidants work by converting deleterious oxidants into less harmful molecules, inactivating them by adding H^+ , interrupting chain reactions, or repairing damaged biomolecules. Furthermore, they exert their effects by inhibiting lipid peroxidation, scavenging ROS, preventing peroxidation chain reactions in lipid

oxidation, and actively participating in the conversion of peroxides to non-radical products. The level and composition of biological antioxidant defence systems differ from tissue to tissue and cell to cell (Klran et al., 2023). Antioxidants are classified according to several characteristics. One classification is function-based, which classifies antioxidants into primary and secondary groupings (Riveros et al., 2022). Another one classifies biological antioxidants into enzymatic and non-enzymatic groups (Moharram and Youssef, 2015; Riveros et al., 2022). Also, antioxidants can be classified as endogenous or exogenous (Riveros et al., 2022). **Figure 2.5** presents the different classification of antioxidants.

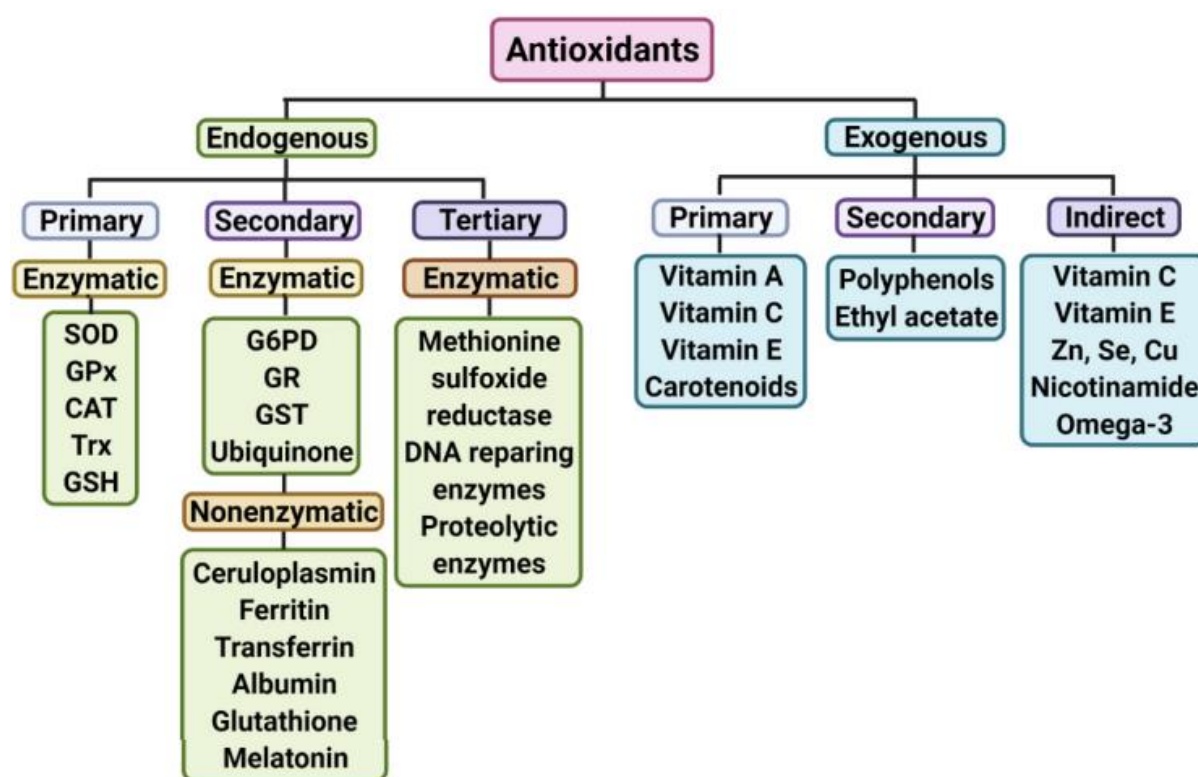


Figure 2.5: Classification of antioxidants (*adopted without permission from Riveros et al., 2022*). SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; Trx, thioredoxin; GR, glutathione reductase; GSH, reduced glutathione; G6PD, glucose 6 phosphate dehydrogenase; GST, glutathione S transferase.

Enzymatic antioxidants are generated in the human body and are classified as primary and secondary antioxidants (Mamta et al., 2014). Primary antioxidants enzymes are the first line of defence against ROS, which include superoxide dismutase (SOD), catalase (CAT), and

glutathione peroxidase (GPx). The enzymes operate synergistically and complementarily in biological systems (Mamta et al., 2014; Kurutas, 2016) as shown in **Figure 2.6**. Secondary antioxidant enzymes include glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH). G6PDH generates NADPH. GR is required to recycle the reduced glutathione (GSH) using GR and NADPH (Mamta et al., 2014; Kurutas, 2016).

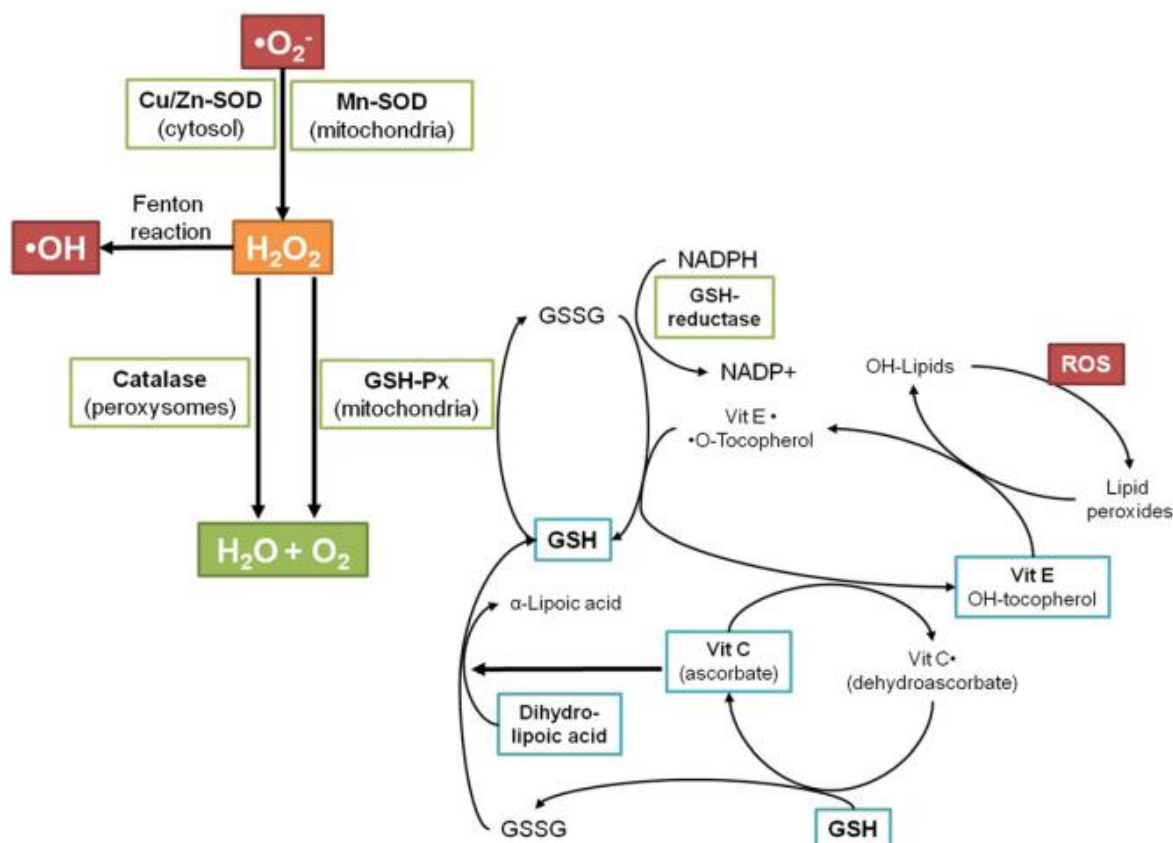


Figure 2.6: Biological antioxidant defences (*adopted without permission from Kurutas, 2016*).

Some of these antioxidants, such as enzymes, low-molecular-weight molecules, and enzyme co-factors are produced endogenously (Bunaciu et al., 2012; Riveros et al., 2022). Non-enzymatic antioxidants are obtained through diet. Dietary antioxidants are categorized into many groups, with dietary polyphenols being the most prominent. Polyphenols are compounds made up of phenolic acids and flavonoids. Vitamins, carotenoids, organosulfur compounds, and minerals are among the various types of dietary antioxidants (Bunaciu et al., 2012; Riveros et al., 2022). Below are the different biological antioxidants that comprise the biological antioxidant defence systems, as well as their modes of action.

The enzyme superoxide dismutase (SOD) eliminates superoxide ion ($O_2^{\cdot-}$) by reducing it to hydrogen peroxide (Riveros et al., 2022) (**Figure 2.6**), and thus mitigates superoxide ion-mediated cellular damage. Superoxide dismutase is also known to compete with nitric oxide for superoxide anion, thus preventing nitric oxide depletion and nitric oxide-mediated production of peroxynitrite (Chakraborty et al., 2009).

Catalase is a peroxisome-specific enzyme that mediates the conversion of hydrogen peroxide to water and molecular oxygen (Ally et al., 2020; Riveros et al., 2022) (**Figure 2.6**). As a result, it limits the cellular accumulation of hydrogen peroxide, thus preventing cellular toxicity. This also starves the Fenton reaction of hydrogen peroxide, thus limiting the production of free radicals (Nandi et al., 2019).

The reduced form of glutathione (GSH) is used in glutathione systems. GSH is produced from glutamate, cysteine, and glycine by two catalytic enzymes, known as glutamylcysteine ligase and GSH synthetase (Kunter et al., 2020). Through direct and indirect enzymatic reactions, GSH can scavenge free radicals. GSH is oxidized to glutathione di-sulphide (GSSG) in such a process (Kunter et al., 2020). Interestingly, glutathione reductase enzyme can recycle glutathione from GSSG (**Figure 2.6**). The antioxidant capability of cells can be assessed by the GSH/GSSG ratio, which is thought to fluctuate between 10:1, respectively, under normal circumstances (Kunter et al., 2020). Glutathione peroxidase (GPx) enzymes have peroxidase activity. GPx enzymes use glutathione as a reductant agent to facilitate hydrogen peroxide reduction (**Figure 2.6**).

Non-enzymatic antioxidants, commonly referred to as secondary antioxidants, are divided into two categories, namely endogenous and exogenous. Many of them can be gotten from dietary sources. Non-enzymatic endogenous antioxidants function as a second line of defence against free radicals. These non-enzymatic endogenous antioxidants include, thiols (glutathione, lipoic acid, N-acetyl cysteine), L-arginine, NADPH and NADH, ubiquinone (coenzyme Q10), melatonin, uric acid, bilirubin, metal binding proteins, albumin (copper), ceruloplasmin (copper), metallothionein (copper), ferritin (iron), myoglobin (iron) and transferrin (iron) (Bunaciu et al., 2012). They help in the proper functioning of the antioxidant enzyme systems.

Vitamins, including vitamins A, C, E, and B, are micronutrients that are essential for the efficient functioning of the body's antioxidant defence system. They cannot be synthesized in our bodies and must therefore be supplemented in our diet. (Mamta et al., 2014). Vitamin A helps the immune system because of its antioxidant characteristics. It comes in three precursors,

namely retinol, 3,4-didehydroretinol, and 3-hydroxyretinol. Sweet potatoes, carrots, milk, egg yolks, and mozzarella cheese are the primary sources of this (Mamta et al., 2014). Vitamin C is a water-soluble vitamin found in fruits (mostly citrus), vegetables, grains, beef, chicken, fish, etc. It helps in the prevention of some DNA damages caused by free radicals. These damages may contribute to aging and the development of illnesses such as cancer, heart disease, and arthritis. (Mamta et al., 2014). Vitamin E is a fat-soluble vitamin that comprises of eight distinct forms, namely α -, β -, γ -, and δ -tocopherol and α -, β -, γ -, and δ -tocotrienol. α -Tocopherol is the most bioavailable amongst the different forms of vitamin E. It is an important lipid-soluble antioxidant that reacts with lipid radicals, thus protecting the membranes from lipid peroxidation. This reaction results in the production of tocopheroxyl radicals, which can be reduced to tocopherol through reduction by other antioxidants, such as ascorbate and retinol. (Mamta et al., 2014)

Carotenoid compounds include beta-carotene, lycopene, lutein, and zeaxanthin. They are pigmented fat-soluble compounds found in fruits and vegetables (Mamta et al., 2014). β -carotene is typically present in foods with a radish-orange-green colour, including carrots, sweet potatoes, apricots, pumpkin etc, as well as certain green and leafy vegetables. Green leafy foods are high in lutein (Hamid et al., 2010). Lutein is well recognized for its role in protecting the retina from free radical damage and preventing atherosclerosis (Sikora et al., 2008). Although lycopene, lutein, canthaxanthin and zeaxanthin do not have provitamin A activity, β carotene has been identified as a vitamin A precursor (Fang et al., 2002). Tomatoes are high in lycopene. Lycopene is a powerful antioxidant that has been proven to be important in eliminate the deleterious singlet oxygen found in tomatoes, watermelon, guava, papaya, apricots, pink grapefruit, and some other foods. (Mamta et al., 2014).

Dietary polyphenols are one of the most important antioxidants obtained from plants. They are potent radical scavengers due to their ability to donate electrons or transfer hydroxy H-atom, thus forming stable phenoxy radicals (Chen et al., 2020).

2.6 Polyphenols

Polyphenols are organic compounds present in a variety of plants, including fruits, vegetables, cereals, and beverages. Dietary polyphenols may contribute to bitterness, astringency, flavour, smell, and oxidative stability (Pandey and Rizvi, 2009). Phenolic compounds are found throughout the plant kingdom and include simple molecules like phenolic acids, as well as

complex polymeric compounds like tannins and lignans (Losada-Barreiro et al., 2022). Polyphenols are categorized into various groups based on the amount of phenol rings they contain and the structural components that hold these rings together. Phenolic is one of the most important classes. The primary category of phenols includes phenolic acids, flavonoids, stilbenes, and lignans (Spencer et al., 2008). Flavonoids and phenolic acids are the most common polyphenol classes found in plant-based diet, and many studies have shown that these phenols can ameliorate the risk of oxidative stress-related diseases such as inflammation and cancer by modulating several relevant biological processes (Ristow et al., 2011; Marroco et al., 2017; Roy et al., 2017). Indeed, the bioavailable phenolic compounds found in these foods have been shown to increase plasma antioxidant capacity, both in the short term when consumed infrequently and in the long term when consumed frequently (Hackman et al., 2008).

2.6.1 Dietary polyphenols as natural antioxidants in disease management

Noncommunicable diseases are the world's leading causes of mortality (Beaglehole et al., 2011). Oxidative stress is thought to be one of the most important underlying causes of present-day major public health issues, such as type 2 diabetes, cardiovascular disease, cancer, as well as age-related illnesses (Jayedi et al., 2018).

Though the body has an inbuilt defence mechanism to scavenge disease causing ROS, antioxidants available in fruits and vegetables still remain relevant sources of dietary antioxidants for improving the bodies antioxidant status and preventing oxidative stress and associated disorders (Sridevi et al., 2018). Whole grains, fruits, and vegetables are the main sources of naturally occurring antioxidants. Dietary antioxidants are known to have the ability to lower disease risk. The significance of the afore mentioned is reinforced by epidemiological data showing an inverse relationship between regular fruit and vegetable consumption and the likelihood of developing one or more of the common illnesses, including cardiovascular, neurodegenerative, oncologic and metabolic diseases (Yahia et al., 2019). This suggests that bioactive plant-based compounds could beneficially influence the underlying pathological mechanisms of neurodegenerative, metabolic and vascular diseases (Islam et al., 2017; Lapuente et al., 2019; Sharifi-Rad et al., 2020; Grodzicki et al., 2020). **Table 2.1** presents the dietary antioxidants found in some fruits.

Table 2.1: Fruits as sources of antioxidant principles

Fruits	Phytochemical class	Phytochemical subclass	Antioxidant compounds
Mango	Tetrapenoids	Anthocyanins, Carotenoids	Cyanidin, delphinidin, α -carotene, β -carotene, β - cryptoxanthin, lycopene, xanthophylls
Apricot	Tetrapenoids, Flavonoids	Carotenoids, Flavonols, Dihydrochalcones	α -carotene, β -carotene, lycopene, Quercetin, kaempferol, Phloretin
Pomegranate	Phenolic acid	Hydroxybenzoic acids	Gallic acid, ellagic acid
Papaya	Tetrapenoids, Organic acids	Carotenoids, Aldonic acids	β -carotene, β -cryptoxanthin, xanthophylls, Vitamin C
Berries	Phenolic acids, Flavonoids, Stilbenoids	Hydroxybenzoic acids, Flavanols, Anthocyanins	Gallic acid, Catechin, Quercetin, kaempferol, Cyanidin, delphinidin, Resveratrol, pterostilbene, piceatanno
Apple	Triterpenoids, Flavonoids Phenolic acids	Sterols, Anthocyanins, Flavanols, Dihydrochalcones, Hydroxycinnamic acids, Salicylates	Campesterol, β -sitosterol, Cyanidin, delphinidin, Catechin, Quercetin, kaemferol, Phloretin, Ferulic acid, chlorogenic acid
Orange	Monoterpenoids, Tetrapenoids	Carotenoids	α -carotene, β -carotene, β - cryptoxanthin, lycopene, xanthophylls
Banana	Phenolic acids, Flavonoids, Lignans, Triterpenoids	Hydroxybenzoic acids, Flavanols, Sterols,	Gallic acid, Catechin, epicatechin, epigallocatechin, Myricetin, Pinorensinol, Campesterol
Grape	Phenolic acids, Flavonoids,	Hydroxybenzoic acid, Hydroxycinnamic acid, Flavanols, Anthocyanins	Gallic acid, protocatechuic acid, Caffeic acid, p- coumaric acid, ferulic acid, chlorogenic acid, Catechin, epicatechin, epigallocatechin, Cyanidin, delphinidin

Adopted without permission from Afam et al., 2021.

Advances in research has shown that the non-edible wastes, including the peels of many fruits contain substantially more phenolics and other phytochemicals than their respective edible tissues (Wadhwa et al., 2015; Sagar et al., 2018; Rafiq et al., 2019), which could be useful in the management of several chronic diseases, including diabetes, hypertension, obesity and cardiovascular complications. This is relevant for a country like South Africa, where under-utilized fruit wastes (peels, seeds, pomace, etc.), which may be of medicinal and bio-economic relevance are generated yearly.

2.7 Fruit wastes

Fruits and vegetables account for more than 42 percent (%) of food waste worldwide, while being the most eaten food (Coman et al., 2020; Pushparaj et al., 2022; Ganesh et al., 2022). Thus, every year, an enormous quantity of fruit wastes, such as peels, seeds, pomace and rind, are produced because of global fruit consumption and processing (Zhu et al., 2023). South Africa generates a significant amount of fruit waste annually, primarily from the citrus and deciduous fruit industries (Khan et al., 2015). The disposal of by-products and waste generated by the fruit processing industries is a global problem. Most of the wastes end up in landfills, which cause environmental and socioeconomic concerns (Giroto et al., 2015). Fruit wastes contribute to severe environmental challenges, such as water and soil pollution, eutrophication, global warming, green house effects, and other health issues if not appropriately managed because of their high biodegradability and fermentability (de Medeiros et al., 2020). Greenhouse gases, such as CO₂ and methane are produced during fruit wastes biodegradation, which contributed to the greenhouse effect (Khan et al., 2015).

2.7.1 Fruit wastes as potential sources of antioxidant principles

Underutilized wastes (peels, seeds, pomace, etc.) from various fruits, are potential sources of numerous bioactive compounds, including phenolics, flavonoids, tannins, and limonoids, which are uncommon in other plants. These bioactive principles can exhibit biological activities particularly antioxidant and anti-inflammatory properties (Ali et al., 2022; Hussain et al., 2022). **Table 2.2** summarizes the antioxidant and anti-inflammatory potentials of some fruit wastes and their phytochemical profiles.

Table 2.2: Antioxidant and anti-inflammatory potentials of the fruit wastes and their phytochemicals

Fruit scientific name (common name)	Parts (wastes)	Antioxidant properties	Phytochemical profile	References
<i>Citrus × sinensis</i> (L.) Osbeck (Sweet orange)	Peel	• <i>In vitro</i> radical scavenging and Fe³⁺ reducing activity	Narirutin, sinensetin, nobiletin, 4',5,6,7-tetramethoxyflavone, and 3,3',4',5,6,7-hexamethoxyflavone	Long et al., 2021
		• Suppressed LPS and IFN-γ induced NO production in RAW 264.7 cells • Reduced expression of iNOS and COX-2 in RAW 264.7 cells • Suppressed t-BPH-induced oxidative stress and cytotoxicity in HepG2 cells by increasing antioxidant enzymes and GSH and suppressing ROS production, LPO, caspase-3 activation and pro-apoptotic signalling.	Narirutin, hesperidin, nobiletin and tangeretin	Chen et al., 2012; Chen et al., 2017
		4 wks adm. (50 mg/kg bw, p.o.) of extracts in male Wistar rats ameliorated N-acetyl-p-aminophenol-induced liver damage by reducing serum ALT, AST, ALP, inflammatory marker (TNF-α and IL-4), hepatic LPO and pro-apoptotic activity, increasing hepatic GSH and antioxidant enzyme activity and improving hepatic histopathological deteriorations.	Diosmin, gallic acid, naringin, rutin, hesperidin, quercetin, naringenin and hesperetin	Ahmed et al., 2019
	Seed	• <i>In vitro</i> radical scavenging activity • <i>In vitro</i> inhibitory activity on BSA glycation	Gallic acid, 3,4-dihydroxybenzoic acid, (+)-catechin, 1,2-dihydroxybenzene, syringic acid, caffeic acid, rutin trihydrate, p-coumaric acid, trans-ferulic acid, resveratrol,	Shakthi Deve et al., 2014; Al Juhaime et al., 2018

Fruit scientific name (common name)	Parts (wastes)	Antioxidant properties	Phytochemical profile	References
			quercetin, kaempferol, apigenin-7-glucoside, isorhamnetin, naringenin and trans-cinnamic acid	
<i>Citrus unshiu</i> (Yu.Tanaka ex Swingle) Marcow (Naartjie)	Peel	• <i>In vitro</i> radical scavenging and Fe³⁺ reducing activity • Suppressed H₂O₂-induced oxidative damage in neuronal cells (HT22 cells) by reducing cytotoxicity or cell death and expression of pro-apoptotic signalling proteins (p-JNK, p-p38, caspase 3 and Bcl-2) • Suppressed LPS-induced inflammation in RAW 264.7 macrophages by suppressing NO production, iNOS and COX-2 expression and the secretion of inflammatory cytokines (IL-6, TNF-α and PGE2)	Narirutin, narigenin, prunin, nobiletin, rutin, neoponcirin, tangeretin, hesperetin-7-O-glucoside, sinensetin, hesperetin and hesperidin	Cho et al., 2015; Kim et al., 2019; Kim and Lim, 2020
<i>Citrus × paradisi</i> Macfad (Grapefruit)	Peel	15 days supplementation of diet containing 8% of peel powder ameliorated TNBS-induced colitis in male Wistar rats by reducing colonic and serum expression of pro-inflammatory markers and improving colonic and serum antioxidant status	Protocatechuic acid, isorhamnetin-3, kaempferol-3, feruloyl, caffeoyl, coumaroyl, myricetin-3, quercetin-3 and, laricitrin-3 hexosides; quercetin-3 and isorhamnetin-3 glucuronides; quercetin-3-rutinoside; caftaric, coutaric, ferulic, syringic, coumaric and fertaric acids; epicatechin, catechin, resveratrol, procyanidin and viniferin	Maurer et al., 2020
<i>Citrus × aurantiifolia</i> (Christm.)	Peel	• <i>In vitro</i> radical scavenging activity	Gallic acid, 3,4-dihydroxybenzoic acid, (+)-catechin, 1,2-	Al Juhaimi et al., 2018a

Fruit scientific name (common name)	Parts (wastes)	Antioxidant properties	Phytochemical profile	References
Swingle (Key lime)			dihydroxybenzene, syringic acid, caffeic acid, rutin trihydrate, p-coumaric acid, trans-ferulic acid, resveratrol, quercetin, kaempferol, apigenin-7-glucoside, isorhamnetin, naringenin and trans-cinnamic acid	
<i>Citrullus lanatus</i> (Thunb.) Matsum. & Nakai (Watermelon)	Peel	• <i>In vitro</i> radical scavenging and Fe³⁺ reducing activity • Improved antioxidant status of STZ-induced diabetic male SD rats • Reduced hepatic, renal and cardiac LPO in rats fed with atherogenic diet.	Dihydroferulic acid 4-sulfate, isolariciresinol 9-O-beta-D-glucoside, citric acid, ascorbic acid gingerol and curcumenol	Parmar and Kar, 2008; Rezq, 2017; Jibril et al., 2019
	Seed	• <i>In vitro</i> radical scavenging and Fe³⁺ reducing activity	1,9-Dimethyluric acid, citric acid, dihydrocaffeic acid 3-O-glucuronide, 6-hydroxyluteolin 7-rhamnoside, 3-ppihydroxy-2'-deoxymugineic acid, curcumenol and arjunolic acid	Jibril et al., 2019
<i>Mangifera indica</i> L. (Mango)	Seed kernel	• <i>In vitro</i> radical scavenging activity • <i>In vitro</i> Fe³⁺ reducing activity • Dose-dependently suppressed H₂O₂-induced toxicity of human umbilical vein endothelial cells	Mangiferin, Quinic acid, β-glucogallin, theogallin, 2-hydroxy-3,4-dimethoxybenzoic acid, 1,2,3,4-tetragalloyl-α-D-glucose, 7,8,4'-trihydroxyflavanone and 6-paradol	Lasano et al., 2019; Luo et al., 2012
<i>Musa acuminata</i> Colla (Banana)	Peel	• DPPH and superoxide ion radical scavenging activity • Mitigated H₂O₂-mediated human red blood cell haemolysis • Increased lysozyme, leucocyte phagocytic, immunoglobulin M and	Alkaloids, catechin and quercetin	Sundaram et al., 2011; Giri et al., 2016; Vijay et al., 2022

Fruit scientific name (common name)	Parts (wastes)	Antioxidant properties	Phytochemical profile	References
		plasma antioxidant enzyme activities; reduced the expression of inflammatory markers (IL-10 and NF-kB) in Labeo rohita fingerlings		
<i>Persea americana</i> Mill. (Avocado)	Seed	• ABTS, DPPH, superoxide and peroxy radicals scavenging and Fe³⁺ reducing antioxidant activity • Modulation of anti-inflammatory response in murine macrophage cells	Trans-5-O-caffeoyl-D-quinic acid, procyanidin B1, catechin and epicatechin	Tremocoldi et al., 2018
<i>Ananas comosus</i> (L.) Merr. (Pineapple)	Peel	• <i>In vitro</i> radical scavenging activity	Ferulic acid, gallic acid, epicatechin, syringic acid, phenylacetic acid, malonic acid, succinic acid, glyceric acid and glucaric acid	Azizan et al., 2020
<i>Carica papaya</i> L. (Papaya)	Seed	• <i>In vitro</i> radical scavenging and Fe³⁺ reducing activity • exerted immunomodulatory and anti-inflammatory effects by IgM and IgG and reducing serum inflammatory cytokines (IL-1β, IL-6, IL-10, IL-12 and TGF-β1) in bacteria infected mice • protected against STZ-induced diabetic myocardial damage in rats by improving myocardial antioxidant status.	Syringic acid hexoside, 5-hydroxy caffeic quinic acid, 5-hydroxy feruloyl quinic acid, acetyl p-coumaroyl quinic acid, feruloyl quinic acid, p-coumaroyl trimethyl glycoside, kaempferol-3-O-rhamnoside, n-methyl feruloyl quinic acid, quercetin-3-O-glycoside, quercetin-3-O-rhamnoside, chlorogenic acid and cyanidin-3-O-glucose	Zunjar et al., 2015; Amin et al., 2019; 2021
<i>Malus domestica</i> Borkh (Apple)	Seed	• <i>In vitro</i> radical scavenging and Fe³⁺ reducing activity	Protocatechuic acid, caffeic acid, quercetin, hyperin, procyanidin B2, chlorogenic acid, (+)catechin, (-)epicatechin and phloridzin	Xu et al., 2016

Fruit scientific name (common name)	Parts (wastes)	Antioxidant properties	Phytochemical profile	References
<i>Prunus domestica</i> L. (European plum)	Seed or pit	• <i>In vitro</i> oxygen and DPPH radical scavenging and Fe³⁺ reducing activity • Xanthine oxidase inhibitory activity	p-Hydroxybenzoic acid, vanillin, vanillic acid, 3,4-dihydroxybenzoic acid, gallic acid and syringic acid	Khallouki et al., 2012
<i>Prunus armeniaca</i> L. (Apricot)	Seed or pit	• <i>In vitro</i> radical scavenging activity • Ameliorated dimethylnitrosamine-induced hepatic fibrosis in rats by improving hepatic histology and antioxidant status	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	Abdel-Rahman, 2011; Al Juhaimi et al., 2018b
<i>Prunus cerasus</i> L. (Sour Cherry) and <i>Prunus persica</i> (L.) Batsch 1801 (Peach)	Seed or pit	• <i>In vitro</i> scavenger of ABTS and AAPH radicals • <i>In vitro</i> inhibition of methylglyoxal induced BSA glycation and AGEs production	p-Hydroxybenzoic, syringic acid, vanillic acid, p-coumaric, caffeic acid, ferulic acid, protocatechuic acid, gallic acid and sinapinic acid	Mesías et al., 2013

Abbreviations: AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride; ABST, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); Adm; administration; AGE, advanced glycation end-products; ALT, alanine transaminase; ALP, alkaline phosphatase; AST, aspartate transaminase; BSA, bovine serum albumin; COX-2, cyclooxygenase-2; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GSH, reduced glutathione; LPO, lipid peroxidation; LPS, lipopolysaccharide; NO, nitric oxide; ROS, reactive oxygen species; SD, Sprague-Dawley; STZ, streptozotocin; t-BPH, tert-butyl hydroperoxide; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

For the purpose of the present project, this thesis will be focusing on pomegranate (*Punica granatum* L.), a fruit with numerous benefits on metabolic, vascular, and oxidative health.

2.8 Pomegranate

Pomegranate is a deciduous and ornamental plant (**Figure 2.7**) that belongs to the family of Lythraceae (**Table 2.3**) and is indigenous to Central Asia (Fawole and Opara, 2016). Due to its

hardy nature, the plant thrives and is extensively cultivated in many tropical and sub-tropical regions of the world, including South Africa (Dhinesh and Ramasamy, 2016). Pomegranate fruit is highly nutritious, has high economic value and contains several active components with potent pharmacological benefits (Dhinesh and Ramasamy, 2016).

Table 2.3: Taxonomic classification of Pomegranate (*Punica granatum* L.)

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 2.7: Pomegranate tree with fruits.

The pomegranate fruit, often known as balausta, is a pulpy berry that is crowned by the persistent calyx. It comprises of small seeds (prismatic shape) that have fleshy/pulpy cover (testa or arils), woody tegmen without albumin, straight embryo and cotyledons that are all enclosed in the inside of the leathery peel (Melgarejo et al., 2020) (**Figure 2.8**).

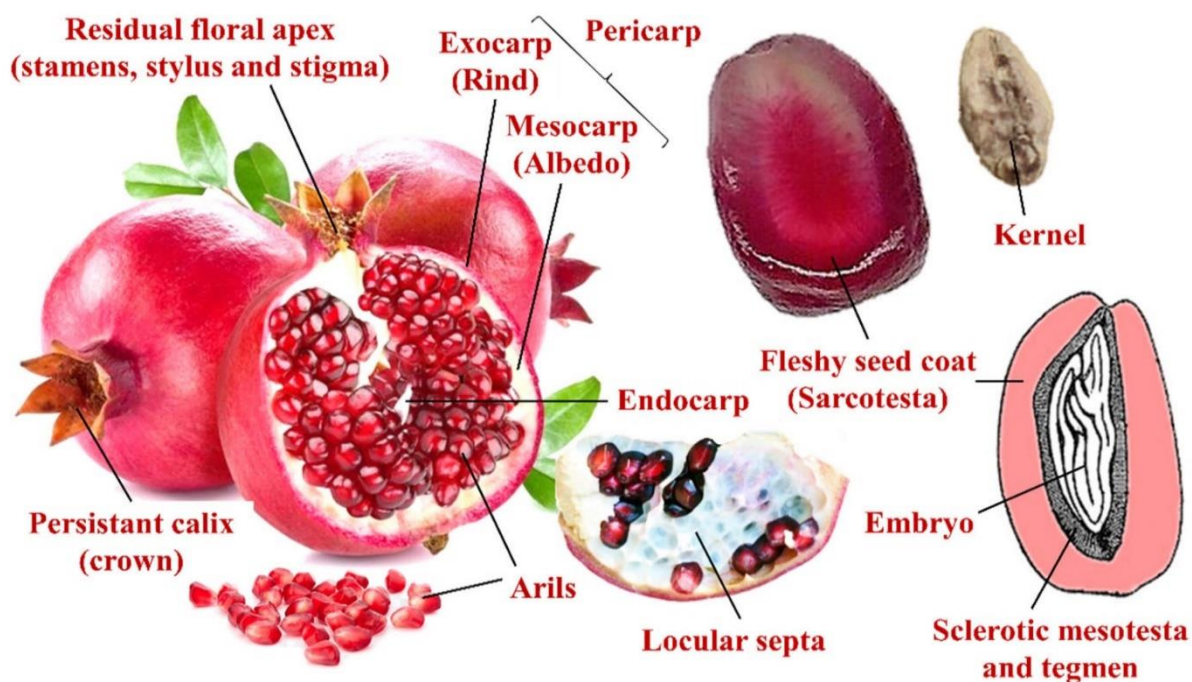


Figure 2.8: Pomegranate fruit and its parts (*adopted without permission from Montefusco et al., 2021*).

2.8.1 Pomegranate fruit medicinal potential and phytochemical profile

Pomegranate fruit has been linked to several health benefits, including oxidative and vascular health benefits (Kandylis and Kokkinomagoulos, 2020; Giménez-Bastida et al., 2021). Documented evidence has reported the antioxidant, anti-inflammatory, antidiabetic, anti-obesogenic and cardioprotective potential of the fruit (Kandylis and Kokkinomagoulos, 2020; Benedetti et al., 2023). The health benefits of the fruit, particularly the antioxidant benefits have been linked to the presence of several phytochemical constituents, mainly polyphenols, including the predominant ellagitannin constituents (Giménez-Bastida et al., 2021). Ellagitannins are hydrolysable tannins found in pomegranate fruit, which comprise punicalagins, punicalin, gallic acid, ellagic acid, and related glycosides (Altieri et al., 2019).

Pomegranate fruit also contains phenolic acids and flavonoids including gallic acid, catechins, esculetin, hesperidin, rutin, naringenin, kaempferol, apigenin, rhamnetin, quercitrin, and quercetin, which contribute to its antioxidant effects (Zeghad et al., 2019). **Table 2.4** shows the phytochemicals that have been identified in pomegranate fruit.

Table 2.4: Different classes of phytochemicals identified from pomegranate

Class of phytochemical	Reported compounds or phytochemicals
Ellagitannins, gallotannins and derivatives	Brevifolin, Brevifolin carboxylic acid, Brevifolin carboxylic acid 10-monopotassium sulphate, Castalagin, Casuariin, Casuarinin, Corilagin, Isocorilagin, Hippomanin A, Gemin D, Diellagic acid rhamnosyl(1→4) glucopyranoside, 1,2-Di-O-galloyl-4,6-O-(S)-hexahydroxydiphenoyl β-D-glucopyranoside, Ellagic acid, 3,30 -Di-O-methylellagic acid, 3,30 ,40 -Tri-O-methylellagic acid, 3-O-Methylellagic acid, 4,40 -Di-O-methylellagic acid, 30 -O-Methyl-3,4-methylenedioxy-ellagic acid, Eschweilenol C (Ellagic acid 4-O-α-L-rhamnopyranoside), Ethyl brevifolincarboxylate, Eucalbanin B, Eucarpanin T1 , Pomegraniin A, Pomegraniin B, Gallagic acid, Gallic acid 3-O-β-D-(60 -O-galloyl)-glucopyranoside, 6-O-Galloyl-2,3-(S)-hexahydroxydiphenoyl-D-glucose, 5-Galloylpunicacortein D, 2-O-Galloylpunicalin (2-O-Galloyl-4,6-(S,S)-gallagyl-D-glucose), Granatin A, Granatin B, 2,3-(S)-Hexahydroxydiphenoyl-D-glucose, Lagerstannin B, Lagerstannin C, 3-O-Methylellagic acid 4-O-α-L-rhamnopyranoside, 3,40 -O-Dimethylellagic acid 4-O-α-L-rhamnopyranoside, Oenothien B, Pedunculagin I, Pedunculagin II, 1,2,3,4,6-Penta-O-galloyl-β-D-glucose, 3,4,8,9,10-Pentahydroxydibenzo [b,d] pyran-6-one (Urolithin M-5), Phyllanthusiin E, Pomegranate, Punicacortein A, Punicacortein B, Punicacortein C, Punicacortein D, Punicafolin, Punicalagin A, Punicalagin B, Punicalin, Punicatannin A, Punicatannin B, Punigluconin, Strictinin [1-O-Galloyl-4,6-(S)-hexahydroxydiphenoyl-D-glucose], Tellimagrandin I, Tercatain [1,4-Di-O-galloyl-3,6-(R)-hexahydroxydiphenoyl-β-glucopyranose], Terminalin (Gallagyl dilactone), 1,2,4,6-Tetra-O-galloyl-β-D-glucose, 1,2,3-Tri-O-galloyl-β-glucopyranose, 1,2,4-Tri-O-galloyl-β-glucopyranose, 1,2,6-Tri-O-galloyl-β-glucopyranose, 1,3,4-Tri-O-galloyl-β-glucopyranose, 1,4,6-Tri-O-galloyl-β-glucopyranose, 3,4,6-Tri-O-galloyl-β-glucopyranose, Valoneic acid dilactone
Flavonoids	Hovetrichoside C, Phloretin, Phlorizin, Eriodictyol-7-O-α-L-arabinofuranosyl (1-6)-β-D-glucoside, Granatumflavanyl xyloside, Naringin (Naringenin-7-O-rhamnoglucoside), Naringenin-40methyl ether

	7-O-α-L-arabinofuranosyl(1-6)-β-D-glucoside, Pinocembrin, Punicaf flavanol, Apigenin, Apigenin 4-O-β-glucopyranoside, Luteolin, Luteolin 3-O-β-glucopyranoside, Luteolin 4-O-β-glucopyranoside, Cynaroside (Luteolin 7-O-glycoside), Luteolin 3-O-β-xylopyranoside, Tricetin, Daidzein, Genistein, Amurensin (Noricaritin 7-β-D-glucopyranoside), Kaempferol, Astragalin (Kaempferol 3-O-glucoside), Kaempferol-3-O-rhamnoglucoside, Myricetin, Phellatin, Quercetin, Hirsutrin (Quercetin-3-O-glucoside), Quercimeritrin (Quercetin-7-O-glucoside), Quercetin 3-O-rhamnoside, Rutin (Quercetin-3-O-rutinoside), Quercetin-3,40-dimethyl ether 7-O-α-L-arabinofuranosyl(1-6)-β-D-glucoside, Cyanidin, Chrysanthemin (Cyanidin-3-O-glucoside), Cyanin (Cyanidin-3,5-di-O-glucoside), Antirrhinin (Cyanidin-3-O-rutinoside), Catechin-cyanidin-3-hexoside, Delphinidin, Myrtillin (Delphinidin-3-O-glucoside), Delphinidin-3,5-di-O-glucoside, Pelargonidin, Callistephin (Pelargonidin-3-O-glucoside), Pelargonin (Pelargonidin-3,5-di-O-glucoside), Catechin, Epicatechin, Epicatechin gallate, Epigallocatechin-3-O-gallate, Gallocatechin-(4$\rightarrow$8)-catechin, Gallocatechin-(4$\rightarrow$8)-gallocatechin, Catechin-(4$\rightarrow$8)-gallocatechin, Procyanidin A2, Procyanidin B1, Procyanidin B2, Procyanidin B3
Lignans	Conidendrin, Isohydroxymatairesinol, Isolariciresinol, Matairesinol, Medioresinol, Phylligenin, Pinoresinol, Secoisolariciresinol, Syringaresinol, Pomegralignan, Punicatannin C
Triterpenoids and phytosterols	Asiatic acid, Betulinic acid (Betulic acid), Friedooleanan-3-one (Friedelin), Maslinic acid, Oleanolic acid, Punicanolic acid, Ursolic acid, Campesterol, Cholesterol, Daucosterol, β-Sitosterol, β-Sitosterol laurate, β-Sitosterol myristate, Stigmasterol
Fatty acids and lipids	Caproic acid (Hexanoic acid), Caprylic acid (Octanoic acid), Capric acid (Decanoic acid), Lauric acid (Dodecanoic acid), Myristic acid (Tetradecanoic acid), Myristoleic acid (9-cis-Tetradecanoic acid), Palmitic acid (Hexadecanoic acid), Palmitoleic acid (Hexadec-9-enoic acid), Punicic acid (9Z, 11E, 13Z-octadecatrienoic acid), Linoleic acid (cis, cis-9,12-Octadecadienoic acid), α-Linolenic acid (All-cis-9,12,15-octadecatrienoic acid), γ-Linolenic acid (All-cis-6,9,12-octadecatrienoic acid), Oleic acid (9Z-octadecenoic acid), Stearic acid (Octadecanoic acid), α-Eleostearic acid (9Z, 11E, 13E-octadecatrienoic acid), β-Eleostearic acid (9E, 11E, 13E-octadecatrienoic acid), Catalpic acid (9E, 11E, 13Z-octadecatrienoic acid), Arachidic acid (Eicosanoic acid), Gadoleic acid (9Z-icosenoic acid), Behenic acid (Docosanoic acid), Nervonic acid (cis-15-Tetracosenoic acid), 1-O-9E,11Z,13E-Octadecatrienoyl glycerol, 1-O-Isopentyl-3-O-octadec-2-enoyl glycerol, Tri-O-punicylglycerol, Di-O-punicyl-O-octadeca-8Z, 11Z, 13E-trienylglycerol, N-palmitoyl cerebroside

Organic acids and phenolic acids	Ascorbic acid, Citric acid, Fumaric acid, L-Malic acid, Oxalic acid, Quinic acid, Succinic acid, Tartaric acid, Caffeic acid, Chlorogenic acid, Cinnamic acid, o-Coumaric acid, p-Coumaric acid, cis-p-Coumaric acid, Coumaric acid, 7,8-Dihydroxy-3-carboxymethylcoumarin-5-carboxylic acid, Ferulic acid, Gallic acid, Methyl gallate, Neochlorogenic acid (5-O-Caffeoylquinic acid), Protocatechuic acid, Vanillic acid, Coniferyl 9-O-[β -D-apiofuranosyl(1 $\rightarrow$ 6)]-O- β -D-glucopyranoside, Sinapyl 9-O-[β -D-apiofuranosyl(1 $\rightarrow$ 6)]-O- β -D-glucopyranoside
Others	Catechol, Coumestrol, Icariside D1, Phenylethylrutinoside, Syringaldehyde

Adopted without permission from Wu and Tian, 2017.

The above-mentioned polyphenols, including ellagitannins, phenolic acids and some flavonoids, account for a large amount of the fruit's antioxidant properties and are more abundant in the fruit peel and juice than in other portions of the fruit (Ismail et al., 2012; Marra et al., 2022; Yaritz et al., 2022).

