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„Acute impact of pomegranate juice on total antioxidant capacity of plasma (FRAP) in healthy female adults. "

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„*Life is nothing but an electron looking for a place to rest*.”

Albert Szent-György

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List of Content

List of illustrations

List of tables

List of acronyms and abbreviations

ANOVA – Analysis of variance

Aqua dest. – *Aqua destillata* (distilled water)

BIA – Bio Impedance Analysis

BMI – Body Mass Index

°C – Degrees Celsius

CAT – Catalase

CGA – Chlorogenic acid

CMH - 2,2,6,6-tetramethylpiperidine

CV – Coefficient of variation

df – Degrees of freedom

DNA – Desoxyribonucleic acid

DPPH - 2,2-diphenyl-1-picryl-hydrazyl-hydrate

ECG – Electrocardiogram

EDTA – Ethylenediaminetetraacetate

EPR – Electron paramagnetic resonance

ET – Electron transfer

FCR – Folin Ciocalteau reagent

Fe^{2+} - Ferrous Ions

Fe^{3+} - Ferric Ions

$FeCl_3 *_6H_2O$ – Iron-(III)-chloride hexahydrate

$FeSO_4*_7H_2O$ – Ferrous-(II)-sulphate heptahydrate

FRAP – Ferric reducing ability of plasma / Ferric reducing antioxidant power

GAE – Gallic acid equivalents

GPX – Glutathione peroxidase

GSH – Glutathione

HAT – Hydrogen atom transfer

HbA1c – Haemoglobin A1c

HCl – Hydrochloric acid

HDL – High density lipoprotein

HIIT – High intensity interval training

H_2O_2 – Hydrogen peroxide

HOCl – Hypochlorous acid

HPLC – High-performance liquid chromatography

KCl – Potassium chloride

LDL – Low density lipoprotein

MDA – Malondialdehyde

NaCl – Sodium chloride

NADPH – Nicotinamide adenine dinucleotide phosphate

Na_2HPO_4 – Di-sodium-hydrogen phosphate

NaOH – Sodium hydroxide

NEAC – Non enzymatic antioxidant capacity

NFκ-B – Nuclear factor kappa B

NO – Nitric oxide

NO_2^* - Nitric oxide radical

NOS – Nitric oxide synthase

O_2^{*-} - Superoxide radical

$^1O_2$ – Singlet oxygen

O_3 – Ozone

OH – Hydroxyl

OH* - Hydroxyl radical

OJ – Orange juice

OS – Oxidative stress

PBS – Phosphate-buffered saline

PC – Protein carbonyls

RCT – Randomised control trial

rmANOVA – repeated measurement analysis of variance

RNA – Ribonucleic acid

RNS – Reactive nitrogen species

RO* - Alkoxyl radical

RO_2^* - Peroxyl radical

ROS – Reactive oxygen species

RPE – Rate of Perceived Exertion

SET – Single electron transfer

SOD – Superoxide dismutase

TEAC – Trolox equivalent antioxidant capacity

TPTZ – 2,4,6-Tris(2-pyridyl)-s-triazine

UA –Uric acid

XO – Xanthine oxidase

Abstract

This master's thesis aimed to assess the impact of pomegranate juice on the total antioxidant capacity of plasma (FRAP) after physical activity in female participants. Pomegranate juice is a source of polyphenols, and it is known for its high antioxidant capacity. This might have positive effects on the scavenging of oxidants in the organism. 20 healthy women, aged 20 to 37, participated in a randomised intervention study conducted by the faculty of nutritional sciences of the University of Vienna. Further intervention beverages were orange juice, coffee, matcha, a sugar-sweetened soft drink, milk, and water. In addition, other oxidative stress markers, such as reactive oxygen species (ROS), protein carbonyls (PC), malondialdehyde (MDA), high-sensitive Interleukin 6 (HS-IL 6), and high-sensitive C reactive protein (HS-CRP) were determined. Since it was a clinical study conducted with female participants, estradiol-17β was measured. The participants received 250 mL of pomegranate juice two hours before a high-intensity interval training (HIIT). Blood samples (venous and capillary) were taken two hours before, immediately before and after the HIIT, and 15 minutes after the exercise. Between the first and the second blood draw, the participants received the intervention beverage. The consumption of 250 mL pomegranate juice before a HIIT did significantly affect the FRAP levels in healthy women. Increased FRAP values could be shown after drinking pomegranate juice, water, orange juice, matcha, coffee, and milk. Therefore, it can be assumed that a wide range of polyphenolic and other antioxidant compounds in beverages have a positive influence on the overall antioxidant capacity in plasma.

Zusammenfassung

Die vorliegende Masterarbeit beschäftigt sich mit dem Einfluss von Granatapfelsaft auf die gesamt antioxidative Kapazität im Plasma (FRAP) nach sportlicher Betätigung bei weiblichen Studienteilnehmerinnen. Granatapfelsaft ist reich an Polyphenolen und die antioxidative Kapazität des Saftes ist sehr hoch. Dies könnte positive Effekte auf die Bindung von Oxidantien im Organismus haben. Um dies zu überprüfen, wurden 20 gesunde Frauen im Alter von 20 bis 37 Jahren innerhalb einer randomisierten Interventionsstudie, durchgeführt an der Fakultät für Ernährungswissenschaften der Universität Wien, untersucht. Neben der Bestimmung des FRAP-Wertes wurden weitere Marker für oxidativen Stress, wie Reaktive Sauerstoffspezies (ROS), Proteincarbonyle (PC), Malondialdehyd (MDA), hoch-sensitives Interleukin 6 (HS-IL 6) und hoch-sensitives C-reaktives Protein (HS-CRP) bestimmt. Da es sich um ein weibliches Studienkollektiv handelt, wurde zusätzlich Estradiol-17β, gemessen. Die Teilnehmerinnen erhielten 250 mL Granatapfelsaft zwei Stunden vor einem Training mit hoher Intervallintensität (*engl. High intensity intervall training -HIIT-*). Venöse und kapillare Blutabnahmen erfolgten zwei Stunden vor, direkt vor und direkt nach dem Training und nochmals 15 Minuten nach dem Training. Zwischen der ersten und zweiten Blutabnahme bekamen die Probandinnen das Interventionsgetränk verabreicht. Es zeigte sich, dass der Konsum von 250 mL Granatapfelsaft vor einem HIIT einen signifikanten Einfluss auf die gesamt antioxidative Kapazität bei gesunden Frauen hat. Es konnten steigende FRAP-Werte nach dem Konsum von Granatapfelsaft, Wasser, Orangensaft, Matcha, Kaffee, und Milch gezeigt werden. Schlussfolgernd ist davon auszugehen, dass verschiedenste polyphenolische und andere antioxidativ wirkende Verbindungen in den Getränken einen positiven Einfluss auf die gesamt antioxidative Kapazität in Plasma haben.