2.8.2 Antioxidant and phytochemical profiles of pomegranate peel

Several studies have reported that the fruit's peel contains ellagitannins, flavonoids, and phenolic acids, which may be useful in managing oxidative stress and mitigating tissue damage (Akuru et al., 2022; Marra et al., 2022; Mo et al., 2022). Maximizing the therapeutic potential of the fruit's peel may potentially reduce environmental biomass and pollution caused by the fruit's wastes. The fruit's peel has been proven to have powerful radical scavenging activity (Li et al., 2006; Akuru et al., 2022; Marra et al., 2022), and stronger than the edible pulp (Li et al., 2006). The peel was demonstrated to prevent lipid peroxidation induced by oxidative stress in both hepatocytes and isolated rat liver tissues (Akuru et al., 2022).

The peel has been shown to exhibit hepatoprotective effect in rats by improving oxidative stress and reducing carbon tetrachloride-induced liver oxidative damage in rats (Chidambara et al., 2002). Indeed, phenolic compounds from pomegranate peel (ellagic acid, punicalin, and punicalagin) have been found to lower oxidative stress and inflammatory markers in oxidatively challenged rats (Sun et al., 2017). In rats, the peel powder feeding protected against dextran sulfate sodium-induced intestinal and hepatic oxidative stress by reducing small intestinal and hepatic lipid peroxidation and increasing GSH content and GSH/GSSG ratio (Al-

Gubory et al., 2021). Also, pomegranate 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 and improving renal and hepatic tissue histology, and antioxidant status (El Bohi et al., 2021). Polysaccharide isolate from pomegranate peel exerted immunomodulatory effects in cyclophosphamide-induced immunosuppressed mice by increasing lymphocytes proliferation, immunoglobins and cytokines secretion, as well as antioxidant status (Wu et al., 2019). **Table 2.5** summarizes the antioxidant, anti-inflammatory and tissue protective potentials of pomegranate peel, as well as the phytochemical profile.

Table 2.5: The antioxidant and tissue protective potential and phytochemical profile of pomegranate fruit peel

Pharmacological action	Tested samples	Phytochemical profile	References
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
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	Polysaccharide isolate		Wu et al., 2019

Pharmacological action	Tested samples	Phytochemical profile	References
cyclophosphamide-induced immunosuppressed mice increased splenic LDH and acid phosphatase activity, lymphocytes proliferation, immunoglobins and cytokines secretion and antioxidant status.			
<i>In vitro</i> ABTS and hydroxyl radicals scavenging activity	Aqueous extract	Punicalin, pedunculagin (I and III isomers), ellagic acid, ellagic acid-deoxyhexoside, punicalagin (α and β isomers), ellagic acid-hexoside and gallic acid	Hernández-Corroto et al., 2019
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
<i>In vitro</i> DPPH and NO radical scavenging activity; <i>In vitro</i> Fe ³⁺ reducing activity; Increased GSH and reduced LPO in hepatocytes induced with oxidative stress	Acetone extract	Catechin, ferulic acid, vanillic acid, gallic acid, caffeic acid, vanillin, epicatechin and methyl gallate	Chukwuma et al., 2020
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

Pharmacological action	Tested samples	Phytochemical profile	References
<i>In vitro</i> DPPH and ABTS radicals scavenging activity; Improved serum biochemical markers of kidney and liver damage in alloxan-induced diabetic and HFD-fed male Wistar rats	75% methanol extract	Gallic acid, protocatechuic, catechin, p-hydroxybenzoic acid, caffeic acid, ferulic acid, punicalagin, vanillic acid, p-coumaric acid, ellagic acid, cinnamic acid, naringin, rutin, quercetin, Naringenin, hesperetin, kaempferol, apigenin, rhamnetin, hesperidin and glycoside derivatives of some flavonoids	El-Hadary and Ramadan, 2019
<i>In vitro</i> DPPH radical and H ₂ O ₂ scavenging activity; <i>In vitro</i> Fe ³⁺ reducing activity	Water and methanol extracts	Punicalagins, rutin, quercetin, gallic acid and ellagic acid	Middha et al., 2013

Abbreviations: Adm., administration; CRP, C-reactive protein; GSH, reduced glutathione; GSSG, oxidized glutathione; HFD, high-fat-diet; LDH, lactate dehydrogenase, LPO, lipid peroxidation; PTP1B, Protein tyrosine phosphatase 1B; ROS, reactive oxygen species; SD, Sprague Dawley.

As mentioned previously, documented evidence suggests that phenolics, including ellagitannins and derivatives largely influence the antioxidant potency of pomegranate peel (Akuru et al., 2022; Marra et al., 2022; Mo et al., 2022). Moreover, phenolic principles (ellagic acid, punicalin, and punicalagin) from pomegranate peel has been shown to reduce oxidative stress and inflammatory makers in oxidatively stressed mice (Sun et al., 2017). Also, 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).

While the peel of pomegranate fruit is rich in antioxidant principles, including the constituent ellagitannins, it is imperative to adopt appropriate extraction approaches to optimally recover these bioactive principles without sacrificing their functional and chemical integrity. This is

because extraction methods can influence the recovery and bioactive potencies of phytochemicals.

2.9 Extraction methods and their influence on bioactive phytochemicals

Extraction in phytochemistry is a crucial initial step for recovering biological and organic compounds from plant in the preparation of nutraceutical products (Feng et al., 2020). The purpose of extraction is to recover as much of the target bioactive compound(s) as possible, while minimizing the recovery of other undesired compounds (Feng et al., 2020). There are various extraction methods used to extract valuable bioactive compounds from plants. **Table 2.6** presents a summary of the different methods employed for phytochemical extraction, which include traditional and contemporary techniques.

Table 2.6: Traditional and modern methods employed for phytochemical extraction

Traditional Extraction methods	Modern Extraction methods
Soxhlet Extraction	Ultrasonic-assisted Extraction
Soaking Extraction	Subcritical
Maceration extraction	Supercritical CO ₂
Ultra-sonication	Enzyme-assisted extraction
Turbo- fast blending	Pressure assisted extraction
Solvent permeation	Microwave assisted extraction

Adopted without permission from Nour et al., 2021.

The extraction method used is determined by the type of extraction, preparation procedure, and energy consumption (Azmir et al., 2013). It must be selected to minimize loss and energy consumption, while increasing the extraction yield of the desired compounds (Usman et al., 2022). The extraction process affects the physicochemical properties of the plant extract and the quality and quantity of the desired compound (Xia et al., 2006; Kumar et al., 2020). Also, the pre-preparation steps, such as drying and grinding done before extraction influences the efficiency of the extraction method (Chuo et al., 2022). The extraction method selected should depend on the solubility and the nature of the targeted bioactive compound in different solvents

(Bart and Pilz, 2011). There are major factors that play a role in the mass transfer and extraction of polyphenols. These include pressure, sample particle size, temperature, pH of the solution, solvent type, time, and others (Usman et al., 2022). For the purpose of this project, temperature and extraction solvents are the key variables for pomegranate peel extraction.

2.9.1 Influence of different solvents on polyphenol extraction of pomegranate fruit peel

Altemimi et al. (2017) reported that the solvent of choice must share the same polarity as the solute for extraction procedures to effectively dissolve it. Different extraction techniques are therefore required for varied solutes, which varies in polarity (Hayes and Breakley, 2017). The polarity of the solvent(s) influences the selectivity of the partition system and the distribution of various phenolic species in the extract species (Jessop et al., 2012). The solvent's capacity to form hydrogen bonds is essential for solvation and the release of matrix bound species (Jessop et al., 2012). The hydrogen bond network of the matrix may be destabilized more easily, and target compounds can be solvated more effectively using a solvent that has a higher matrix diffusivity (Alara et al., 2021). The yield, chemical profile, and antioxidant activity of phenolic preparations are strongly influenced by solvents with various polarity (Xu & Chang, 2007).

Phenolic compounds are often polar and hence more hydrophilic than lipophilic; polar protic solvents generally yield more effective polyphenol extraction outcomes. As a result, aliphatic alcohols (such as methanol and ethanol) and polar organic solvents (such as acetone and ethyl acetate) are the most often used methods for extracting phenolics from plant byproducts (Garcia-Salas et al., 2010; Tsao, 2010).

Previous data have shown that the type of solvent extraction strongly influences the chemical and antioxidant profiles of pomegranate fruit peel extracts (Chidambara et al., 2002; Li et al., 2006; Middha et al., 2013; Chukwuma, et al., 2020; Akuru et al., 2022). Particularly, the acetone and methanol solvents extraction of pomegranate peel yielded better phenolic recovery and bioactivity than some other organic solvent extraction and/or water extraction (Zhang et al., 2007; Abid et al., 2017; Khalil et al., 2017; 2018; Thitipramote et al., 2019; Karthikeyan and Vidya, 2019; Chukwuma, et al., 2020; Akuru et al., 2022; Kumar et al., 2022).

Although organic solvents extraction, such as acetone and methanol extraction appear to yield better phenolic recovery and bioactivity from pomegranate peel than water extraction, the later has always been perceived as the safest and least expensive extraction method in folk medicine,

and thus commonly used in Chinese traditional medicine (Yang & Ross, 2010). It is, therefore, imperative to explore methods that can be used to improve the recovery and bioactivity of polyphenols from pomegranate peel when conducting water/aqueous extraction. A previous study has suggested that using fresh fruit peel for water extraction may be promising for recovering ellagitannins from pomegranate fruit waste, perhaps to a commercial scale (Seeram et al., 2005). Nevertheless, temperature-dependent factors remain relevant in phytochemical water extraction. It is known that heat facilitates the infusion of phytochemicals present in plant materials, and thus is commonly employed in Chinese traditional medicine (Yang and Ross, 2010).

2.9.2 Influence of temperature on aqueous polyphenol extraction from pomegranate fruit peel

The recovery of phenolic compounds from plant materials is influenced by extraction time and temperature, which reflects the conflicting actions of solubilization and analyte degradation by oxidation (Robards, 2003). An increase in the extraction temperature can promote higher analyte solubility by increasing both solubility and mass transfer rate (Dai & Mumper, 2010). In addition, the viscosity and the surface tension of the solvents are decreased at higher temperature, which helps the solvents to reach the sample matrices, improving the extraction rate (Dai & Mumper, 2010). However, many phenolic compounds are easily hydrolysed and oxidized. Long extraction times and high temperature increase the chance of oxidation and degradation of phenolics, which decrease the yield and antioxidant potencies of phenolics in the extracts (Dai & Mumper, 2010).

Furthermore, pre-extraction drying of pomegranate peel are also influenced by temperature and time variables. Calín-Sánchez et al. (2013) investigated the effect different types of drying methods (freeze drying, convective drying and vacuum–microwave drying) on the antioxidant and phenolic profiles of pomegranate rind and observed that higher phenolic contents required freeze drying or soft conditions (50 °C) of convective drying. Kumar et al. (2022) also reported that freeze-drying method is required to optimally extract bioactive compounds such as ellagic acid, gallic acid, quercetin, and punicalagin from pomegranate peel relative to tray-oven and sun drying.

2.10 Problem statement

To benefit from the bioactive phytochemical profile of pomegranate peel, it is important to optimally recover the bioactive polyphenols without sacrificing their functional properties. Many variables including extraction temperature and extraction solvent can influence the degree of recovery of bioactive phenolics, including ellagitannins from pomegranate peel, as well as their bioactivity profiles. In fact, many studies have investigated how these extraction variables affect the phenolic profiles and bioactivity of pomegranate peel.

Documented evidence has shown that extracting pomegranate peel with organic solvents, such as acetone and methanol can afford appreciable phenolic and bioactivity profiles (Zhang et al., 2007; Abid et al., 2017; Khalil et al., 2017; 2018; Thitipramote et al., 2019; Karthikeyan and Vidya, 2019; Chukwuma, et al., 2020; Akuru et al., 2022; Kumar et al., 2022). However, these reports lack statistical validation to correlate the influence of the solvent extraction on the phytochemical and antioxidant profile of pomegranate peel extraction. Thus, may not be able to ascertain the solvent extraction for optimum ellagitannins and antioxidant profile.

Furthermore, reports have suggested that using fresh fruit peel for aqueous/water extraction may be promising for recovering ellagitannins from pomegranate fruit wastes, perhaps to a commercial scale (Seeram et al., 2005). However, how extraction temperature influences the ellagitannin recovery and antioxidant profile remains elusive. In fact, there are hardly studies that have statistically correlated the influence of aqueous extraction temperature or type of organic solvent extraction to the antioxidant and ellagitannins profiles of pomegranate peel.

2.11 Aims and objectives

The aim of this study was to investigate the influence of aqueous extraction temperature or type of organic solvent extraction on the antioxidant and ellagitannins profile of pomegranate peel.

The objectives include the following:

- Employ different extraction temperature and organic solvents to extract pomegranate peel and recover ellagitannins
- Determine the antioxidant profiles of the extracts
- Use LC-MS to characterize the ellagitannin profiles of the extracts

- Use advanced chemometric models to correlate the influence of the extraction methods on the ellagitannins and antioxidant profiles of the extracts.

CHAPTER 3

MATERIALS AND METHODS

3.1 Fruit procurement and removal of peel

Class 1 (processing grade) “Wonderful” variety of pomegranate fruits were procured from Sonlia Fruit Packhouse (Wellington, Western Cape Province, South Africa) on 03 May 2021. The fruits were washed, and the peel was thoroughly removed.

3.1.1 Preparation of dried peel and solvent extraction

The fruit peel was dried at 37 °C until all the moisture were completely removed and a constant dry weight was attained. The dried peel was pulverized using a Kinematica POLYMIX® PX-MFC 90 D milling machine equipped with a blade grinding attachment [Lasec (Pty) Ltd, Cape Town, South Africa]. About 250 g of the pulverized dried peel was separately extracted using 1 L of methanol, ethanol, acetone and water. The extraction was done at room temperature on an orbital shaker [OrbiShake, Model 262, Labotec (Pty) Ltd, Johannesburg, South Africa] set at 125 rpm for 72 hours. The fruit peel debris was removed by filtering with a filter paper. The filtrate of the organic extracts was concentrated using a Buchi Rotavapor® R-300 [Labotec (Pty) Ltd, Johannesburg, South Africa] and dried under a fume hood. The filtrate of the water extract was dried using a Martin Christ Alpha 1-2 LDplus Freeze Dryer (Separations, Johannesburg, South Africa). The recovered extracts were stored in air-tight vials at -20 °C until they were used.

3.1.2 Preparation of fresh peel extracts at different temperatures

Approximately 1 kg of the fresh peel was separately soaked in 2.5 L of distilled water at different temperatures (25 °C, 37 °C, 50 °C, 65 °C, 78 °C and 95 °C) for 1 h using metallic containers with fitted lids. Thereafter, the peel and the incubation water were blended and filtered with a clean cheese cloth to remove the debris. The filtrate was filtered again with a filter paper to remove any remaining residue. The filtered extracts were freeze-dried using a Martin Christ Alpha 1-2 LDplus Freeze Dryer (Separations, Johannesburg, South Africa). During the freeze drying, the vacuum pressure was typically lower than 1 mbar, while the ice

condenser temperature ranged between 53 and 57 °C. The recovered extracts were stored in air-tight vials at -20 °C until they were used.

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 h 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 Antioxidant analyses

The antioxidant effect of the extracts was analysed at 45 µg/mL using the following *in vitro* and cell-based experimental models described below. The activity of the extracts was compared to an equivalent concentration of the positive controls (ascorbic acid and Trolox) or standard phytoconstituents (punicalagin A+B and ellagic acid).

3.3.1 Radical scavenging action

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS^{•+}) scavenging effects of the extracts were determined using methods described in previous reports (Motlounq et al., 2020) and expressed as percentage radical scavenging activity.

For the 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 the 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.

For the ABTS^{•+} 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 µL of the extracts or standards (ascorbic acid and Trolox) or their solvents (control) with 125 µL 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^{•+} and DPPH radical scavenging activities (%) were computed using the following formula:

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

3.3.2 Oxygen radical absorbance capacity (ORAC) assay

A previous method (Ou et al., 2013) was slightly modified to perform this assay. 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) or the standards (punicalagin A+B and ellagic acid) at the tested concentration (45 $\mu\text{g/mL}$ 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:

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

3.3.3 Fe^{3+} reducing activity (FRAP) assay

A previous method (Motlounge et al., 2020) was slightly modified to perform this assay. First, 25 μL of the tested samples or positive controls (ascorbic acid and Trolox) (45 $\mu\text{g/mL}$ in reaction mixture) or increasing concentrations (3.75 – 60 $\mu\text{g/mL}$ in reaction mixture) of gallic acid standard, 25 μL of 0.2 M phosphate buffer (pH 6.6) and 25 μL of 1% potassium ferricyanide were mixed in wells of a 96-well clear bottom plate. The mixture was incubated for 20 min at 50 °C, before successively adding 25 μL of 10% trichloroacetic acid, 100 μL of distilled water and 50 μL of 0.1% FeCl_3 solution. Thereafter, absorbance was measured at 700 nm and blanked with the sample blank. The Fe^{3+} reducing activity was determined from a gallic acid standard curve and computed as mg/g equivalent of the gallic acid (mg/g GAE) using the following formula:

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

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 *in vitro* inhibition on FeSO₄-induced 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 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 °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 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”.

3.3.5 Measurement of inhibition on lipid peroxidation in Chang liver cells

Chang liver cells (ATCC® CCL-13™) were supplied by ATCC (Virginia, USA) and the experiment was performed in accordance with a published protocol (Akuru et al., 2022), while adding slight modifications. Cell culturing was carried out using an EMEM medium supplemented with fetal bovine serum at a concentration of 10%. Trysinization was performed when the cells attained confluence of 85%. The cells were resuspended in the culture media and 200 μL of the cell suspension was aliquoted into designated wells of a 96-well plate with each well having a cell density of 8000 cells/well. After a 36-h incubation period (at 5% CO_2 and 37 °C) in a NÜVE EC 160 CO_2 incubator (Ankara, Turkey), the spent medium in the wells of the plate was aspirated and the attached cells in the wells were treated with the tested samples dissolved in the medium (45 $\mu\text{g/mL}$ in 200 μL incubation medium). The plate was incubated for 30 min, before addition of 50 μL of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solution at a concentration of 1 mM in incubation medium to induce oxidative stress. Some wells were used as the normal control (control) and negative control. The normal control (control) wells contained untreated cells, the sample solvent/vehicle in place of the sample and 50 μL of sterile water in place of the $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solution. The negative control wells contained untreated cells, sample solvent/vehicle in place of the sample and 50 μL of the $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solution. The plate was incubated for 1 h after adding the $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solution. Thereafter, the incubation medium in each well of the plate was replaced with 250 μL of cold 50 mM sodium phosphate buffer containing 0.5% v/v Triton X-100 (pH 7.5) to lyse the cells. A gentle tapping of the plate was done for 10 – 15 min to facilitate cell lysis. The cell debris was pelleted by centrifuging for 1 min at 5000 x g and 4 °C. Aliquots (100 μL) of the supernatant in each well of the plate were used to measure lipid peroxidation.

Into designated vials, 100 μL of the supernatant or standards (malondialdehyde at concentrations of 0, 7.5, 15, 22.5, 30, and 45 μM in the assay volume), 500 μL of 0.25% w/v thiobarbituric acid, 200 μL of acetic acid and 200 μL of distilled water were successively added. The vials were heated for 50 min in a boiling water bath, allowed to cool and then centrifuged for 10 min at 3500 x g. Spectroscopic absorption at 532 nm was measured in aliquots (200 μL) from each vial. Lipid peroxidation was computed using extrapolated values from a malondialdehyde linear plot. The anti-lipid peroxidative activity (%) of the tested samples were calculated relative to the controls (normal and negative) using the formula shown below:

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

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

3.4 Crude recovery of tannins/ellagitannins from extracts

An Amberlite® XAD16N resin (product code: XAD16; Sigma Aldrich, Johannesburg, South Africa) was used to purify the tannins in the extracts. The methods previously reported by Seeram et al. (2005) was adopted with little modifications as presented in **Figure 3.1**.

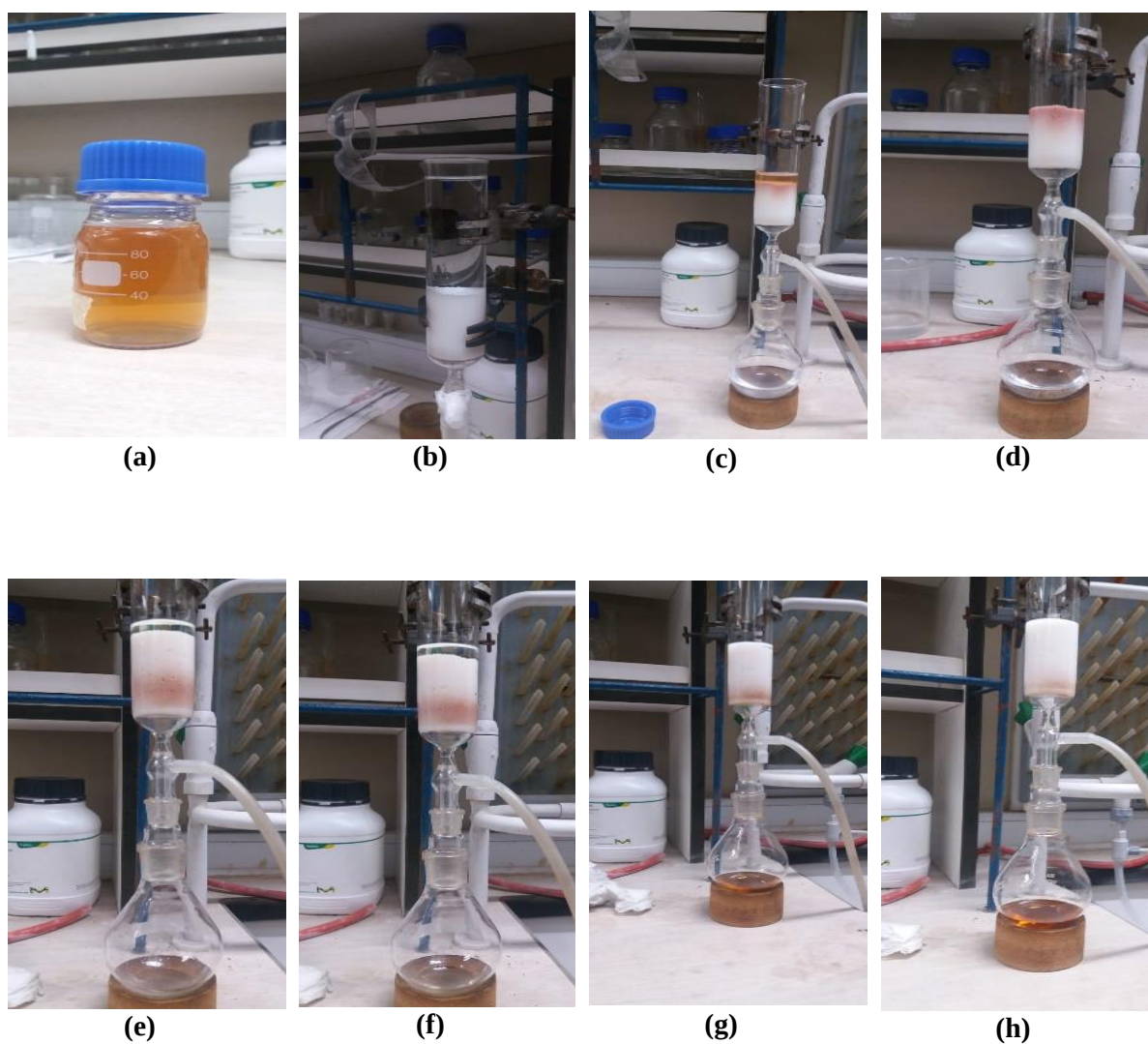


Figure 3.1: Images showing the crude purification process of tannins from the extracts using XAD16N resin.

Approximately 1.52 g of the extracts was dissolved in 100 mL of distilled water and filtered with a filter paper. The filtrate was collected as a yellowish-brown liquid. Approximately 35 g of the XAD16N resin was first washed thrice with absolute methanol. The resin was allowed to equilibrate in distilled water for 12 hours using a sealed flash column as shown in **Figure 3.1a**. Thereafter, the distilled water was eluted/removed using a water jet vacuum pump and extract filtrate was gradually loaded onto the XAD16N resin in the flash column as shown in **Figure 3.1b**. The column was continuously eluted with distilled water using the water jet vacuum pump until the eluent consistently remained colourless and all the tannins in the extract were trapped in the XAD16N resin (**Figure 3.1c**). The column was vacuum aspirated to dry up the remaining water. Next the trapped tannins were gradually eluted using absolute methanol as shown in **Figures 3.1d – e**. The eluent was air-dried in a fume hood. The recovered tannins fractions were weighed, and the percentage yield was computed using the formula below. The fractions were stored at -20 °C until further analysis.

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

3.5 Liquid chromatography - mass spectroscopic (LC-MS) quantification of ellagitannins

The LCMS analysis was done using a Waters Synapt G2 (Waters Corporation, MA, USA), ESI probe, ESI Pos, Cone Voltage 15 V. Liquid chromatography was operated with an Acquity binary solvent manager. A HSS T3 column with dimension of 2.1 x 150mm and particle size of 1.8µm (Waters Corporation, MA, USA) was used for chromatographic separation. The mobile phase was made up of A (0.1% formic acid in HPLC grade water) and B (0.1% formic acid in HPLC grade acetonitrile) solvents. The flow rate for mobile phase was 0.25 mL/min. A gradient chromatographic condition was applied as follows: 100% of A and 0% of B for 10 min; then 72% of A and 28% of B for 21 min; then 60% of A and 40% of B for 50 sec; then 0% of A and 100 % of B for 2 min. Chromatographic separation was done at 30 °C. Samples were reconstituted in 1 mL of 50%:50% acetonitrile and water, centrifuged at 14,000 rpm for 5 min and filtered using a 0.22 µm filter. A 20 µL injection volume was applied. A UV wavelength of 254 nm was used to record the chromatographic signals. Puniculagin A, puniculagin B and ellagic acid in the ellagitannins fraction were identified and quantified from

calibration curves established using a range of calibration standards prepared from stock solutions of Puniculagin A+B (product code: PHL80524, Sigma Aldrich, Johannesburg, South Africa) and ellagic acid (product code: 14668, Sigma Aldrich, Johannesburg, South Africa). The other compounds (granatin A, corilagin, casuarinin, geraniin and quercetin hexoside) in the tannin fractions were identified and quantified in a relative fashion against ellagic acid. They were identified based on accurate mass elemental composition and quantified using extracted ion chromatography against ellagic acid. The quantification was presented as a triplicate analysis.

3.6 Statistical analysis

Statistical analysis was done on the Window's version 27.0 of IBM SPSS (IBM Corp, Armonk, NY, USA). Multiple comparison of data averages among the groups (extracts or standards) was done for the different studied parameters using the Tukey multiple *post-hoc* test and one-way analysis of variance (ANOVA). Statistically significant difference (p) was set at $p < 0.05$ when comparing the mean values of the different groups.

3.7 Advanced chemometrics analysis

Pearson's bivariate correlation analysis was used to estimate the direction and magnitude of the linear correlation between all pairs of studied variables. Principal component analysis (PCA) and orthogonal partial least squares (OPLS) analysis were performed using SIMCA-P+ 16.0 software (Umetrics, MKS Instruments Inc., Sweden) as described by Gbashi et al. (2020). Data preprocessing for multivariate chemometric analysis involved mean-centering and unit variate scaling of the entire dataset. For OPLS analysis, each observation in the data was assigned to a class depending on the extraction solvent (methanol, ethanol, acetone, and water) or temperature (25, 37, 50, 65, 78, and 95 °C). After labelling, the data were categorized into X variables (predictors) and Y variables (responses). The predictors included ellagic acid, casuarinin, punicalagin A, punicalagin B, ellagitannin/tannin yield, corilagin, granatin A, geraniin, and quercetin hexoside. These are the extracted analytes quantified in each sample extract. The response variables consisted of the antioxidant assays: ABTS, ORAC, TPC, TFC, FRAP, DPPH, anti-linoleic acid, and anti-lipid.

The adequacy of the fitted chemometric models was assessed by evaluating the goodness-of-fit, goodness-of-prediction, and several other model fit parameters. The portion of X variation explained in a specific component using the X model (R^2X) was examined. The combined fractions of variation in the X data modelled up to the specified component using the X model [$R^2X(\text{cum})$] was also evaluated. Similarly, the R^2Y i.e., the fraction of explained Y variation in the component using the Y model was evaluated. Also, the cumulative fraction of variation in the Y data modelled up to the specified component using the Y model [$R^2Y(\text{cum})$] was assessed. Other model fit parameters investigated included: R^2 (the portion of variation in Y data modelled by the X model in the component), $R^2(\text{cum})$ (combined fractions of modelled variation in the Y data using the X model up to the specified component). The root-mean-standard error for cross-validation (RMSECV), root mean square error of estimation (RMSEE), and coefficient of determination (R^2) of the predicted versus the observed response values were also evaluated. Rule 1 (R1), Rule 2 (R2), or Rule 3 (R3) were applied to establish the statistical significance of model components. When $Q^2 > \text{Limit}$, the component is regarded as significant for R1. For R2, a component is considered significant if $Q^2V > \text{Limit}$. A component is designated undetermined (U) for R3 in terms of PCA when it is insignificant. Nonetheless, if the succeeding component is significant and has similar eigenvalues (5% tolerance) to the previous component, then both components are regarded as significant, and the component labelled U is relabelled R5. The SIMCA® manual details the computations and determinations of the Limits for both PCA and OPLS, as well as a description of the model validation parameters (Sartorius Stedim, 2018).

CHAPTER 4

EFFECT OF SOLVENT EXTRACTION ON THE ANTIOXIDANT AND PHYTOCHEMICAL PROFILE OF ELLAGITANNINS FROM “WONDERFUL” POMEGRANATE PEEL: AN ADVANCED CHEMOMETRICS ANALYSIS

Prescript

This chapter reports the influence of extraction solvent on the antioxidant, phenolic and ellagitannin profiles of dried pomegranate peel extracts using advanced chemometrics correlational models. **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>. The above-mentioned publication was adopted in writing the content of this chapter.

4.1 Abstract

Ellagitannins are the predominant bioactive tannins present in the peel of pomegranate. Studies have explored different extraction solvents to maximize recovery of the bioactive phytochemicals in pomegranate peel but lack proper statistical correlation between the extraction solvent, phytochemistry and bioactivity. This study employed advanced chemometrics models, including principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) in determining the solvent extraction (among methanol, ethanol, acetone and water) that will yield optimum antioxidant and ellagitannins profiles from “Wonderful” pomegranate peel. Acetone extraction had the highest ($p < 0.05$) total phenol (TFC) and flavonoid contents and strongest ($p < 0.05$) Fe^{3+} reducing effect, while methanol extraction had the strongest ($p < 0.05$) radical scavenging activity. The oxygen radical absorbance capacity (ORAC) and anti-lipid peroxidative activities of both solvent extractions outperformed that of the ethanol and water extractions. Tannins purified using Amberlite® XAD16N resin was highest for acetone and methanol extractions. LCMS-quantified ellagitannins in the tannins varied for the different extractions. PCA and OPLS models adequately characterized, described and predicted the variation and patterns in the antioxidant and ellagitannins datasets (up to 95% for PCA). OPLS bi-plot showed that the high ellagic acid constituents and total tannins yield of the methanol extraction influenced its potent radical scavenging activity, while the ellagitannin constituents including punicalagins, granatin A, geraniin and casuarinin influenced the high TFC, as well as the potent Fe^{3+} reducing, ORAC and anti-lipid peroxidative activities of acetone extraction. Acetone and methanol extraction of pomegranate peel are promising for optimum ellagitannins recovery and antioxidant profile.

Key words: *Pomegranate peel, solvent extraction, antioxidant, ellagitannins, advanced chemometrics analysis.*

4.2 Introduction

Pomegranate fruit is well recognized for its benefits on oxidative and cardiovascular health (Singh et al., 2014). It, also, has antibacterial, anti-diabetic, anti-atherosclerotic, anti-inflammatory, and anti-allergic potentials (Singh et al., 2014). These medicinal properties can be attributed to the fruit's many secondary metabolites - the most significant being the

ellagitannins (punicalagin and ellagic acid), as well as other polyphenols like flavonoids, anthocyanidins, flavonones, catechins and phenolic acids (Middha et al., 2013; Qua et al., 2021; Akuru et al., 2022). These phenolic compounds account for up to 92% of the fruits antioxidant property and are mostly concentrated in the fruit peel and juice compared to other parts of the fruit (Ismail et al., 2012; Yaritz et al., 2022). Evidence of the radical scavenging property of pomegranate peel has been documented (Middha et al., 2013) and in some cases was stronger than that of the pulp (Li et al., 2006). In rats the peel improved antioxidant status, thus protecting against hepatic oxidative damage caused by tetrachloromethane (Chidambara et al., 2002).

The rising fruit production and commercialization of pomegranate causes accumulation of the fruit's wastes, particularly the peels (Bertolo et al., 2021). To avoid wastage, reduce environmental biomass and maximize the therapeutic potential of the fruit's wastes, the fruit's peel has been researched as a possible source of antioxidants (Chidambara et al., 2002; Li et al., 2006; Middha et al., 2013; Chukwuma et al., 2020; Akuru et al., 2022). The results offer a justification for the potential relevance of the peel in managing oxidative stress and insults.

Several solvent extracts have been explored for extracting phytoconstituents from pomegranate peel with promising antioxidant and other bioactivity profiles. This is because solvent polarity largely influences the type of compounds extracted from a plant material, which consequently influences the antioxidant and other bioactive profile of the resulting extracts (Singh et al., 2014). Previous data have shown that the type of solvent extraction strongly influences the chemical and antioxidant profile pomegranate fruit peel extracts (Chidambara et al., 2002; Li et al., 2006; Middha et al., 2013; Chukwuma et al., 2020; Akuru et al., 2022). However, these studies lack a proper statistical correlation between the extraction solvent, phytochemistry and antioxidant activity. The studies lack statistical validation to determine the influence of the solvent extraction on the phytochemical and antioxidant profile of pomegranate peel extracts. Thus, may not be able to ascertain the solvent extraction for optimum ellagitannins and antioxidant profile. Therefore, this study was done to determine the effect of different solvent extraction on the antioxidant and phytochemical profile of ellagitannins from pomegranate peel using advanced chemometrics analyses. This will potentially provide a more reliable information regarding what extraction solvent system for pomegranate peel will lead to optimum ellagitannins and antioxidant profile.

4.3 Materials and methods

For the materials and methods of this chapter, please refer to **sub-sections 3.1 to 3.8** of chapter 3.

4.4 Results

4.4.1 Total phenol and total flavonoid contents

The total phenol content (TFC) of the acetone extract was significantly ($p < 0.05$) higher than that of the other extracts (**Figure 4.1a**). The ethanol extract had the least TFC, which was significantly ($p < 0.05$) lower than that of the methanol and water extracts. The total flavonoid content of the acetone extract was more than 2 folds compared to that of the other extracts (**Figure 4.1b**).

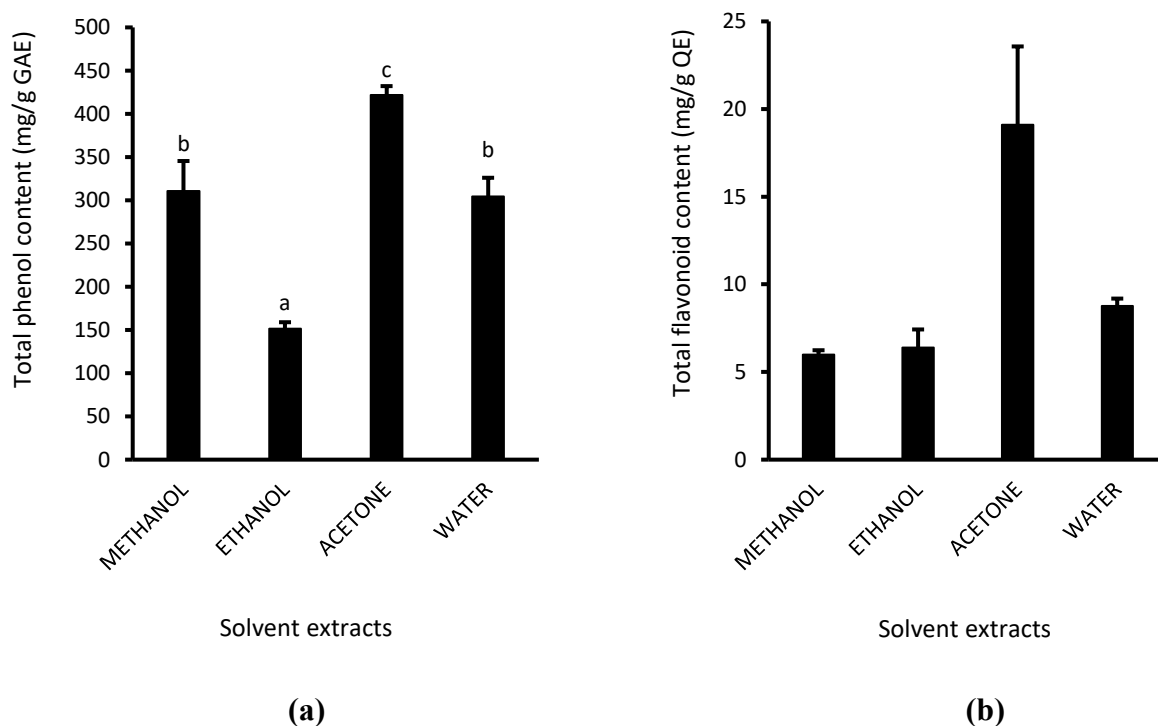


Figure 4.1: (a) Total phenol content and (b) total flavonoid content of the different solvent extracts of the dry peel. Data are presented as mean $\pm$ SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

4.4.2 Fe³⁺ reducing and radical scavenging antioxidant properties

Although not as potent as ascorbic acid, the Fe³⁺ reducing activity of the acetone extract was significantly ($p < 0.05$) stronger than that of Trolox, as well as the other extracts (methanol, ethanol and water extracts) (**Figure 4.2**). The methanol and water extracts, however, outperformed the ethanol extracts.

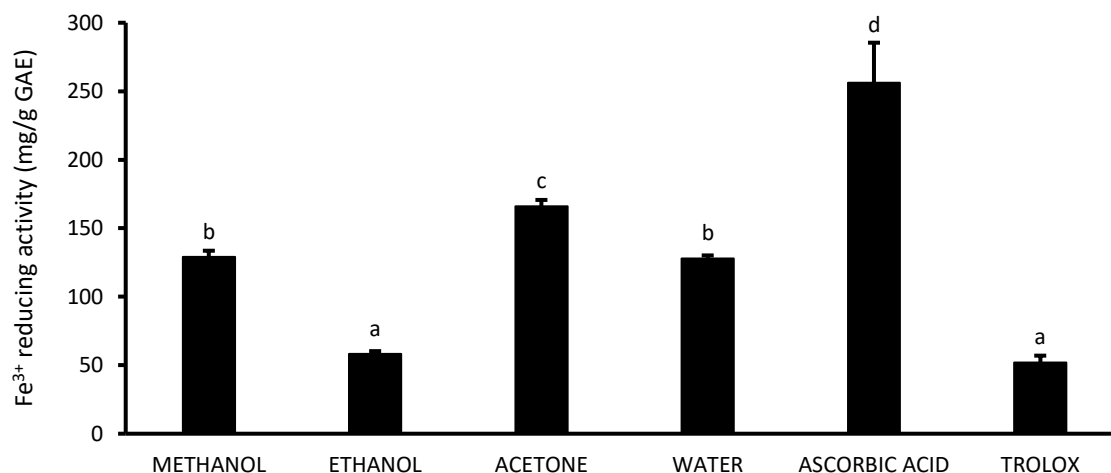


Figure 4.2: Fe³⁺ reducing activity of the different solvent extracts of the dry peel. Data are presented as mean $\pm$ SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

4.4.3 Radical scavenging activity

The DPPH radical scavenging activity of the methanol extract was statistically comparable to that of ascorbic acid and Trolox (**Figure 4.3**) and significantly ($p < 0.05$) stronger than the activities of the other extracts. The ethanol extract had the least potent DPPH radical scavenging activity, which was significantly ($p < 0.05$) less potent than that of the methanol and water extracts. The ABTS radical scavenging activity of the extracts followed similar trend as their DPPH radical scavenging activity (**Figure 4.3**).

The oxygen radical absorbance capacity (ORAC) of the acetone and methanol extracts was notably stronger than that of the other extracts and statistically comparable to the activity of ellagic acid and punicalagin A+B standards (**Figure 4.4**). Among all the extracts, the acetone extracts had the most potent ORAC.

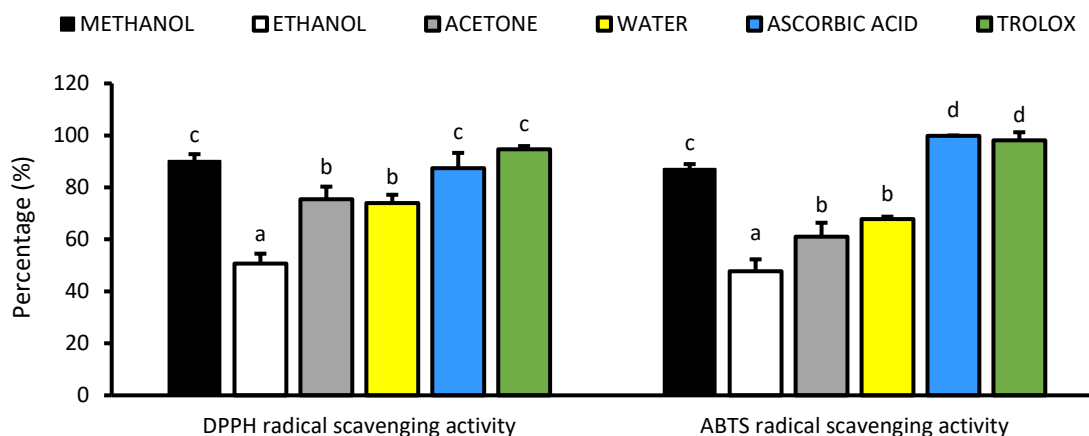


Figure 4.3: Radical scavenging activity of the different solvent extracts of the dry peel. Data are presented as mean $\pm$ SD of triplicate analysis. For each analysis, the letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

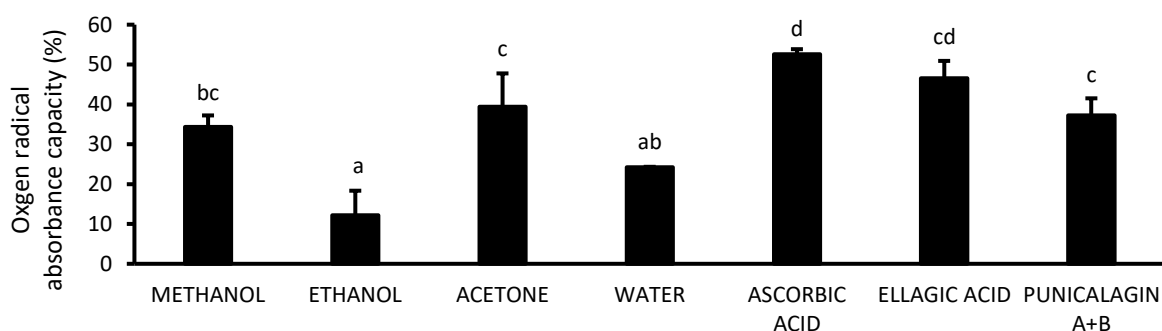


Figure 4.4: Oxygen radical absorbance capacity (ORAC) of the different solvent extracts of the dry peel. Data are presented as mean $\pm$ SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

4.4.4 Anti-lipid peroxidative antioxidant properties

The ability of the extracts to inhibit linoleic acid peroxidation *in vitro* (Figure 4.5) and lipid peroxidation in Chang liver cells (Figure 4.6) is shown in Figure 3. The acetone and methanol extracts were the most potent among the extracts. Their activity was statistically comparable to that of ascorbic acid, Trolox, ellagic acid and punicalagin A+B standards.

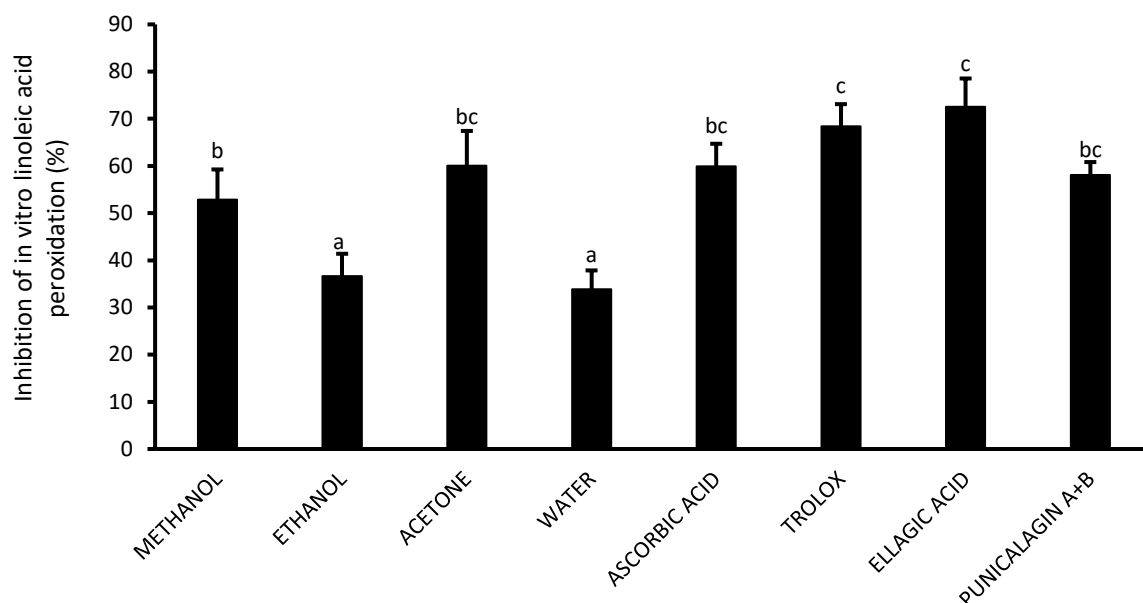


Figure 4.5: Inhibitory effect of the dry peel solvent extracts on *in vitro* FeSO₄-induced linoleic acid peroxidation. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, the letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

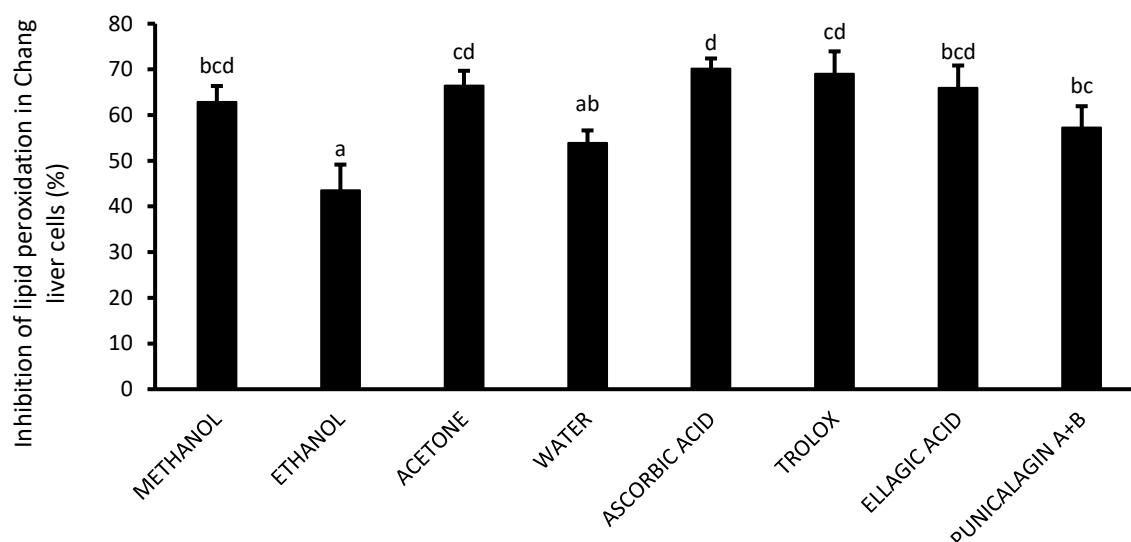


Figure 4.6: Inhibitory effect of the dry peel solvent extracts on FeSO₄-induced lipid peroxidation in Chang liver cells. Data are presented as mean $\pm$ SD of triplicate analysis. For each assay, the letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

4.4.5 Total tannin yields and ellagitannins phytochemical profile

The yield of total tannins from the extracts is presented in **Table 4.1**. The yield from the methanol and acetone extracts was significantly higher than that of the ethanol and water extracts, with the ethanol extract having the least yield. LCMS-quantified phytochemicals ellagitannins included punicalagin A, punicalagin B, ellagic acid, granatin A, corilagin, casuarinin and geraniin (**Table 4.1** and **Appendix 1**). The above mentioned ellagitannin constituents were more mostly predominant in the acetone extracts compared to the other extracts, and in some case, significantly ($p < 0.05$).

Table 4.1: The yield and phytochemical profile of ellagitannins from the different solvent extracts of dry pomegranate peel

Extracts	Yield of total tannins (%)	LCMS-quantified ellagitannin constituents in the purified total tannins from extracts (mg/L)							
		Punicalagin A	Punicalagin B	Ellagic acid	Granatin A	Corilagin	Casuarinin	Geraniin	Quercetin hexoside
METHANOL	47.4 ± 3.77 ^c	143 ± 8.31 ^a	140 ± 18.9 ^a	32.5 ± 3.21 ^b	22.6 ± 3.72	6.17 ± 0.72	4.74 ± 2.42	4.42 ± 1.57	9.68 ± 1.65
ETHANOL	36.9 ± 2.47 ^b	215 ± 10.5 ^b	190 ± 13.2 ^{ab}	21.6 ± 2.11 ^a	24.3 ± 6.02	4.17 ± 1.04	6.57 ± 2.05	5.69 ± 0.80	13.8 ± 0.88
ACETONE	44.0 ± 2.97 ^b	261 ± 18.3 ^b	247 ± 12.8 ^b	29.7 ± 5.47 ^{ab}	47.0 ± 4.32	5.18 ± 2.38	8.03 ± 1.46	10.6 ± 1.86	15.8 ± 1.91
WATER	16.8 ± 1.30 ^a	150 ± 4.90 ^a	149 ± 6.43 ^a	28.3 ± 2.27 ^{ab}	44.8 ± 12.1	3.80 ± 0.40	4.23 ± 0.29	6.33 ± 1.70	9.73 ± 1.62

Data are presented as mean ± SD of triplicate analysis. For each parameter, the letters appearing as superscripts in the cells represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

4.4.6 Advanced chemometrics analysis

The PCA scores plot showed a clear clustering pattern of the various observation classes in the data (**Figure 4.7**). A total of five principal components were generated from the analysis, with the first two components describing 73% of the patterns in the data (PC1 = 41.7%, PC2 = 31.3%) and predicting 53.9% of the variation in the data by cross-validation (**Table 4.2**). Overall, the five components of the PCA model could explain up to 95.4% of the variation in the obtained data (**Table 4.2**).

The OPLS scores plot (**Figure 4.8a**) showed distinct grouping patterns for all observation classes. On the OPLS biplot (**Figure 4.8b**), the predictors (i.e., X variables) are coloured purple and the responses (i.e., Y variables) are coloured black. The grouping patterns on this plot showed the closeness of the water and ethanol extracts (**Figures 4.7** and **4.8**), indicating the

inherent similarities of these two groups of samples. Also, it can be seen that acetone was very distinct from the other three classes.

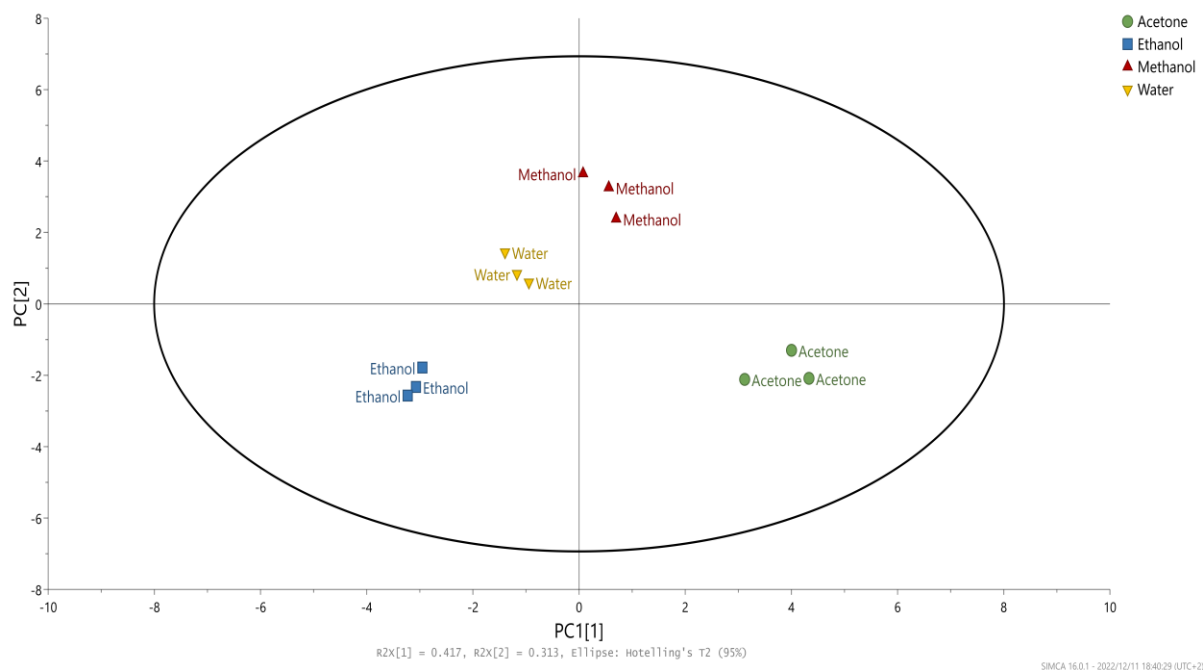
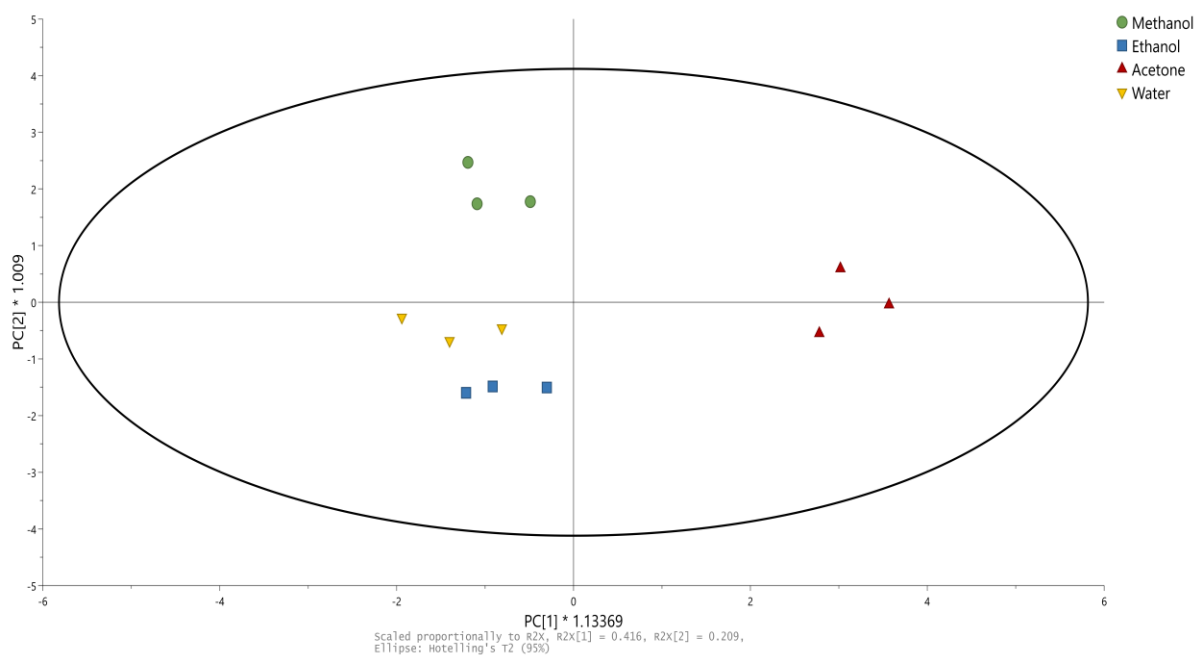
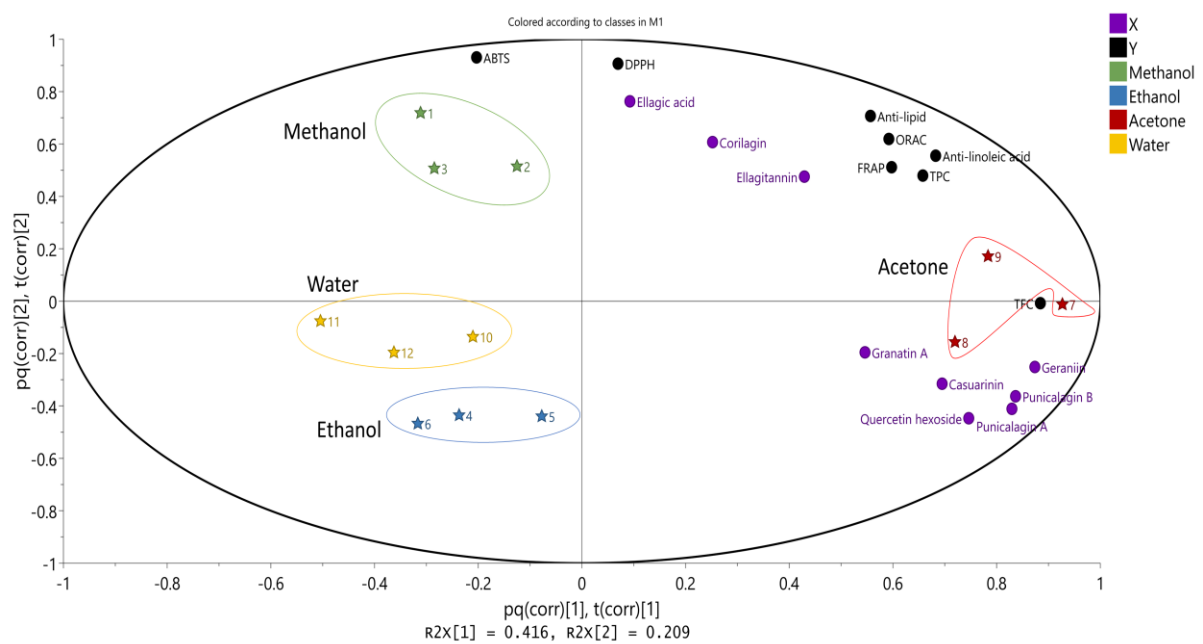


Figure 4.7: Scores plot of unsupervised linear transformation and multivariate dimensional reduction analysis of the data based on PCA.

Further, it can be deduced from this plot that ABTS and DPPH radical scavenging properties were the strongest Y variables contributing to the differential clustering of the methanol extracts compared to the other extracts. TFC had the highest influence on the differential clustering of the acetone extracts. TPC, FRAP, and *in vitro* anti-linoleic acid peroxidation also strongly contributed to the spatial distribution of acetone extracts. None of the variables were distributed close to the ethanol and water class along PC1 axis (i.e., x-axis). Also, none of the variables were located within the same quadrant with the two sample classes (water and ethanol). The biplot showed a clustered grouping of TFC and a number of predictors including granatin A, geraniin, casuarinin, punicalagin A, quercetin hexoside, and punicalagin B. This cluster was also in and around the acetone sample class. it can also be deduced from **Figure 4.8b** that ABTS and DPPH bioactivities are strongly linked to the presence of ellagic acid, corilagin, and tannins yield. The phytochemical content in the water and ethanol extracts appeared not to potently influence their bioactivity (**Table 4.1** and **Figure 4.8b**).



(a)



(b)

Figure 4.8: (a) Scores plot of supervised linear transformation, dimensionality reduction, and modelling of the patterns in the data based on OPLS and (b) OPLS biplot showing the clustering of sample classes and spatial distribution the X and Y variables.

Table 4.2: PCA modelling parameters and summary of fit

Component	R2X	R2X(cum)	Eigenvalue	Q2	Limit	Q2(cum)	Significance	Iterations
1	0.417	0.417	5.01	0.113	0.134	0.113	R2	31
2	0.313	0.731	3.76	0.337	0.144	0.412	R1	10
3	0.124	0.854	1.48	0.169	0.156	0.511	R1	15
4	0.0616	0.916	0.739	-	0.17	0.506	R2	20
5	0.0378	0.954	0.453	0.0108	0.188	0.539	R2	13

The HCA dendrogram (**Figure 4.9**) identifies groupings of similar sample classes, where observations in a cluster are more similar than those in separate clusters. Thus, in terms of similarity of the extraction solvents (in the context of tannins recovery, ellagitannins profile and antioxidant properties), the HCA dendrogram indicates that water extracts were more similar to ethanol extracts, which was more similar to methanol extracts, which was in turn the closest to acetone extracts. A pattern which is also corroborated on the PCA and OPLS scores plots, as well as the OPLS biplot.

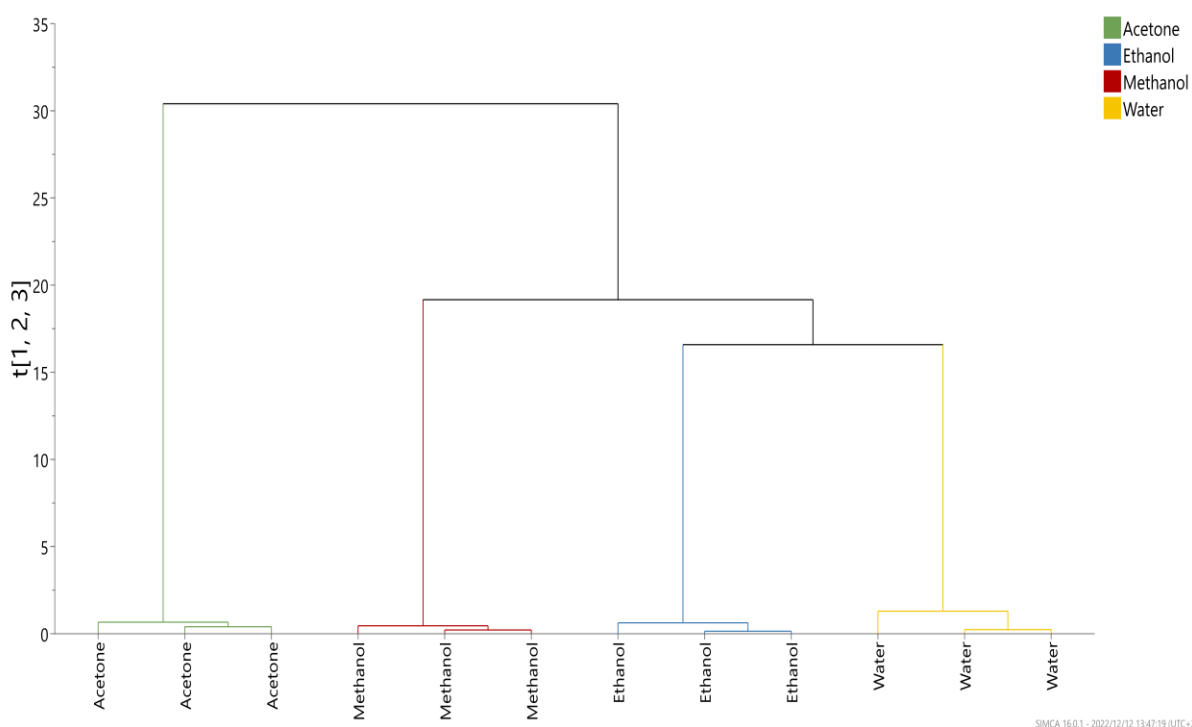


Figure 4.9: HCA dendrogram showing the similarities/relatedness of the various observation classes.

Indeed, there exist an empirical relationship between extracted compounds and the bioactivities of the respective extracts, a phenomenon well-captured by the OPLS analysis as it reveals that the X model could predict up to 62.3% of the patterns in the response variables (**Table 4.3**). The variable contributions plot (**Figure 4.10**) for TPC show that ellagic acid, granatin A, geraniin, and corilagin appeared to be the strongest predictors of TPC and TFC. It appeared that ellagic acid had the highest effect on FRAP, DPPH, ABTS, ORAC, and cellular anti-lipid peroxidation (**Figure 4.10**). Total tannins yield had the strongest impact on *in vitro* anti-linoleic acid peroxidation followed closely by ellagic acid and corilagin. Overall, punicalagin A, geraniin, and punicalagin B were the most important variables (in descending order) in the construction of the OPLS model and description of the patterns in the entire dataset (**Figure 4.11**).

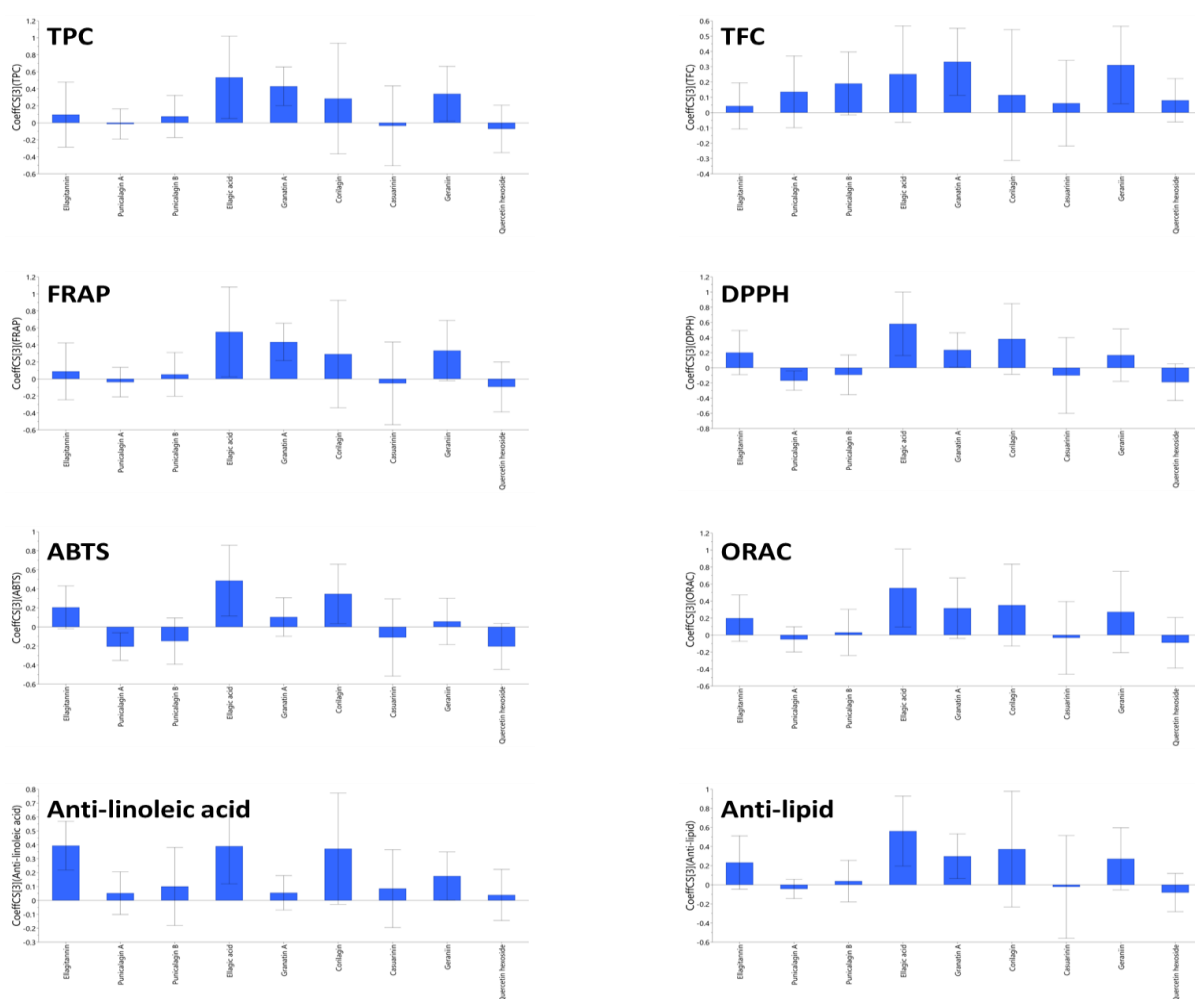


Figure 4.10: Variable contribution plot for each response variable.

Table 4.3: OPLS-DA modelling parameters and summary of fit

Component	R2X	R2X(cum)	R2	R2(cum)	Q2	Limit	Q2(cum)	R2Y	R2Y(cum)	Significance
Model		0.804		0.862			0.628		0.949	
Predictive		0.804		0.862			0.628		0.949	
P1	0.416	0.416	0.597	0.597	0.0358	0.01	0.0358	0.646	0.646	R1
P2	0.209	0.625	0.223	0.82	0.497	0.01	0.533	0.244	0.89	R1
P3	0.179	0.804	0.042	0.862	0.0956	0.01	0.628	0.0588	0.949	R1

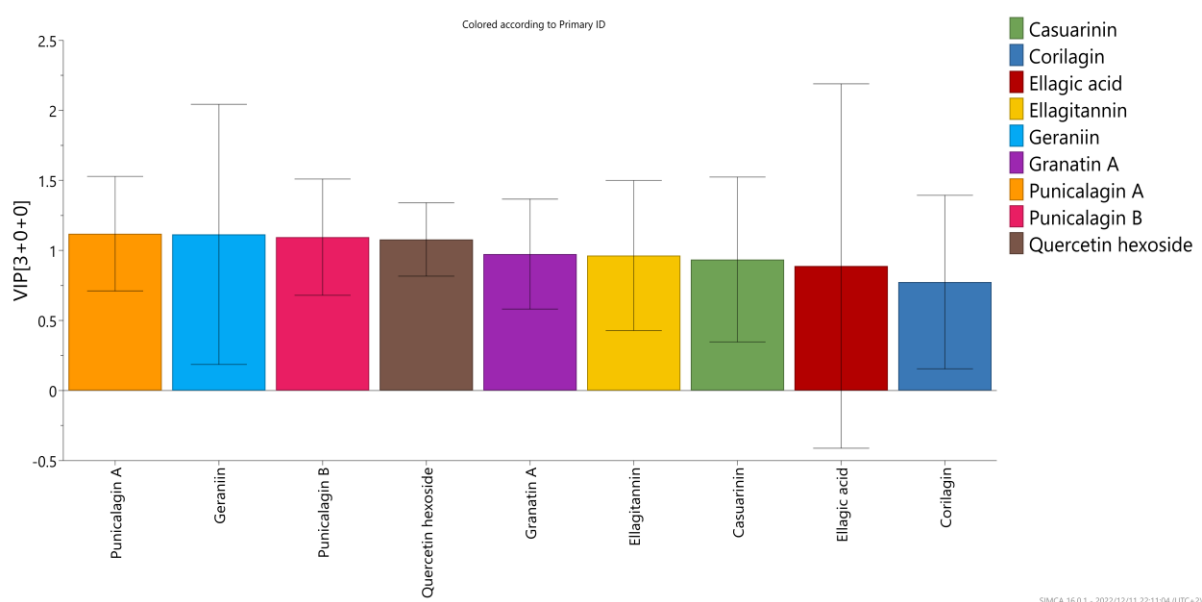


Figure 4.11: Variable importance plot (VIP) based on OPLS.

4.4.7 Advanced chemometric model overview, evaluation, and validation

It is important to validate any fitted model in order to ascertain its adequacy, efficiency, and predictive ability where applicable. The goodness-of-fit, goodness-of-prediction, and other relevant model fit parameters for the PCA and OPLS models are presented in (Tables 4.2 and 4.3). Model fit diagnostics show that all PCA and OPLS generated components were significant (Tables 4.2 and 4.3). The OPLS algorithm generated a total of 3 PCs from the dataset and could describe over 80% of the variation in the X data [$R^2X(\text{cum})=0.804$] and 86.2% of the variation in the Y data [$R^2(\text{cum})=0.862$]. The first two components had a $R^2X(\text{cum})$ value of 62.5%, $R^2(\text{cum})$ value of 82.0%, R^2Y value of 89%, and $Q^2(\text{cum})$ value of 53.3%. The Y model described 94.9% of the variation in the response variables ($R^2Y=0.949$). The *goodness-of-*

prediction parameter $Q^2(\text{cum})$ indicated a predictive capability of 62.8% for the model, i.e., the X model had a cumulative prediction accuracy of approximately 63% based on cross-validation. Interestingly, the fractions of Y variation predicted by the X model in components 2 and 3 (PC2 and PC3) were higher (Q^2 49.7 and 9.6%, respectively) than that of PC1 (Q^2 3.6%). Results of regression of observations versus the corresponding predictions using the X model for each response variable were as follows: TPC (R^2 : 0.87, RMSEE: 43.62, RMSECV: 64.33), TFC (R^2 : 0.85, RMSEE: 2.67, RMSECV: 3.11), FRAP (R^2 : 0.88, RMSEE: 16.33, RMSECV: 25.12), DPPH (R^2 : 0.90, RMSEE: 5.58, RMSECV: 8.36), ABTS (R^2 : 0.95, RMSEE: 4.08, RMSECV: 6.47), ORAC (R^2 : 0.77, RMSEE: 6.68, RMSECV: 9.38), *in vitro* anti-linoleic acid peroxidation (R^2 : 0.82, RMSEE: 6.21, RMSECV: 6.67), and cellular anti-lipid peroxidation (R^2 : 0.86, RMSEE: 4.32, RMSECV: 5.08).

4.5 Discussion

The peel of pomegranate has been shown to contain rich phytochemical profile, which could be of medicinal relevance if not wasted. Ellagitannins are the one of the predominant tannins present in the peel of pomegranate, which have been documented to have medicinal relevance (Middha et al., 2013). Different studies have explored different extraction solvents to maximize recovery of the bioactive phytochemicals in pomegranate peel but lack a proper statistical correlation between the extraction solvent, phytochemistry and bioactivity. The present study used advanced chemometric models to correlate between the solvent extraction used, bioactivity and ellagitannins recovery from the peel of “Wonderful” variety pomegranate, with the aim of ascertaining the extraction solvent with optimum ellagitannins and antioxidant profiles.

Data showed that the acetone extracts had the highest TPC and TFC (**Figure 4.1**), which could be attributed to its suitable polarity for extracting polyphenols and flavonoids (Tsao, 2010). The methanol extracts also had an appreciable TPC content, which is linked to its compatible polarity with polyphenols. The TPC and TFC of these extracts may be partly influential in their potent radical scavenging/quenching effects (**Figures 4.3b and c**). This is due to the ability of polyphenols to form stable phenoxy radicals by donating electron or transferring hydrogen atom, which are able to react with and quench the deleterious effects of other radicals (Platzer et al., 2022). In both *in vitro* and Chang liver cells experimental models, the acetone and methanol extracts outperformed the ethanol and water extracts in inhibiting FeSO_4 -induced

linoleic acid or lipid peroxidation (**Figures 4.5 and 4.6**). Their potent effects may be attributed to their rich tannins yield, which also had higher constituents of ellagic acid and punicalagin A+B (**Table 4.1**) relative to other extracts. Moreover, these constituents also demonstrated appreciable anti-lipid peroxidative effects (**Figures 4.5 and 4.6**), while ellagitannins-rich dietary supplement from pomegranate, as well as ellagic acid have been shown to have potent anti-lipid peroxidative activity in obese animals (Heber, 2007) and human subjects (Xu et al., 2021).

PCA and OPLS are advanced dimensionality reduction algorithms that emphasize differences or similarities between data observations by constructing a smaller number of interpretable, linearly uncorrelated variables [latent variables or principle components (PCs)] from the dataset (Worley and Powers, 2013; Gbashi et al., 2020). In order to detect any outliers and have a global appraisal and overview of the inherent patterns in the dataset without any reference to pre-assigned observation class labels, PCA was performed. Overall, the five components of the PCA model could explain up to 95.4% of the variation in the obtained data (**Table 4.2**). OPLS was further adopted to scrutinize the data for inherent discriminatory patterns since it is a supervised learning algorithm and can aggressively differentiate between sample groups following training on the labelled data. Clearly distinct grouping patterns were observed for all the observation classes (**Figures 4.7 and 4.8a; Table 4.3**).

The biplot combines the information from the scores plot with information from the loadings plot, thus further elucidating information on data distribution patterns, observation classes, and variable relationships. The distinct distribution of the acetone extract (**Figure 4.8b**) suggests that extracts from this solvent performed uniquely in terms of the monitored parameters (i.e., X and Y variables). The differential clustering of the acetone extracts was influenced by its anti-lipid peroxidative and ORAC activities, which can be attributed to its higher TFC, TPC and ellagitannins constituents (punicalagin, granatin A, geraniin and casuarinin) (**Figure 4.8b**). Ellagic appears to be most influencing component influencing the remarkable radical scavenging effect of the methanol extract, which further influenced its distribution in the biplot (**Figure 4.8b**). The high tannins yield, as well as some key ellagitannins constituents, such as ellagic acid and corilagin appear as the most predominant components influencing the antioxidant effects of the extracts (**Figure 4.10**). Moreover, the higher tannins yield and ellagitannins composition of the acetone and methanol extract (**Table 4.1**) correlates with the stronger antioxidant profiles of these extracts compared to that of the water and ethanol extracts (**Figures 4.2 – 4.6**).

It is important to note that the computed values obtained from the evaluation and validation of the advanced chemometrics models indicate strong linearity and correlation between the predicted and observed values and demonstrate a good predictability of the response variables by the model. Therefore, it can be said that the OPLS model was able to adequately characterize, describe, and predict the variation and patterns in the dataset.

4.6 Conclusion

It can be concluded that the ethanol and water extracts overall had lower yield of total tannins and contained lower amounts of key influencing ellagitannins, particularly, ellagic acid and corilagin, which are located the furthest away from these observation classes on the biplot. Consequently, ethanol and water extracts nominally possessed the least bioactivities of the solvent extracts. It can be deduced from the biplot that tannins crudely purified from the acetone extracts nominally contained the highest amounts of most of the ellagitannins and thus showed the highest bioactivity of all the solvent extracts. Methanol extracts also, had promising bioactivity, which may be largely influenced by the high ellagic acid content in the tannins purified from it. It is, however, important to note that there may be other phytochemicals influencing the antioxidant capacities of the acetone and methanol extracts, which have not been profiled in the present study. In the context of ellagitannins from pomegranate peel, acetone and methanol solvent extraction may be promising for optimum ellagitannins recovery and antioxidant profile.

CHAPTER 5

MODELLING THE INFLUENCE OF EXTRACTION TEMPERATURE ON THE ELLAGITANNINS AND ANTIOXIDANT PROFILE OF “WONDERFUL” POMEGRANATE PEEL USING ADVANCED CHEMOMETRICS ANALYSIS

Prescript

This chapter reports the influence of extraction temperature on the antioxidant, phenolic and ellagitannin profiles of fresh pomegranate peel water extracts using advanced chemometrics correlational models. **Setlhodi R**, Mashile B, Izu GO, Gbashi S, Mashele SS, Bonnet SL, Makhafola TJ, Chukwuma CI*. (2023). *Modelling 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>. The above-mentioned publication was adopted in writing the content of this chapter.

5.1 Abstract

Pomegranate peel contains ellagitannins as antioxidant principles but the aqueous extraction temperature for optimum ellagitannins and antioxidant profile remains elusive. This study employed advanced chemometrics models, including principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) to determine the influence of extraction temperatures (25 – 95 °C) during a 1 h period on the antioxidant profile of the peel's aqueous extract, as well as the profile of the constituent ellagitannins recovered using Amberlite® XAD16N resin and characterized by LC-MS. Extraction at 78 °C had the most potent radical scavenging potency, oxygen radical absorbance capacity (ORAC) and anti-lipid peroxidative activity, which was consistent with its highest ellagitannins constituents. Pearson's bivariate correlation depicted strong positive correlations ($r = 0.7 - 0.9$) between the ellagitannins and anti-lipid peroxidative profile of the extracts. PCA and OPLS models adequately characterized and predicted the variation and patterns in the antioxidant and phytochemical datasets (up to 93.6%). OPLS biplot showed distinct clustering pattern of the antioxidant and ellagitannins variables around 78 °C extraction temperature, suggesting the observable influence of the ellagitannins on the potent antioxidant profile of the 78 °C extraction. The variable contribution plots, as well as the obtained antioxidant data suggest punicalagins, granatin A and ellagic acid as notable influencing ellagitannins. It is safe to speculate that extraction at 78 °C optimally influenced the ellagitannins and antioxidant profile of pomegranate fruit peel. Thus, this aqueous extraction temperature may be promising for optimally recovering the ellagitannins from pomegranate peel, without sacrificing the antioxidant capacity.

Key words: *Pomegranate peel, aqueous extraction, antioxidant, ellagitannins, advanced chemometrics analysis.*

5.2 Introduction

Pomegranate (*Punica granatum* L.) fruit is widely consumed globally for its appreciable taste. It has, however, been linked to several health benefits, including oxidative and vascular health benefits (Kandylis and Kokkinomagoulos, 2020; Giménez-Bastida et al., 2021). The health benefits have been majorly attributed to the presence of polyphenols, including the predominant ellagitannins constituents (Giménez-Bastida et al., 2021). Ellagitannins are

hydrolysed tannins common in pomegranate fruit, which include constituents like punicalagins, punicalin, gallagic acid, ellagic acid and relative glycosides (Altieri et al., 2019). Phenolic acids and flavonoids including catechins, esculetin, hesperidin, rutin, naringenin, kaempferol, apigenin, rhamnetin, quercitrin and quercetin have also been reported in pomegranate fruit (Zeghad et al., 2019;), which partly influence its antioxidant health benefit. These polyphenols, including the ellagitannins account for a high percentage of the fruit's antioxidant property and are mostly concentrated in the fruit peel and juice compared to other parts of the fruit (Ismail et al., 2012; Yaritz et al., 2022; Marra et al., 2022).

Documented evidence has shown that the fruit's peel contains ellagitannins, flavonoids and phenolic acids and may be useful in managing oxidative stress (Akuru et al., 2022; Marra et al., 2022; Mo et al., 2022). Thus, maximizing the therapeutic potential of the fruit's wastes and potentially reducing environmental biomass and pollution caused by the fruit's peel. The potent radical scavenging activity of the fruit's peel has been demonstrated (Li et al., 2006; Akuru et al., 2022; Marra et al., 2022), and in some instances has been shown to be stronger than that of the edible pulp ((Li et al., 2006). In both hepatocytes and isolated rat liver tissues, the peel was shown to reduced lipid peroxidation caused by oxidative stress (Akuru et al. 2022). In rats the peel was shown to improve oxidative stress marker and hepatic oxidative damage caused by carbon tetrachloride, suggesting the hepatoprotective effect of the peel (Chidambara et al., 2002). In fact, phenolic principles (ellagic acid, punicalin, and punicalagin) from pomegranate peel has been shown to reduce oxidative stress and inflammatory makers in oxidatively stressed mice (Sun et al., 2017).

Although, different organic solvent extracts have been explored for recovering the antioxidant principles in pomegranate peel, water extraction has always been perceived as the safest in folk medicine and thus commonly used in Chinese traditional medicine (Yang and Ross, 2010). Moreover, other common solvents like ethanol used in folk medicine may be more costly than water and may pose toxicity concerns. Despite, the preferred safety profile of water extraction, acetone extraction of pomegranate peel has been shown to result in better antioxidant profile; perhaps, due to a better phytochemical profile (Chukwuma et al., 2020; Akuru et al., 2022). Hence, it is imperative to explore methods that could improve the antioxidant and phytochemical profiles of pomegranate aqueous peel extract.

Previous studies investigated the effect of different drying and extraction conditions/methods on the phytochemical and antioxidant profiles of pomegranate peel. Calín-Sánchez et al. (2013)

investigated the effect different types of drying methods (freeze drying, convective drying and vacuum–microwave drying) on the antioxidant and phenolic profiles of pomegranate rind and peel. However, they did not determine or investigate the extraction method for optimum ellagitannins recovery and antioxidant profile. Their study is also void of advanced statistical correlation models to statistical correlate between the studied variables. Alexandre et al. (2017) used Box–Behnken design to investigate the effect of high-pressure extraction on the antioxidant and bioactive phytochemical profile on pomegranate peel. However, their study was limited to the peel material that was dried and did not investigate the influence of varying extraction temperature on the ellagitannins and antioxidant profile of pomegranate peel. A previous study has suggested that using fresh fruit peel for aqueous extraction may be promising for recovering ellagitannins from pomegranate fruit waste, perhaps to a commercial scale (Seeram et al., 2005). However, how extraction temperature influences the ellagitannin recovery and antioxidant profile remains elusive. It is known that heat facilitates the infusion of phytochemicals present in plant materials and thus is commonly employed in Chinese traditional medicine (Yang and Ross, 2010).

It is, however, important to conduct temperature-dependent extraction at optimum temperatures that can preserve the structural and functional properties of the bioactive principles, particularly the ellagitannins constituents in pomegranate peel. Too much heat may alter the ellagitannins integrity/profile, thus compromising the antioxidant profile (Sójka et al., 2019). Conversely, insufficient heat may not provide the optimum heat condition for optimum ellagitannins extraction and antioxidant profile (Yang and Ross, 2010). To date no study has determined or investigated the optimum temperature for aqueous extraction of pomegranate peel that will yield optimum antioxidant and ellagitannins profile. Therefore, this study was done to investigate the influence of temperature on the antioxidant and ellagitannins profile of pomegranate peel water extraction by employing advanced chemometrics models and analyses. The models include Pearson's bivariate correlation, principal component analysis (PCA) and orthogonal projections to latent structures (OPLS), which for the first time was used to correlate and model the influence of temperature on the antioxidant and ellagitannins profile of pomegranate peel. Antioxidant models used in this study, including oxygen radical absorbance capacity (ORAC) and *in vitro* and Chang liver cells anti-lipid peroxidation assays are more advanced than those employed in the studies reported by Calín-Sánchez et al. (2013) and Alexandre et al. (2017).

5.3 Materials and methods

For the materials and methods of this chapter, please refer to **sub-sections 3.1 to 3.8** of chapter 3.

5.4 Results and discussion

In the present study, the optimum aqueous extraction temperature for pomegranate peel that will optimally recover the ellagitannins and preserve their antioxidant profile was studied using advanced chemometrics models. The data suggest a temperature dependent influence on the ellagitannins and antioxidant profile of pomegranate peel during aqueous extraction.

5.4.1 Influence of extraction temperature on the phytochemical profile of pomegranate peel

The TPC and TFC dropped as extraction temperature was increased from room temperature to 37 °C (**Figures 5.1**).