1 Introduction

Since the beginning of the 20th century free radicals are known in chemistry. Later pioneering work by D. Gilbert and R. Gersham recognized that these radicals are also present in the biological environment and can cause deleterious damage (Kohen et al. 2002). Increasing health awareness and the desire for self-optimization in the modern Western world allows the research field around free radicals, antioxidants and sports to continue to grow (Gomez-Cabrera et al. 2008 and Kristensen et al. 2015). Different factors are known to increase the production of ROS (reactive oxygen species) or RNS (reactive nitrogen species). Within the physiological range, this can be described as a common phenomenon and not detrimental (Sorg, 2004). Only through the development of oxidative stress, which is an imbalance between formation and neutralisation of ROS, lipids (i.e., cell membranes), proteins, and DNA can be damaged. Previous studies have shown that oxidative stress is involved in a wide range of diseases such as cardiovascular diseases (e.g., ischaemia, atherosclerosis), and neurodegenerative diseases (e.g., Alzheimer, Parkinson) (Sorg, 2004). Oxidants also play a role in ageing processes as free radicals continuously impact cells and tissues (Sorg, 2004). Antioxidants are known to neutralize ROS if consumed in adequate amounts. Scientists disagree about the quantity and origin (natural or synthetic) of antioxidants, necessary to yield health benefits. The negative effects of dietary supplements with high doses of (synthetic) antioxidants have been discussed in recent publications (Poljsak et al. 2013). The objective of this master's thesis is to investigate the impact of acute consumption of naturally occurring antioxidants, particularly polyphenols in pomegranate juice, on total plasma antioxidant capacity in healthy women. To increase oxidative stress, the women were instructed to perform a high intensity resistance training, which served as a stress model. Physical exercise can be an exogenous source of ROS (Poljsak et al. 2013). In the literature, many interventional studies could be found. The outstanding point of this project is that the participants are female adults, whereas in previous studies women were rarely represented. The interventional study of Ammar (2017) examined the mitigating effects of pomegranate juice (containing 2.56 g total phenols per 500 mL) on oxidative stress responses after weightlifting training sessions in nine elite male weightlifters. It was reported that pomegranate juice can potentially reduce oxidative stress by enhancing enzymatic (i.e., catalase [CAT] and glutathione peroxidase [GPx])

and non-enzymatic antioxidants (i.e., uric acid [UA] and total bilirubin) responses after intense weightlifting exercises. Also, a significant decreasing effect on the lipid oxidation biomarker Malondialdehyde (MDA) was observed (Ammar et al. 2017). In addition, Al Dujaili (2016) was able to show that the consumption of 500 mL pomegranate juice (containing total phenols of 1685 mg GAE / L) can reduce the acute increase in oxidative stress, caused by physical activity, in overweight and obese people of women and men. Based on these studies it is reasonable to expect an effect from pomegranate juice to reduce oxidative stress, however, it is very difficult to find comparable data in the multitude of literature as there are many ways to measure oxidative stress biomarkers. Therefore, in the present work, a comprehensive method for determining the antioxidant capacity was used: Ferric Reducing Ability of Plasma or Ferric Reducing Antioxidant Power -FRAP. This assay allows to obtain a comprehensive perspective on the non-enzymatic reducing capacity of plasma (Benzie and Devaki, 2017). Unlike other more complex methods, the FRAP assay is not dependent on preparatory enzymatic or non-enzymatic methods to generate free radicals for evaluation of the antioxidant capacity of plasma, nor isolation of plasma fractions such as LDL is required (Pulido et al. 2000). The major advantage of this assay is the high reproducibility with an intra- and interassay coefficient of variation lower than 5% (Firuzi et al. 2005).

1.1 Reactive oxygen species

Reactive oxygen species (ROS) are generated when a small percentage of oxygen is not completely reduced within the normal cellular respiration chain in the metabolism of the cell. These oxygen intermediates are commonly known as ROS. It is also possible that reactive species derive from nitrogen. Then they are called reactive nitrogen species (RNS) (Peternelj, 2011). ROS generated in biological systems as byproducts of metabolism can be superoxide radicals (O_2^{*-}), hydroxyl radicals (OH*), nitric dioxide radicals (NO_2^*), alkoxyl (RO*), peroxyl radicals (RO_2^*), and singlet oxygen ($^1O_2$) (Pizzino et al. 2017).

1.2 Oxidative Stress

Oxidative stress describes a circumstance in which the production and detoxification of ROS are in disequilibrium. Low levels of ROS are generated under normal physiological conditions. The main endogenous source of ROS is recognized to be enzymes such as nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), nitric oxide synthase (NOS), and