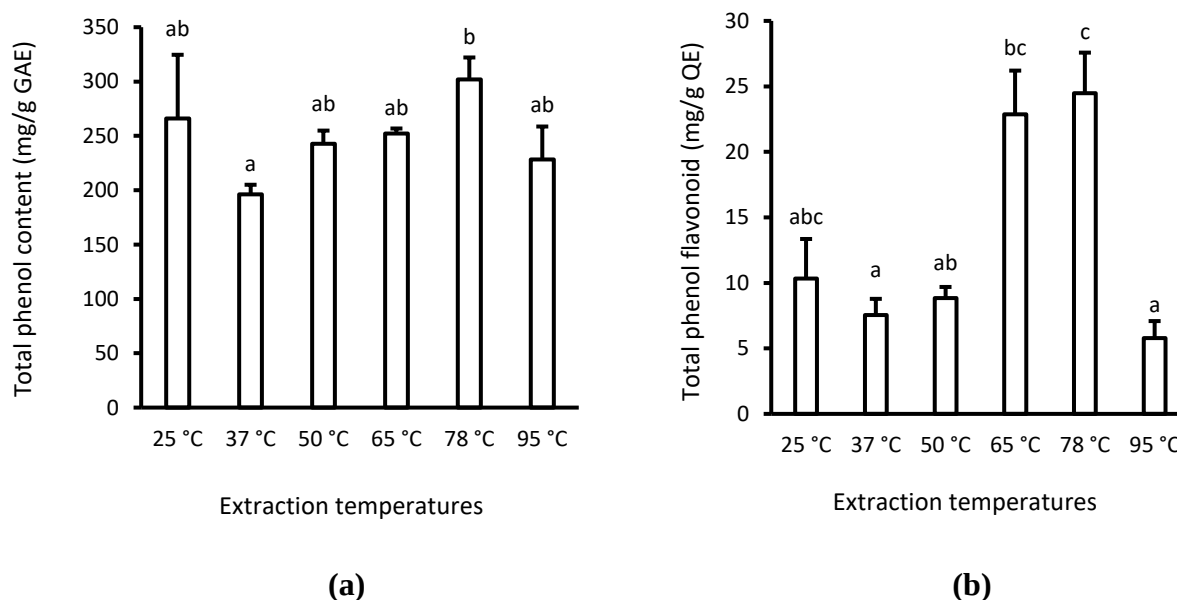


Figure 5.1: (a) Total phenol content and (b) total flavonoid content of the different water extracts from the fresh peel. Data are presented as mean $\pm$ SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

Considering that extraction was done on fresh fruit peel, this drop in TPC and TFC may be attributed to enzymes like polyphenol oxidases (PPOs), which degrades polyphenols (Sikora et al., 2019). PPOs are optimally active at about 35 – 37 °C (Sikora et al., 2019), thus actively degrading polyphenols and reducing the TPC and TFC at 37 °C extraction temperature. As the extraction temperature increases to 50, 65 and 78 °C, the TPC and TFC gradually increase and peaked at 78 °C, which was significantly ($p < 0.05$) higher compared to 37 °C (**Figures 5.1**). It is plausible that there was inactivation and denaturing of PPOs as temperature increased from 37 to 50 °C and beyond, which reflects the gradually increasing TPC and TFC. Also, temperature-dependent release of insoluble bound phenolic compounds or lignin breakdown may contribute to the gradual increase in TPC and TFC (Irakli et al., 2018) as the extraction temperature increased. However, as extraction temperature increased to 95 °C, the TPC and TFC dropped or reduced notably, which may be attributed to thermal degradation of polyphenols in the extract. Moreover, it has been documented that high TPC is obtained at extraction temperature between 60 and 80 °C, while temperatures above 80 °C can lead to thermal degradation of the polyphenols and reduced TPC (Antony and Farid, 2022).

The yield of the purified tannins/ellagitannins from the extracts, as well as the LC-MS-quantified ellagitannins (punicalagin A and B, granatin A, corilagin, casuarinin and geraniin) also followed a similar trend as the TPC and TFC, with the extraction done at 78 °C having the highest concentrations of the ellagitannins compared to the extraction done at other temperatures (**Table 5.1** and **Appendix 1**).

Table 5.1: The yield and phytochemical profile of ellagitannins yield from the different fresh pomegranate peel water extracts

Extraction at different temperatures	Yield of total tannins (%)	LCMS-quantified ellagitannin constituents in the purified total tannins from extracts (mg/L)							
		Punicalagin A	Punicalagin B	Ellagic acid	Granatin A	Corilagin	Casuarinin	Geraniin	Quercetin hexoside
25 °C	34.2 ± 2.69 ^b	268 ± 21.5 ^a	257 ± 20.5 ^a	12.4 ± 2.74 ^a	75.8 ± 9.64 ^a	3.50 ± 0.52 ^a	6.27 ± 0.41 ^a	9.75 ± 2.71 ^a	20.4 ± 1.45 ^{ab}
37 °C	21.7 ± 1.90 ^a	464 ± 15.4 ^b	439 ± 47.3 ^b	28.0 ± 6.70 ^b	117 ± 8.24 ^b	6.02 ± 0.81 ^{ab}	9.76 ± 1.45 ^a	14.5 ± 2.12 ^{ab}	30.7 ± 5.77 ^c
50 °C	18.5 ± 1.69 ^a	571 ± 11.7 ^c	502 ± 5.72 ^{bc}	24.9 ± 1.54 ^b	126 ± 2.35 ^b	6.14 ± 0.77 ^{ab}	11.0 ± 2.41 ^a	19.3 ± 3.91 ^b	28.3 ± 3.31 ^{bc}
65 °C	34.7 ± 4.35 ^b	523 ± 31.4 ^c	526 ± 49.7 ^c	31.9 ± 5.42 ^{bc}	128 ± 6.57 ^b	7.01 ± 2.29 ^{ab}	8.71 ± 2.25 ^a	21.2 ± 1.31 ^{bc}	33.9 ± 4.18 ^c
78 °C	38.6 ± 2.94 ^b	782 ± 34.0 ^d	717 ± 18.7 ^d	40.5 ± 3.54 ^c	171 ± 13.7 ^c	8.64 ± 2.44 ^b	24.1 ± 2.85 ^b	26.5 ± 4.03 ^c	48.5 ± 5.42 ^d
95 °C	18.8 ± 2.99 ^a	277 ± 13.5 ^a	238 ± 23.1 ^a	7.15 ± 2.37 ^a	59.6 ± 12.4 ^a	3.07 ± 0.91 ^a	6.02 ± 1.07 ^a	10.9 ± 2.06 ^a	15.5 ± 3.18 ^a

Data are presented as mean ± SD of triplicate analysis. For each parameter, the letters appearing as superscripts in the cells represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

It has been documented that temperature elevation increases the yield of ellagic acid/ellagitannins extraction from pomegranate husk, which explains the temperature dependent increase in tannin/ellagitannins yield (Muñiz-Márquez et al., 2021). However, the notable drop in tannin yield or ellagitannins concentration beyond 78 °C (at 95 °C extraction temperature) (**Table 5.1**) may be attributed to temperature-dependent degradation of ellagitannins. Moreover, it has been reported that ellagitannins are relatively stable in acidic conditions but could be degraded at temperatures beyond 80 °C (Sójka et al., 2019).

5.4.2 Influence of extraction temperature on the antioxidant profile of pomegranate peel

The Fe^{3+} reducing and radical scavenging activity, as well as the ORAC showed an observable reduction moving from extraction at 25 °C to extraction 37 °C (**Figures 5.2 – 5.4**), which may be linked to the observed depleted TPC and TFC (**Figures 5.1a and b**). Polyphenols have the ability to form stable phenoxy radicals by donating electrons or transferring H-atom, thus are potent radical scavengers (Chen et al., 2020)

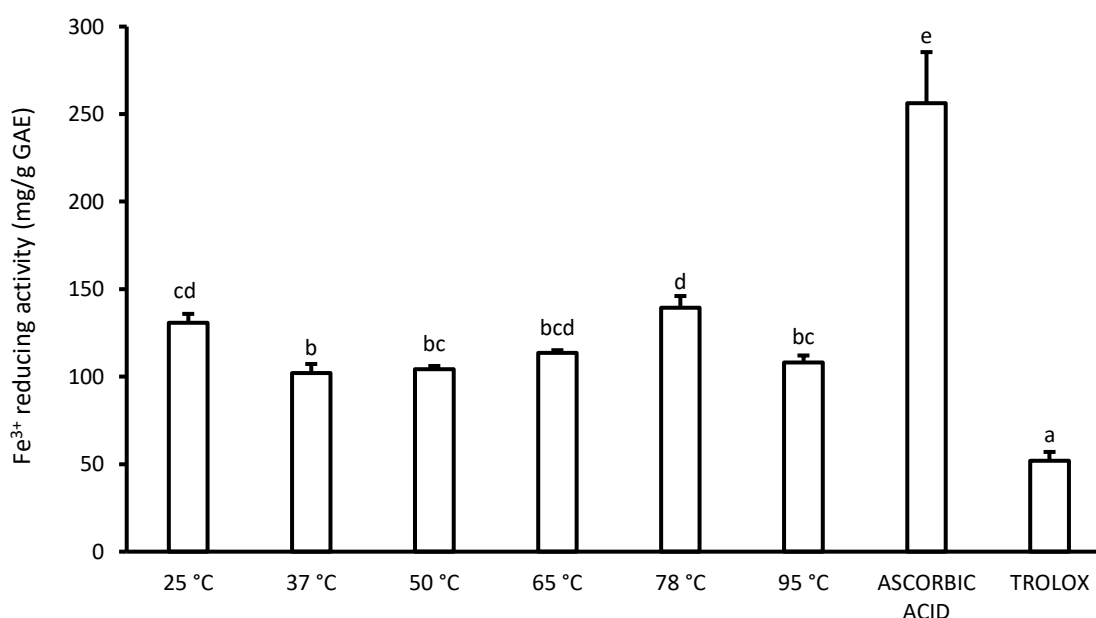


Figure 5.2: Fe^{3+} reducing activity of the different water extracts from the fresh peel. Data are presented as mean $\pm$ SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

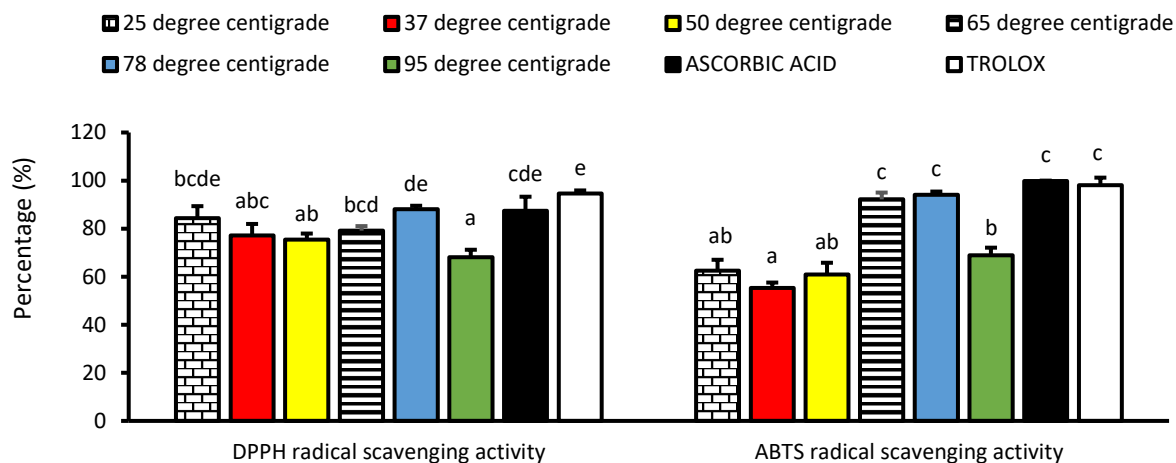


Figure 5.3: Radical scavenging activity of the different water extracts from the fresh peel. Data are presented as mean $\pm$ SD of triplicate analysis. For each analysis, the letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

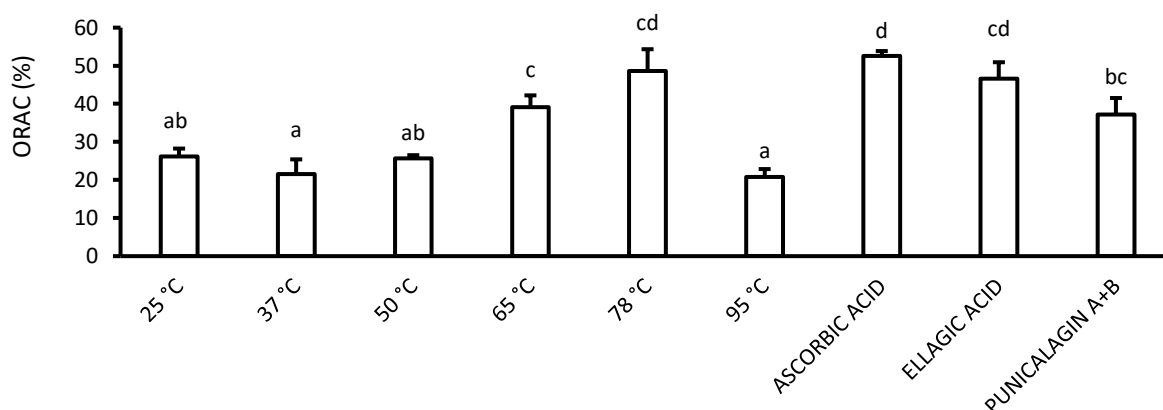
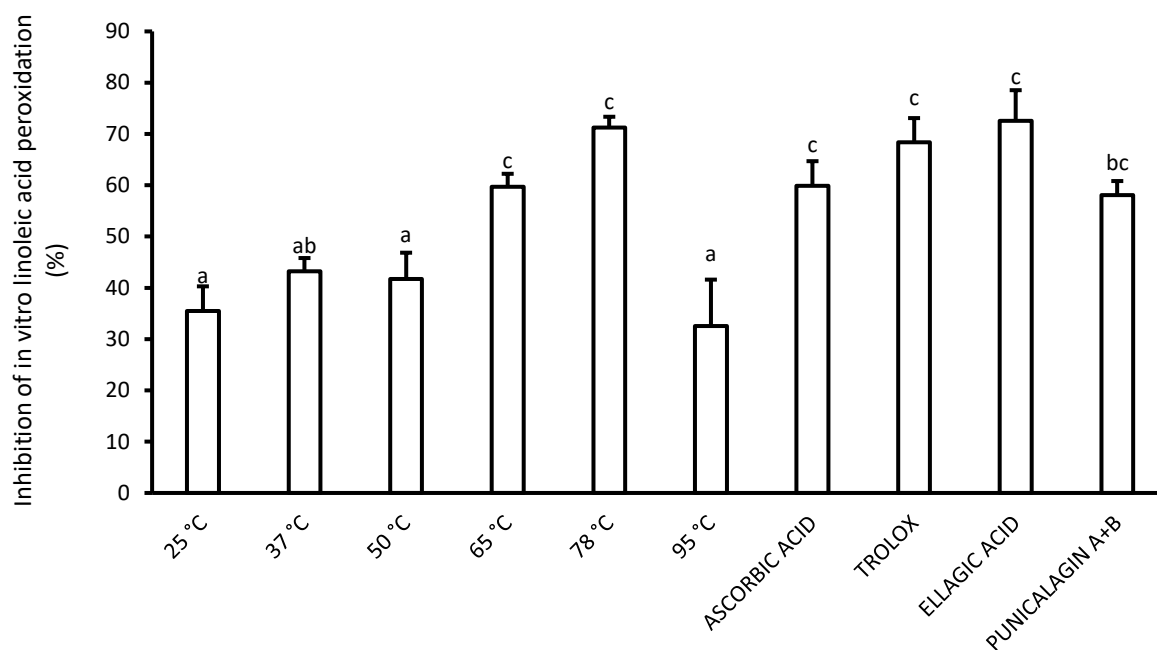
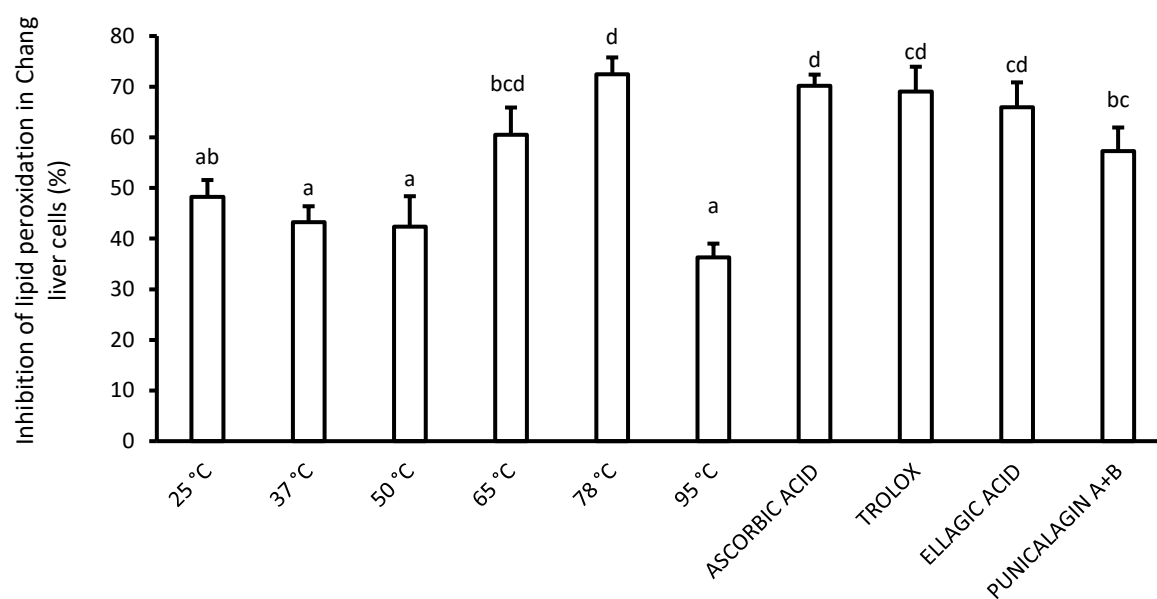


Figure 5.4: Oxygen radical absorbance capacity (ORAC) of the different water extracts from the fresh peel. Data are presented as mean $\pm$ SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters.

Beyond 37 °C, the radical scavenging and Fe^{3+} reducing effect of the extracts gradually increased and peaked at 78 °C, which is consistent with the profile of TPC and TFC (**Figure 5.1**) and that of the predominant ellagitannins (punicalagins, ellagic acid and granatin A) in the extracts (**Table 5.1**).



(a)



(b)

Figure 5.5: Inhibitory effect of the fresh peel water extracts on (a) *in vitro* FeSO₄-induced linoleic acid peroxidation and (b) FeSO₄-induced lipid peroxidation in Chang liver cells. 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.

The peel extract obtained at 78 °C had *in vitro* and cellular anti-lipid peroxidative activity that was more potent than that of the other extracts and did not differ significantly ($p > 0.05$) when compared to that of ascorbic acid and Trolox (**Figure 5.5**), which suggest the potential of the extract obtained at 78 °C to mitigate oxidative insult. The higher ellagitannins content of the peel extract obtained at 78 °C (**Table 5.1**) appeared to influence its stronger anti-lipid peroxidative activity relative to the other extracts. Moreover, the predominant ellagitannins (punicalagin and ellagic) showed promising *in vitro* and cellular anti-lipid peroxidative activity (**Figure 5.5**), while ellagitannins-rich dietary supplement from pomegranate, as well as ellagic acid have been shown to have potent anti-lipid peroxidative activity in obese animals (Xu et al., 2021) and human subjects (Heber et al., 2007). Data suggest that high extraction temperatures beyond 80 – 100 °C may compromise the structural and antioxidant profiles of phenolics and ellagitannins in pomegranate peel, while temperatures between 70 and 78 °C may be considered as optimum temperatures for aqueous extraction of pomegranate peel in the context of antioxidant and ellagitannins profile.

5.4.3 Correlation analysis

The phytochemistry and bioactivities of different temperature-controlled aqueous extracts were evaluated. The Pearson's correlation coefficients (**Figure 5.6** and **Appendix 1**) indicate varying degrees of strength of linear association between all pairwise combinations of the variable in the dataset. It can clearly be observed that the ORAC and anti-lipid peroxidation effects of the extracts strongly correlates ("r" ranging from 0.7 to 0.9) with the tannin yield of the extracts, as well as the concentration of the quantified ellagitannin constituents present in the extracts. This strongly buttresses the possible notable contribution of the ellagitannins in pomegranate peel in its anti-lipid peroxidative potential, which has been previously reported in obese animals (Xu et al., 2021) and human subjects (Heber et al., 2007). Moreover, punicalagin A+B and ellagic acid standards (particularly ellagic acid), which are notable chemical constituents of pomegranate ellagitannins showed promising ORAC and anti-lipid peroxidative potential that were statistically comparable to known antioxidants (ascorbic acid and Trolox) (**Figures 5.4 and 5.5**).

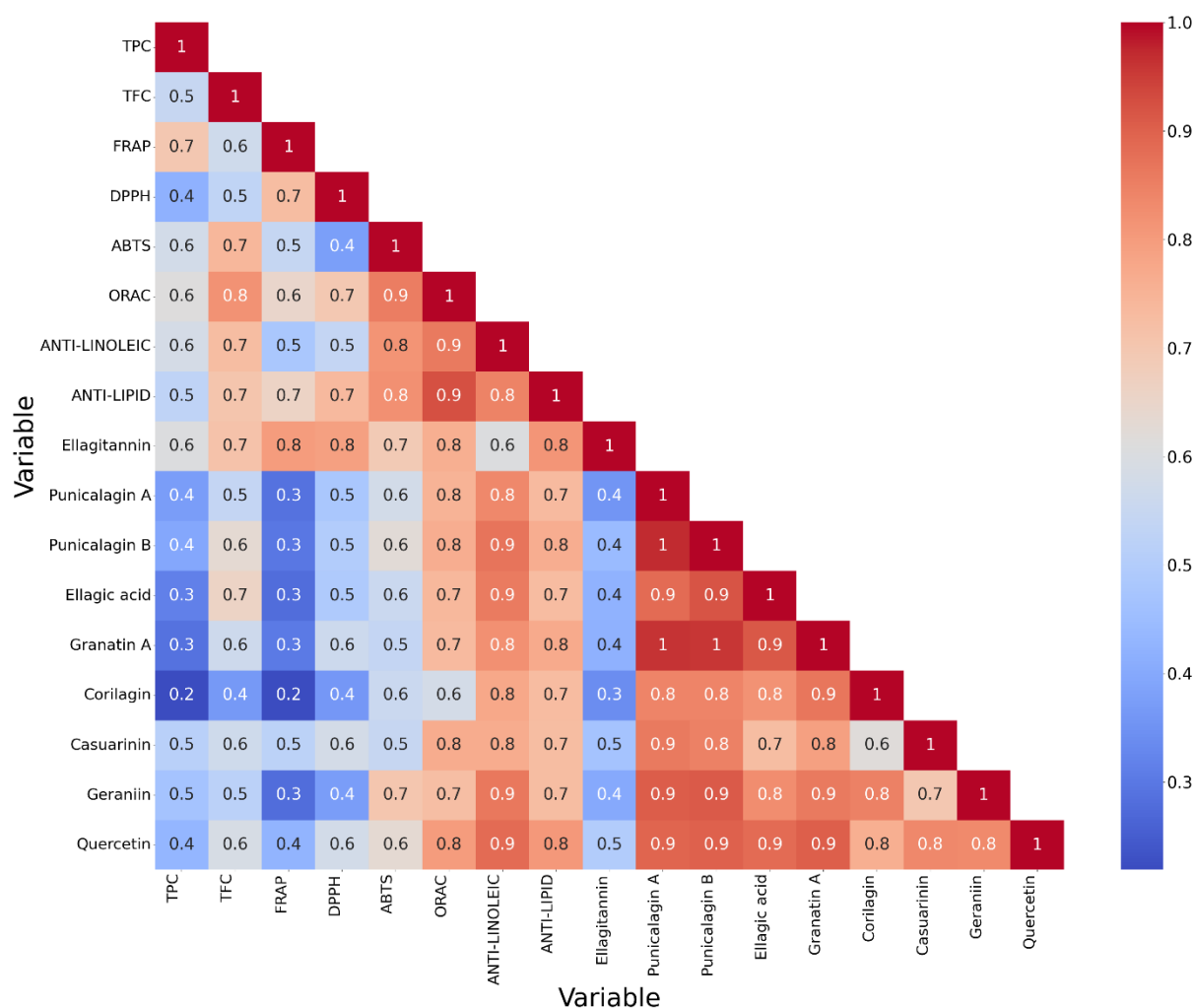
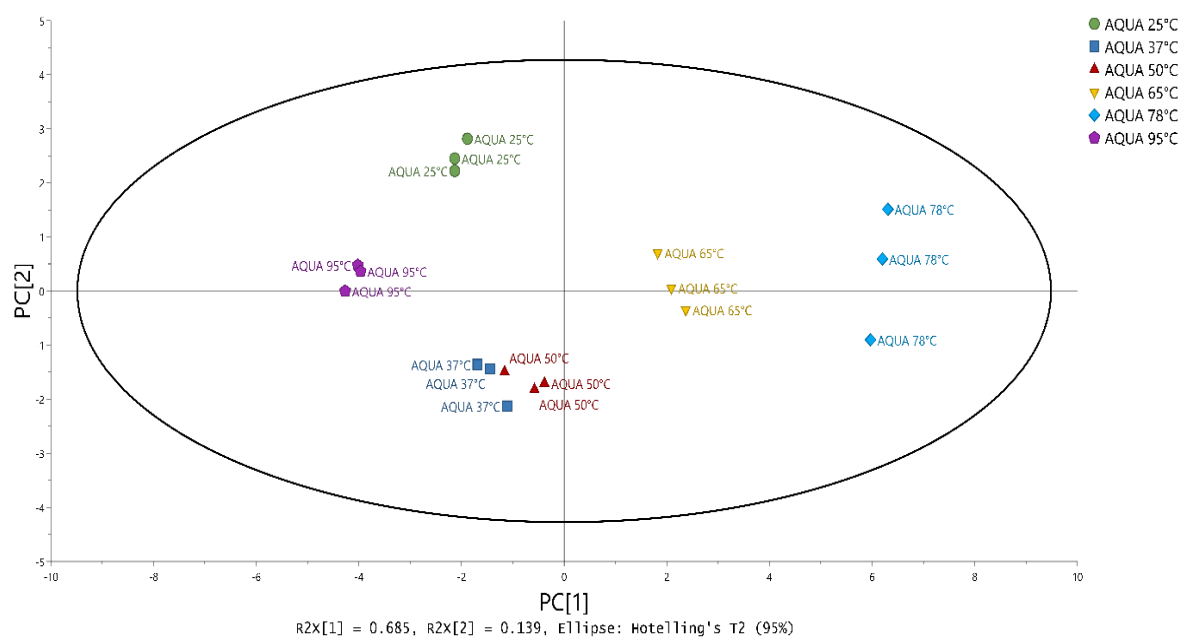


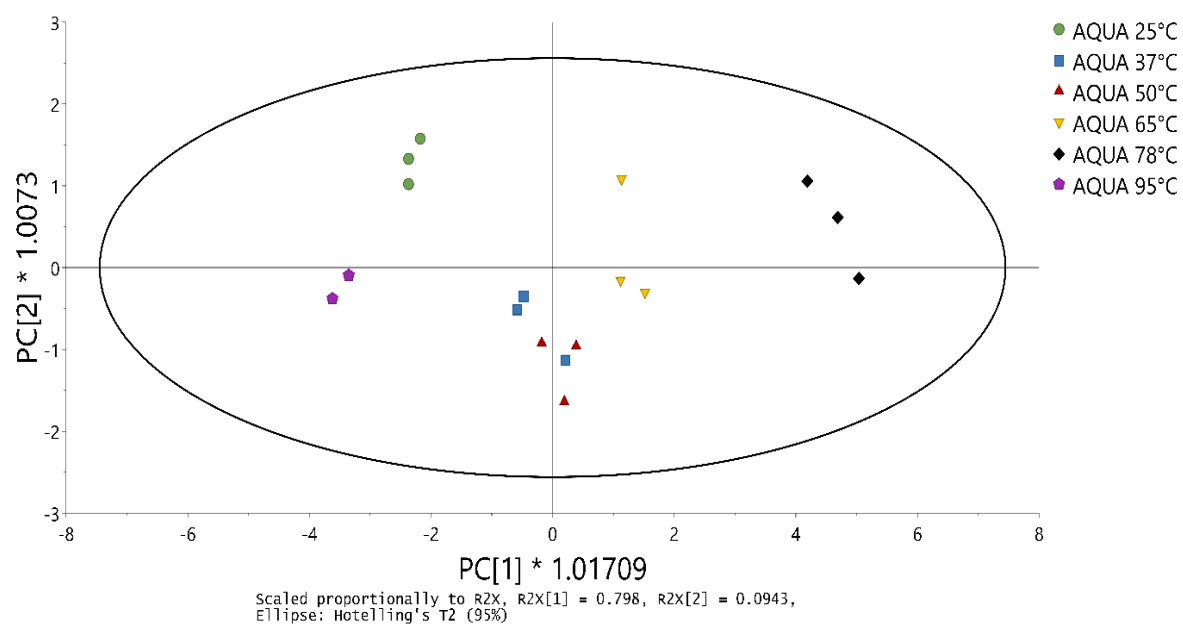
Figure 5.6: Pearson's bivariate correlation matrix between data variables.

5.4.4 Advanced chemometric analysis

The data was further interrogated using PCA and OPLS algorithms to extract maximum differential and explanatory information, identify corresponding variables (phytochemicals and antioxidant potency), as well as build predictive models to describe the relationship between extracted compounds and various antioxidant effects in different aqueous extraction temperatures. Through the use of latent variables or principal components, PCA and OPLS techniques project the prevailing trends in the multidimensional dataset onto a small number of representative virtual variables (Wheelock & Wheelock, 2013; Gbashi et al., 2017). PCA provided an initial dataset overview. From the PCA results, no outliers were detected, i.e., all samples were within the Hotelling T2 95% confidence ellipse (**Figure 5.7a**).



(a)



(b)

Figure 5.7: (a) PCA scores plot of unsupervised linear transformation and multivariate dimensional reduction analysis of the data, (b) OPLS scores plot of supervised linear transformation, dimensionality reduction, and modelling of the patterns in the data.

Also, the PCA model revealed high explained variation [$R^2X(\text{cum}) = 0.824$] and strong predictive patterns [$Q^2(\text{cum}) = 0.723$] in the dataset (Table 2). The PCA scores plot (**Figure 5.7a**) also showed clear clusters of the samples indicating extraction temperature results in differential metabolite recovery patterns and antioxidant potency of the various extracts.

OPLS modelling was used to look for linear relationships between the predictors (metabolites) and response variables (antioxidant capacities), and to establish the significance of the variables in explaining the overall patterns in the data. **Figure 5.8** shows the OPLS biplot and the HCA dendrogram, which suggest the similarities and differences in the sample groups.

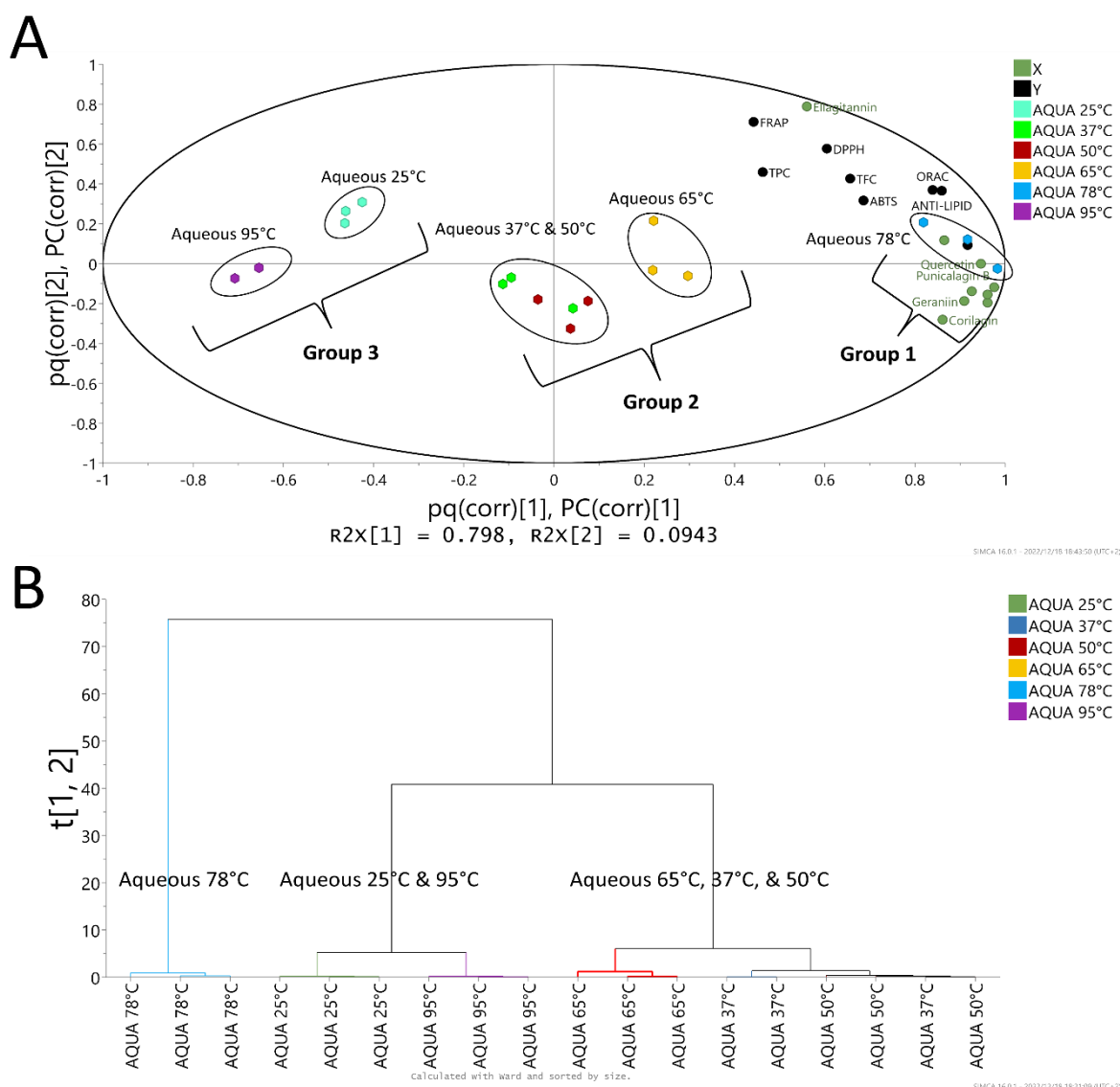


Figure 5.8: (a) OPLS biplot and (b) HCA dendrogram of the different sample variables and classes.

The biplot overlays data distribution patterns from both the loadings plot and the scores plot (**Figure 5.8a**). The loadings consist of the weights of the original data variables while the new coordinates for the observations are the scores. The scores plot shows how the observations are divided into groups while the loadings show how the original variables relate to the scores (Wheelock & Wheelock, 2013).

On the biplot, the X variables are coloured green, the Y variables are coloured black, and observations belonging to the extraction conditions are also coloured differently. A distinct grouping pattern can be visualized on the plot, with the 78 °C aqueous extracts standing out and being the most alienated from the other extracts (**Figures 5.7b** and **5.8a**). The HCA dendrogram (**Figure 5.8b**) further emphasizes the clustering pattern observed on the PCA scores plot and the OPLS biplot. In this plot, the clusters are shown along the horizontal axis, while the vertical axis denotes the separation or dissimilarity between groups. A vertical line is broken into two lines on the diagram to symbolise each joining (fusion) of the two clusters. The separation (difference) between the two clusters is shown by the split's vertical location, which is depicted by a short bar. This plot reveals three major clusters which are Cluster 1: 78 °C, Cluster 2: 25 and 95 °C, and Cluster 3: 37, 50, and 65 °C. Essentially, samples within the same cluster may share inherent similarities and are more closely related to adjacent clusters in order of hierarchy. It is safe to speculate that extraction at 78 °C notably and optimally influenced the ellagitannins and antioxidant profile of pomegranate fruit peel.

The Y variables, including *in vitro* anti-linoleic acid peroxidation, TFC, ORAC, ABTS, and cellular anti-lipid peroxidation were closely grouped around the 78 °C samples and largely within the same quadrant, which were clustered around the ellagitannins (X variables) (**Figure 5.8a**), suggesting that the distinct antioxidant potential of the extract at 78 °C was largely influenced by its ellagitannins profile. Since PC1 explains most of the variation in the dataset (79.8%), separation along its axis (i.e., x-axis) could describe differences in the sample groups. In this regard, the aqueous extract at 78 °C was more potent than the other extracts, while the extract at 95 °C was the least potent for the investigated antioxidant assays. This implies that the extract at 78 °C contained highest amounts, while the extract at 95 °C contained the lowest amounts of the bioactive phenolics and ellagitannins, thus explaining their contrasting antioxidant profiles (**Figures 5.3 – 5.5**).

The variable contribution plot further provides information on the influence of the ellagitannins on the antioxidant profile of the potent extracts (**Figures 5.9** and **5.10**). The higher

tannin/ellagitannins yield of the extract at 78 °C, relative to the other extracts notably contributed to its antioxidant profile (**Figure 5.9**).

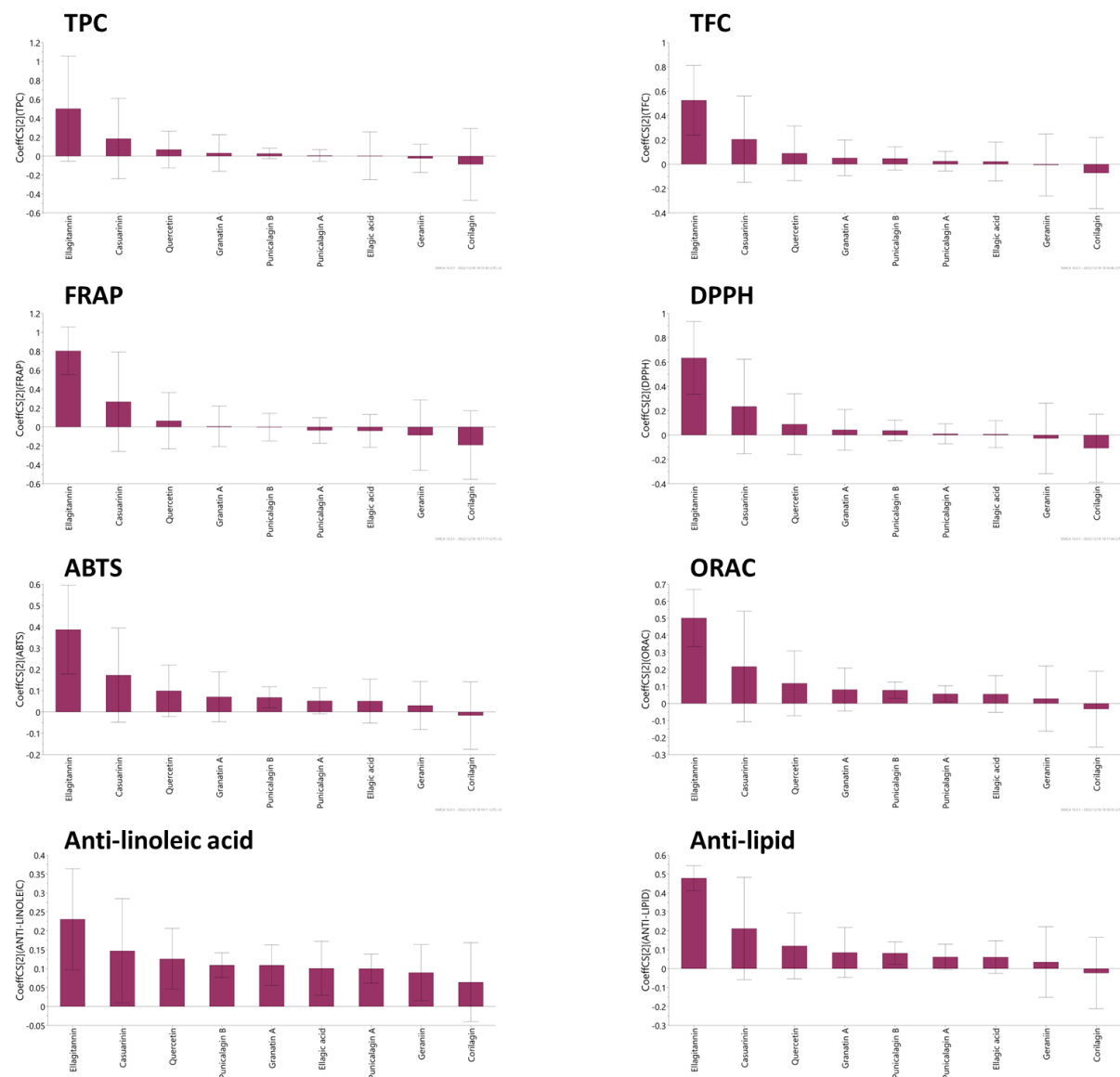


Figure 5.9: Contribution of each predictor variable on the specific response variable.

Most of the identified ellagitannins fairly influenced the ORAC and anti-lipid peroxidative capacities of the extracts, with casuarinin appearing to be most influential. Although, with a relatively high standard error (**Figure 5.9**). Perhaps, because its concentration in the extract at 78 °C was double the amount in any other extract (**Table 5.1**). Punicalagin, on the other hand, appears to consistently influence the ORAC and anti-lipid peroxidative capacities of the

extracts with a fairly low standard error, which is consistent with the observed ORAC and anti-lipid peroxidative effect shown by punicalagin A+B standard (**Figures 5.4 and 5.5**). In summary, however, punicalagin, granatin A and ellagic acid are among the top five ellagitannins influencing the antioxidant profile of the extracts (**Figure 5.10**). Moreover, ellagic acid was shown to exhibit appreciable ORAC and anti-lipid peroxidative effect (**Figures 5.4 and 5.5**).

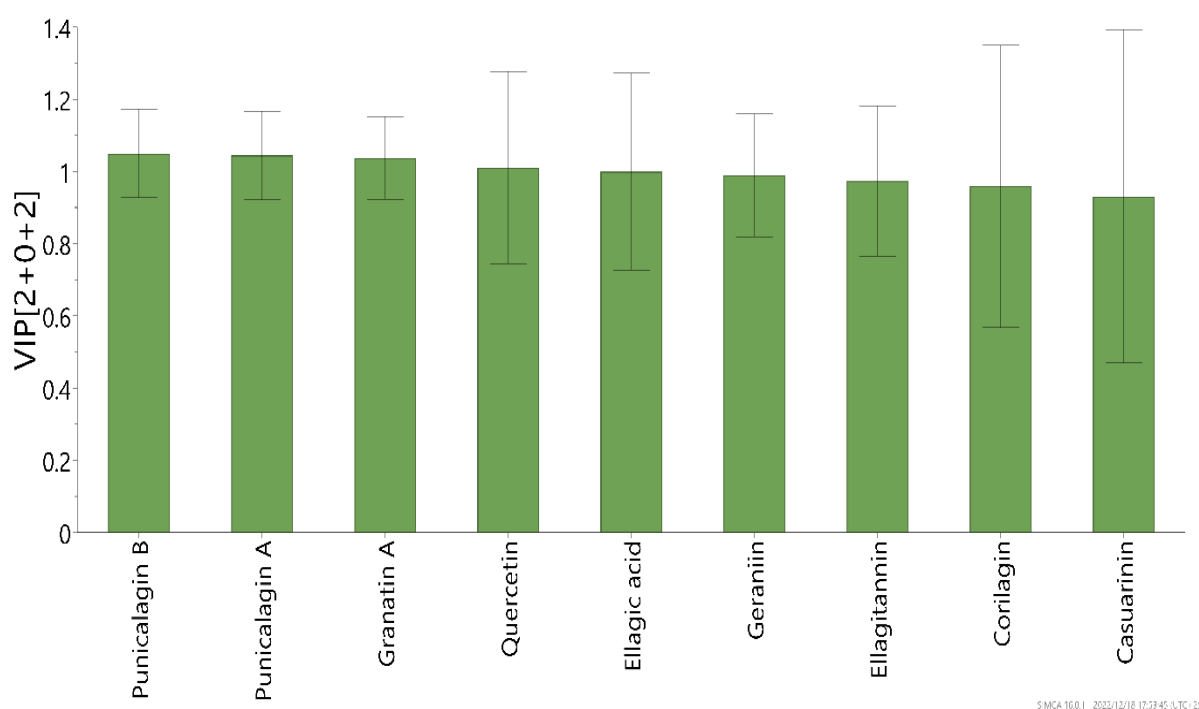


Figure 5.10: OPLS variable importance plot.