xanthine oxidase (XO) (Powers et al. 2008). Due to their impaired electron in their outer orbital, ROS are unstable, highly reactive and stimulate oxidation reactions. ROS do not need to be exclusively reactive species but also can be some non-radical derivates of oxygen. These can be hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), and nitric oxide (NO) (Peternelj, 2011). ROS are not always deleterious. Low and therefore physiological levels of ROS are required for intracellular signalling cascades, redox regulations of protein phosphorylation, ion channels, and transcription factors (Valko et al. 2006). Physiological levels of ROS in healthy subjects range from 1.84 to 2.04 µM / min (Mrakic-Sposta et al. 2014). The transmission of signals between cells is crucial for normal neural activity and is mainly supported by H_2O_2 and NO (Pizzino et al. 2017). Additionally, moderate levels of ROS can be seen as a physiological stimulus for endogenous antioxidant defence systems (Peternelj, 2011). For example, one important adaptation mechanism involves H_2O_2. This radical induces gene expression of antioxidant enzymes (named in 1.4 Antioxidants) via nuclear factor κ-B (NFκ-B) in muscle cells which leads to improved ability in scavenging ROS (Li et al. 2021). On the other hand, excessive levels of ROS exposure exceed endogenous adaptations and promote oxidative damage on lipids and proteins including protein oxidation, DNA or RNA strand breakage, lipid peroxidation, or formation of advanced glycosylation end-products. These mechanisms typically increase with age and are related to neurodegenerative disease (Pohl and Lin, 2018). To protect the cell against oxidant-mediated damage there is a complex defence system of antioxidants. Furthermore, the antioxidant capacity of the muscle plays an important role in quenching ROS (Powers et al. 2004). Scientists agree, that under physiological conditions, the balance between pro-oxidants and antioxidants is slightly on the pro-oxidant side which is fundamental for essential cell processes (Pohl et al. 2018). The processes described up to this point refer to the endogenous sources of ROS. There are also exogenous sources of ROS. These include environmental influences such as ultraviolet rays, water- and air pollution, alcohol, drugs, tobacco, pesticides, paracetamol, and others (Phaniendra et al. 2014).

1.3 Important Radicals

As described earlier, oxygen-derived oxidants, also called pro-oxidants in a biological environment can cause deleterious damage to lipids, DNA, and proteins (Kohen et al. 2002). Some of the most important reactive species will now be explained in more detail.

1.3.1 Superoxide-Ions (O_2^{*-})

O_2^{*-} can be formed by NADPH oxidase and xanthine oxidase within complex I of the mitochondrial electron transport chain (Valko et al. 2006). By generating OH^-, O_2^{*-} indirectly initiates peroxidation of phospholipids (Li et al. 2021). O_2^{*-} in general, is considered to be relatively membrane impermeable and unreactive compared with other radical species. But through various reactions, O_2^{*-} can become very reactive (Powers et al. 2008). Dismutation is the most important reaction of superoxide radicals. In the latter reaction, two superoxide radicals react with each other, one is oxidized to oxygen, and the other is reduced to hydrogen peroxide in an acidic environment (Kohen et al. 2002).

$$O_2^{*-} + O_2^{*-} + 2H^+ \rightarrow H_2O_2 + O_2$$ [Dismutation reaction] (Powers et al. 2008)

1.3.2 Hydrogen Peroxide (H_2O_2)

The result of dismutation of O_2^{*-}, H_2O_2, can be easily dissolved in water and also can penetrate biological membranes. Even in relatively low concentrations (10 µM), it can cause damage to the cell (Kohen et al. 2002). H_2O_2 plays a major role in the *Fenton Reaction*. In 1894 the foundation for one of the most important explanations of oxidative damage was discovered. Fenton described the interaction between Fe^{2+} and H_2O_2 in acidic environments. Within this Fenton reaction, H_2O_2 and Fe^{2+} lead to the formation of the most reactive radical *in vivo*, HO*.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^*$$ [Fenton reaction] (Kohen et al. 2002)

Most of the transition metals, except zinc, can convert relatively stable oxidants into radicals. Copper and especially iron are the most abundant transition metals for this reaction (Kohen et al. 2002).

1.3.3 Hydroxyl Radical (OH*)

In contrast to superoxide radicals, OH* radicals are extremely reactive and relatively stable. As a short-lived species, OH^* poses a high affinity for other molecules and biological components

in its direct environment (Kohen et al. 2002). Hydroxyl radicals are generated by the interaction between hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$) (Powers et al. 2016). In 1934 Haber and his student Weiss published the *Haber-Weiss reaction* as a concept for the generation of highly reactive hydroxyl radicals *in vivo* (Kehrer 2000). Later biochemical work found that the Haber-Weiss reaction depends on a catalyst for oxygen-transferring properties (transition metal ions, particularly iron) to take place in biological systems. The now so-called *iron-catalysed Haber-Weiss reaction* makes use of the Fenton reaction (see 1.3.2 Hydrogen Peroxide (H2O2)). Because iron salts are present at low concentrations in biological systems the iron-catalysed Haber-Weiss reaction is recognized as the main reaction for generating $OH^{\bullet}$ *in vivo* (Kehrer 2000).

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{Fe^{3+}/Fe^{2+}} OH^{\bullet} + OH^- + O_2 \text{ [Haber-Weiss reaction]}$$

(Kanti Das et al. 2014).

Professor Haber's early research proved to be the cornerstone and at the same time the footing for understanding the mechanisms of free radical formation (Kehrer 2000).

1.4 Antioxidants

The Institute of Medicine of the National Academy of Sciences defines an antioxidant as "a dietary antioxidant is a substance in foods that significantly decreases the adverse effects of reactive oxygen species, reactive nitrogen species, or both on normal physiological function in humans" (Institute of Medicine, 1998). An Antioxidant in general is a substance of endogenous or exogenous origin. They act as a scavenger and reduce oxidative stress and its following damaging processes (Poljsak et al. 2013). In the human organism, there are three different antioxidant defence systems. These are non-enzymatic, enzymatic, and dietary antioxidants (for an overview see Appendix I). Enzymatic antioxidants are superoxide dismutase (SOD), peroxidases, like glutathione peroxidase (GPx), and catalase (CAT). Non-enzymatic antioxidants are glutathione (GSH), UA, lipoic acid, bilirubin, and coenzyme Q_{10}. These arise endogenously and are mostly byproducts of the cellular metabolism. The most common dietary antioxidants are vitamin E (tocopherols, tocotrienols, and α-tocopherols), Vitamin C (ascorbic acid), and carotenoids (β-carotene). Recently, also various polyphenolic compounds received increasing attention (Peternelj et al. 2011 and He et al. 2017). There have been critical discussions regarding the use of antioxidant supplements. An increasing number of papers have shown that administration of antioxidants may prevent physiological adaptations to ROS

(Vivekananthan et al. 2003 and Ristow et al. 2009). As described above, ROS are also acting as signals that regulate molecular pathways which are important for muscle cell adaptation to exercise. Externally supplied antioxidants could interrupt these adaptations (Gomez-Cabrera et al. 2008). It is important to differentiate between synthetic and strong antioxidants, and antioxidants from natural origin. On the market, there is a wide range of vitamins and extracts advertised as antioxidants. The effectiveness has not been adequately tested, particularly in humans (Peternelj, 2011). Therefore, Peternelj's review (2011) concludes that a diet rich in antioxidants is preferable to supplements because whole foods like fruits, vegetables, nuts, and whole grains provide healthy levels of antioxidants and phytochemicals (Peternelj, 2011). Table 1 below is intended to give an idea of how healthy levels of antioxidants should be classified.