5.4.5 Model overview, evaluation, and validation

When numerical models are fitted to empirical data, it is necessary to evaluate how fit the model is to ensure that the model behaviour is reasonable and that any predicted or fitted parameter values may be interpreted and used with confidence. Model fit parameters for both PCA and OPLS models are presented in Tables 5.2 and 5.3. PCA generated two latent variables which combined, described 82.4% of the variation in the entire dataset, with a predictive capacity of 72.3%. PC1 modelled 68.5% of the variation in the dataset while PC2 explained 13.9% of the variation in the data. All generated latent variables are significant (**Table 5.2**).

Table 5.2: PCA modelling parameters and summary of fit

Primary ID		R2VX	R2VX(cum)	Q2VX	Q2 limit	Q2VX(cum)
<i>Total</i>	<i>Comp 1</i>	<i>0.685191</i>	<i>0.685191</i>	<i>0.618699</i>	<i>0.108025</i>	<i>0.618699</i>
	<i>Comp 2</i>	<i>0.139213</i>	<i>0.824404</i>	<i>0.272691</i>	<i>0.114187</i>	<i>0.722676</i>
TPC	Comp 1	0.344271	0.344271	0.253445	0.108025	0.253445
	Comp 2	0.244753	0.589024	0.031283	0.114187	0.276799
TFC	Comp 1	0.582139	0.582139	0.360532	0.108025	0.360532
	Comp 2	0.0966583	0.678797	0.041494	0.114187	0.387066
FRAP	Comp 1	0.348633	0.348633	0.269087	0.108025	0.269087
	Comp 2	0.484575	0.833209	0.560992	0.114187	0.679123
DPPH	Comp 1	0.460869	0.460869	0.417135	0.108025	0.417135
	Comp 2	0.164683	0.625552	0.096908	0.114187	0.47362
ABTS	Comp 1	0.629428	0.629428	0.589435	0.108025	0.589435
	Comp 2	0.0534932	0.682921	0.013503	0.114187	0.594979
ORAC	Comp 1	0.860704	0.860704	0.827949	0.108025	0.827949
	Comp 2	0.0507649	0.911469	0.19962	0.114187	0.862294
Anti-linoleic	Comp 1	0.895341	0.895341	0.85556	0.108025	0.85556
	Comp 2	0.0032216	0.898562	-0.08565	0.114187	0.843189
Anti-lipid	Comp 1	0.860302	0.860302	0.825013	0.108025	0.825013
	Comp 2	0.0341192	0.894422	0.241578	0.114187	0.867286
Ellagitannin	Comp 1	0.487474	0.487474	0.419182	0.108025	0.419182
	Comp 2	0.384826	0.8723	0.54912	0.114187	0.738121
Punicalagin A	Comp 1	0.804255	0.804255	0.768588	0.108025	0.768588
	Comp 2	0.148628	0.952883	0.70926	0.114187	0.932719
Punicalagin B	Comp 1	0.849989	0.849989	0.82349	0.108025	0.82349
	Comp 2	0.113947	0.963936	0.72083	0.114187	0.950724
Ellagic acid	Comp 1	0.77182	0.77182	0.727932	0.108025	0.727932
	Comp 2	0.109404	0.881224	0.345866	0.114187	0.822031

Primary ID		R2VX	R2VX(cum)	Q2VX	Q2 limit	Q2VX(cum)
Granatin A	Comp 1	0.802621	0.802621	0.757407	0.108025	0.757407
	Comp 2	0.133613	0.936234	0.573621	0.114187	0.896563
Corilagin	Comp 1	0.621759	0.621759	0.457406	0.108025	0.457406
	Comp 2	0.189866	0.811625	0.366858	0.114187	0.656461
Casuarinin	Comp 1	0.726118	0.726118	0.63688	0.108025	0.63688
	Comp 2	0.0061637	0.732282	-0.00427	0.114187	0.635329
Geraniin	Comp 1	0.758138	0.758138	0.732661	0.108025	0.732661
	Comp 2	0.105765	0.863903	0.274824	0.114187	0.806132
Quercetin hexoside	Comp 1	0.844388	0.844388	0.796175	0.108025	0.796175
	Comp 2	0.0421359	0.886524	0.139237	0.114187	0.824555

Table 5.3: OPLS modelling parameters and summary of fit

Component	R2X	R2X(cum)	R2	R2(cum)	Q2	Limit	Q2(cum)	R2Y	R2Y(cum)	Significance
Model		0.892		0.695			0.611		0.936	
Predictive		0.892		0.695			0.611		0.763	
P1	0.798	0.798	0.645	0.645	0.44	0.01	0.44	0.688	0.688	R1
P2	0.0943	0.892	0.0498	0.695	0.171	0.01	0.611	0.0748	0.763	R1
Orthogonal in Y (OPLS)									0.173	
O1								0.107	0.107	R1
O2								0.066	0.173	R1

Similar to the results of PCA, OPLS analysis generated two components which were all significant (**Table 5.3**). The X model described 89.2% of the X variation [R2X(cum)=0.892] and 69.5% of the Y variation [R2(cum)=0.695], while the Y model described 93.6%

[R2Y(cum)=0.936] of the variation in the response variables. The total orthogonal variation in the response variable i.e., orthogonal in Y, was 17.3%. The cumulative predictive power of the OPLS model was 61.1% [Q2(cum)=0.611]. Regressing the predicted values versus the observed values yielded R2 values ranging from 0.42 to 0.87, RMSEE values ranging from 4.22 to 33.35, and RMSECV values ranging from 4.22 to 34.45 (**Figure 5.11**). Indeed, all the model fit parameters indicate that our fitted models sufficiently capture the patterns in the data and adequately describe the empirical relationship between the predictors and response variables.

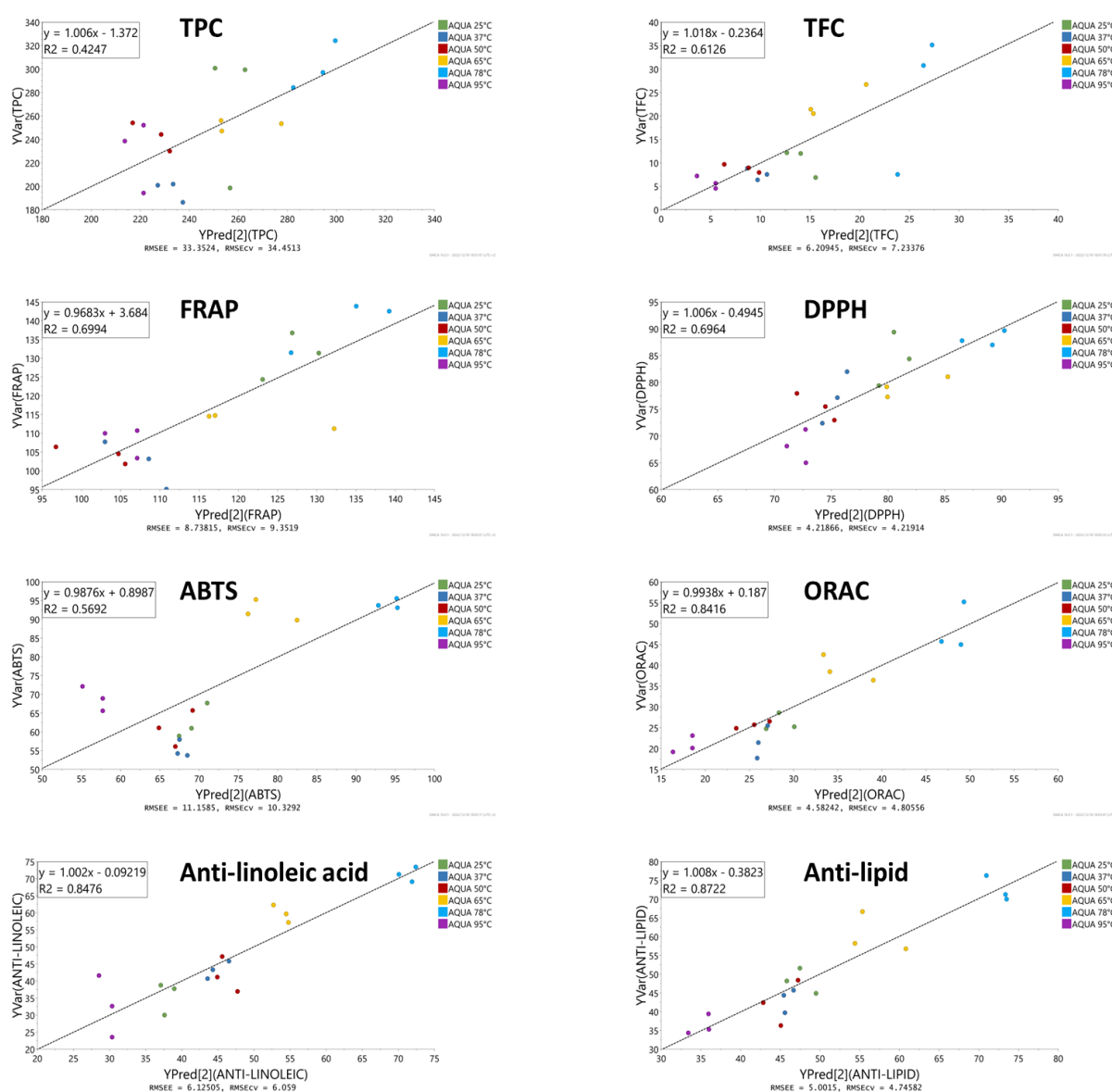


Figure 5.11: OPLS predicted versus observed values.

5.5 Conclusion

The present study reveals a temperature dependent influence on the antioxidant and ellagitannins profile of pomegranate peel subjected to aqueous extraction. The strongest antioxidant potential and optimum ellagitannins yield was obtained at 78 °C extraction temperature. The obtained OPLS biplot from chemometric analysis showed a clustering pattern of antioxidant and ellagitannins variables distinctly around 78 °C extraction temperature, suggesting an observable influence of the phytochemicals on the potent antioxidant profile of extracts obtained at 78 °C relative to the other extracts. The variable contribution plots, as well as the obtained antioxidant data suggest punicalagin, granatin A and ellagic acid as notable ellagitannins influencing the potent antioxidant action of the pomegranate peel extracts. It is safe to speculate that aqueous extraction at 78 °C notably and optimally influenced the ellagitannins and antioxidant profile of pomegranate fruit peel. Thus, may be a promising aqueous extraction temperature for optimally recovering the ellagitannins from pomegranate peel, without sacrificing the antioxidant capacity, which may be of medicinal relevance in managing oxidative stress in many diseases.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

6.1 General discussion

Pomegranate fruit has been widely consumed because it is palatable and has beneficial effects on oxidative, metabolic and vascular health (Kandylis and Kokkinomagoulos, 2020; Giménez-Bastida et al., 2021). This has been attributed to its phenolic constituents, including bioactive ellagitannins (Giménez-Bastida et al., 2021). Although underutilized, the peel of pomegranate also contains these beneficial ellagitannins (Yaritz et al., 2022; Marra et al., 2022; Akuru et al., 2022; Mo et al., 2022), which could be of medicinal relevance in managing oxidative stress and associated insults. By altering variables such as temperature and solvent system, we investigated how these ellagitannins can be optimally recovered from the fruit's peel, without compromising their antioxidant potencies. Advanced chemometrics correlational models/analyses, including principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) were employed to conducting the investigation.

It was observed using acetone and methanol to extracts the dry peel resulted in extracts with higher phenolic contents (**Figure 4.1**) and ellagitannins profiles (**Table 4.1**), as compared to extracting the dry peel with water and ethanol. It is plausible that this higher phytochemical further influenced the more potent radical scavenging and anti-lipid peroxidative capacities of the acetone and methanol extracts relative to the water and ethanol extracts (**Figures 4.3 – 4.6**). Phenolic compounds are often polar and hence more hydrophilic than lipophilic. Thus, polar protic solvents generally yield more effective polyphenol extraction outcomes. As a result, aliphatic alcohols (such as methanol) and polar organic solvents (such as acetone and ethyl acetate) are the most often used for extracting phenolics from plant byproducts (Garcia-Salas et al., 2010; Tsao, 2010), which explains why the acetone and methanol extracts were more potent antioxidants with better phytochemical profiles. Moreover, acetone and methanol solvent extractions of pomegranate peel have been shown to yield better phenolic recovery and bioactivity than some other organic solvents extraction and/or water extraction (Zhang et al., 2007; Abid et al., 2017; Khalil et al., 2017; 2018; Thitipramote et al., 2019; Karthikeyan and Vidya, 2019; Chukwuma, et al., 2020; Akuru et al., 2022; Kumar et al., 2022).

The distinct distribution of the acetone extract (**Figure 4.8b**) suggests that extracts from this solvent performed uniquely in terms of the monitored parameters (i.e., X and Y variables). The differential clustering of the acetone extracts was influenced by its anti-lipid peroxidative and ORAC activities, which can be attributed to its higher TFC, TPC and ellagitannins constituents (punicalagin, granatin A, geraniin and casuarinin) (**Figure 4.8b**). Ellagic appears to be most influencing component influencing the remarkable radical scavenging effect of the methanol extract, which further influenced its distribution in the biplot (**Figure 4.8b**).

In the context of temperature dependent water/aqueous extraction, fresh peel of pomegranate fruit was used, since ellagitannins and punicalagins have been previously recovered from pomegranate husk by a fairly quick and reproducible method (Seeram et al., 2005). Generally, a consistent trend in the phenolic (**Figure 5.1**) and ellagitannin profiles (**Table 5.1**) was observed as extraction temperature was varied, which correlated with antioxidant profiles of the extracts (**Figures 5.2 – 5.5**). As extraction temperatures increased from room temperature (25 °C) to 37 °C, the polyphenols and ellagitannin contents, as well as the antioxidant capacity decreased, but gradually increased as the temperature increased beyond 37 °C and peaks at 78 °C. At 95 °C, there was a sharp decline in the polyphenols and ellagitannin contents, as well as the antioxidant capacity of the extract. A closer look at this trend suggests that enzymes like polyphenol oxidases (PPOs), which degrade polyphenols (Sikora et al., 2019) may play a key role, since the water extraction was done using freshly removed pomegranate fruit peel. The enzymes are known to be optimally active at around 37 °C, which suggests why the polyphenol content, as well as the antioxidant profiles of the extract reduced when temperature was increased from 25 °C to 37 °C. Also, temperatures beyond 37 °C may have compromised the integrity and functionality of the PPOs, thus the polyphenol content, as well as the antioxidant profiles were gradually recovered as extraction temperatures increased to 50 °C. Beyond this temperature, it is believed that heat gradually played a role in facilitating phenolic extraction, thus potentiating stronger antioxidant effects, which peaked at 78 °C. Moreover, it has been documented that high TPC is obtained at extraction temperature between 60 and 80 °C, while temperatures above 80 °C can lead to thermal degradation of the polyphenols and reduced TPC (Antony and Farid, 2022). Hence, there was a decline in the polyphenols and ellagitannin contents, as well as the antioxidant capacity of the extract when extraction was done at 95 °C.

The PCA scores plot (**Figure 5.7a**) also showed clear clusters of the samples indicating extraction temperature results in differential metabolite recovery patterns and antioxidant potency of the various extracts. A distinct grouping pattern can be visualized on the plot, with

the 78 °C aqueous extracts standing out and being the most alienated from the other extracts (**Figures 5.7b** and **5.8a**). The HCA dendrogram (**Figure 5.8b**) further emphasizes the clustering pattern observed on the PCA scores plot and the OPLS biplot. The Y variables, including *in vitro* anti-linoleic acid peroxidation, TFC, ORAC, ABTS, and cellular anti-lipid peroxidation were closely grouped around the 78 °C samples and largely within the same quadrant, which were clustered around the ellagitannins (X variables) (**Figure 5.8a**), suggesting that the distinct antioxidant potential of the extract at 78 °C was largely influenced by its ellagitannins profile. It is safe to speculate that extraction at 78 °C notably and optimally influenced the ellagitannins and antioxidant profile of pomegranate fruit peel. Most of the identified ellagitannins fairly influenced the ORAC and anti-lipid peroxidative capacities of the extracts. punicalagin, granatin A and ellagic acid are among the top five ellagitannins influencing the antioxidant profile of the extracts (**Figure 5.10**).

Comparing the dry peel solvent extractions to the fresh peel water extractions reveals a new observation. It was observed that the fresh peel water extractions, particularly at 65 - 78 °C temperature range yielded higher ellagitannins relative to the dry peel solvent extractions (**Tables 4.1** and **5.1**), suggesting that temperature-dependent water extraction of pomegranate peel may be more promising for ellagitannins recovery from pomegranate peel. Moreover, documented evidence suggests that using fresh removed by-products of pomegranate for aqueous extraction may be promising for recovering ellagitannins from pomegranate fruit waste, perhaps to a commercial scale (Seeram et al., 2005). It is however important to mention that the higher ellagitannins content of the fresh peel relative to the dry peel extracts was not replicated or mimicked in the in profiles, perhaps due to variability in experimental conditions, since the effects of extraction temperature (fresh peel) or extraction solvents (dry peel) on the antioxidant profiles of pomegranate peel was investigated separately.

6.1 General conclusion

In the context of ellagitannins from pomegranate dry peel, acetone and methanol solvent extraction may be promising for optimum ellagitannins recovery and antioxidant profile. Conversely, when conducting aqueous/water extraction, conducting fresh peel extraction at 78 °C may be promising for optimum ellagitannins recovery and antioxidant profile. It is also safe to speculate that fresh peel water extraction at optimum temperatures may recover more ellagitannins that extracting using the dry peel of pomegranate fruit. Thus, may be useful in

optimally recovering the ellagitannins from pomegranate peel, without sacrificing the antioxidant capacity, which may be of medicinal relevance in managing oxidative stress in many diseases.

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APPENDIX

CC_1CC_1

CC_CUT_220620_2 Sm (Mn, 2x3)

1: TOF MS ES-
BPI
4.09e3

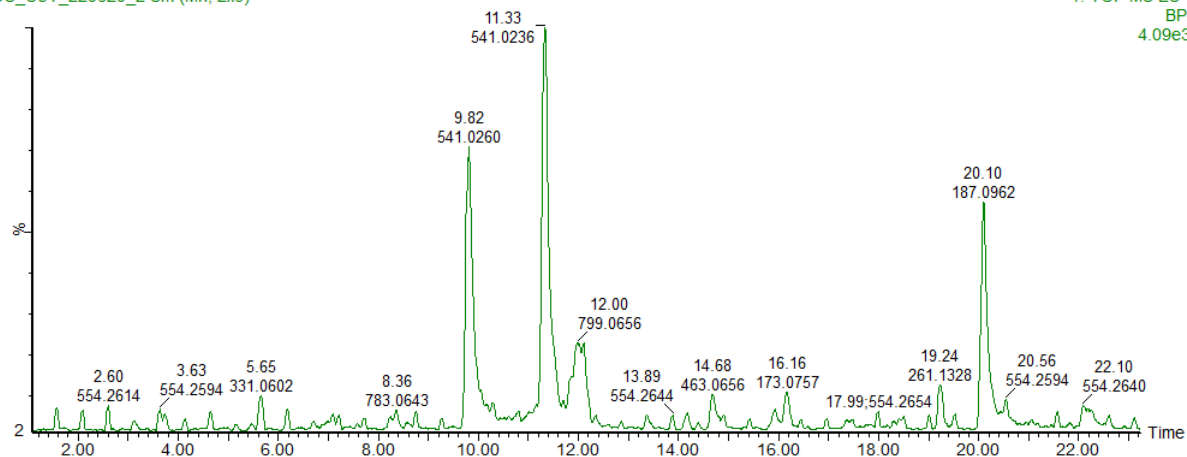
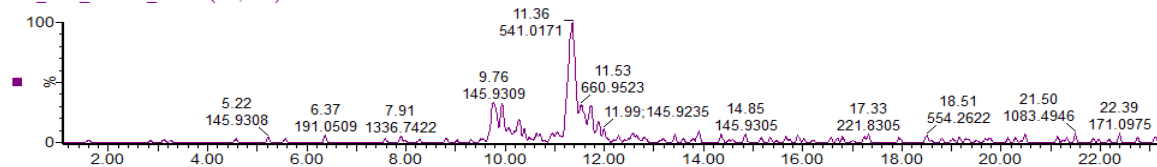


Figure: Sample chromatogram showing peaks for Puniculagin A (9.82 mins), Puniculagin B (11.33 mins) and Granatin A (12.00 mins)

100 ppm punicalagin

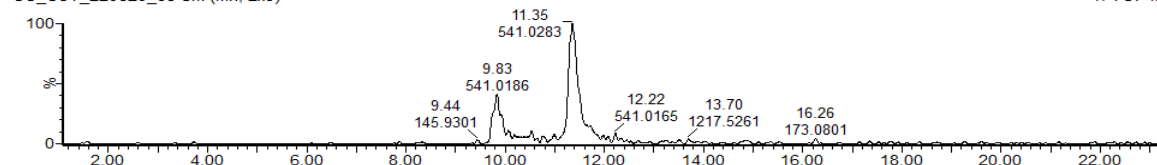
CC_CUT_220620_34 Sm (Mn, 2x3)

1: TOF MS ES-
1083
128



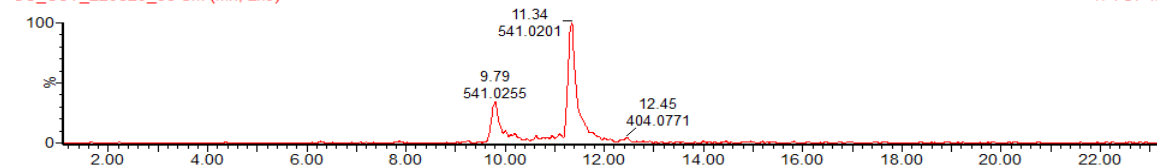
CC_CUT_220620_35 Sm (Mn, 2x3)

1: TOF MS ES-
1083
364



CC_CUT_220620_36 Sm (Mn, 2x3)

1: TOF MS ES-
1083
836



CC_CUT_220620_37

1: TOF MS ES-
1083
1.73e4

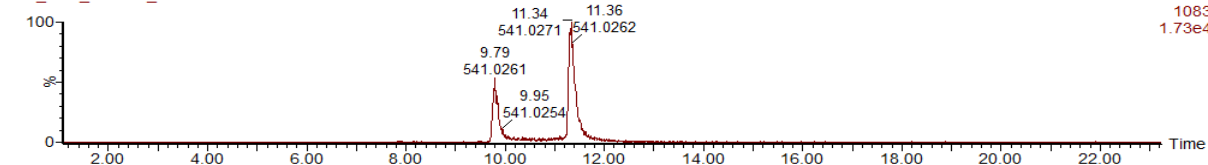


Figure: Chromatograms for punicalagin A and B standard

10 ppm ellagic acid

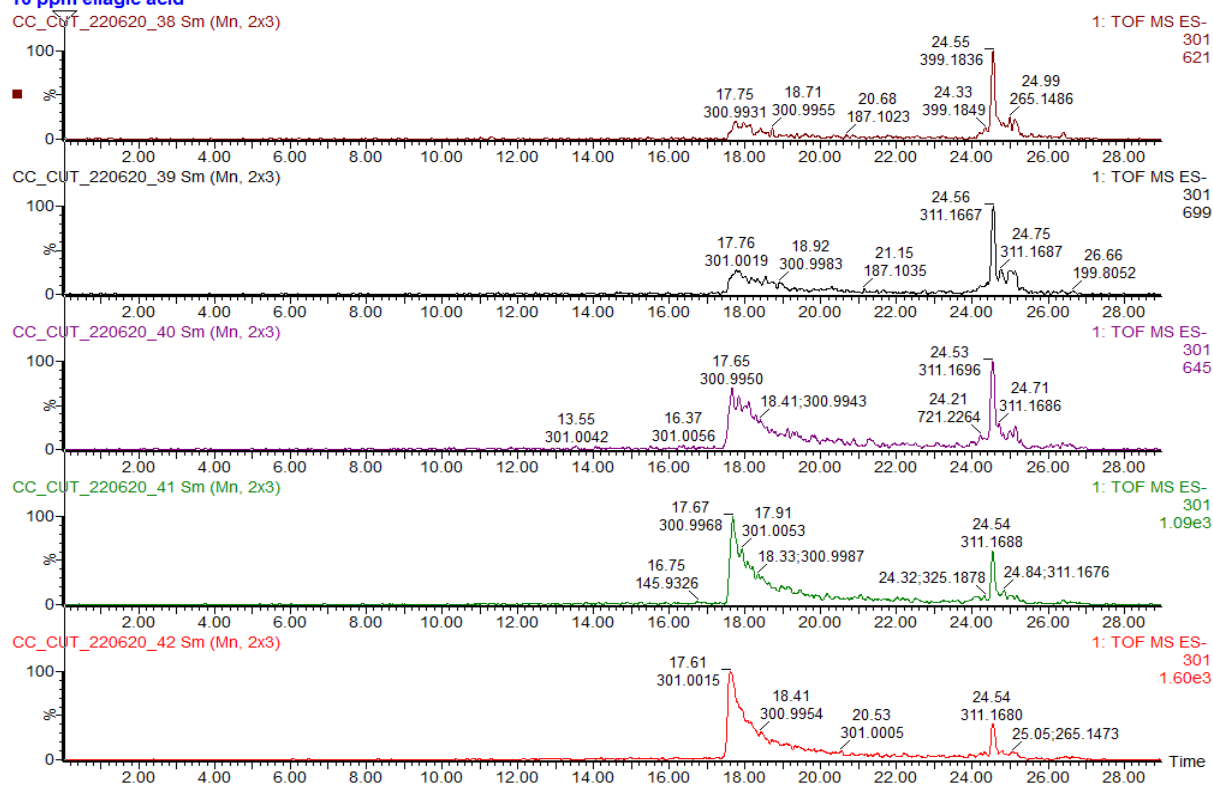
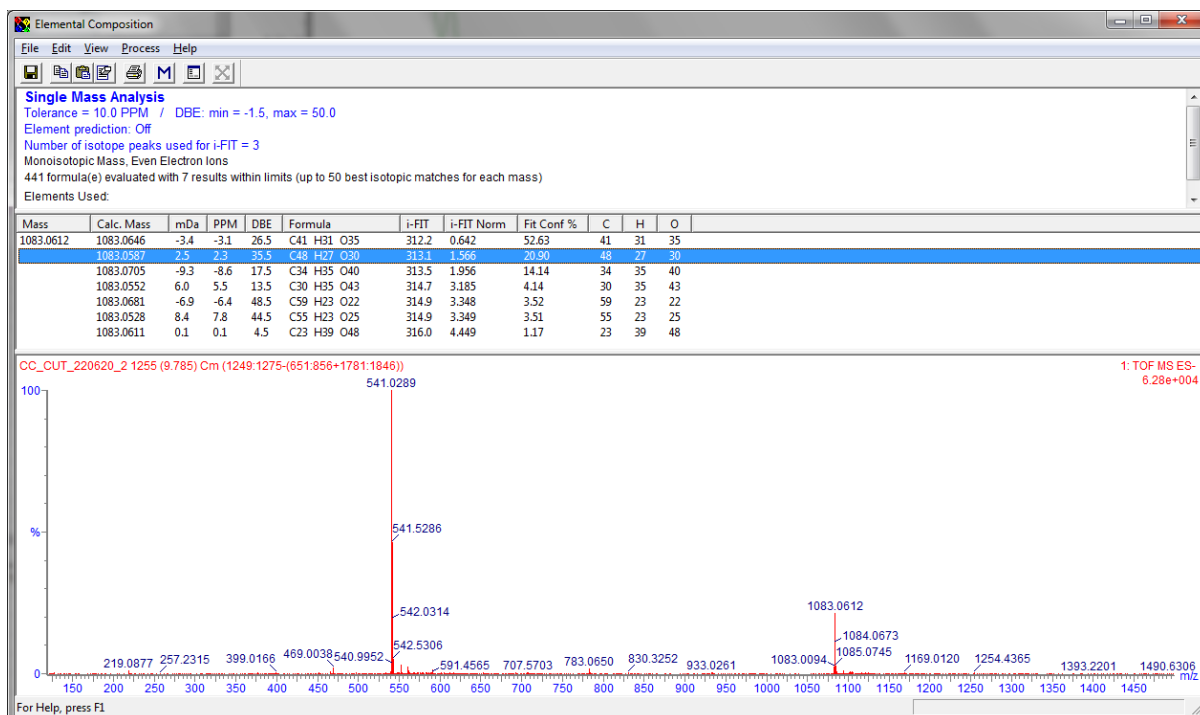
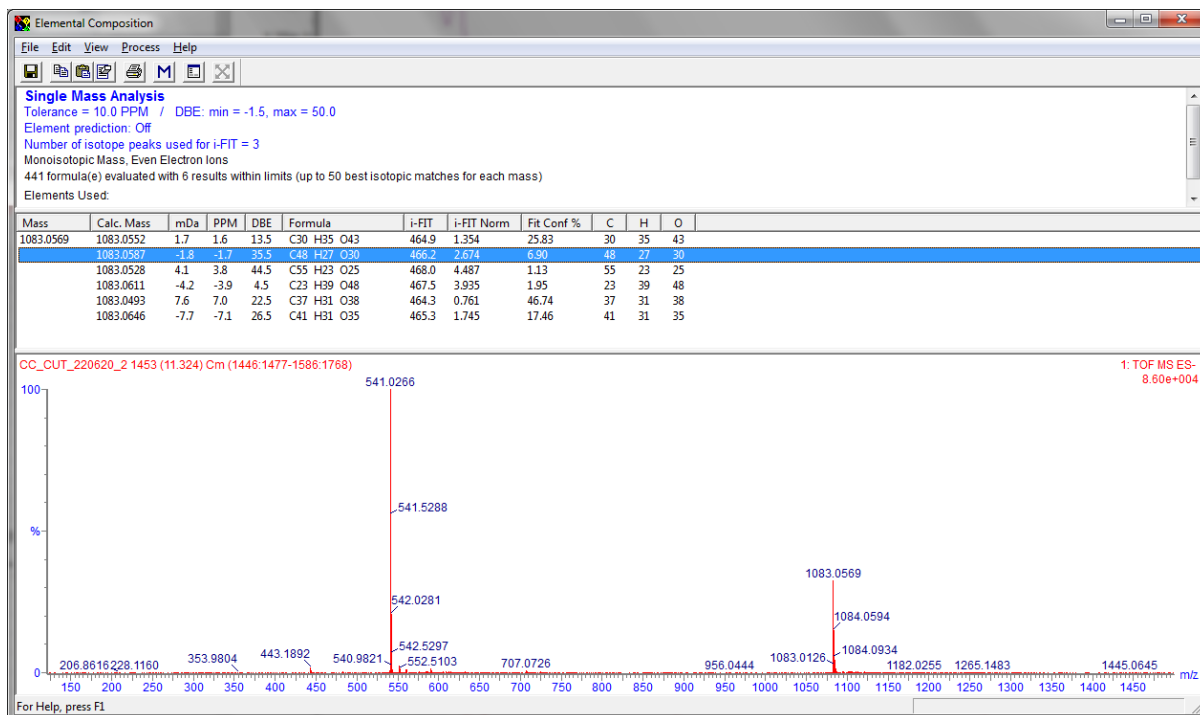


Figure: Chromatograms for ellagic acid standard

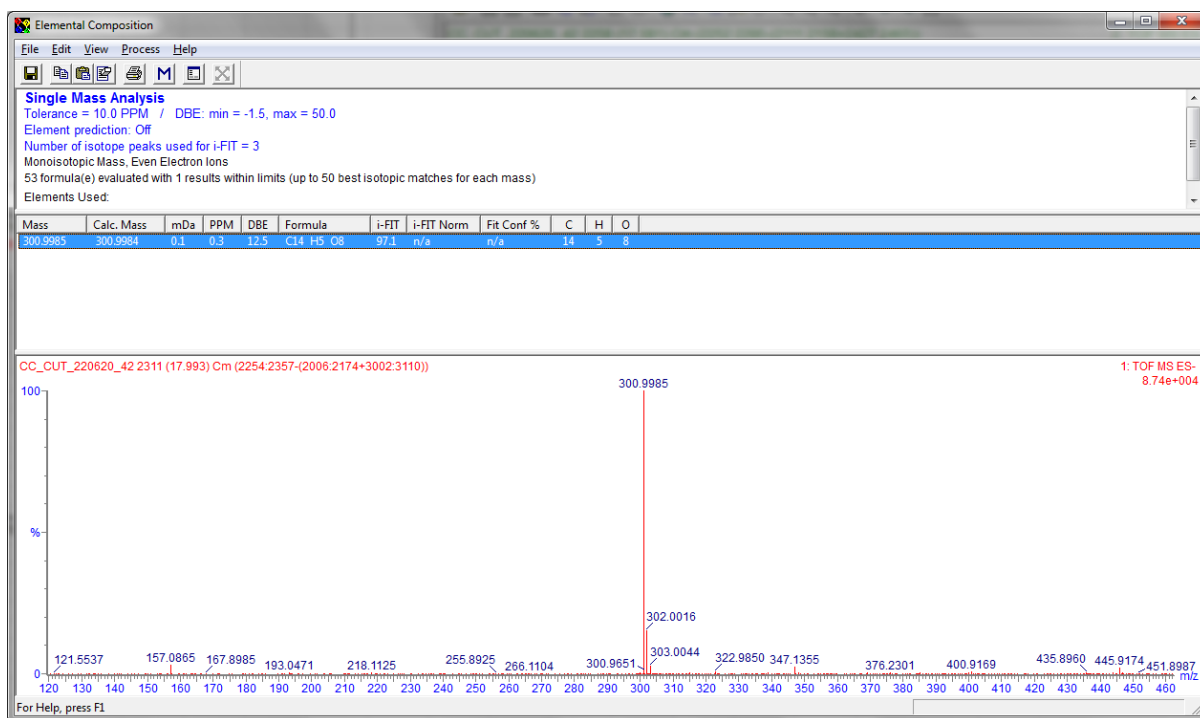
Below are the Single Mass Analysis of each of the analytes/compounds identified in the ellagitannins from the different solvent extracts:



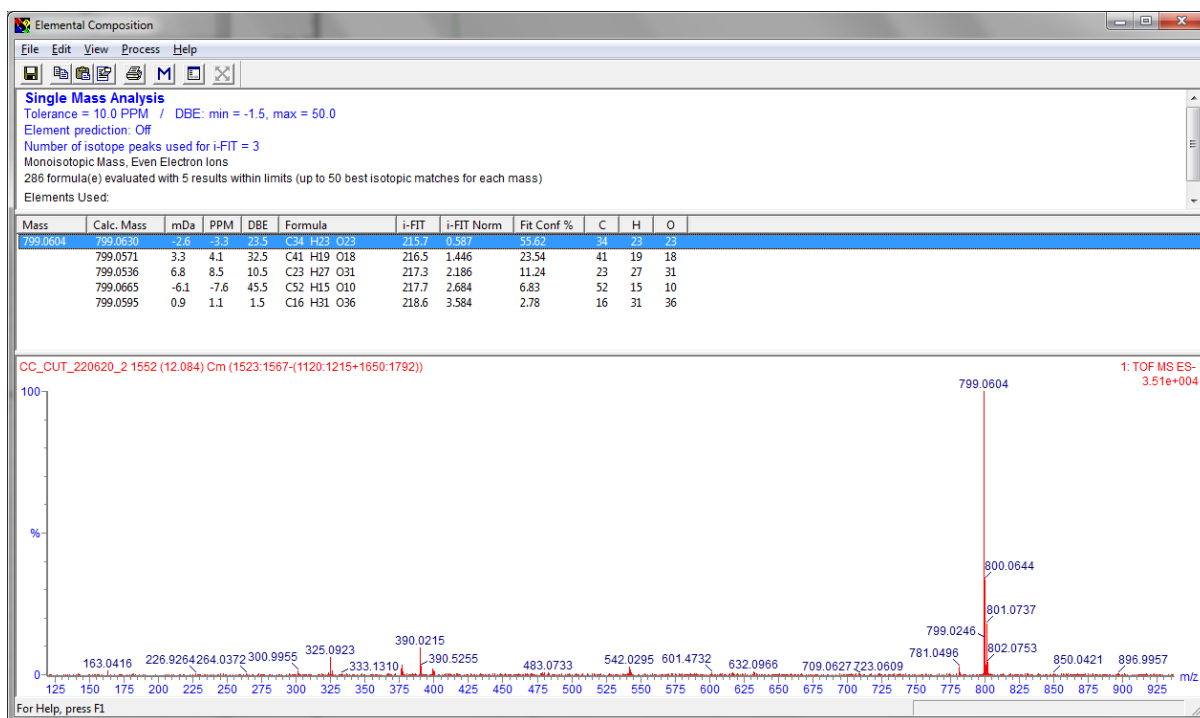
Punicalagin A



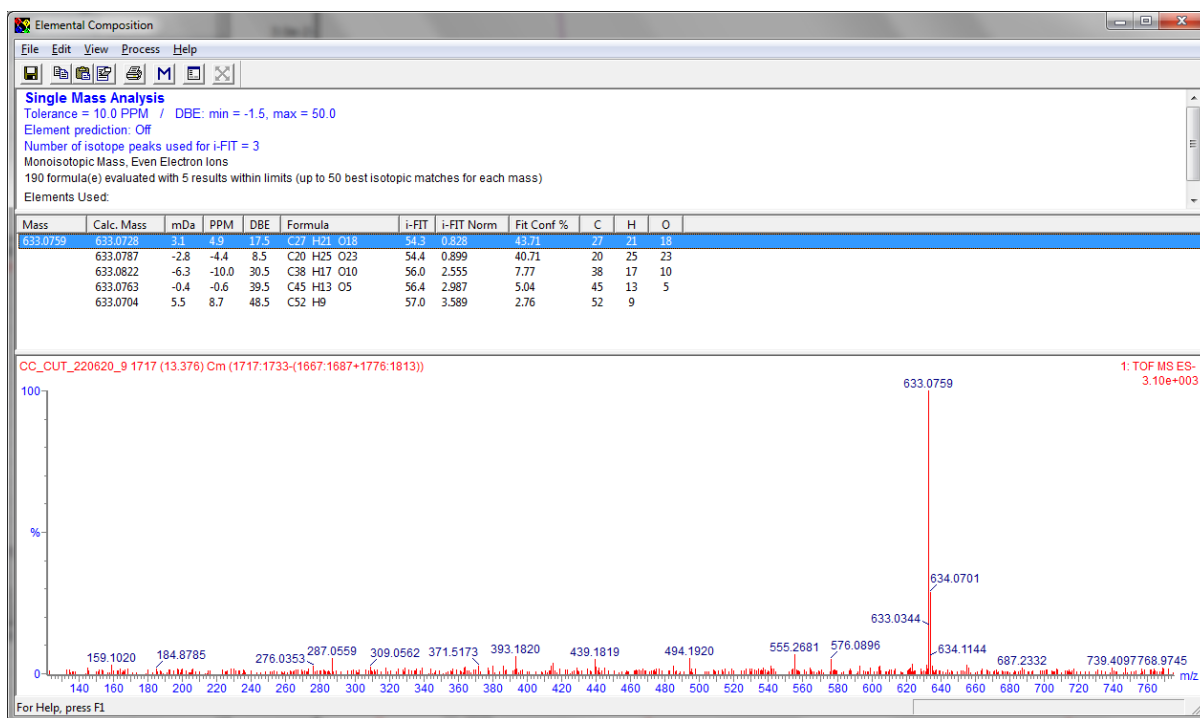
Punicalagin B



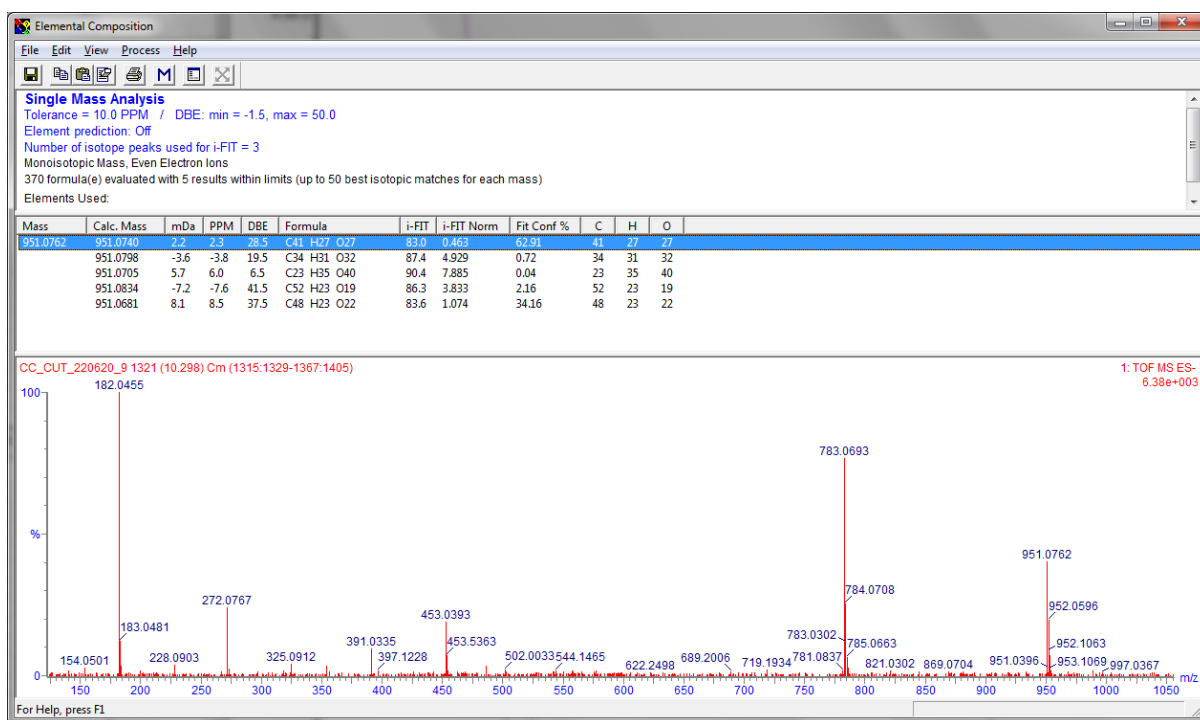
Ellagic acid



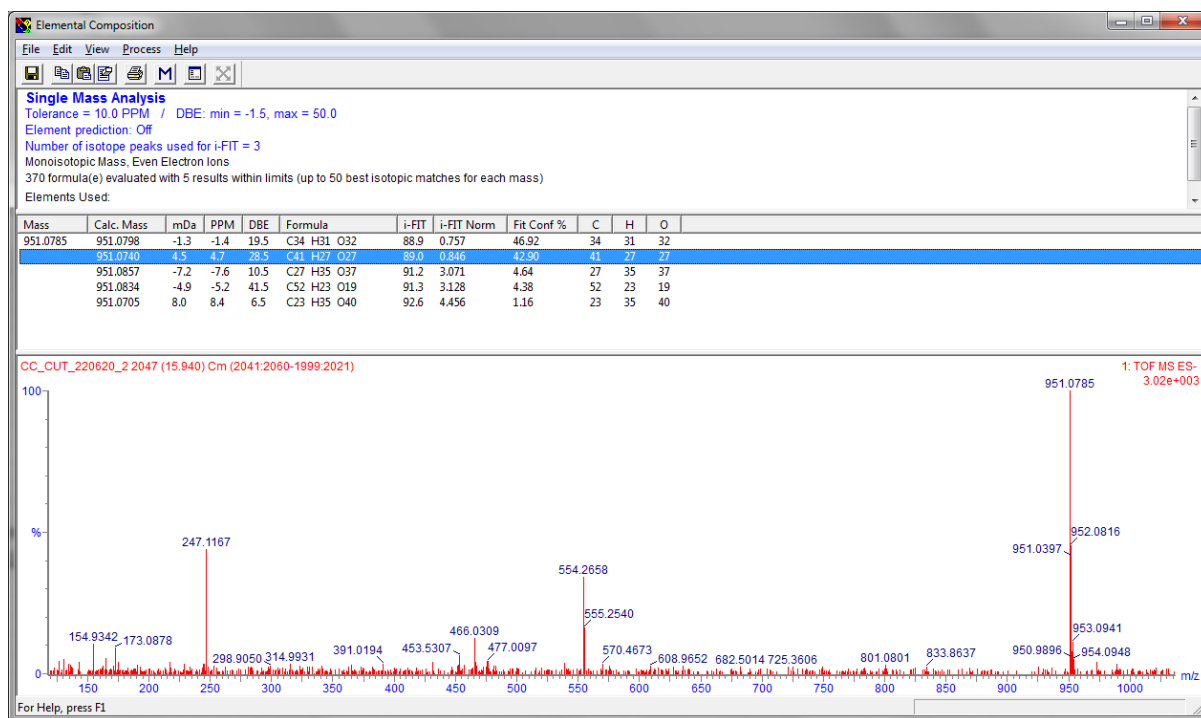
Granatin A



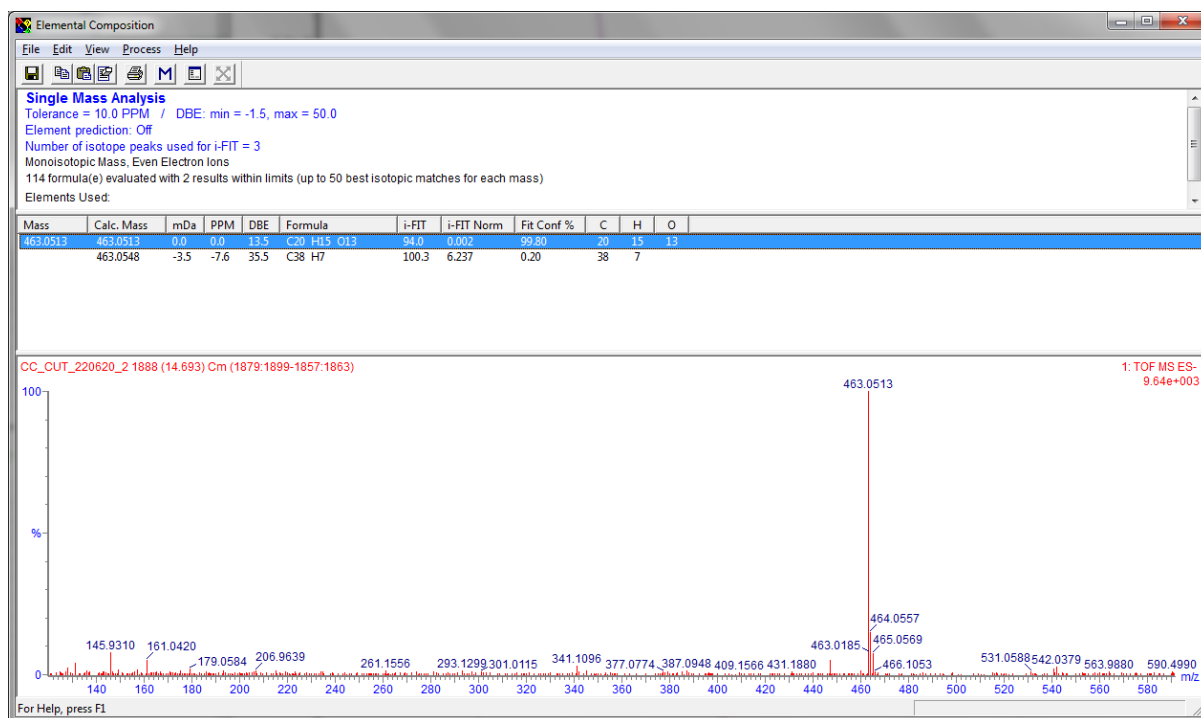
Corilagin



Casuarinin



Geraniin



Quercetin hexoside



Effect of solvent extraction on the antioxidant and phytochemical profiles of ellagitannins from “wonderful” pomegranate peel: an advanced chemometrics analysis

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Abstract

Ellagitannins are the predominant bioactive tannins present in the peel of pomegranate. Studies have explored different extraction solvents to maximize recovery of the bioactive phytochemicals in pomegranate peel but lack proper statistical correlation between the extraction solvent, phytochemistry and bioactivity. This study employed advanced chemometrics models, including principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) in determining the solvent extraction (among methanol, ethanol, acetone and water) that will yield optimum antioxidant and ellagitannins profiles from “Wonderful” pomegranate peel. Acetone extraction had the highest ($p < 0.05$) total phenol (TFC) and flavonoid contents and strongest ($p < 0.05$) Fe^{3+} reducing effect, while methanol extraction had the strongest ($p < 0.05$) radical scavenging activity. The oxygen radical absorbance capacity (ORAC) and anti-lipid peroxidative activities of both solvent extractions outperformed that of the ethanol and water extractions. Tannins purified using Amberlite® XAD16N resin were highest for acetone and methanol extractions. LCMS-quantified ellagitannins in the tannins varied for the different extractions. PCA and OPLS models adequately characterized, described and predicted the variation and patterns in the antioxidant and ellagitannins datasets (up to 95% for PCA). OPLS bi-plot showed that the high ellagic acid constituents and total tannins yield of the methanol extraction influenced its potent radical scavenging activity, while the ellagitannin constituents including punicalagins, granatin A, geraniin and casuarinin influenced the high TFC, as well as the potent Fe^{3+} reducing, ORAC and anti-lipid peroxidative activities of acetone extraction. Acetone and methanol extraction of pomegranate peel are promising for optimum ellagitannins recovery and antioxidant profile.

Keywords Pomegranate peel · Solvent extraction · Antioxidant · Ellagitannins · Advanced chemometrics analysis

Introduction

Pomegranate fruit is well recognized for its benefits on oxidative and cardiovascular health [1]. It, also, has antibacterial, anti-diabetic, anti-atherosclerotic, anti-inflammatory and anti-allergic potentials [1]. These medicinal properties can be attributed to the fruit's many secondary metabolites—the most significant being the ellagitannins (punicalagin and ellagic acid), as well as other polyphenols like flavonoids, anthocyanidins, flavonones, catechins and phenolic acids [2–4]. These phenolic compounds account for up to 92% of the fruits antioxidant property and are mostly concentrated in the fruit peel and juice compared to other parts of the fruit [5, 6]. Evidence of the radical scavenging property of pomegranate peel has been documented [3] and in some cases was stronger than that of the pulp [7]. In rats the peel improved

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antioxidant status, thus protecting against hepatic oxidative damage caused by tetrachloromethane [8].

The rising fruit production and commercialization of pomegranate causes accumulation of the fruit's wastes, particularly the peels [9]. To avoid wastage, reduce environmental biomass and maximize the therapeutic potential of the fruit's wastes, the fruit's peel has been researched as a possible source of antioxidants [3, 4, 7, 8, 10]. The results offer a justification for the potential relevance of the peel in managing oxidative stress and insults.

Several solvent extracts have been explored for extracting phytoconstituents with promising antioxidant and other bioactivity profiles from pomegranate peel. This is because solvent polarity largely influences the type of compounds extracted from a plant material, which consequently influences the antioxidant and other bioactivity profiles of the resulting extracts [1]. Previous data have shown that the type of solvent extraction strongly influences the chemical and antioxidant profiles of pomegranate fruit peel extracts [3, 4, 7, 8, 10]. However, these studies lack a proper statistical correlation between the extraction solvent, phytochemistry and antioxidant activity. The studies lack statistical validation to determine the influence of the solvent extraction on the phytochemical and antioxidant profile of pomegranate peel extracts. Thus, may not be able to ascertain the solvent extraction for optimum recovery of antioxidant ellagitannins from pomegranate peel. Therefore, this study was done to determine the effect of different solvent extraction on the antioxidant and phytochemical profiles of ellagitannins from pomegranate peel using advanced chemometrics analyses. This will potentially provide a more reliable information regarding what extraction solvent system will facilitate optimum recovery of antioxidant ellagitannins from pomegranate peel.

Materials and methods

Fruit procurement and removal of peel

Class 1 (processing grade) "Wonderful" variety of pomegranate fruits were procured from Sonlia Marketing, a subsidiary of Sonlia Fruit Packhouse (Wellington, Western Cape Province, South Africa) on 03 May 2021. The fruits were washed, and the peel was thoroughly removed. The fruit peel was dried at 37 °C until all the moisture were completely removed and a constant dry weight was attained.

Preparation of dried peel and solvent extraction

The dried peel was pulverized using a Kinematica POLY-MIX® PX-MFC 90 D milling machine equipped with a blade grinding attachment [Lasec (Pty) Ltd, Cape Town,

South Africa]. About 250 g of the pulverized dried peel was separately extracted using 1 L of methanol, ethanol, acetone and water. The extraction was done at room temperature on an orbital shaker [OrbiShake, Model 262, Labotec (Pty) Ltd, Johannesburg, South Africa] set at 125 rpm for 72 h. The fruit peel debris was removed by filtering with a filter paper. The filtrate of the organic extracts was concentrated using a Buchi Rotavapor® R-300 [Labotec (Pty) Ltd, Johannesburg, South Africa] and dried under a fume hood. The filtrate of the water extract was dried using a Martin Christ Alpha 1–2 LDplus Freeze Dryer (Separations, Johannesburg, South Africa). The recovered extracts were stored in air-tight vials at – 20 °C until they were used.

Total phenol and total flavonoid contents measurement in extracts

The total phenol content (TPC) and total flavonoid content (TFC) of the extracts were measured using a previously reported protocol [10] and expressed as mg/g gallic acid equivalent (GAE) and mg/g quercetin equivalent (QE), respectively. The extracts were tested at 45 µg/mL.

Antioxidant analyses

The antioxidant effect of the extracts was analysed at 45 µg/mL using the following in vitro and cell-based experimental models described below. The activity of the extracts was compared to an equivalent concentration of the positive controls (ascorbic acid and Trolox) or standard phytoconstituents (punicalagin A + B and ellagic acid).

Radical scavenging action

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS*+) scavenging effects of the extracts were determined using methods described in a previous report [11] and expressed as percentage radical scavenging activity.

Oxygen radical absorbance capacity (ORAC) assay

A previous method [12] was slightly modified to perform this assay. 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-methylpropionamide) 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) or the standards (punicalagin A + B and ellagic acid) at the tested concentration (45 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 fluorescence (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:

$$\text{ORAC (\%)} = \frac{\text{Slope}_{\text{Control}} - \text{Slope}_{\text{Test}}}{\text{Slope}_{\text{Control}}} \times 100$$

Fe³⁺ reducing activity

A previous method [11] was slightly modified to perform this assay. First, 25 µL of the tested samples or positive controls (ascorbic and Trolox) (45 µg/mL in reaction mixture) or increasing concentrations (3.75–60 µg/mL in reaction mixture) of gallic acid standard, 25 µL of 0.2 M phosphate buffer (pH 6.6) and 25 µL of 1% potassium ferricyanide were mixed in the wells of a 96-well clear bottom plate. The mixture was incubated for 20 min at 50 °C, before successively adding, 25 µL of 10% trichloroacetic acid, 100 µL of distilled water and 50 µL of 0.1% FeCl₃ solution. Thereafter, absorbance was measured at 700 nm and blanked with the sample blank. The Fe³⁺ reducing activity was determined from a gallic acid standard curve and computed as mg/g equivalent of the gallic acid (mg/g GAE) using the following formula:

$$\text{FRAP (mg/gGAE)} = \frac{C \times SV}{M},$$

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”.

Measurement of inhibitory action on FeSO₄-induced linoleic acid peroxidation in vitro

To perform this assay, the method reported by Ramorobi et al. (2022) [13], which was modified from the method reported by Choi et al. (2002) [14] was adopted.

Measurement of inhibitory action on FeSO₄-induced lipid peroxidation in Chang liver cells

The protocol reported previously [4] was slightly modified to perform this assay. First, Chang liver cells (ATCC® CCL-13™, ATCC, Virginia, USA) were grown in an EMEM media, which contained 10% FBS. When the cell confluence reached 85%, the cells were trypsinized and plated into a 96-well plate with 200 µL aliquot of the culturing medium. The cell density in each well of the plate was about 8000 cells. The plate was incubated for 36 h in a CO₂ incubator (NÜVE EC 160, Ankara, Turkey) set at 5% CO₂ supply and 37 °C temperature, during which the cells attached to the plate. After the incubation, the medium in the wells was removed by aspiration. Thereafter, the tested samples were dissolved in fresh culturing medium, which was added to designated wells of the plate. The tested samples or the positive controls (ascorbic acid and Trolox) or the standards (punicalagin A + B and ellagic acid) were added to wells at the tested concentration concentration (45 µg/mL in the incubation medium). Some wells were assigned as the control and negative control, which contained the solvent (water or 0.5% DMSO) used in dissolving the tested samples. The plate was incubated for 30 min. Thereafter, 50 µL of FeSO₄·7H₂O solution (1 mM in incubation medium) was aliquoted into the wells containing the tested samples and the wells that were assigned as the negative control to initiate oxidative stress. An equivalent volume of distilled water was aliquoted into the wells that were assigned as the control. Immediately, the plate was put into the CO₂ incubator and kept there for 1 h. Thereafter, the medium in the wells was removed by aspiration and replaced with 250 µL of cold lysis buffer (50 mM sodium phosphate buffer with 0.5% v/v Triton X-100, pH 7.5). A gentle agitation was applied to lyse the cells in the wells. Next, the plate was immediately centrifuged for 1 min at 5000 g and 4 °C to pellet the cell debris. The supernatant was quickly collected and kept on ice. Immediately, lipid peroxidation was measured in the supernatant as described below.

Approximately 100 µL of the supernatant or malondialdehyde standards (0, 7.5, 15, 22.5, 30, and 45 µM in the assay volume) was put into designated vials. Next, 0.25% w/v thiobarbituric acid, 20% v/v acetic acid, and distilled water were aliquoted into the vials at a volume ratio of 500 µL:200 µL:200 µL, respectively. A boiling water bath was then used to heat all the vials for 50 min. After heating, the vials were cooled under room temperature and centrifuged for 10 min at 3500 g and ambient temperature. Two hundred microlitres of the supernatant in each vial was aliquoted into a 96-well plate and absorbance was read at 532 nm. A malondialdehyde standard curve was used to compute the lipid peroxidation, which was estimated as thiobarbituric acid reactive substances. The inhibitory activity (%) of the tested samples on lipid peroxidation was calculated as follows:

Inhibition (%)

$$= \frac{(E_{\text{Negative control}} - E_{\text{Normal control}}) - (E_{\text{Test}} - E_{\text{Normal control}})}{(E_{\text{Negative control}} - E_{\text{Normal control}})} \times \frac{100}{1},$$

where, “*E*” means “Extrapolated equivalent malondialdehyde concentration”.

Crude recovery of tannins from extracts

Tannins were recovered from the extracts using an Amberlite® XAD16N resin (product code: XAD16; Sigma Aldrich, Johannesburg, South Africa) according to a protocol slightly modified from a previous article [15]. First 1.52 g of the extracts was dissolved in 100 mL of distilled water and filtered with a filter paper. The filtrate was collected as a yellowish-brown liquid (Fig. 1a). Approximately 35 g of the XAD16N resin was first washed twice with methanol and then equilibrated with distilled water in a sealed flash column for 12 h (Fig. 1b). After the 12 h equilibration, the

flash column was connected to a water jet vacuum pump and the distilled water was completely removed using the water jet vacuum pump. Next, the extract solution was gradually loaded onto the XAD16N resin in the flash column (Fig. 1c). The column was continuously eluted with distilled water using the water jet vacuum pump until the eluent consistently remained colourless and all the tannins in the extract were trapped in the XAD16N resin (Fig. 1d). The column was vacuum aspirated to dry up the remaining water. Next the trapped ellagitannins/tannins were gradually eluted using methanol (Figs. 1e – h). The eluent was air-dried in a fume hood. The recovered tannins fractions were weighed, and the percentage yield was computed using the formula below. The fractions were stored at -20 °C until further analysis.

Yield (%)

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

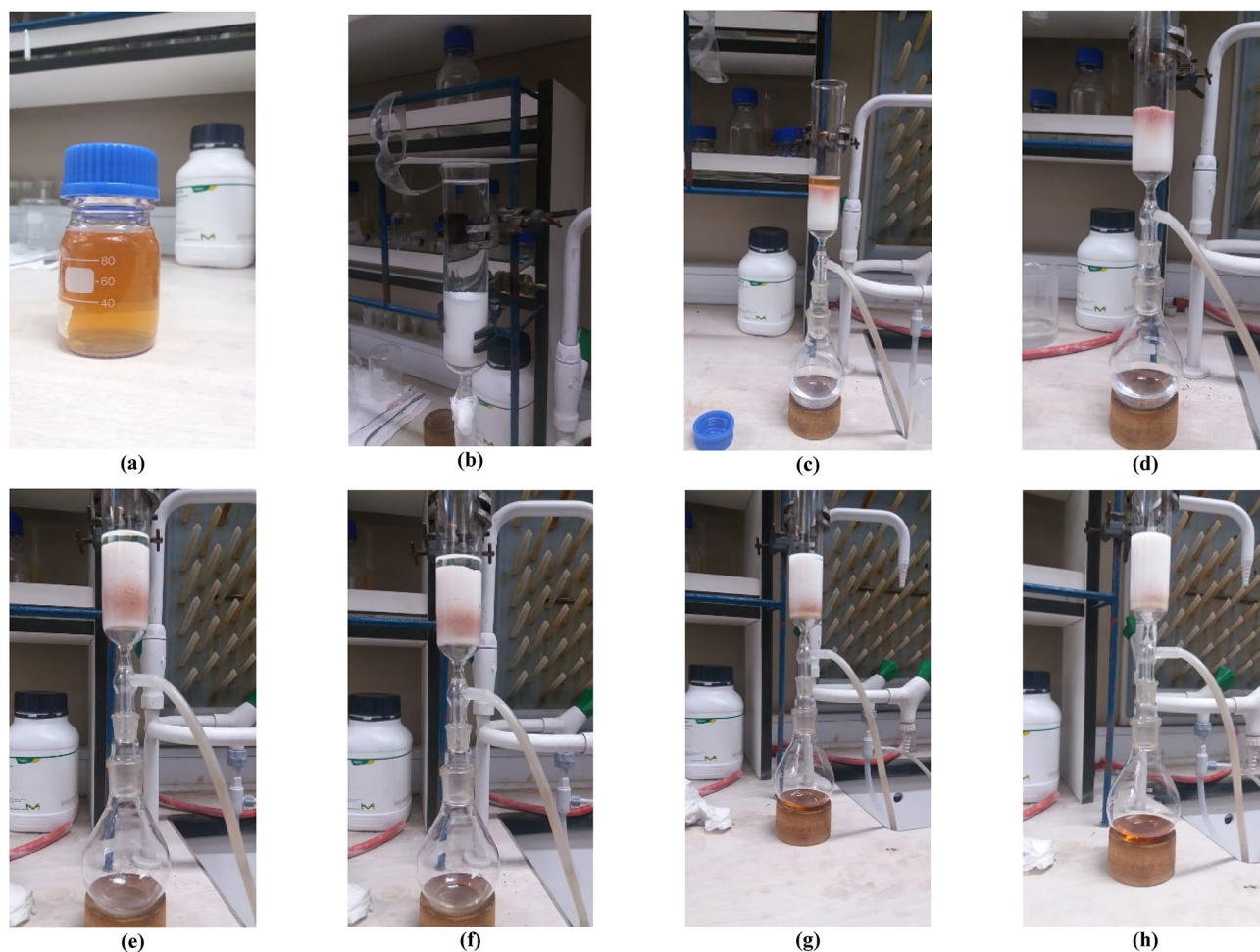


Fig. 1 Images showing the crude purification process of tannins from the extracts using XAD16N resin

Liquid chromatography—mass spectroscopic (LCMS) quantification of ellagitannins

The LCMS analysis was done using a Waters Synapt G2 (Waters Corporation, MA, USA), ESI probe, ESI Pos, Cone Voltage 15 V. Liquid chromatography was operated with an Acquity binary solvent manager. A HSS T3 column with dimension of 2.1×150 mm and particle size of $1.8 \mu\text{m}$ (Waters Corporation, MA, USA) was used for chromatographic separation. The mobile phase was made up of A (0.1% formic acid in HPLC grade water) and B (0.1% formic acid in HPLC grade acetonitrile) solvents. The flow rate for mobile phase was 0.25 mL/min. A gradient chromatographic condition was applied as follows: 100% of A and 0% of B for 10 min; then 72% of A and 28% of B for 21 min; then 60% of A and 40% of B for 50 s; then 0% of A and 100% of B for 2 min. Chromatographic separation was done at 30°C . Samples were reconstituted in 1 mL of 50%:50% acetonitrile and water, centrifuged at 14,000 rpm for 5 min and filtered using a $0.22 \mu\text{m}$ filter. A 20 μL injection volume was applied. A UV wavelength of 254 nm was used to record the chromatographic signals. Punicalagin A, punicalagin B and ellagic acid in the ellagitannins fraction were identified and quantified from calibration curves established using a range of calibration standards prepared from stock solutions of Punicalagin A + B (product code: PHL80524, Sigma Aldrich, Johannesburg, South Africa) and ellagic acid (product code: 14,668, Sigma Aldrich, Johannesburg, South Africa). The other compounds (granatin A, corilagin, casuarinin, geraniin and quercetin hexoside) in the tannins fractions were identified and quantified in a relative fashion against ellagic acid. They were identified based on accurate mass elemental composition and quantified using extracted ion chromatography against ellagic acid. The quantification was presented as a triplicate analysis.

Statistical analysis

Statistical analysis was done on the Window's version 27.0 of IBM SPSS (IBM Corp, Armonk, NY, USA). Multiple comparison of data averages among the groups (extracts or standards) was done for the different studied parameters using the Tukey multiple post hoc test and one-way analysis of variance (ANOVA). Statistically significant difference (p) was set at $p < 0.05$ when comparing the mean values of the different groups.

Advanced chemometrics analysis

Advanced chemometrics models were employed to scrutinize the data to extract maximum useful information and interpret the patterns and distribution in the data. Principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) were performed using

SIMCA-P + 16.0 software (Umetrics, MKS Instruments Inc., Sweden) as described by Gbashi et al. (2020) [16]. Prior to PCA and OPLS analysis, the data were mean-centered, and Unit-Variance scaled since both PCA and OPLS are sensitive to the scale of the input variables. Further, for OPLS, the data were classified into X variable (i.e., predictors) and Y variables (i.e., responses). The X variables are the independent predictors, which included ellagitannins or total tannins yield, punicalagin A, punicalagin B, ellagic acid, granatin A, corilagin, casuarinin, geraniin and quercetin hexoside. These are essentially the extracted components/metabolites from the peel biomass present in the solvent extracts. The Y variables consisted of the various investigated antioxidant assays, namely TPC, TFC, Fe^{3+} reducing activity (FRAP), DPPH and ABTS radical scavenging activity, oxygen radical absorbance capacity (ORAC), in vitro anti-linoleic acid peroxidation and anti-lipid peroxidation in Chang liver cells. Class labels were accordingly assigned to the observations for extracts obtained from the various extraction solvents, namely methanol, ethanol, water, and acetone. This was crucial because OPLS is a supervised model and requires information about observation classes in order to learn the patterns in the data. This essentially enables the algorithm to better approximate the relationships in the data and improve the interpretability and predictive capability of the model.

Model fit evaluation and validation was performed by assessing various modelling diagnostic parameters such as R^2X (fraction of explained X variation in the component using the X model), $R^2X(\text{cum})$ (combined fractions of X variation modelled up to the specified component using the X model), R^2Y (fraction of explained Y variation in the component using the Y model), $R^2Y(\text{cum})$ (combined fractions of Y variation modelled up to the specified component using the Y model), R^2 (fraction Y variation modelled by the X model in the component), $R^2(\text{cum})$ (cumulative fractions of explained variation in the Y data using the X model up to the specified component). The coefficient of determination (R^2), root mean square error of estimation (RMSEE), and root-mean-standard error for cross-validation (RMSECV) for the observed versus predicted values of the response variables were also evaluated. The statistical significances of model components were determined based on Rule 1 (R_1), Rule 2 (R_2), or Rule 3 (R_3). For R_1 , a component is considered significant when $Q^2 > \text{Limit}$. For R_2 , a component is significant if $Q^2V > \text{Limit}$. For R_3 , in terms of PCA, a component is labeled undecided (U) when it is insignificant. However, if the subsequent component is significant and has similar eigenvalues (tolerance of 5%) as the previous component, then both components are considered significant, and the component labeled undecided is re-labeled R_5 . The specific calculations and determinations of the Limits for both PCA and OPLS, as well as a description of the model validation parameters are described in the SIMCA® manual [17].

Results

Total phenol and total flavonoid contents

The total phenol content (TFC) of the acetone extract was significantly ($p < 0.05$) higher than that of the other extracts (Fig. 2a). The ethanol extract had the least TFC, which was significantly ($p < 0.05$) lower than that of the methanol and water extracts. The total flavonoid content of the acetone extract was more than 2 folds compared to that of the other extracts (Fig. 2b).

Fe³⁺ reducing and radical scavenging antioxidant properties

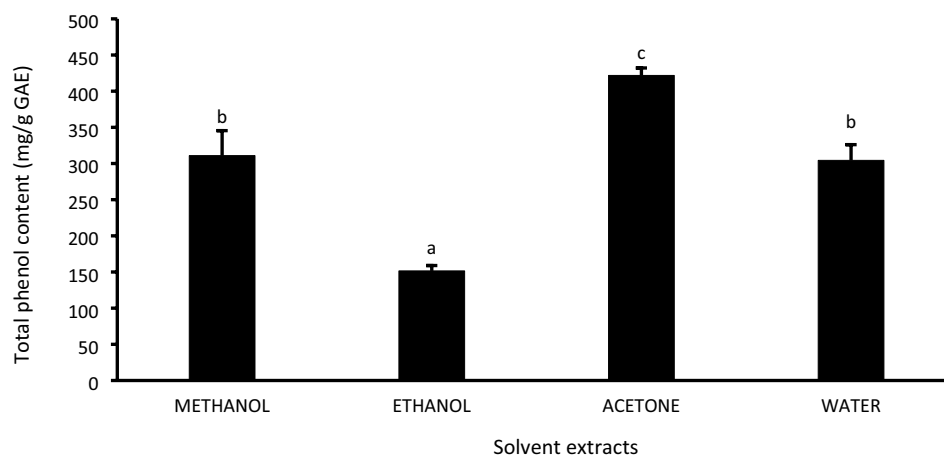
Although not as potent as ascorbic acid, the Fe³⁺ reducing activity of the acetone extract was significantly ($p < 0.05$)

stronger than that of Trolox, as well as the other extracts (methanol, ethanol and water extracts) (Fig. 3a). The methanol and water extracts, however, outperformed the ethanol extracts.

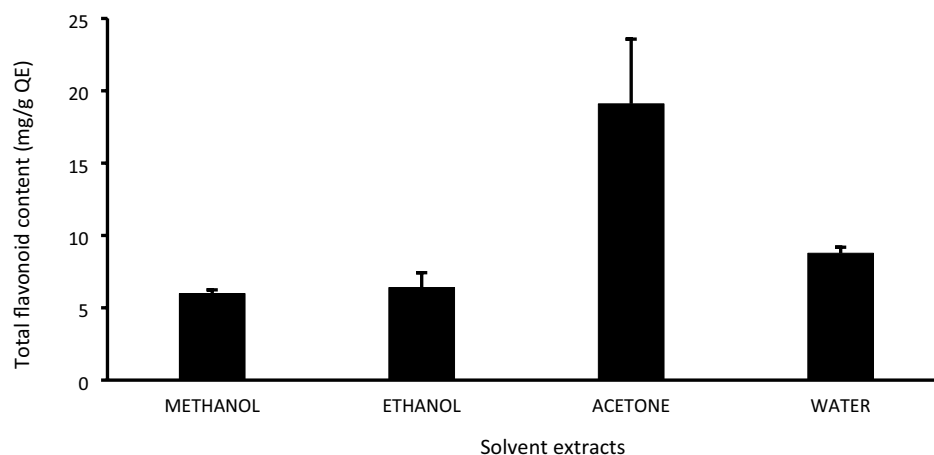
The DPPH radical scavenging activity of the methanol extract was statistically comparable to that of ascorbic acid and Trolox (Fig. 3b) and significantly ($p < 0.05$) stronger than the activities of the other extracts. The ethanol extract had the least potent DPPH radical scavenging activity, which was significantly ($p < 0.05$) less potent than that of the methanol and water extracts. The ABTS radical scavenging activity of the extracts followed similar trend as their DPPH radical scavenging activity (Fig. 3b).

The oxygen radical absorbance capacity (ORAC) of the acetone and methanol extracts was notably stronger than that of the other extracts and statistically comparable to the activity of ellagic acid and punicalagin A + B standards. Among

Fig. 2 **a** Total phenol content and **b** total flavonoid content of the different solvent extracts. Data are presented as mean $\pm$ SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters

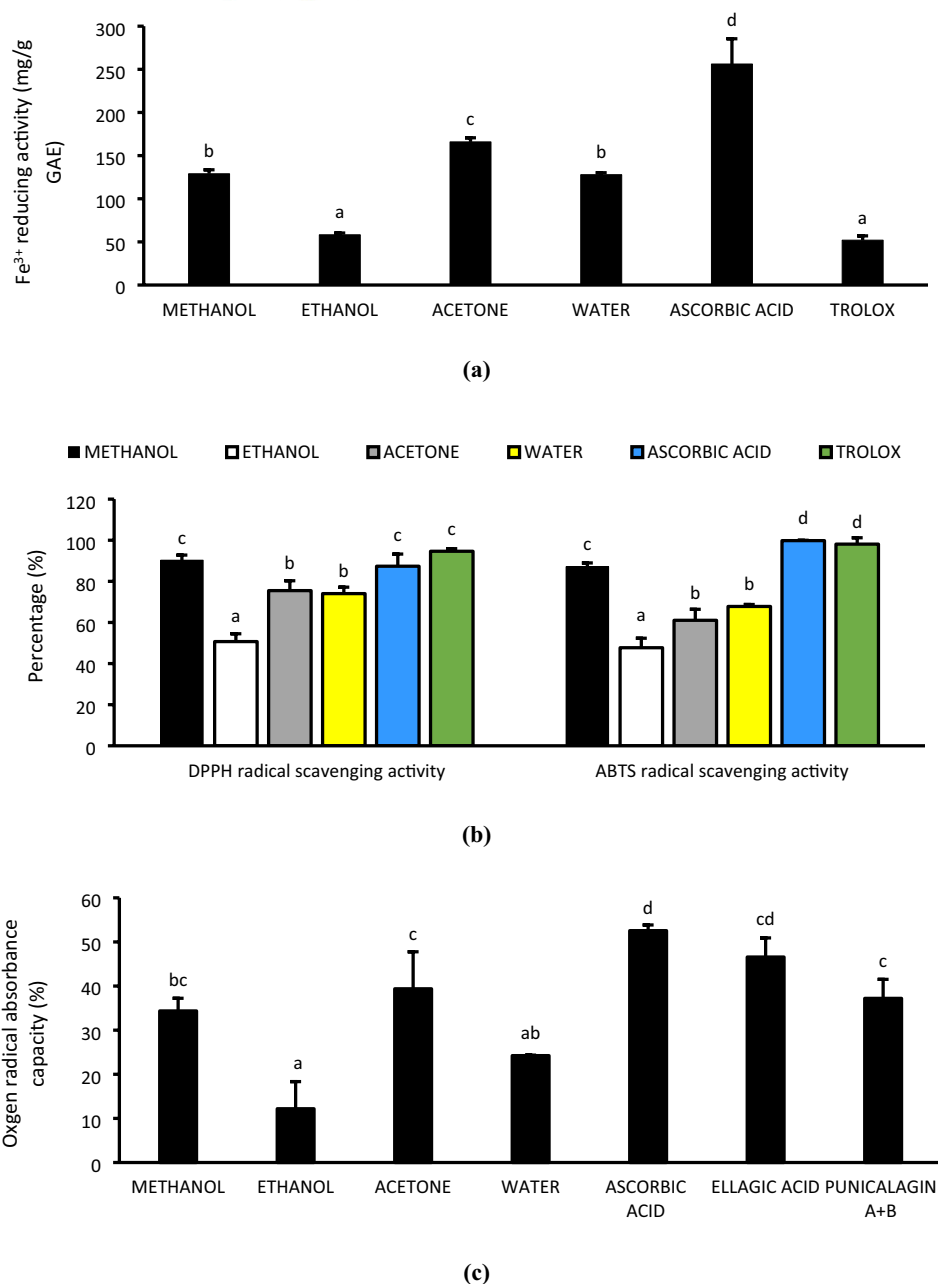


(a)



(b)

Fig. 3 **a** Fe^{3+} reducing, **b** DPPH and ABTS radicals scavenging and **c** oxygen radical absorbance antioxidant capacities of the different solvent extracts. 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



all the extracts, the acetone extracts had the most potent ORAC.

Anti-lipid peroxidative antioxidant properties

The ability of the extracts to inhibit linoleic acid peroxidation in vitro (Fig. 4a) and lipid peroxidation in Chang liver cells (Fig. 4b) is shown in Fig. 4. The acetone and methanol extracts were the most potent among the extracts. Their activity was statistically comparable to that of ascorbic acid, Trolox, ellagic acid and punicalagin A + B standards.

Total tannin yields and ellagitannins phytochemical profile

The yield of total tannins from the extracts is presented in Table 1. The yield from the methanol and acetone extracts was significantly higher than that of the ethanol and water extracts, with the ethanol extract having the least yield. LCMS-quantified phytochemicals included punicalagin A, punicalagin B, ellagic acid, granatin A, corilagin, casuarinin, geraniin and quercetin hexoside (Table 1 and supplementary material). The ellagitannin constituents in the above mentioned phytochemicals were more mostly predominant

Fig. 4 Inhibitory effect of the solvent extracts on **a** in vitro linoleic acid peroxidation and **b** lipid peroxidation in Chang liver cells. 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

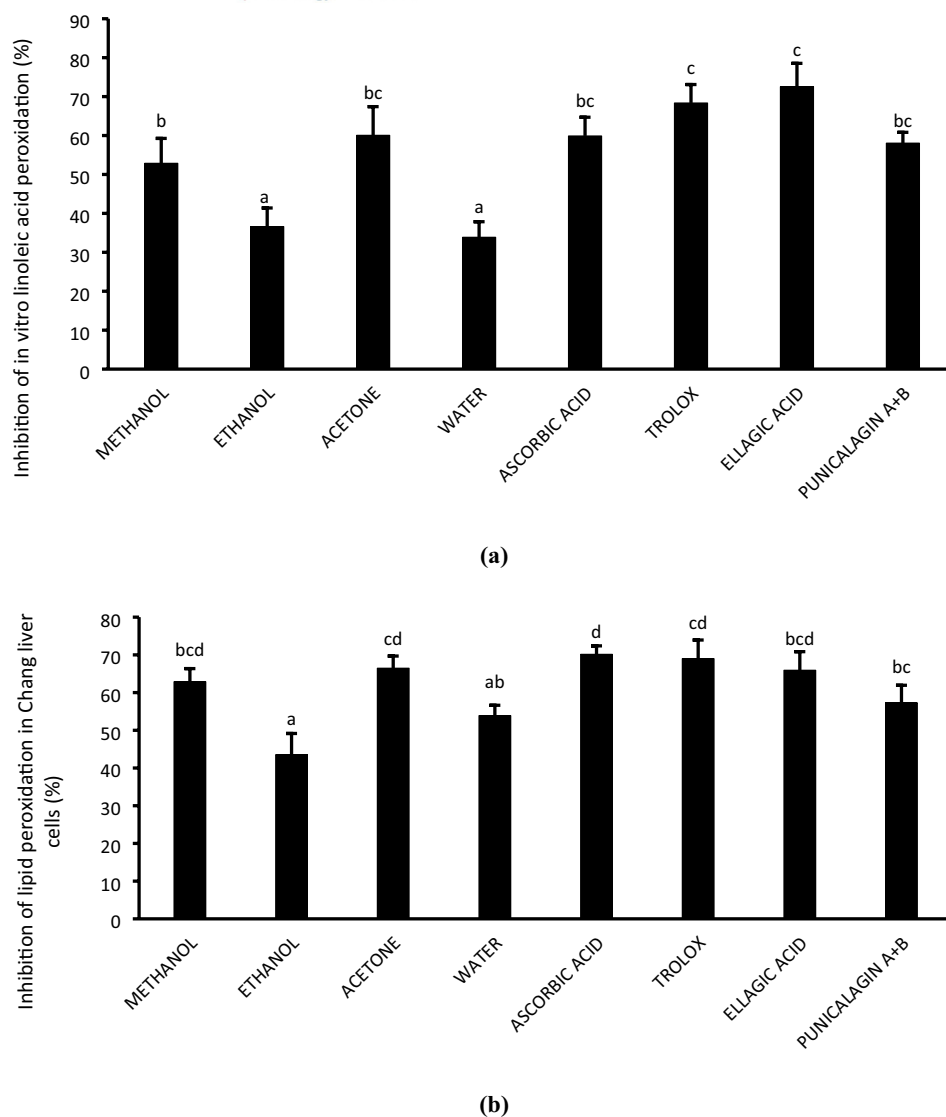


Table 1 The yield and phytochemical profile of ellagitannins from the different solvent extracts

Extracts	Yield of total tannins (%)	LCMS-quantified ellagitannin constituents in the purified total tannins from extracts (mg/L)							
		Punicalagin A	Punicalagin B	Ellagic acid	Granatin A	Corilagin	Casuarinin	Geraniin	Quercetin hexoside
METHANOL	47.4 $\pm$ 3.77 ^c	143 $\pm$ 8.31 ^a	140 $\pm$ 18.9 ^a	32.5 $\pm$ 3.21 ^b	22.6 $\pm$ 3.72	6.17 $\pm$ 0.72	4.74 $\pm$ 2.42	4.42 $\pm$ 1.57	9.68 $\pm$ 1.65
ETHANOL	36.9 $\pm$ 2.47 ^b	215 $\pm$ 10.5 ^b	190 $\pm$ 13.2 ^{ab}	21.6 $\pm$ 2.11 ^a	24.3 $\pm$ 6.02	4.17 $\pm$ 1.04	6.57 $\pm$ 2.05	5.69 $\pm$ 0.80	13.8 $\pm$ 0.88
ACETONE	44.0 $\pm$ 2.97 ^b	261 $\pm$ 18.3 ^b	247 $\pm$ 12.8 ^b	29.7 $\pm$ 5.47 ^{ab}	47.0 $\pm$ 4.32	5.18 $\pm$ 2.38	8.03 $\pm$ 1.46	10.6 $\pm$ 1.86	15.8 $\pm$ 1.91
WATER	16.8 $\pm$ 1.30 ^a	150 $\pm$ 4.90 ^a	149 $\pm$ 6.43 ^a	28.3 $\pm$ 2.27 ^{ab}	44.8 $\pm$ 12.1	3.80 $\pm$ 0.40	4.23 $\pm$ 0.29	6.33 $\pm$ 1.70	9.73 $\pm$ 1.62

Data are presented as mean $\pm$ SD of triplicate analysis. For each parameter or column, statistical comparison was done across the different extraction temperatures. Within a given column, significant difference ($p < 0.05$) occurs between any two cells, when there is no common superscript letter

Fig. 5 **a** Scores plot of unsupervised linear transformation and multivariate dimensional reduction analysis of the data (PCA), **b** scores plot of supervised linear transformation, dimensionality reduction, and modelling of the patterns in the data (OPLS) and **c** OPLS biplot showing the clustering of sample classes and spatial distribution the X and Y variables

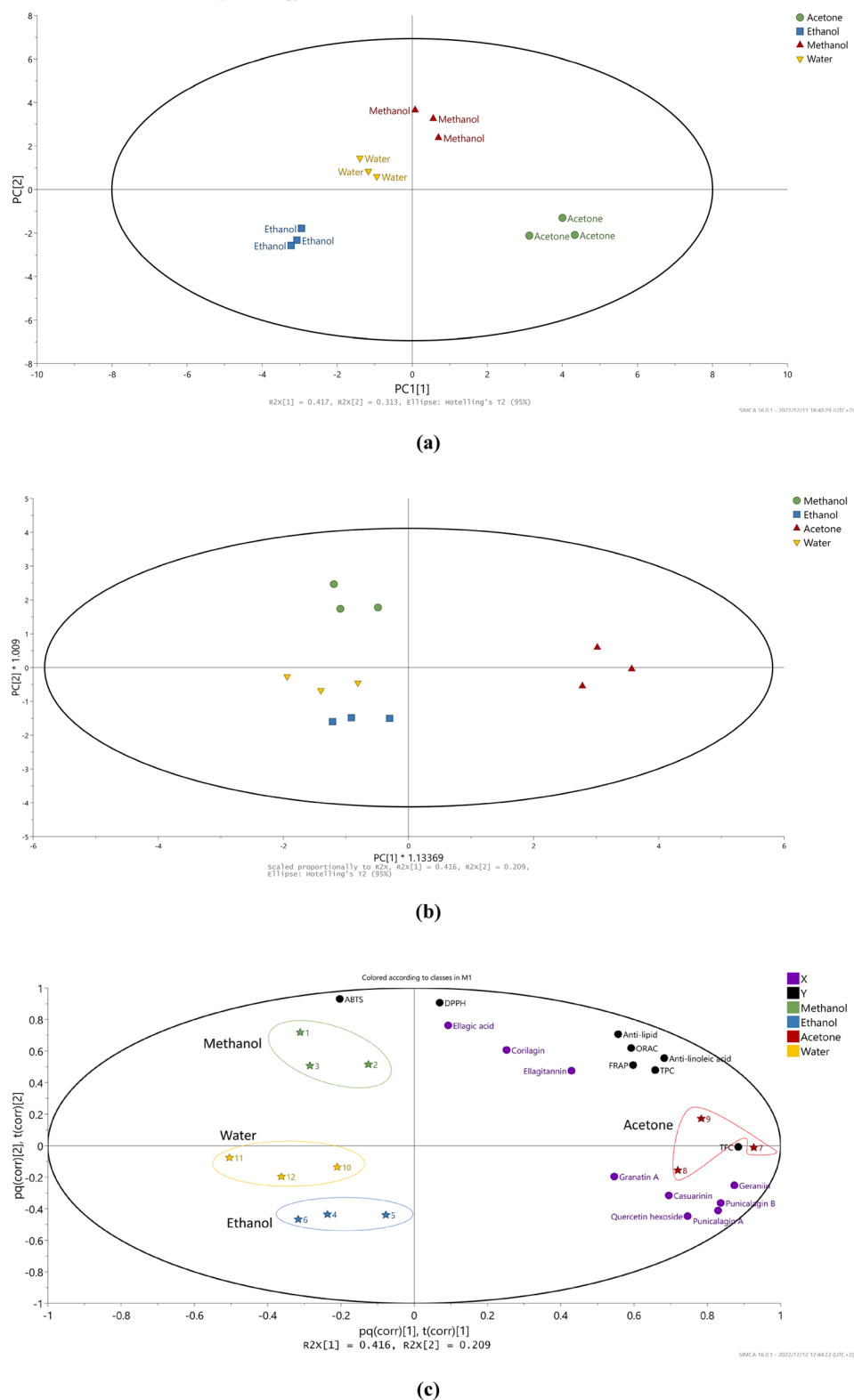


Table 2 PCA modelling parameters and summary of fit

Component	R2X	R2X(cum)	Eigen value	Q2	Limit	Q2(cum)	Significance	Iterations
1	0.417	0.417	5.01	0.113	0.134	0.113	R2	31
2	0.313	0.731	3.76	0.337	0.144	0.412	R1	10
3	0.124	0.854	1.48	0.169	0.156	0.511	R1	15
4	0.0616	0.916	0.739	-0.0108	0.17	0.506	R2	20
5	0.0378	0.954	0.453	0.0666	0.188	0.539	R2	13

in the acetone extracts compared to the other extracts, and in some case, significantly ($p < 0.05$).