Antioxidant	**Maximum levels for nutritional supplements (mg / day)**	**Average amounts supplied by diet (mg / day)**
Vitamin A	0.2	1.0
Vitamin C	250	97
Vitamin E	30	8.8
Selenium	0.045	Not available
Zinc	6.5	8.4
Beta-carotene	3.5	Not available
Other carotenoids (Lycopene, Lutein, Zeaxanthin, Astaxanthin)	Not available	Not available
Secondary plant ingredients (Anthocyanins, Bioflavonoids, Resveratrol)	Not available	Not available
Adapted from	*Bundesinstitut für Risikobewertung, 2021*	*Krems et al. 2013. Nationale Verzehrsstudie II*

Table 1: Safe upper limits of antioxidants.

Comparison of the maximum permitted amounts of antioxidants in supplements and the estimated amounts of antioxidants supplied by diet.

Many antioxidative substances have been shown to have significant effects *in vitro*. In contrast, dietary antioxidants focuses on the effects of such substances when consumed by humans (Sen, 1995).

1.4.1 Mechanisms of antioxidants

Another classification of antioxidants can be made based on the mechanism of the compounds. Herein two categories arise, preventive antioxidants, such as SOD, catalase, peroxidase, and transferrin. And chain-breaking antioxidants such as vitamin C, vitamin E, UA, bilirubin, and polyphenols. The former is able to inhibit the formation of reactive oxygen species, while the latter scavenges oxygen radicals and thereby breaks radical chain sequences (Ou et al. 2002). The chain-breaking mechanism occur through two possible pathways (Figure 1). The first one involves a hydrogen atom transfer (HAT), and the second pathway is the single electron pathway (SET) (Ou et al. 2002) – which is the mechanism applicable to FRAP.

1.4.1.1 Hydrogen atom transfer (HAT)

HAT is one possible mechanism of antioxidants to scavenge free oxygen radicals. The oxygen radical extracts hydrogen from the antioxidant, through bond dissociation enthalpy, leading to the formation of a stable antioxidant radical (Ou et al. 2002).

1.4.1.2 Single electron transfer (SET)

The single electron transfer is the second possible pathway for the de-activation of free radicals by antioxidants, using the ionization potential and transferring the ion from the radical to the antioxidant (Beya et al. 2021).

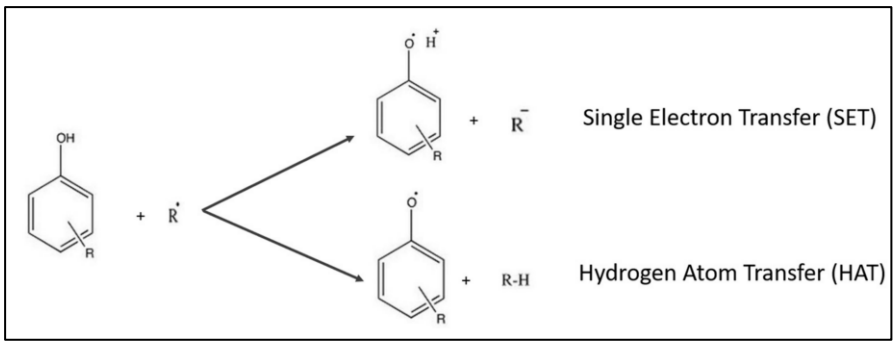

Figure 1: Single Electron Transfer (SET) and Hydrogen Atom Transfer (HAT)
(Beya et al. 2021)

1.5 Phytochemicals

Pomegranate juice contains numerous secondary plant compounds or phytochemicals (see chapter 2) of which polyphenols are the generic term. For the classification of all chemical compounds see Appendix II Polyphenols with natural origin include simple molecules

(phenolic acids, phenylpropanoids, and flavonoids) and highly polymerized compounds (lignins, melanins, and tannins) (Vuida-Martos et al. 2010). In general, polyphenols are chemically viewed as aromatic alcohols with an aromatic benzene ring and an alcoholic hydroxyl group (-OH). If a chemical structure consists of more than two of these rings and two to four -OH groups, then it is described as polyphenol. Especially, the -OH groups are responsible for the specific chemical properties. The hydrogen from the -OH group can easily be split off radically. This means that they easily give up electrons and therefore act as reductants or antioxidants. In addition, polyphenols can form hydrogen bonds with other classes of substances through their -OH groups. Here, they interact primarily with proteins and act as adhesives (Kuhnert, 2013). The bioavailability of a substance is the limiting factor for its effectiveness. According to Kuhnert (2013), the more polar the polyphenol, the better the effect as an antioxidant. This competes with bioavailability. Polar (water-soluble) compounds can only overcome the natural lipophilic cell wall with difficulty and are therefore only absorbed in small amounts (Kuhnert, 2013). There are only a few studies examining the correlation between health effects and the bioavailability of polyphenols. The review by Di Lorenzo (2021) calculated the total polyphenol intake to be at 0.9 g per day in young people, adults, and the elderly. Interestingly, the participants in this underlying study achieved a total polyphenol intake of around 0.96 g after consumption of 250 mL of dm Bio pomegranate juice, based on the calculation in chapter 2.1 assuming a total polyphenol content of 3862.10 µg GAE / mL. Anthocyanins which are one of the most abundant polyphenols in pomegranate juice have the lowest bioavailability of all polyphenols. Only 0.003% and 0.002% of delphinidin glycosides and cyanidin glycosides are excreted in the urine after 120 minutes of consumption and therefore appear to be negligible (Di Lorenzo et al. 2021). Flavonoids are a subgroup of polyphenols, which are bioactive compounds widely found in pomegranates and other fruits and vegetables. Flavonoids represent a very large group among polyphenols which consists of flavonols, anthocyanins, isoflavonoids, flavanones and flavones. The antioxidant activity of flavonoids is based on the total number of hydroxyl groups and the arrangement of the functional groups (He et al. 2017). These characteristics enable the quenching of OH*, H_2O_2 and O_2^{*-} (Powers et al. 2004). Flavonoids are differentiated according to their degree of oxidation. This results in chemical names such as flavanols, flavanones, and flavanolones. Tannins, as

they occur in pomegranates and pomegranate juice, are formed by the oxidation of flavanols. A distinction is made between hydrolysable and non-hydrolysable tannins (Kuhnert, 2013).

1.6 Physiological changings during physical exercise

Research in the field of generation of reactive oxygen species due to physical exercise started in the fifties and has grown steadily in the last decades. In the 1970s, scientists proved that an exhaustive and intense muscle contraction results in oxidative stress in the human organism (Dillard et al. 1978). One of the early studies that investigated the correlation between physical exercise and increased levels of ROS was by Davies et al. 1982. The group directly determined the load of free radicals in tissues by electron paramagnetic- and spin resonance-spectroscopy. They showed a three-fold increase in free radical levels in the muscle and liver of rats exercised on a treadmill in rats (Davies et al. 1982). Later, *in vivo* studies in humans helped to understand this issue, because during exercise, the metabolism of mitochondria of exercising muscle cells increases oxygen consumption 100- to 200-fold (Sen, 1995). It has been demonstrated that moderate physical activity also leads to the generation of ROS in humans and plays a physiological role in the adaptation to exercise due to the upregulation of endogenous antioxidant enzymes (Gomez-Cabrera et al. 2008). From this perspective, at moderate levels, free radicals can act as signal molecules to enhance oxidative defences, but also can be deleterious when the organism is exposed to too high levels. The hormesis theory can thus also be applied to the ROS-generating effects of exercise (Ji et al. 2006). Hormesis (Figure 2) describes a dose-response relationship in which moderate levels or doses of a substance are beneficial and high levels or doses of a substance are inhibitory and toxic (Mattson, 2008). The group of Skenderi et al. (2008) showed that well-trained athletes have the ability to increase their antioxidant capacity by up to 25 % during an ultramarathon race. ROS act as vasodilators and are discussed to be an important part of the vasodilatory response during exercise (Peternelj, 2011). Another benefit of slightly increased ROS levels during exercise is the ability to efficiently counteract insulin resistance induced by increased induction of ROS-dependent molecular regulators of insulin sensitivity (Ristow et al. 2009).

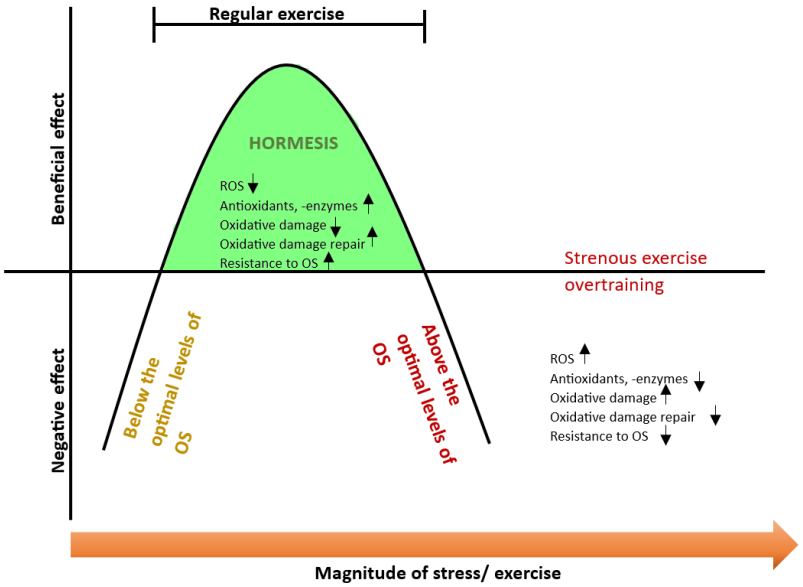

Figure 2: Hormesis of exercise

OS – oxidative stress (Adapted from Pingitore et al. 2015)

2 Product Knowledge: Pomegranate Juice

In order to get a brief idea about pomegranate juice as a product, this chapter deals with some basic information about the pomegranate fruit and -juice.

2.1 Composition and manufacturing of Pomegranate Juice

The pomegranate juice in general is extracted from the Pomegranate fruit (*Punica granatum L.*) which belongs to the family of Punicaceae and is cultivated in India, Spain, Israel, and the United States. In recent years the juice gained economic importance for these countries of cultivation. Johanningsmeier et al. (2011) report increasing sales from 2001 ($84.507) to 2005 ($66 million) for pomegranate juice in the United States. Even before this increasing consumption, pomegranate juice was said to have various beneficial medical effects (Basu and Penugonda, 2009). Compared to other fruit juices, red wine, and green tea, the antioxidant capacity of pomegranate juice is the highest. Antioxidant capacity can be defined as the ability of a substance to reduce pro-oxidants and oxidants (Ronald et al. 1999). In comparison to red wine and green tea, the antioxidant capacity of pomegranate juice is three times higher (Mertens-Talcott et al. 2006). Pomegranate juice is rich in ellagitannins, especially punicalagins, flavonoids, like anthocyanins, and other polyphenols (Ammar et al. 2017). In ancient times pomegranate was named a ‘healing food’ nowadays it is commonly a