Advanced chemometrics analysis

The PCA scores plot showed a clear clustering pattern of the various observation classes in the data (Fig. 5a). A total of five principal components were generated from the analysis, with the first two components describing 73% of the patterns in the data (PC1 = 41.7%, PC2 = 31.3%) and predicting 53.9% of the variation in the data by cross-validation (Table 2). Overall, the five components of the PCA model could explain up to 95.4% of the variation in the obtained data (Table 2).

The OPLS scores plot (Fig. 5b) showed distinct grouping patterns for all observation classes. On the OPLS biplot (Fig. 5c), the predictors (i.e., X variables) are coloured purple and the responses (i.e., Y variables) are coloured black. The grouping patterns on this plot showed the closeness of the water and ethanol extracts (Figs. 5a and c), indicating the inherent similarities of these two groups of samples. Also, it can be seen that acetone was very distinct from the other three classes. Further, it can be deduced from this plot that ABTS and DPPH radical scavenging properties were the strongest Y variables contributing to the differential clustering of the methanol extracts compared to the other extracts. TFC had the highest influence on the differential clustering of the acetone extracts. TPC, FRAP and in vitro anti-linoleic acid peroxidation also strongly contributed to the spatial distribution of acetone extracts. None of the variables were distributed close to the ethanol and water class along PC1 axis (i.e., x-axis). Also, none of the variables were located within the same quadrant with the two sample classes (water and ethanol). The biplot showed a clustered grouping of TFC and a number of predictors including granatin A, geraniin, casuarinin, punicalagin A, quercetin hexoside and punicalagin B. This cluster was also in and around the acetone sample class. It can also be deduced from Fig. 5c that ABTS and DPPH bioactivities are strongly linked to the presence of ellagic acid, corilagin and tannin yield. The phytochemical content in the water and ethanol extracts appeared not to potentially influence their bioactivity (Table 1 and Fig. 5c).

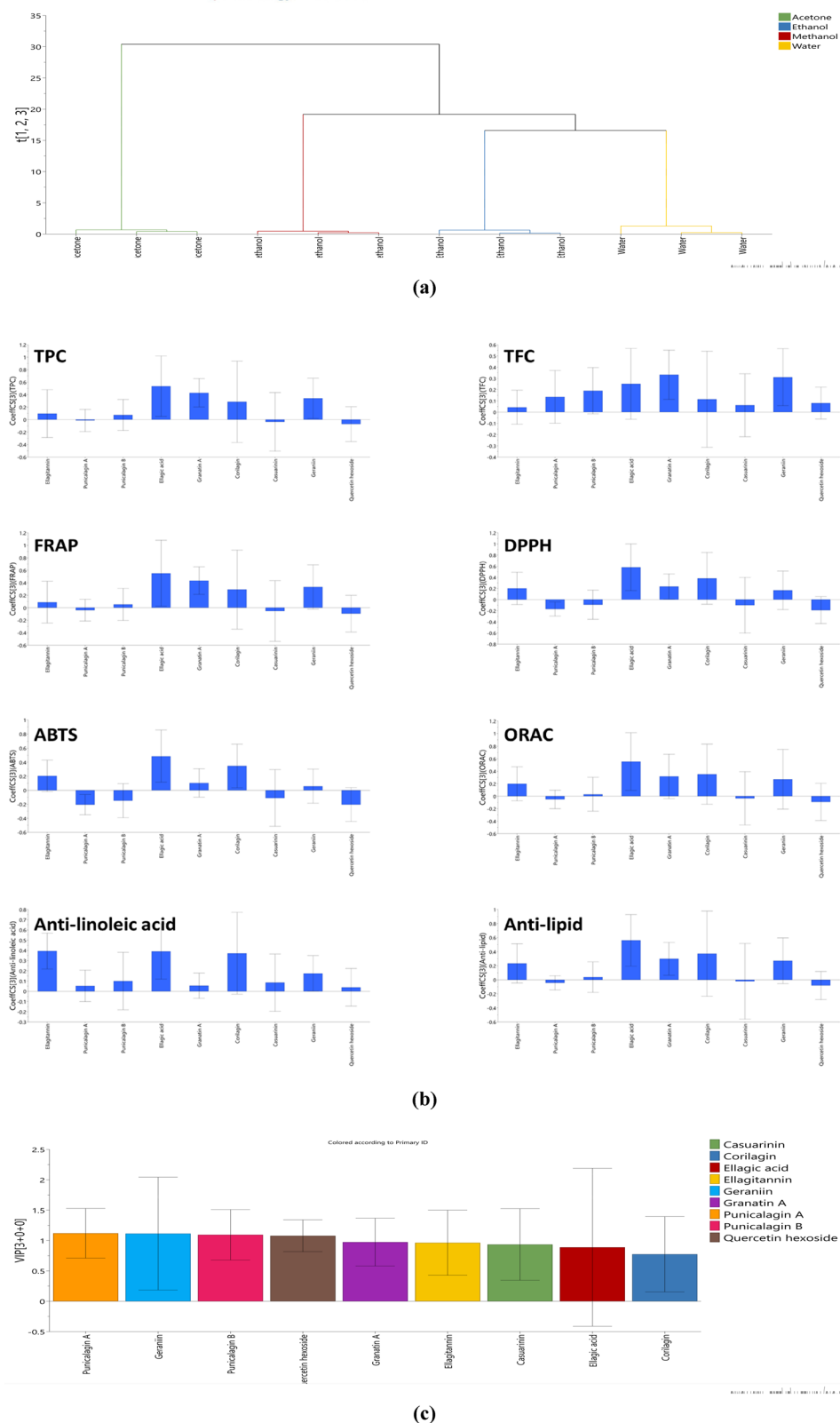
The HCA dendrogram (Fig. 6a) identifies groupings of similar sample classes, where observations in a cluster are more similar than those in separate clusters. Thus, in terms of similarity of the extraction solvents (in the context of tannins recovery, ellagitannins profile and antioxidant properties), the HCA dendrogram indicates that water extracts were more similar to ethanol extracts, which was more similar to methanol extracts, which was in turn the closest to acetone extracts. A pattern which is also corroborated on the PCA and OPLS scores plots, as well as the OPLS biplot.

Indeed, there exist an empirical relationship between extracted compounds and the bioactivities of the respective extracts, a phenomenon well-captured by the OPLS analysis as it reveals that the X model could predict up to 62.3% of the patterns in the response variables (Table 3). The variable contributions plot (Fig. 6b) for TPC show that ellagic acid, granatin A, geraniin and corilagin appeared to be the strongest predictors of TPC and TFC. It appeared that ellagic acid had the highest effect on FRAP, DPPH, ABTS, ORAC and cellular anti-lipid peroxidation (Fig. 6b). Total tannins yield had the strongest impact on in vitro anti-linoleic acid peroxidation followed closely by ellagic acid and corilagin. Overall, punicalagin A, geraniin and punicalagin B were the most important variables (in descending order) in the construction of the OPLS model and description of the patterns in the entire dataset (Fig. 6c).

Model overview, evaluation, and validation

It is important to validate any fitted model in order to ascertain its adequacy, efficiency and predictive ability, where applicable. The goodness-of-fit, goodness-of-prediction and other relevant model fit parameters for the PCA and OPLS models are presented in (Tables 2 and 3). Model fit diagnostics show that all PCA and OPLS generated components were significant (Tables 2 and 3). The OPLS algorithm generated a total of 3 PCs from the dataset and could describe over 80% of the variation in the X data [$R2X(cum)=0.804$] and 86.2% of the variation in the Y data [$R2(cum)=0.862$]. The first two components had a $R2X(cum)$ value of 62.5%, $R2(cum)$ value of 82.0%, $R2Y$ value of 89% and $Q2(cum)$ value of 53.3%. The Y model described 94.9% of the variation in the response variables ($R2Y=0.949$). The

Fig. 6 **a** HCA dendrogram showing the similarities/relatedness of the various observation classes, **b** variable contribution plot for each response variable and **c** variable importance plot (VIP) based on OPLS



goodness-of-prediction parameter $Q^2(\text{cum})$ indicated a predictive capability of 62.8% for the model, i.e., the X model had a cumulative prediction accuracy of approximately

63% based on cross-validation. Interestingly, the fractions of Y variation predicted by the X model in components 2 and 3 (PC2 and PC3) were higher (Q^2 49.7 and 9.6%,

Table 3 OPLS-DA modelling parameters and summary of fit

Component	R2X	R2X(cum)	R2	R2(cum)	Q2	Limit	Q2(cum)	R2Y	R2Y(cum)	Significance
Model		0.804		0.862			0.628		0.949	
Predictive		0.804		0.862			0.628		0.949	
P1	0.416	0.416	0.597	0.597	0.0358	0.01	0.0358	0.646	0.646	R1
P2	0.209	0.625	0.223	0.82	0.497	0.01	0.533	0.244	0.89	R1
P3	0.179	0.804	0.042	0.862	0.0956	0.01	0.628	0.0588	0.949	R1

respectively) than that of PC1 (Q2 3.6%). Results of regression of observations versus the corresponding predictions using the X model for each response variable were as follows: TPC (R2: 0.87, RMSEE: 43.62, RMSECV: 64.33), TFC (R2: 0.85, RMSEE: 2.67, RMSECV: 3.11), FRAP (R2: 0.88, RMSEE: 16.33, RMSECV: 25.12), DPPH (R2: 0.90, RMSEE: 5.58, RMSECV: 8.36), ABTS (R2: 0.95, RMSEE: 4.08, RMSECV: 6.47), ORAC (R2: 0.77, RMSEE: 6.68, RMSECV: 9.38), in vitro anti-linoleic acid peroxidation (R2: 0.82, RMSEE: 6.21, RMSECV: 6.67) and cellular anti-lipid peroxidation (R2: 0.86, RMSEE: 4.32, RMSECV: 5.08).

Discussion

The peel of pomegranate has been shown to have a rich phytochemical profile, which could be of medicinal relevance if not wasted. Ellagitannins are the predominant tannins present in the peel of pomegranate, which have been documented to have medicinal relevance [3]. Different studies have explored different extraction solvents to maximize recovery of the bioactive phytochemicals in pomegranate peel but lack a proper statistical correlation between the extraction solvent, phytochemistry and bioactivity. The present study used advanced chemometric models to correlate between the solvent extraction used, bioactivity and ellagitannins recovery from the peel of “Wonderful” variety pomegranate, with the aim of ascertaining the extraction solvent that will optimally recover antioxidant ellagitannins.

Data showed that the acetone extracts had the highest TPC and TFC (Fig. 2), which could be attributed to its suitable polarity for extracting polyphenols and flavonoids [18]. The methanol extracts also had an appreciable TPC content, which is linked to its compatible polarity with polyphenols. The TPC and TFC of these extracts may be partly influential in their potent radical scavenging/quenching effects (Fig. 3). This is due to the ability of polyphenols to form stable phenoxy radicals by donating electron or transferring hydrogen atom, which are able to react with and quench the deleterious effects of other radicals [19]. In both in vitro and Chang liver cells experimental models, the acetone and methanol extracts outperformed the ethanol and water

extracts in inhibiting FeSO₄-induced linoleic acid or lipid peroxidation (Fig. 4). Their potent effects may be attributed to their rich tannins yield, which also had higher constituents of ellagic acid and punicalagin A/B (Figure Table 1) relative to the other extracts. Moreover, these constituents also demonstrated appreciable anti-lipid peroxidative effects (Fig. 4), while ellagitannin-rich dietary supplement from pomegranate, as well as ellagic acid have been shown to have potent anti-lipid peroxidative activity in obese animals [20] and human subjects [21].

PCA and OPLS are advanced dimensionality reduction algorithms that emphasize differences or similarities between data observations by constructing a smaller number of interpretable, linearly uncorrelated variables [latent variables or principle components (PCs)] from the dataset [16, 22]. In order to detect any outliers and have a global appraisal and overview of the inherent patterns in the dataset without any reference to pre-assigned observation class labels, PCA was performed. Overall, the five components of the PCA model could explain up to 95.4% of the variation in the obtained data (Table 2). OPLS was further adopted to scrutinize the data for inherent discriminatory patterns since it is a supervised learning algorithm and can aggressively differentiate between sample groups following training on the labelled data. Clearly distinct grouping patterns were observed for all the observation classes (Figs. 5a and b; Table 3).

The biplot combines the information from the scores plot with information from the loadings plot, thus further elucidating information on data distribution patterns, observation classes and variable relationships. The distinct distribution of the acetone extract (Fig. 5c) suggests that extracts from this solvent performed uniquely in terms of the monitored parameters (i.e., X and Y variables). The differential clustering of the acetone extracts was influenced by its anti-lipid peroxidative and ORAC activities, which can be attributed to its higher TFC, TPC and ellagitannin constituents (punicalagin, granatin A, geraniin and casuarinin) (Fig. 5c). Ellagic appears to be most influencing component influencing the remarkable radical scavenging effect of the methanol extract, which further influenced its distribution in the biplot (Fig. 5c). The high tannins yield, as well as some key ellagitannins constituents, such as ellagic acid and corilagin

appear as the most predominant components influencing the antioxidant effects of the extracts (Fig. 6b). Moreover, the higher tannins yield and ellagitannins composition of the acetone and methanol extract (Table 1) correlates with the stronger antioxidant profiles of these extracts compared to that of the water and ethanol extracts (Figs. 3 and 4).

It is important to note that the computed values obtained from the evaluation and validation of the advanced chemometrics models indicate strong linearity and correlation between the predicted and observed values and demonstrate a good predictability of the response variables by the model. Therefore, it can be said that the OPLS model was able to adequately characterize, describe, and predict the variation and patterns in the dataset.

Conclusion

It can be concluded that the ethanol and water extracts overall had lower yield of total tannins and contained lower amounts of key influencing ellagitannins, particularly, ellagic acid and corilagin, which are located the furthest away from these observation classes on the biplot. Consequently, ethanol and water extracts nominally possessed the least bioactivities of the solvent extracts. It can be deduced from the biplot that tannins crudely purified from the acetone extracts nominally contained the highest amounts of most of the ellagitannins and thus showed the highest bioactivity of all the solvent extracts. Methanol extracts also, had promising bioactivity, which may be largely influenced by the high ellagic acid content in the tannins purified from it. It is, however, important to note that there may be other phytochemicals influencing the antioxidant capacities of the acetone and methanol extracts, which have not been profiled in the present study. In the context of ellagitannins from pomegranate peel, acetone and methanol solvent extraction may be promising for optimum ellagitannins recovery and antioxidant profile.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00217-023-04255-4>.

Author contributions Conceptualization: CIC; Data curation: RS, BM, GOI; Funding acquisition: CIC; Methodology: RS, BM, GOI, SG, SSM, SLB, TJM and CIC; Resources: SSM, TJM and CIC; Software: SG, SLB and CIC; Supervision: SSM and CIC; Formal analysis: RS, BM, GOI, SG and CIC; Writing—original draft: RS; Writing—review & editing: BM, GOI, SG, SSM, SLB, TJM and CIC.

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Data Availability The data of this study is available from the corresponding author on request.

Declarations

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

Compliance with Ethics requirements This article complies with the applicable ethics requirements.

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Modeling the Influence of Extraction Temperature on the Ellagitannin and Antioxidant Profiles of “Wonderful” Pomegranate Peel Using Advanced Chemometrics Analysis

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Abstract

Pomegranate peel contains ellagitannins as antioxidant principles but the aqueous extraction temperature for optimum ellagitannins recovery and antioxidant profile remains elusive. This study employed advanced chemometrics models, including principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) to determine the influence of different extraction temperatures (25–95 °C) during a 1 h period on the antioxidant profile of the peel's aqueous extract, as well as the profile of the constituent ellagitannins recovered using Amberlite® XAD16N resin and characterized by LC–MS. Extraction at 78 °C had the most potent radical scavenging potency, oxygen radical absorbance capacity (ORAC), and anti-lipid peroxidative activity, which was consistent with its highest ellagitannins constituents relative to other extraction temperatures. Pearson's bivariate correlation depicted strong positive correlations ($r=0.7\text{--}0.9$) between the ellagitannins and the anti-lipid peroxidative profile of the extracts. PCA and OPLS models adequately characterized and predicted the variation and patterns in the antioxidant and phytochemical datasets (up to 93.6%). OPLS biplot showed a distinct clustering pattern of the antioxidant and ellagitannins variables around 78 °C extraction temperature, suggesting the observable influence of the ellagitannins on the potent antioxidant profile of the 78 °C extraction. The variable contribution plots, as well as the obtained antioxidant data, suggest punicalagins, granatin A, and ellagic acid as notable influencing ellagitannins. It is safe to speculate that extraction at 78 °C optimally influenced the ellagitannins and antioxidant profile of pomegranate fruit peel. Thus, this aqueous extraction temperature may be promising for optimally recovering the ellagitannins from pomegranate peel, without sacrificing the antioxidant capacity.

Keywords Pomegranate peel · Aqueous extraction · Antioxidant · Ellagitannins · Advanced chemometrics analysis

Introduction

Pomegranate (*Punica granatum* L.) fruit is widely consumed globally for its appreciable taste. It has, however, been linked to several health benefits, including oxidative and vascular health benefits (Giménez-Bastida et al., 2021; Kandylis & Kokkinomagoulos, 2020). The health benefits have been majorly attributed to the presence of polyphenols, including the predominant ellagitannin constituents (Giménez-Bastida et al., 2021). Ellagitannins are hydrolyzable tannins common in pomegranate fruit, which include constituents such as punicalagins, punicalin, gallagic acid, ellagic acid, and relative glycosides (Altieri et al., 2019). Phenolic acids and flavonoids including catechins, esculetin, hesperidin, rutin, naringenin, kaempferol, apigenin, rhamnetin, quercitrin, and quercetin have also been reported in pomegranate fruit (Zeghad et al., 2019), which partly influence its antioxidant benefits.

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These polyphenols, including the ellagitannins, account for a high percentage of the fruit's antioxidant property and are mostly concentrated in the fruit peel and juice compared to other parts of the fruit (Ismail et al., 2012; Marra et al., 2022; Yartz et al., 2022).

Documented evidence has shown that the fruit's peel contains ellagitannins, flavonoids, and phenolic acids and may be useful in managing oxidative stress (Akuru et al., 2022; Marra et al., 2022; Mo et al., 2022), thus maximizing the therapeutic potential of the fruit's wastes and potentially reducing environmental biomass and pollution caused by the fruit's peel. The potent radical scavenging activity of the fruit's peel has been demonstrated (Akuru et al., 2022; Li et al., 2006; Marra et al., 2022), and in some instances has been shown to be stronger than that of the edible pulp (Li et al., 2006). In both hepatocytes and isolated rat liver tissues, the peel was shown to reduce lipid peroxidation caused by oxidative stress (Akuru et al., 2022). In rats, the peel was shown to improve oxidative stress marker and hepatic oxidative damage caused by carbon tetrachloride, suggesting the hepatoprotective effect of the peel (Chidambara et al., 2002). In fact, phenolic principles (ellagic acid, punicalin, and punicalagin) from pomegranate peel have been shown to reduce oxidative stress and inflammatory makers in oxidatively stressed mice (Sun et al., 2017).

Although different organic solvent extracts have been explored for recovering the antioxidant principles in pomegranate peel, water extraction has always been perceived as the safest in folk medicine and thus commonly used in Chinese traditional medicine (Yang & Ross, 2010). Moreover, other common solvents like ethanol used in folk medicine may be more costly than water and may pose toxicity concerns. Despite the preferred safety profile of water extraction, acetone extraction of pomegranate peel has been shown to result in a better antioxidant profile, perhaps due to a better phytochemical profile (Akuru et al., 2022; Chukwuma et al., 2020). Hence, it is imperative to explore methods that could improve the antioxidant and phytochemical profiles of pomegranate aqueous peel extract.

Previous studies investigated the effect of different drying and extraction conditions/methods on the phytochemical and antioxidant profiles of pomegranate peel. Calín-Sánchez et al. (2013) investigated the effect of different types of drying methods (freeze drying, convective drying, and vacuum–microwave drying) on the antioxidant and phenolic profiles of pomegranate rind and peel. However, they did not determine or investigate the extraction method for optimum ellagitannins recovery and antioxidant profile. Their study is also void of advanced statistical correlation models to statistically correlate between the studied variables. Alexandre et al. (2017) used Box–Behnken design to investigate the effect of high-pressure extraction on the antioxidant and bioactive phytochemical profile on pomegranate peel. However,

their study was limited to the peel material that was dried and did not investigate the influence of varying extraction temperature on the ellagitannins and antioxidant profile of pomegranate peel. A previous study has suggested that using fresh fruit peel for aqueous extraction may be promising for recovering ellagitannins from pomegranate fruit waste, perhaps to a commercial scale (Seeram et al., 2005). However, how extraction temperature influences the ellagitannin recovery and antioxidant profile remains elusive. It is known that heat facilitates the infusion of phytochemicals present in plant materials and thus is commonly employed in Chinese traditional medicine (Yang & Ross, 2010).

It is, however, important to conduct temperature-dependent extraction at optimum temperatures that can preserve the structural and functional properties of the bioactive principles, particularly the ellagitannin constituents in pomegranate peel. Too much heat may alter the ellagitannins integrity/profile, thus compromising the antioxidant profile (Sójka et al., 2019). Conversely, insufficient heat may not provide the optimum heat condition for optimum ellagitannins extraction and antioxidant profile (Yang & Ross, 2010). To date, no study has determined or investigated the optimum temperature for aqueous extraction of pomegranate peel that will yield optimum antioxidant and ellagitannin profiles. Therefore, this study was done to investigate the influence of temperature on the antioxidant and ellagitannin profiles of pomegranate peel water extraction by employing advanced chemometrics models and analyses. The models include Pearson's bivariate correlation, principal component analysis (PCA), and orthogonal projections to latent structures (OPLS), which for the first time was used to correlate and model the influence of temperature on the antioxidant and ellagitannin profiles of pomegranate peel. Antioxidant models used in this study, including oxygen radical absorbance capacity (ORAC) and in vitro and Chang liver cells anti-lipid peroxidation assays, are more advanced than those employed in the studies reported by Calín-Sánchez et al. (2013) and Alexandre et al. (2017).

Materials and Methods

Fruit Procurement and Removal of Peel

Class 1 (processing grade) “Wonderful” variety of pomegranate fruits was procured from Sonlia Fruit Packhouse (Wellington, Western Cape Province, South Africa) on 03 May 2021. The fruits were washed.

Preparation of Peel and Extraction

The peel was removed from the fruit. Approximately 1 kg of the fresh peel was separately soaked in 2.5 L of distilled water at different temperatures (25 °C, 37 °C, 50 °C, 65 °C, 78 °C, and 95 °C) for 1 h. Thereafter, the peel and

the incubation water were blended and filtered with a clean cheese cloth to remove the debris. The filtrate was filtered again with a filter paper to remove any remaining residue. The filtered extracts were freeze-dried using a Martin Christ Alpha 1–2 LDplus Freeze Dryer (Separations, Johannesburg, South Africa). During the freeze drying, the vacuum pressure was typically lower than 1 mbar, while the ice condenser temperature ranged between 53 and 57 °C. The recovered extracts were stored in air-tight vials at –20 °C until they were used.

Total Phenol and Total Flavonoid Contents Measurement in Extracts

The total phenol content (TPC) and total flavonoid content (TFC) of the extracts were measured using a previously reported protocol (Chukwuma et al., 2020) and expressed as mg/g gallic acid equivalent (GAE) and mg/g quercetin equivalent (QE), respectively. The extracts were tested at 45 µg/mL.

Antioxidant Analyses

The antioxidant effect of the extracts was analyzed at 45 µg/mL using the following in vitro and cell-based experimental models described below. The activity of the extracts was compared to an equivalent concentration of the positive controls (ascorbic acid and Trolox) or standard ellagitannin constituents (punicalagin A + B and ellagic acid).

Radical Scavenging Action

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS*+) scavenging effects of the extracts were determined using methods described in a previous report (Chukwuma et al., 2021) and expressed as percentage radical scavenging activity.

Oxygen Radical Absorbance Capacity (ORAC) Assay

A previous method (Ou et al., 2013) was slightly modified to perform this assay. 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-methylpropionamide) 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) or the standards (punicalagin A + B and ellagic acid) at the tested concentration (45 µg/mL in assay volume) or their solvents (controls) were added into the wells of the plate. Thereafter, 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:

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

Fe³⁺ Reducing Activity

A previous method (Chukwuma et al., 2021) was slightly modified to perform this assay. First, 25 µL of the tested samples or positive controls (ascorbic acid and Trolox) (45 µg/mL in the reaction mixture) or increasing concentrations (3.75–60 µg/mL in the reaction mixture) of gallic acid standard, 25 µL of 0.2 M phosphate buffer (pH 6.6) and 25 µL of 1% potassium ferricyanide were mixed in wells of a 96-well clear bottom plate. The plate was incubated for 20 min at 50 °C, before successively adding 25 µL of 10% trichloroacetic acid, 100 µL of distilled water, and 50 µL of 0.1% FeCl₃ solution. Thereafter, absorbance was measured at 700 nm and blanked with the sample blank. The Fe³⁺ reducing activity was determined from a gallic acid standard curve and computed as the mg/g equivalent of the gallic acid (mg/g GAE) using the following formula:

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

where, “C” is the concentration (mg/mL) extrapolated from the 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.”

Measurement of Inhibitory Action on FeSO₄-Induced Linoleic Acid Peroxidation In Vitro

To perform this assay, the method reported by Ramorobi et al. (2022), which was modified from the method reported by Choi et al. (2002) was adopted.

Measuring the Anti-Lipid Peroxidative Capacity of Samples in Chang Liver Cells

Chang liver cells (ATCC[®] CCL-13[™]) were supplied by ATCC (Virginia, USA), and the experiment was performed according to a published protocol (Akuru et al., 2022), while adding slight modifications. Cell culturing was carried out using an EMEM medium supplemented with fetal bovine serum at a concentration of 10%. Trysinization was performed when the cells attained a confluence of 85%. The cells were resuspended in the culture media, and 200 μ L of the cell suspension was aliquoted into designated wells of a 96-well plate with each well having a cell density of 8000 cells/well. After a 36-h incubation period (at 5% CO₂ and 37 °C) in a NÜVE EC 160 CO₂ incubator (Ankara, Turkey), the spent medium in the wells of the plate was aspirated, and the attached cells in the wells were treated with the tested samples dissolved in the medium (45 μ g/mL in 200 μ L incubation medium). The plate was incubated for 30 min, before the addition of 50 μ L of FeSO₄·7H₂O solution at a concentration of 1 mM in the incubation medium to induce oxidative stress. Some wells were used as the normal control (control) and negative control. The normal control (control) wells contained untreated cells, the sample solvent/vehicle in place of the sample, and 50 μ L of sterile water in place of the FeSO₄·7H₂O solution. The negative control wells contained untreated cells, the sample solvent/vehicle in place of the sample, and 50 μ L of the FeSO₄·7H₂O solution. The plate was incubated for 1 h after adding the FeSO₄·7H₂O solution. Thereafter, the incubation medium in each well of the plate was replaced with 250 μ L of cold 50 mM sodium phosphate buffer containing 0.5% v/v Triton X-100 (pH 7.5) to lyse the cells. A gentle tapping of the plate was done for 10–15 min to facilitate cell lysis. The cell debris was pelleted by centrifuging for 1 min at 5000×g and 4 °C. Aliquots (100 μ L) of the supernatant in each well of the plate were used to measure lipid peroxidation.

Into designated vials, 100 μ L of the supernatant or standards (malondialdehyde at concentrations of 0, 7.5, 15, 22.5, 30, and 45 μ M in the assay volume), 500 μ L of 0.25% w/v thiobarbituric acid, 200 μ L of acetic acid, and 200 μ L of distilled water were successively added. The vials were heated for 50 min in a boiling water bath, allowed to cool, and then centrifuged for 10 min at 3500×g. Spectroscopic absorption at 532 nm was measured in aliquots (200 μ L) from each vial. Lipid peroxidation was computed using extrapolated values from a malondialdehyde linear plot. The anti-lipid peroxidative activity (%) of the tested samples was calculated relative to the controls (normal and negative) using the formula shown below:

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 “malondialdehyde concentration extrapolated from the malondialdehyde linear plot”.

Crude Recovery of Tannins/Ellagitannins from Extracts

An Amberlite[®] XAD16N resin (product code: XAD16; Sigma Aldrich, Johannesburg, South Africa) was used to purify the tannins in the extracts. The methods previously reported by Seeram et al. (2005) were adopted with little modifications. Approximately 1.52 g of the extracts were dissolved in 100 mL of distilled water and filtered with a filter paper. The filtrate was collected as a yellowish-brown liquid. Approximately 35 g of the XAD16N resin was first washed thrice with absolute methanol. The resin was allowed to equilibrate in distilled water for 12 h using a sealed flash column as shown in Fig. 1a. Thereafter, the distilled water was eluted/removed using a water jet vacuum pump, and the extract filtrate was gradually loaded onto the XAD16N resin in the flash column as shown in Fig. 1b. The column was continuously eluted with distilled water using the water jet vacuum pump until the eluent consistently remained colorless and all the tannins in the extract were trapped in the XAD16N resin (Fig. 1c). The column was vacuum aspirated to dry up the remaining water. Next, the trapped tannins were gradually eluted using absolute methanol as shown in Fig. 1d, e. The eluent was air-dried in a fume hood. The recovered tannin fractions were weighed, and the percentage yield was computed using the formula below. The fractions were stored at –20 °C until further analysis.

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

Liquid Chromatography—Mass Spectroscopic (LC–MS) Quantification of Ellagitannins

The LCMS analysis was done using a Waters Synapt G2 (Waters Corporation, MA, USA), ESI probe, ESI Pos, and cone voltage 15 V. Liquid chromatography was operated with an Acquity binary solvent manager. A HSS T3 column with the dimension of 2.1 × 150 mm and particle size of 1.8 μ m (Waters Corporation, MA, USA) was used for the chromatographic separation. The mobile phase was made up of A (0.1% formic acid in HPLC grade water) and B (0.1% formic acid in HPLC grade acetonitrile) solvents. The flow rate for the mobile phase was 0.25 mL/min. A gradient chromatographic condition was applied as follows: 100% of A and 0% of B for 10 min; then 72% of A and 28% of B for 21 min; then 60% of A and 40% of B for 50 s; then 0% of A and 100% of B for 2 min. Chromatographic separation was done

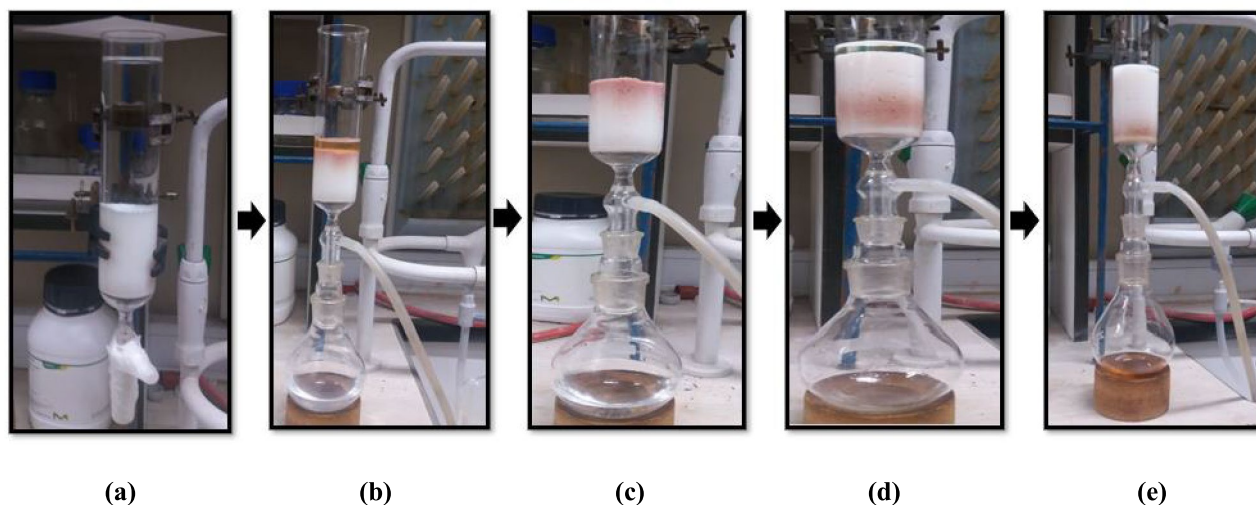


Fig. 1 Flow diagram showing the purification process of tannins from the extracts using XAD16N resin

at 30 °C. Samples were reconstituted in 1 mL of 50%:50% acetonitrile and water, centrifuged at 14,000 rpm for 5 min, and filtered using a 0.22 μ m filter. A 20 μ L injection volume was applied. A UV wavelength of 254 nm was used to record the chromatographic signals. Puniculagin A, puniculagin B, and ellagic acid in the ellagitannins fraction were identified and quantified from calibration curves established using a range of calibration standards prepared from stock solutions of Puniculagin A + B (product code: PHL80524, Sigma Aldrich, Johannesburg, South Africa) and ellagic acid (product code: 14,668, Sigma Aldrich, Johannesburg, South Africa). The other compounds (granatin A, corilagin, casuarinin, geraniin, and quercetin hexoside) in the tannins fractions were identified and quantified using the mass spectrometry data in a relative fashion against ellagic acid. They were identified based on accurate mass elemental composition and quantified using extracted ion chromatography against ellagic acid. The quantification was presented as a triplicate analysis.

Statistical Analysis

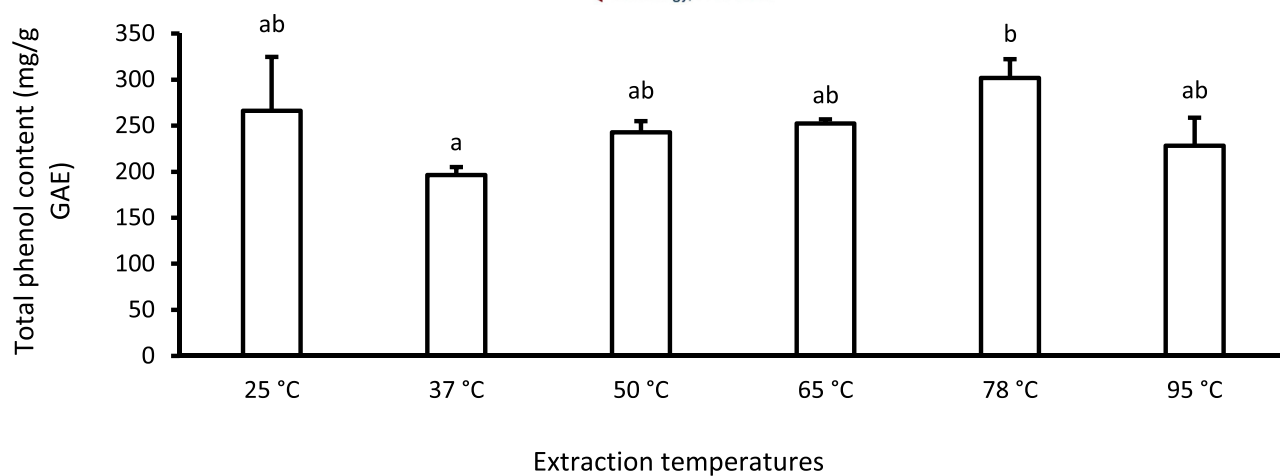
Statistical analysis was done on the Window's version 27.0 of IBM SPSS (IBM Corp, Armonk, NY, USA). Multiple comparisons of data averages among the groups (extracts or standards) were done for the different studied parameters using the Tukey multiple posthoc test and one-way analysis of variance (ANOVA). The statistically significant difference (p) was set at $p < 0.05$ when comparing the mean values of the different groups.

Advanced Chemometrics Analysis

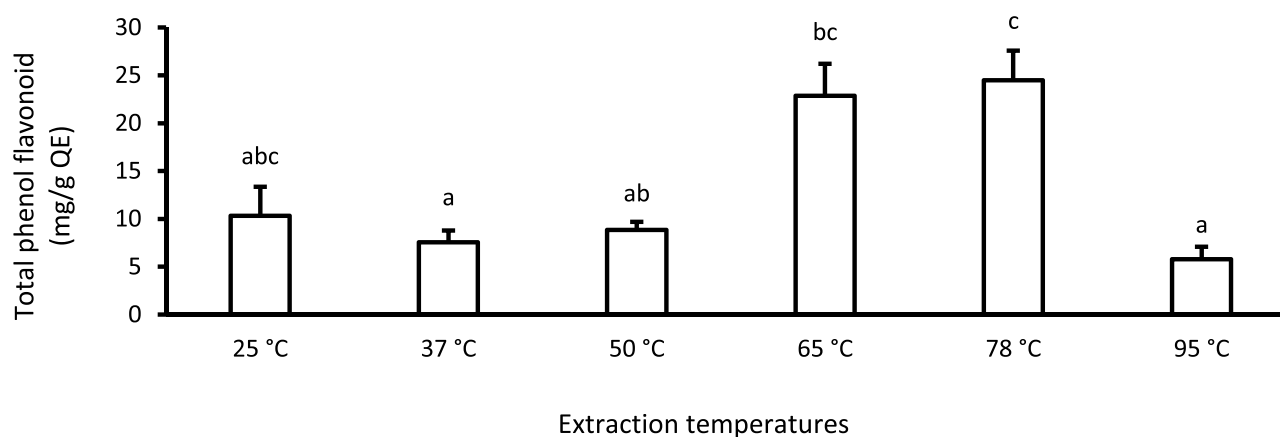
Pearson's bivariate correlation analysis was used to estimate the direction and magnitude of the linear correlation

between all pairs of studied variables. Principal component analysis (PCA) and orthogonal partial least squares (OPLS) analysis were performed using SIMCA-P+ 16.0 software (Umetrics, MKS Instruments Inc., Sweden) as described by Gbashi et al. (2020). Data preprocessing for multivariate chemometric analysis involved mean-centering and unit variate scaling of the entire dataset. For OPLS analysis, each observation in the data was assigned to a class depending on the extraction temperature, namely 25, 37, 50, 65, 78, and 95 °C. After labeling, the data were categorized into X variables (predictors) and Y variables (responses). The predictors included ellagic acid, casuarinin, puniculagin A, puniculagin B, ellagitannin/tannin yield, corilagin, granatin A, geraniin, and quercetin hexoside. These are the extracted analytes quantified in each sample extract. The response variables consisted of the antioxidant assays: ABTS, ORAC, TPC, TFC, FRAP, DPPH, anti-linoleic acid, and anti-lipid.

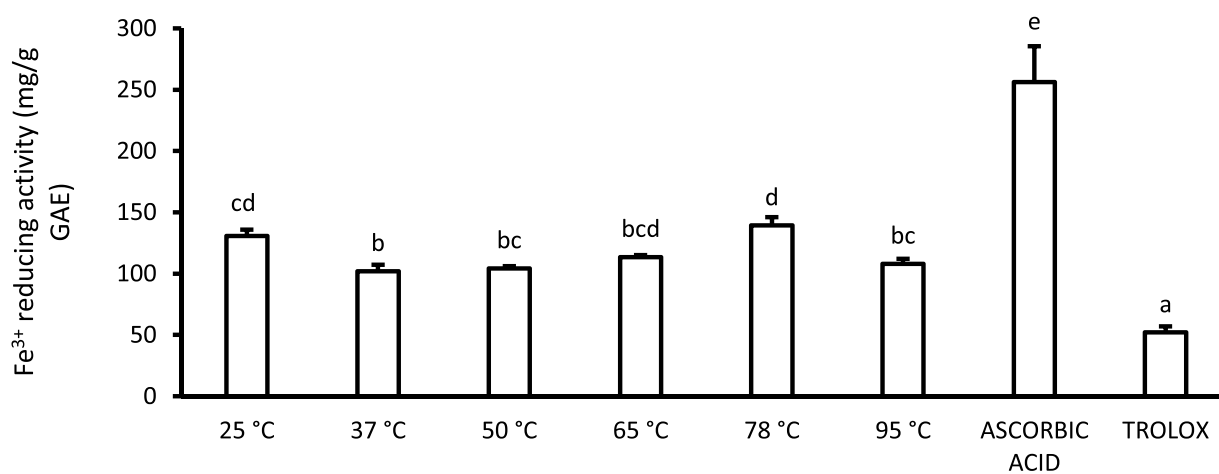
The adequacy of the fitted chemometric models was assessed by evaluating the goodness-of-fit, goodness-of-prediction, and several other model fit parameters. The portion of X variation explained in a specific component using the X model (R^2X) was examined. The combined fractions of variation in the X data modeled up to the specified component using the X model [$R^2X(\text{cum})$] were also evaluated. Similarly, the R^2Y , i.e., the fraction of explained Y variation in the component using the Y model was evaluated. Also, the cumulative fraction of variation in the Y data modeled up to the specified component using the Y model [$R^2Y(\text{cum})$] was assessed. Other model fit parameters investigated included R^2 (the portion of the variation in Y data modeled by the X model in the component) and $R^2(\text{cum})$ (combined fractions of modeled variation in the Y data using the X model up to the specified component). The root-mean-standard error



(a)



(b)



(c)

Fig. 2 **a** Total phenol content, **b** total flavonoid content, and **c** Fe³⁺ reducing the effect of the different extracts. Data are presented as mean $\pm$ SD of triplicate analysis. The letters at the top of the bars represent significant differences ($p < 0.05$) between groups, when there are no similar letters

resent significant differences ($p < 0.05$) between groups, when there are no similar letters

for cross-validation (RMSECV), root mean square error of estimation (RMSEE), and coefficient of determination (R^2) of the predicted versus the observed response values were also evaluated. Rule 1 (R1), rule 2 (R2), or rule 3 (R3) were applied to establish the statistical significance of model components. When $Q^2 > \text{limit}$, the component is regarded as significant for R1. For R2, a component is considered significant if $Q^2_V > \text{limit}$. A component is designated undetermined (U) for R3 in terms of PCA when it is insignificant. Nonetheless, if the succeeding component is significant and has similar eigenvalues (5% tolerance) to the previous component, then both components are regarded as significant, and the component labeled U is relabelled R5. The SIMCA[®] manual details the computations and determinations of the limits for both PCA and OPLS, as well as a description of the model validation parameters (Sartorius Stedim, 2018).

Results and Discussion

In the present study, the optimum aqueous extraction temperature that will optimally recover the ellagitannins from pomegranate peel and preserve their antioxidant profile was studied using advanced chemometrics models. The data suggest a temperature-dependent influence on the ellagitannin and antioxidant profiles of pomegranate peel during aqueous extraction.