‘superfood’. Such a naming partly explains the increasing demand for pomegranate products by the middle 2000s (Krueger, 2012). Whether the term ‘superfood’ is justified or not will become clear in the course of this work. Previous studies examined the antiproliferative, apoptotic and antioxidants effects of pomegranate extracts *in vitro* (Seeram et al. 2005). Most of the *in vivo* clinical studies applied commercial pomegranate juice for examining the benefits on cardiovascular health (Mertens-Talcott, 2006). Commercial pomegranate juice can be gained by pressing whole pomegranate fruits including their peels with hydrostatic pressure so that water-soluble ellagitannins from the rind can pass into the juice. The main amount of ellagitannins are punicalagins (Figure 3). Gil et al. (2000) showed that the high antioxidant capacity of pomegranate juice is primarily due to the high molecular weight of polyphenolic-like tannins, including punicalagins. Punicalagins are extracted in significant levels ranging from 0.017 to 1.5 g / L of pomegranate juice (Mertens-Talcott et al. 2006). The concentration of polyphenols, including punicalagins, are highly dependent on cultivation, processing, and storage conditions. Punicalagins (2,3-hexahydroxy-diphenoyl-4,6-gallagylglucose) are the main ellagitannins in pomegranate juice with almost 70 % (Gil et al. 2000). Ellagitannins occur in fruits and nuts as bioactive compounds and belong to the chemical class of hydrolysable tannins (Seeram et al. 2006). Ellagitannins are enriched in the fruit peels and are extracted in the juice by pressing the whole fruit. Additionally, Pomegranate juice contains other phytochemicals such as anthocyanins, which occur in the fruit arils of pomegranate and lots of other fruits and berries such as blackberries, strawberries, and raspberries and causes the red-purple colour (Gil et al. 2000). Anthocyanins (Figure 4) are the second main phytochemicals in pomegranates. The anthocyanin profile of pomegranate juice consists of Delphinidin-3,5-diglucoside, Cyanidin-3,5-diglucoside, Delphinidin-3-glucoside, Pelargonidin-3,5-diglucoside, Cyanidin-3-glucoside and Pelargonidin-3-glucoside (Krueger, 2012). The research group came to this conclusion after analysing samples of almost 800 pomegranate juices. With high-performance liquid chromatography (HPLC) they showed that 95 % of the nearly 800 pomegranate samples contain only six anthocyanins, previously listed. The review from Kalaycıoğlu et al. (2016) reported a total content of anthocyanins from pomegranate cultivars all over the world ranging from 8.1 to 36.9 mg / 100 mL. The listed anthocyanins are known for their properties in scavenging free radicals and suppressing lipid oxidation *in vitro* (Gil et al. 2000). Unlike other flavonoids, anthocyanidins, aglycons of anthocyanins, do not have a

carboxyl group in the C-ring (Noda et al. 2002). The three major anthocyanidins in pomegranate fruits, delphinidin, cyanidin, and pelargonidin, are shown to have superoxide radical and hydroxyl radical scavenging activity. Noda et al. (2002) found out, that anthocyanins with hydroxyl groups at position 3', 4', and 5' (delphinidin) or 3', and 4' (cyanidin) in the B-ring, are able to chelate with iron, which explains the inhibition of OH* formation within the Fenton reaction. Extensive research on flavonoids and phenolic acids by Rice-Evans et al. (1996) have shown that cyanidin has nearly the same antioxidant activity as quercetin, demonstrating the importance of unsaturation in the C-ring and the resulting electron delocalization.

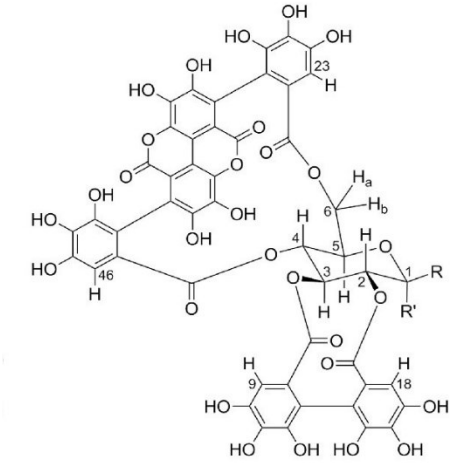

Figure 3: Punicalagin
α-Punicalagin R = H and R' = OH, β*-Punicalagin R = OH and R' = H*

(Boualem et al. 2018)

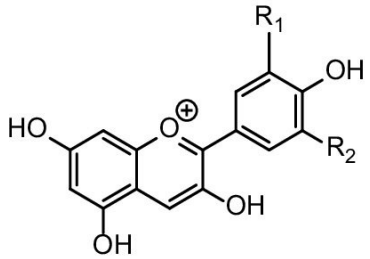

Figure 4: Basic structure of anthocyanin.
Without R_1 and R_2 = Pelargonidin (3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-1-benzopyrylium chloride), R_1 = OH: Cyanidin (3,5,7-Trihydroxy-2-(3,4-dihydroxyphenyl)-1-benzopyrylium chloride), R_2 = OH: Delphinidin (3,5,7-Trihydroxy-2-(3,4,5-trihydroxyphenyl)-1-benzopyrylium chloride) (Dabas, 2016).

Unfortunately, dm Bio could not provide any data on the polyphenols contained in their pomegranate juice. Therefore, the levels of polyphenols were determined using the Folin-

Ciocalteau reagent (FCR) method (see chapter 4.4). Results are reported as gallic acid equivalents (GAE) and are discussed in the chapter 5.1.

2.2 Further Ingredients of pomegranate juice

In addition to phytochemicals, the bioactive compounds, pomegranate juice consists of many other ingredients. With approximately 81% of the total solids, sugar is the predominant solid. The composition of the sugar consists of almost entirely fructose and glucose (Krueger, 2012). The glucose-fructose-ratio ranges according to Zhang et al. 2009 from 0.8 – 1.0, measured with HPLC. In addition, it is known that mannitol naturally occurs in pomegranate juice with levels of a few tenths' percent (Krueger, 2012). Zhang et al. 2009 measured values of mannitol of ≤ 0.03 g / 100 mL. Fructose may have an impact on the UA metabolism. According to Lotito and Frei (2004), it is fructose from fruits, that causes the increase in plasma FRAP via increased UA levels. It can be assumed, that the amounts administered in this study were too small for the described influence. According to literature, potassium was measured with a mean level of 2320 mg / kg. Proline was measured with a mean level of 7.2 mg / kg. Proline is important for authentication because other fruit juices contain much higher proline levels and therefore mixing of the juices could be revealed (Krueger, 2012). Vitamin C is an additional important antioxidant which can be found in pomegranate juice. Concentrations of Vitamin C are dependent on maturity, altitude of cultivation, and variety. Vitamin C concentration increases significantly with maturity and reaches values of 114.33 µg AAE / mL (Ascorbic acid equivalent) (Mphahlele et al. 2014). The protective effects of pomegranates are also due to the antioxidant vitamin C (Rice-Evans et al. 1996). It is worth noting that pomegranate juice contains traces of minerals, such as iron (Fe), calcium (Ca), chloride (Cl), copper (Cu), potassium (K) (mentioned above), magnesium (Mg), manganese (Mn), selenium (Sn) and zinc (Zn) (Kazakos et al. 2020).

2.3 Health benefits of pomegranate juice

As mentioned before (chapter 2), pomegranate juice was used in folk medicine since ancient times. It was applied for eliminating parasites and treating diarrhoea, acidosis, microbial infections, and respiratory pathologies. Based on these old traditions, interest in the study of pomegranates has increased enormously in recent years. Mostly in *vitro*- and *in vivo* studies examined a wide range of functional properties of pomegranate juice. These involve improved oral-, cardiovascular-, and skin health. Pomegranate juice also provides antidiabetic,

antitumoral, antioxidant, antimicrobial, and anti-inflammatory properties (Viuda-Martos, 2010). Previous clinical studies demonstrated that a one-year consumption of pomegranate juice by humans was able to reduce the oxidation of LDL and HDL significantly and a three-year consumption of pomegranate juice by patients with artery stenosis showed to reduce oxidative stress in the patient's blood and decreased atherosclerotic lesion size (Rosenblat et al. 2006). Another clinical study following the gold standard criteria (randomized, double-blind, placebo-controlled) by Sumner et al. (2005) is adding further support to the anti-atherosclerotic, anti-hypertensive, and anti-diabetic effects of pomegranate juice. A consumption of 240 mL pomegranate juice per day for a three-month period led to a significant decrease in stress-induced ischemia in patients with stable coronary heart disease (Sumner et al. 2005).

2.4 Harmful effects of pomegranate juice?

It is not to be neglected that the sugar content accounts for almost the entire carbohydrate content present in pomegranate juice. The World Health Organization (WHO) recommends that adults should not consume more than 10 energy percent and thus no more than 50 g of sugar per day – based on a daily calorie intake of 2000 kcal (Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, 2022). In this study, the participants received one glass of each interventional beverage. A glass is equivalent 250 mL. With one glass of pomegranate juice, the participants already reached more than half of the daily recommended sugar intake (35 g). It is important to keep in mind, that by processing fruits into fruit juices a removal of dietary fibre occurs. Due to the physical disruption, the absorption of fructose is faster when consuming fruit juice compared to the whole fruits (Tetens and Alinia, 2009). In contrast, Rosenblat et al. (2006) demonstrated that the consumption of 50 mL pomegranate juice per day for three months by diabetic patients (non-insulin-dependent) did neither increase serum glucose nor blood HbA1c levels. Most of the studies for examining the beneficial effects as well as most of the toxicity studies of pomegranate juice were conducted on animals. Various research concluded that there is no toxic effect of pomegranate juice or -extracts, supplied in high doses to animals. A few studies were designed for evaluating the safety of pomegranate extract dietary supplements for human subjects. They concluded that there were no serious adverse events in the human study subjects (Viuda-Martos, 2010).

3 FRAP Background Information

The antioxidant capacity is the ability to inhibit or delay the oxidation of various substrates (Halliwell and Gutteridge, 1989). The FRAP (ferric reducing ability of plasma) assay is commonly used for fluids, food components, and plasma and is a simple method for determining total antioxidant capacity. The underlying principle of this assay is a Fe^{3+}/Fe^{2+} redox reaction (Figure 5). Within this reaction, a dark blue-coloured Fe^{2+}-TPTZ complex is formed, proportional to the contained total antioxidants. This modification of colour causes an absorption change which expresses the concentration of Fe^{2+} and can be measured colourimetrically most accurately at a wavelength at 593nm. (Benzie and Strain, 1996). For technical reasons, in the present study, 595nm was chosen. The FRAP assay was first published in 1996 by Benzie and Strain and has since become a commonly used easy method to assess antioxidant power (Benzie and Strain, 1996). The more frequently used term is Ferric Reducing Antioxidant Power assay, as the assay is now used not only to determine the antioxidant capacity of plasma but also of beverages and foods. It is not possible within this assay to identify each antioxidant component on its own, but it directly determines the overall capacity against an oxidant of a compound (Benzie and Devaki, 2017). More specifically, the FRAP assay measures the non-enzymatic antioxidant capacity (NEAC). FRAP does not detect enzymatic antioxidants such as GPx or SOD (Benzie and Devaki, 2017). Antioxidants, which are determined by FRAP assay are ascorbic acid (15%), α-tocopherol (5%), UA (60%), bilirubin (5%), and albumin (10%) with estimated relative contributions to the FRAP values in brackets (Benzie and Strain, 1996). In contrast to other electron transfer (ET) based assays, FRAP is performed under acidic pH conditions (3.6). This causes improved iron solubility and drives the electron transfer to increase the redox potential (Shahidi, 2015). Results of the FRAP assay are reported as Trolox equivalents in mM TEAC

calculated with reference to the Trolox standard or positive control, respectively. (Benzie and Devaki, 2017).