Influence of Extraction Temperature on the Phytochemical Profile of Pomegranate Peel

The TPC and TFC dropped as the extraction temperature was increased from room temperature to 37 °C (Fig. 2a, b). Considering that extraction was done on fresh fruit peel, this drop in TPC and TFC may be attributed to enzymes like polyphenol oxidases (PPOs), which degrade polyphenols (Sikora et al., 2019). PPOs are optimally active at about

35–37 °C (Sikora et al., 2019), thus actively degrading polyphenols and reducing the TPC and TFC at 37 °C extraction temperature. As the extraction temperature increases to 50, 65, and 78 °C, the TPC and TFC gradually increase and peaked at 78 °C, which was significantly ($p < 0.05$) higher compared to 37 °C extraction temperature (Fig. 2a, b). It is plausible that there was inactivation and denaturing of PPOs as temperature increased from 37 to 50 °C and beyond, which reflects the gradually increasing TPC and TFC. Also, the temperature-dependent release of insoluble bound phenolic compounds or lignin breakdown may contribute to the gradual increase in TPC and TFC as the extraction temperature increased (Irakli et al., 2018). However, as the extraction temperature increased to 95 °C, the TPC and TFC dropped or reduced notably, which may be attributed to the thermal degradation of polyphenols in the extract. Moreover, it has been documented that high TPC is obtained at extraction temperature between 60 and 80 °C, while temperatures above 80 °C can lead to thermal degradation of the polyphenols and reduced TPC (Antony & Farid, 2022).

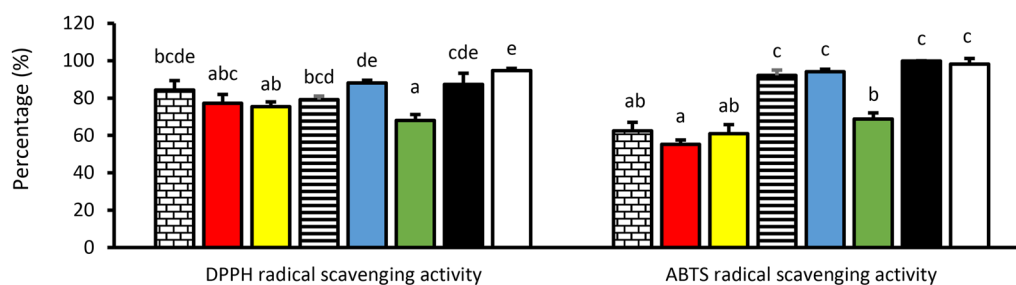
The yield of the purified tannins/ellagitannins from the extracts, as well as the LC–MS-quantified ellagitannins (punicalagin A and B, granatin A, corilagin, casuarinin, and geraniin) also followed a similar trend as the TPC and TFC, with the extraction done at 78 °C having the highest concentrations of the ellagitannins compared to the extraction done at other temperatures (Table 1 and Supplementary Materials). It has been documented that temperature elevation increases the yield of ellagic acid/ellagitannins extraction from pomegranate husk, which explains the temperature-dependent increase in tannin/ellagitannins yield (Muñiz-Márquez et al., 2021). However, the notable drop in tannin yield or ellagitannins concentration beyond 78 °C (at 95 °C extraction temperature) (Table 1) may be attributed to the temperature-dependent degradation of ellagitannins. Moreover, it has been reported that ellagitannins are relatively stable in acidic conditions but could be degraded at temperatures beyond 80 °C (Sójka et al., 2019).

Table 1 The yield and phytochemical profile of ellagitannins from fresh pomegranate peels subjected to aqueous extraction at different temperatures

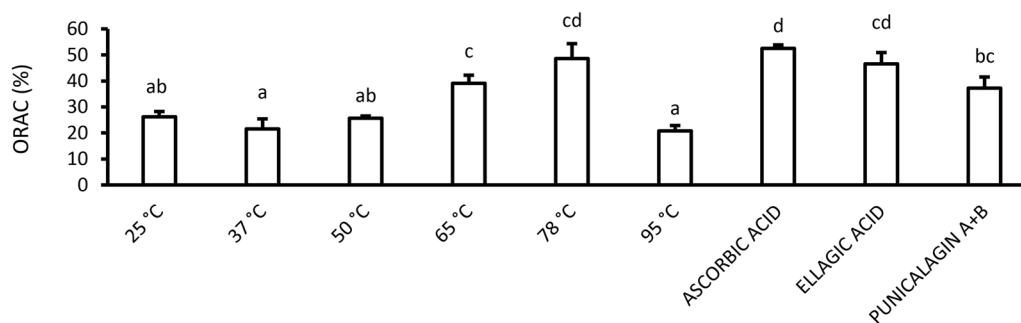
Extraction at different temperatures	Yield of total tannins (%)	LCMS-quantified ellagitannin constituents in the purified total tannins from extracts (mg/L)							
		Punicalagin A	Punicalagin B	Ellagic acid	Granatin A	Corilagin	Casuarinin	Geraniin	Quercetin hexoside
25 °C	34.2 ± 2.69 ^b	268 ± 21.5 ^a	257 ± 20.5 ^a	12.4 ± 2.74 ^a	75.8 ± 9.64 ^a	3.50 ± 0.52 ^a	6.27 ± 0.41 ^a	9.75 ± 2.71 ^a	20.4 ± 1.45 ^{ab}
37 °C	21.7 ± 1.90 ^a	464 ± 15.4 ^b	439 ± 47.3 ^b	28.0 ± 6.70 ^b	117 ± 8.24 ^b	6.02 ± 0.81 ^{ab}	9.76 ± 1.45 ^a	14.5 ± 2.12 ^{ab}	30.7 ± 5.77 ^c
50 °C	18.5 ± 1.69 ^a	571 ± 11.7 ^c	502 ± 5.72 ^{bc}	24.9 ± 1.54 ^b	126 ± 2.35 ^b	6.14 ± 0.77 ^{ab}	11.0 ± 2.41 ^a	19.3 ± 3.91 ^b	28.3 ± 3.31 ^{bc}
65 °C	34.7 ± 4.35 ^b	523 ± 31.4 ^c	526 ± 49.7 ^c	31.9 ± 5.42 ^{bc}	128 ± 6.57 ^b	7.01 ± 2.29 ^{ab}	8.71 ± 2.25 ^a	21.2 ± 1.31 ^{bc}	33.9 ± 4.18 ^c
78 °C	38.6 ± 2.94 ^b	782 ± 34.0 ^d	717 ± 18.7 ^d	40.5 ± 3.54 ^c	171 ± 13.7 ^c	8.64 ± 2.44 ^b	24.1 ± 2.85 ^b	26.5 ± 4.03 ^c	48.5 ± 5.42 ^d
95 °C	18.8 ± 2.99 ^a	277 ± 13.5 ^a	238 ± 23.1 ^a	7.15 ± 2.37 ^a	59.6 ± 12.4 ^a	3.07 ± 0.91 ^a	6.02 ± 1.07 ^a	10.9 ± 2.06 ^a	15.5 ± 3.18 ^a

Data are presented as mean ± SD of triplicate analysis. For each parameter or column, a statistical comparison was done across the different extraction temperatures. Within a given column, a significant difference ($p < 0.05$) occurs between any two cells, when there is no common superscript

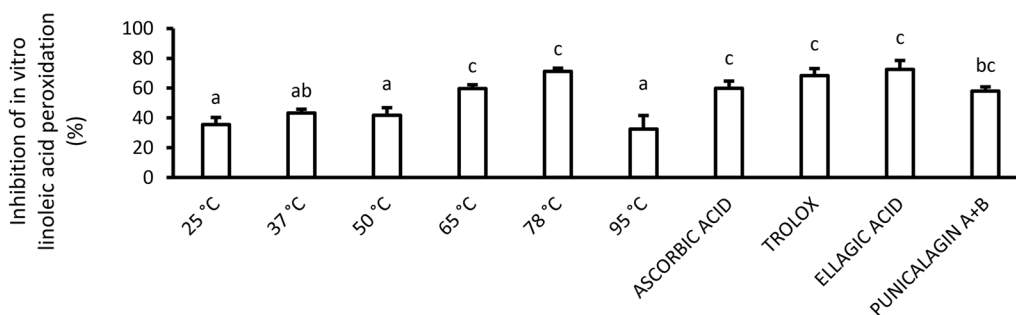
■ 25 degree centigrade ■ 37 degree centigrade ■ 50 degree centigrade ■ 65 degree centigrade
 ■ 78 degree centigrade ■ 95 degree centigrade ■ ASCORBIC ACID ■ TROLOX



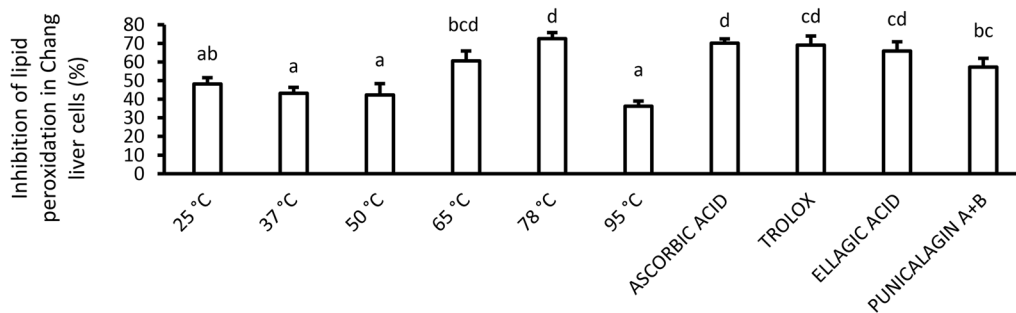
(a)



(b)



(c)



(d)

Fig. 3 **a** Radicals scavenging activity and **b** oxygen radical absorbance capacities (ORAC) of the extracts and their inhibitory effect on **c** in vitro linoleic acid peroxidation and **d** lipid peroxidation in Chang liver cells. 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

Influence of Extraction Temperature on the Antioxidant Profile of Pomegranate Peel

The Fe^{3+} reducing and radical scavenging activity, as well as the ORAC, showed an observable reduction moving from extraction at 25 °C to extraction at 37 °C (Figs. 2c and 3a, b), which may be linked to the observed depleted TPC and TFC (Fig. 2a, b). Polyphenols have the ability to form stable phenoxo radicals by donating electrons or transferring H-atom,

thus are potent radical scavengers (Chen et al., 2020). Beyond 37 °C, the radical scavenging and Fe^{3+} reducing the effect of the extracts gradually increased and peaked at 78 °C, which is consistent with the profile of TPC and TFC (Fig. 2) and that of the predominant ellagitannins (punicalagins, ellagic acid, and granatin A) in the extract obtained at 78 °C (Table 1).

The peel extract obtained at 78 °C had in vitro and cellular anti-lipid peroxidative activity that was more potent than that of the other extracts and did not differ significantly ($p > 0.05$) when compared to that of ascorbic acid and Trolox (Fig. 3c, d), which suggest the potential of the extract obtained at 78 °C to mitigate oxidative insult. The higher ellagitannins content of the peel extract obtained at 78 °C (Table 1) appeared to influence its stronger anti-lipid peroxidative activity relative to the other extracts. Moreover, the predominant ellagitannins (punicalagin and

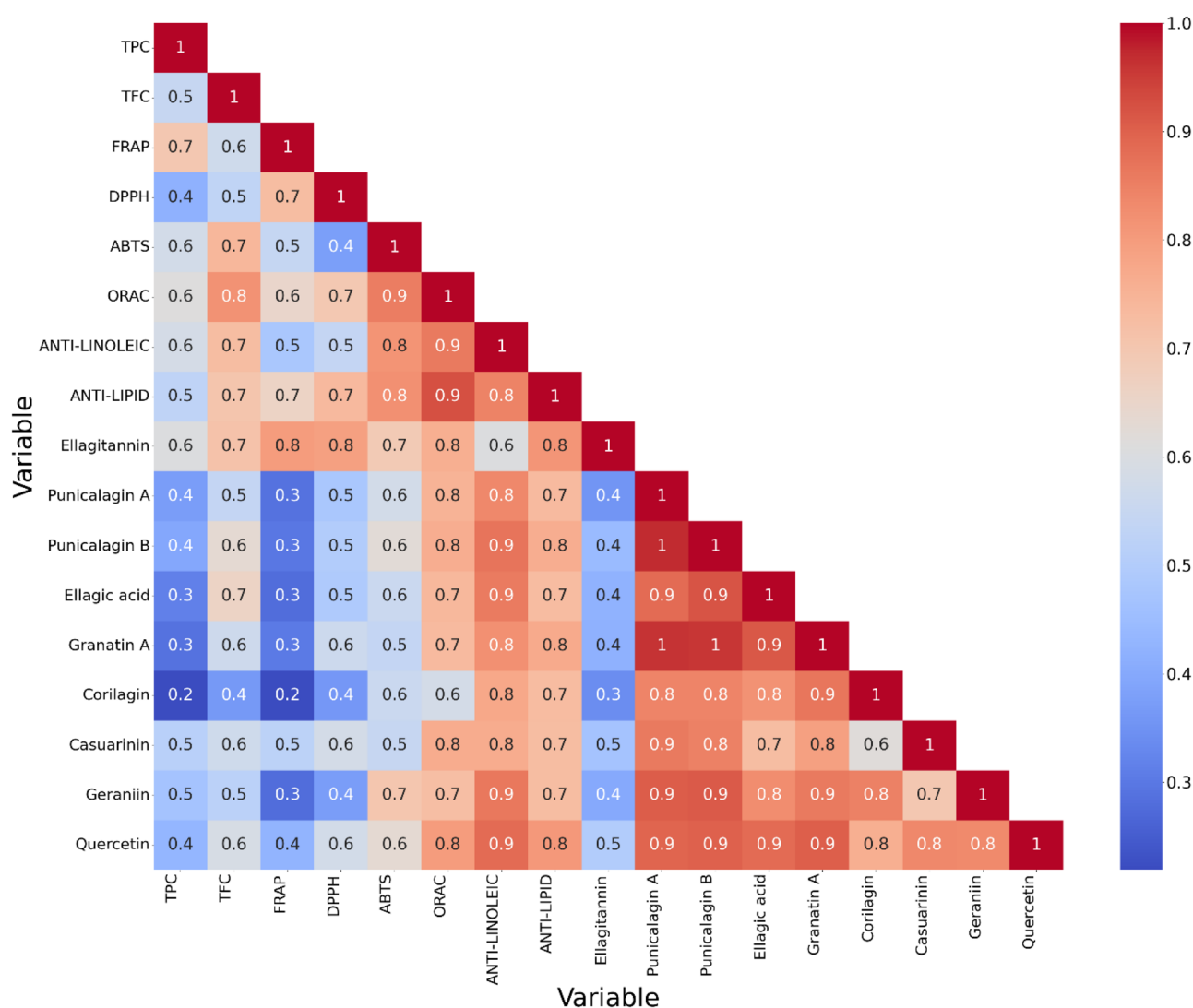
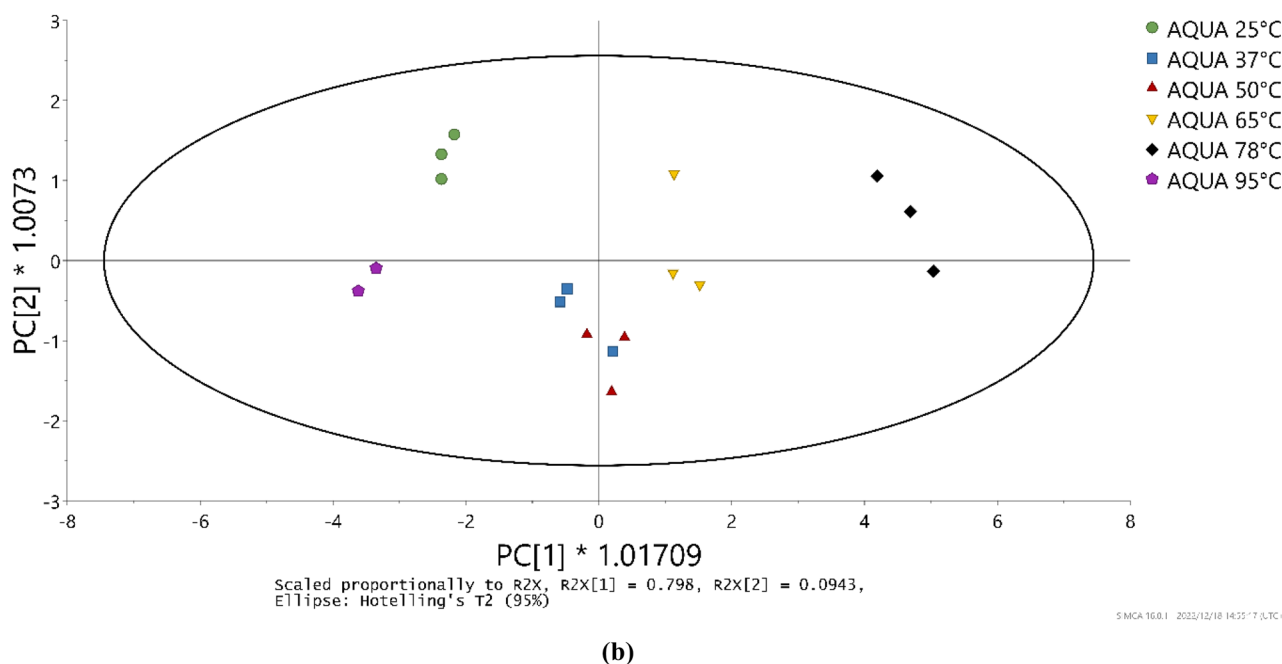
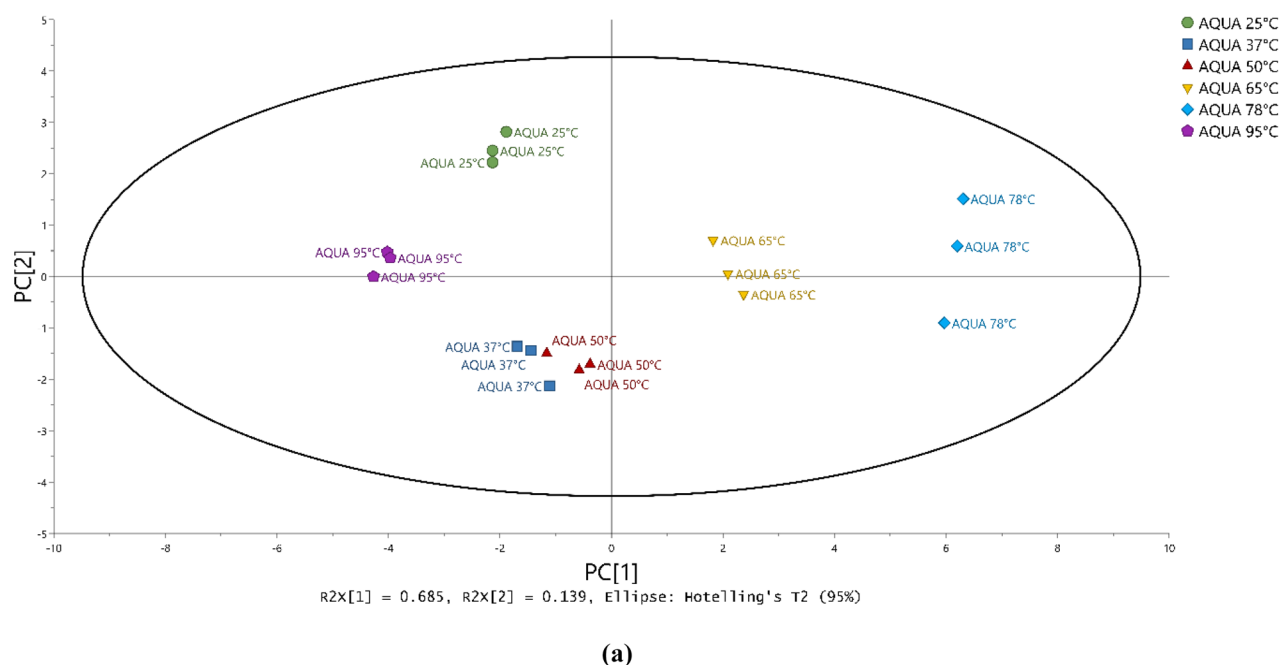


Fig. 4 Pearson's bivariate correlation matrix between data variables

ellagic) showed promising in vitro and cellular anti-lipid peroxidative activity (Fig. 3c, d), while ellagitannins-rich dietary supplement from pomegranate, as well as ellagic acid have been shown to have potent anti-lipid peroxidative activity in obese animals (Xu et al., 2021) and human subjects (Heber et al., 2007). Data suggest that high

extraction temperatures beyond 80–100 °C may compromise the structural and antioxidant profiles of phenolics and ellagitannins in pomegranate peel, while temperatures between 70 and 78 °C may be considered as optimum temperatures for aqueous extraction of pomegranate peel in the context of antioxidant and ellagitannin profile.



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Fig. 5 **a** PCA score plot of unsupervised linear transformation and multivariate dimensional reduction analysis of the data, **b** OPLS score plot of a supervised linear transformation, dimensionality reduction, and modeling of the patterns in the data

Table 2 PCA modeling parameters and summary of fit

Primary ID		R2VX	R2VX(cum)	Q2VX	Q2 limit	Q2VX(cum)
<i>Total</i>	<i>Comp 1</i>	0.685191	0.685191	0.618699	0.108025	0.618699
	<i>Comp 2</i>	0.139213	0.824404	0.272691	0.114187	0.722676
TPC	Comp 1	0.344271	0.344271	0.253445	0.108025	0.253445
	Comp 2	0.244753	0.589024	0.031283	0.114187	0.276799
TFC	Comp 1	0.582139	0.582139	0.360532	0.108025	0.360532
	Comp 2	0.0966583	0.678797	0.041494	0.114187	0.387066
FRAP	Comp 1	0.348633	0.348633	0.269087	0.108025	0.269087
	Comp 2	0.484575	0.833209	0.560992	0.114187	0.679123
DPPH	Comp 1	0.460869	0.460869	0.417135	0.108025	0.417135
	Comp 2	0.164683	0.625552	0.096908	0.114187	0.47362
ABTS	Comp 1	0.629428	0.629428	0.589435	0.108025	0.589435
	Comp 2	0.0534932	0.682921	0.013503	0.114187	0.594979
ORAC	Comp 1	0.860704	0.860704	0.827949	0.108025	0.827949
	Comp 2	0.0507649	0.911469	0.19962	0.114187	0.862294
Anti-linoleic	Comp 1	0.895341	0.895341	0.85556	0.108025	0.85556
	Comp 2	0.0032216	0.898562	-0.08565	0.114187	0.843189
Anti-lipid	Comp 1	0.860302	0.860302	0.825013	0.108025	0.825013
	Comp 2	0.0341192	0.894422	0.241578	0.114187	0.867286
Ellagitannin	Comp 1	0.487474	0.487474	0.419182	0.108025	0.419182
	Comp 2	0.384826	0.8723	0.54912	0.114187	0.738121
Punicalagin A	Comp 1	0.804255	0.804255	0.768588	0.108025	0.768588
	Comp 2	0.148628	0.952883	0.70926	0.114187	0.932719
Punicalagin B	Comp 1	0.849989	0.849989	0.82349	0.108025	0.82349
	Comp 2	0.113947	0.963936	0.72083	0.114187	0.950724
Ellagic acid	Comp 1	0.77182	0.77182	0.727932	0.108025	0.727932
	Comp 2	0.109404	0.881224	0.345866	0.114187	0.822031
Granatin A	Comp 1	0.802621	0.802621	0.757407	0.108025	0.757407
	Comp 2	0.133613	0.936234	0.573621	0.114187	0.896563
Corilagin	Comp 1	0.621759	0.621759	0.457406	0.108025	0.457406
	Comp 2	0.189866	0.811625	0.366858	0.114187	0.656461
Casuarinin	Comp 1	0.726118	0.726118	0.63688	0.108025	0.63688
	Comp 2	0.0061637	0.732282	-0.00427	0.114187	0.635329
Geraniin	Comp 1	0.758138	0.758138	0.732661	0.108025	0.732661
	Comp 2	0.105765	0.863903	0.274824	0.114187	0.806132
Quercetin hexoside	Comp 1	0.844388	0.844388	0.796175	0.108025	0.796175
	Comp 2	0.0421359	0.886524	0.139237	0.114187	0.824555

Correlation Analysis

The phytochemistry and bioactivities of different temperature-controlled aqueous extracts were evaluated. Pearson's correlation coefficients (Fig. 4 and Supplementary Material) indicate varying degrees of strength of linear association between all pairwise combinations of the variable in the dataset. It can clearly be observed that the ORAC and anti-lipid peroxidation effects of the extracts strongly correlate ("r" ranging from 0.7 to 0.9) with the tannin yield of the extracts, as well as the concentration of the

quantified ellagitannin constituents present in the extracts. This strongly buttresses the possible notable contribution of the ellagitannins in pomegranate peel in its anti-lipid peroxidative potential, which has been previously reported in obese animals (Xu et al., 2021) and human subjects (Heber et al., 2007). Moreover, punicalagin A + B and ellagic acid standards (particularly ellagic acid), which are notable chemical constituents of pomegranate ellagitannins showed promising ORAC and anti-lipid peroxidative potential that were statistically comparable to known antioxidants (ascorbic acid and Trolox) (Fig. 3b, c).

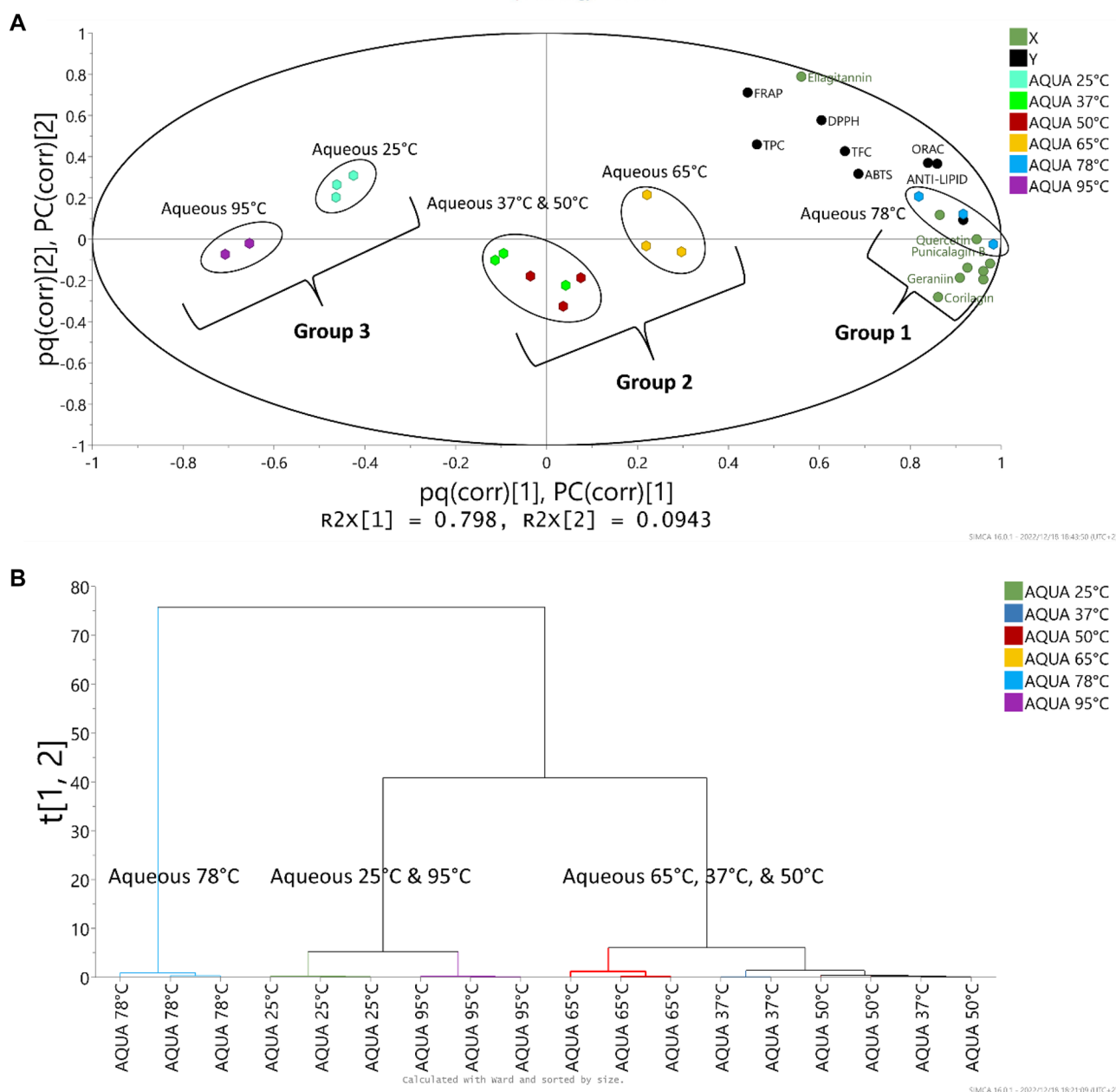


Fig. 6 a OPLS biplot and b HCA dendrogram (b) of the different sample variables and classes

Chemometric Analysis

The data was further interrogated using PCA and OPLS algorithms to extract maximum differential and explanatory information, identify corresponding variables (phytochemicals and antioxidant potency), as well as build predictive models to describe the relationship between extracted compounds and various antioxidant effects in different aqueous extraction temperatures. Through the use of latent variables or principal components, PCA and OPLS techniques project the prevailing trends in the multidimensional dataset onto

a small number of representative virtual variables (Gbashi et al., 2017; Wheelock & Wheelock, 2013).

PCA provided an initial dataset overview. From the PCA results, no outliers were detected, i.e., all samples were within the Hotelling T2 95% confidence ellipse (Fig. 5). Also, the PCA model revealed high explained variation [$R2X(cum)=0.824$] and strong predictive patterns [$Q2(cum)=0.723$] in the dataset (Table 2). The PCA score plot (Fig. 5) also showed clear clusters of the samples indicating extraction temperature results in differential metabolite recovery patterns and antioxidant potency of the various extracts.

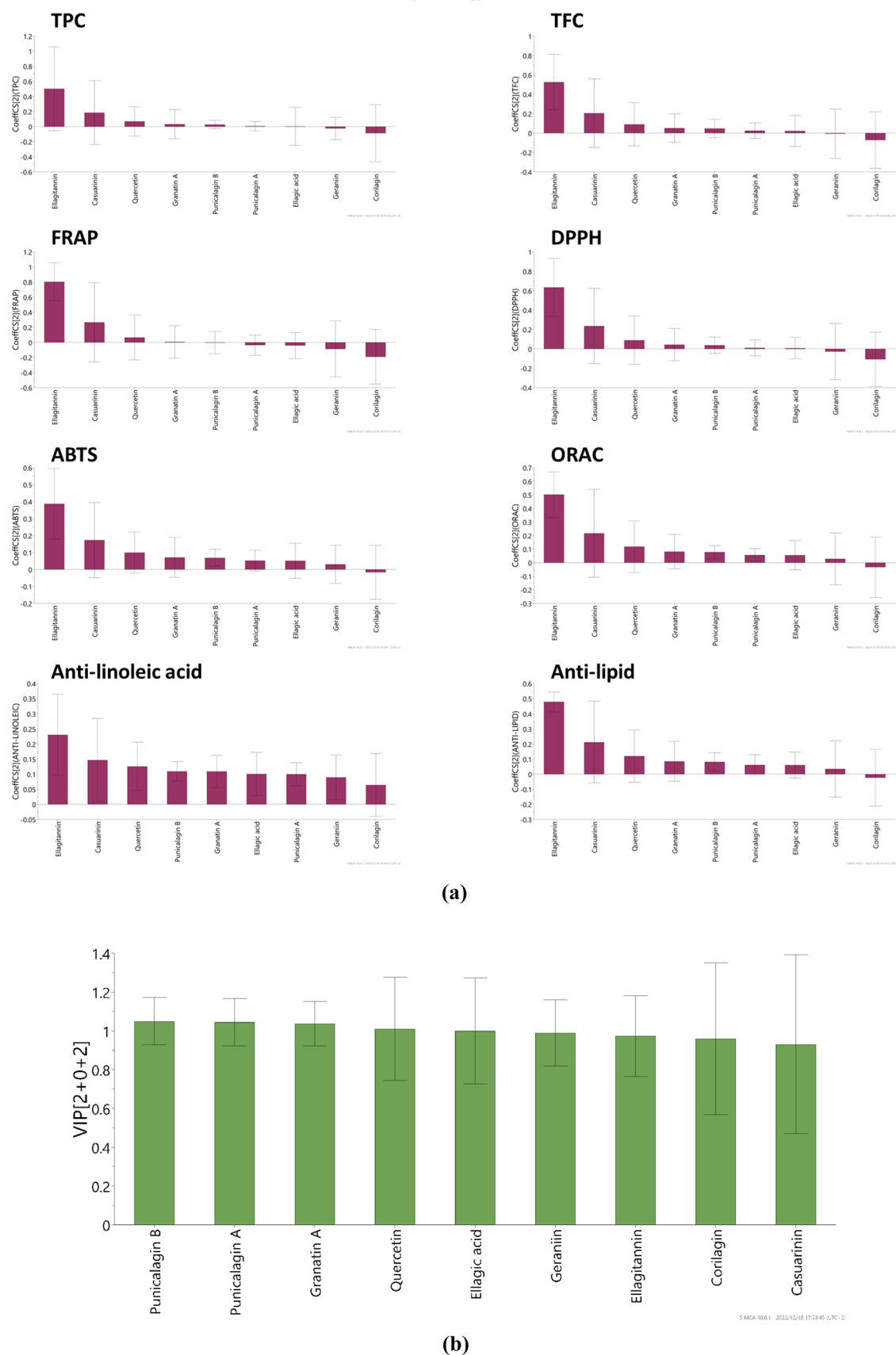


Fig. 7 **a** Contribution of each predictor variable on the specific response variable, **b** OPLS variable importance plot

Table 3 OPLS modeling parameters and summary of fit

Component	R2X	R2X(cum)	R2	R2(cum)	Q2	Limit	Q2(cum)	R2Y	R2Y(cum)	Significance
Model		0.892		0.695			0.611		0.936	
Predictive		0.892		0.695			0.611		0.763	
P1	0.798	0.798	0.645	0.645	0.44	0.01	0.44	0.688	0.688	R1
P2	0.0943	0.892	0.0498	0.695	0.171	0.01	0.611	0.0748	0.763	R1
Orthogonal in Y (OPLS)									0.173	
O1								0.107	0.107	R1
O2								0.066	0.173	R1

OPLS modeling was used to look for linear relationships between the predictors (metabolites) and response variables (antioxidant capacities) and to establish the significance of the variables in explaining the overall patterns in the data. Figure 6 shows the OPLS biplot and the HCA dendrogram, which suggest the similarities and differences in the sample groups. The biplot overlays data distribution patterns from both the loadings plot and the score plot (Fig. 6a). The loadings consist of the weights of the original data variables while the new coordinates for the observations are the scores. The score plot shows how the observations are divided into groups while the loadings show how the original variables relate to the scores (Wheelock & Wheelock, 2013).

On the biplot, the X variables are colored green, the Y variables are colored black, and observations belonging to the extraction conditions are also colored differently. A distinct grouping pattern can be visualized on the plot, with the 78 °C aqueous extracts standing out and being the most alienated from the other extracts (Figs. 6a and 5b). The HCA dendrogram (Fig. 6b) further emphasizes the clustering pattern observed on the PCA score plot and the OPLS biplot. In this plot, the clusters are shown along the horizontal axis, while the vertical axis denotes the separation or dissimilarity between groups. A vertical line is broken into two lines on the diagram to symbolize each joining (fusion) of the two clusters. The separation (difference) between the two clusters is shown by the split's vertical location, which is depicted by a short bar. This plot reveals three major clusters which are cluster 1: 78 °C, cluster 2: 25 and 95 °C, and cluster 3: 37, 50, and 65 °C. Essentially, samples within the same cluster may share inherent similarities and are more closely related to adjacent clusters in order of hierarchy. It is safe to speculate that extraction at 78 °C notably and optimally influenced the ellagitannins and antioxidant profile of pomegranate fruit peel.

The Y variables, including in vitro anti-linoleic acid peroxidation, TFC, ORAC, ABTS, and cellular anti-lipid peroxidation, were closely grouped around the 78 °C samples and largely within the same quadrant, which were clustered

around the ellagitannins (X variables) (Fig. 6a), suggesting that the distinct antioxidant potential of the extract at 78 °C was largely influenced by its ellagitannin profile. Since PC1 explains most of the variation in the dataset (79.8%), separation along its axis (i.e., x-axis) could describe differences in the sample groups. In this regard, the aqueous extract at 78 °C was more potent than the other extracts, while the extract at 95 °C was the least potent for the investigated antioxidant assays. This implies that the extract at 78 °C contained the highest amounts, while the extract at 95 °C contained the lowest amounts of the bioactive phenolics and ellagitannins, thus explaining their contrasting antioxidant profiles (Fig. 3).

The variable contribution plot further provides information on the influence of the ellagitannins on the antioxidant profile of the potent extracts (Fig. 7). The higher tannin/ellagitannins yield of the extract at 78 °C, relative to the other extracts notably contributed to its antioxidant profile (Fig. 7a). Most of the identified ellagitannins fairly influenced the ORAC and anti-lipid peroxidative capacities of the extracts, with casuarinin appearing to be the most influential although with a relatively high standard error (Fig. 7a). Perhaps, because its concentration in the extract at 78 °C was double the amount in any other extract (Table 1). Punicalagin, on the other hand, appears to consistently influence the ORAC and anti-lipid peroxidative capacities of the extracts with a fairly low standard error, which is consistent with the observed ORAC and anti-lipid peroxidative effect shown by punicalagin A + B standard (Fig. 3b, c). In summary, however, punicalagin, granatin A, and ellagic acid are among the top five ellagitannins influencing the antioxidant profile of the extracts (Fig. 7b). Moreover, ellagic acid was shown to exhibit appreciable ORAC and anti-lipid peroxidative effect (Fig. 3b, c).

Model Overview, Evaluation, and Validation

When numerical models are fitted to empirical data, it is necessary to evaluate how fit the model is to ensure that the model behavior is reasonable and that any predicted or fitted parameter values may be interpreted and used with

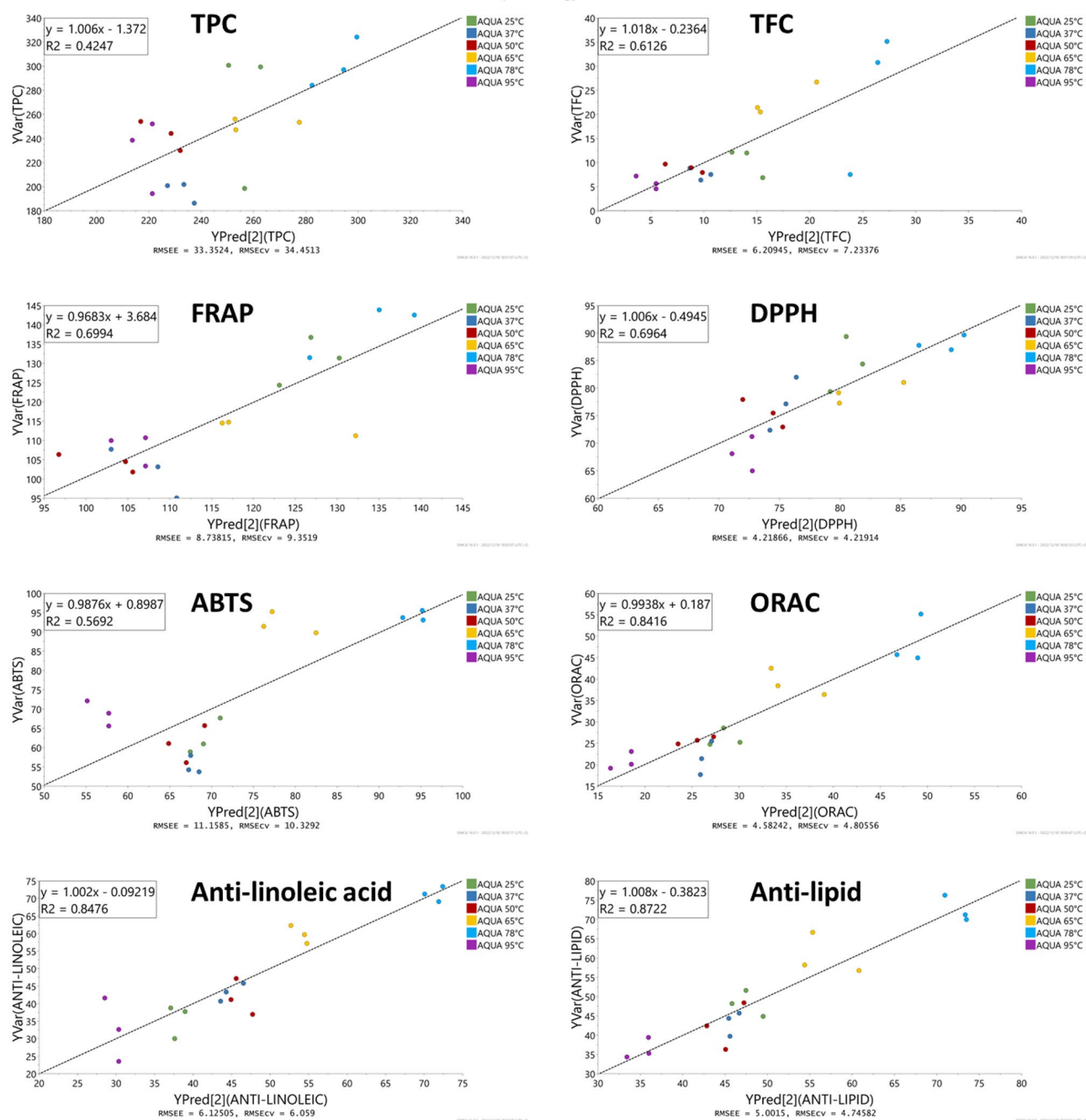


Fig. 8 OPLS predicted versus observed values

confidence. Model fit parameters for both PCA and OPLS models are presented in Tables 2 and 3. PCA generated two latent variables which combined, described 82.4% of the variation in the entire dataset, with a predictive capacity of 72.3%. PC1 modeled 68.5% of the variation in the dataset while PC2 explained 13.9% of the variation in the data. All generated latent variables are significant (Table 2).

Similar to the results of PCA, OPLS analysis generated two components which were all significant (Table 3). The X

model described 89.2% of the X variation [$R^2X(\text{cum})=0.892$] and 69.5% of the Y variation [$R^2(\text{cum})=0.695$], while the Y model described 93.6% [$R^2Y(\text{cum})=0.936$] of the variation in the response variables. The total orthogonal variation in the response variable, i.e., orthogonal in Y, was 17.3%. The cumulative predictive power of the OPLS model was 61.1% [$Q^2(\text{cum})=0.611$]. Regressing the predicted values versus the observed values yielded R^2 values ranging from 0.42 to 0.87, RMSEE values ranging from 4.22 to 33.35, and RMSECV

values ranging from 4.22 to 34.45 (Fig. 8). Indeed, all the model fit parameters indicate that our fitted models sufficiently capture the patterns in the data and adequately describe the empirical relationship between the predictors and response variables.

Conclusion

The present study reveals a temperature-dependent influence on the antioxidant and ellagitannin profile of pomegranate peel subjected to aqueous extraction. The strongest antioxidant potential and optimum ellagitannins yield were obtained at 78 °C extraction temperature. The obtained OPLS biplot from the chemometric analysis showed a clustering pattern of antioxidant and ellagitannins variables distinctly around 78 °C extraction temperature, suggesting an observable influence of the phytochemicals on the potent antioxidant profile of extracts obtained at 78 °C relative to the other extracts. The variable contribution plots, as well as the obtained antioxidant data suggest punicalagin, granatin A, and ellagic acid as notable ellagitannins influencing the potent antioxidant action of the pomegranate peel extracts. It is safe to speculate that aqueous extraction at 78 °C notably and optimally influenced the ellagitannins recovery and antioxidant profile of pomegranate fruit peel. Thus, maybe a promising aqueous extraction temperature for optimally recovering the ellagitannins from pomegranate peel, without sacrificing the antioxidant capacity, which may be of medicinal relevance in managing oxidative stress in many diseases.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11947-023-03104-2>.

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Data Availability The data of this study is available from the corresponding author upon request.

Declarations

Conflict of Interest The authors declare no competing interests.

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