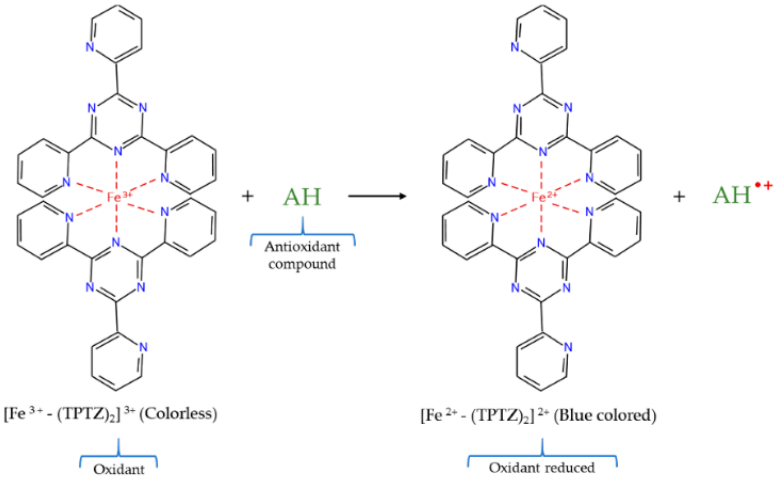

Figure 5: Reduction reaction of FRAP assay.

(Echegaray et al. 2021)

3.1 Advantages of FRAP measurement

The main advantage of FRAP is its relative simplicity, quick implementation with high sample throughput, and cost effectiveness. Furthermore, a wide range of sample types can be tested, and the chemicals used are of low toxicity and high stability (Benzie and Strain, 1996).

3.2 Limitations of FRAP

The FRAP assay does not measure Glutathione (GSH) separately. GSH is an important water-soluble cellular antioxidant produced in the liver. It is important to keep in mind, that not all reductants which are able to reduce Fe^{3+} to Fe^{2+} are antioxidants. In addition, not every antioxidant which is able to reduce pro-oxidants must be able to reduce Fe^{3+} and vice versa. GSH is a good example of this matter (Ronald et al. 1999). It could be proven that compounds such as thiols, glutathione, and proteins are not able to reduce iron (III) within the FRAP assay (Jakubczyk et al. 2020).

Another point to keep in mind is, that FRAP is carried out under acidic conditions, whereas other assays are carried out under neutral (e.g., TEAC) or basic (e.g., total phenols assay by FCR) conditions (Huang et al. 2005). It is known that the pH value has an important effect on the reducing capacity of antioxidants. So, FRAP has the disadvantage, that under acidic conditions, the reducing capacity of antioxidants may be suppressed due to protonation of antioxidant

compounds (Huang et al. 2005). On the other hand, iron is highly soluble in the acidic environment, as discussed earlier (chapter 3). The ideal test plasma samples would be fresh heparinized plasma samples (Benzie and Devaki, 2017). In the underlying work, EDTA plasma was used, which can also be used, however it is not as stable as heparinized plasma (Chung et al. 2001). Some small differences in stability could be found when comparing EDTA-plasma and heparinized plasma. The loss of antioxidant capacity after two hours at room temperature is higher in EDTA-plasma samples (5%) than in heparinized plasma samples (1%). Measuring the FRAP values without any delay, no significant difference between EDTA plasma and heparinized plasma could be observed. Therefore, speed is the key factor when using EDTA plasma for measuring the antioxidant capacity (Benzie and Chung, 1999).

In summary, all the introductory topics represent a broad field of research that will be the subject of this master's thesis. There is a growing body of evidence that whole and natural foods are good sources of antioxidants. It will be clarified whether pomegranate juice, which is considered a natural source of antioxidants, lives up to its position as a superfood. The acute impact of pomegranate juice on the total antioxidant capacity of plasma in healthy female adults will be investigated.

4 Methods

The following chapter, all methods of this underlying master's thesis are presented in more detail.

4.1 Study design

The research project "Acute impact of different beverages on exercise induced oxidative stress and inflammation", led by MSc. M. Gassner is a project of the Faculty of Life Sciences of the University of Vienna at the Department for Nutritional Sciences – Working Group Sports Nutrition, Univ-Prof. Dr. Daniel König. The study is part of the dissertation of M. Gassner and investigates the influence of different beverages on recovery after strength training in women. The aim is to clarify how green tea, pomegranate juice (PJ), coffee, orange juice (OJ), a sugar-sweetened soft drink, water, and estrogen levels affect the generation of ROS and antioxidant capacity after 20 minutes of high intensity resistance training. For this purpose, different inflammation markers (HS-IL6, HS-CRP), MDA, protein carbonyls, radical oxygen formation (ROS), total antioxidant power (FRAP) and estrogen levels were analysed from the blood

samples of the participants. Furthermore, a 24h-Recall was collected on each intervention day, as well as the BORG Scale after each training session. Maximum strength was determined at the beginning of the study. Sample size calculation were carried out by the study manager M. Gassner using G*Power analysis. With an effect size of 0.8 and a common alpha error of 0.05, the sample size was estimated at 20 subjects for an administration of seven beverages plus a safety margin of four, meaning that 24 women were recruited of which 20 should complete successfully. All participants signed a form of consent to participate. The study design was approved by the Ethics Committee of the University of Vienna, reference ID 00743, on 01.12.2021.

4.1.1 Recruitment

The recruitment for this study began in December 2021. The study was open to women aged 18 to 40 with a body mass index (BMI) of 18.5 to 25.0 kg/m^2. In addition, the women were required not to engage in regular strength training (maximum one training per week) or any physical activity (longer than two hours per week) so far. Further inclusion criteria were non-smoker for at least six months, no intolerances to the beverages mentioned above, ability to exercise on an empty stomach, no acute or chronic illnesses, no iron or haemoglobin deficiencies, and a good tolerance to blood sampling. The intervention was brought to attention using social media. For this purpose, a study poster including all relevant information was posted in various social network groups.

At the beginning of the recruitment 51 women were registered, and 20 women completed all seven days of the study.

4.1.2 Intervention

The intervention days took place in the period from 14.04.2022 to 29.11.2022. At the beginning of the study, a preliminary examination was conducted to determine the health suitability of the participants. The participants were not obliged to fast for the initial examination. Weight, height, and waist circumferences were determined. Additionally, an electrocardiogram (ECG) was performed and their body mass index (BMI), body fat mass, lean body mass (LBM), skeletal muscle mass (SMM), and phase angle were measured using a bioelectrical impedance analysis (BIA, Seca-mBCA 515). An initial blood draw was performed to determine health eligibility. This analysis was carried out by a partner laboratory.

The procedure of each intervention day was as follows (Figure 6): after the participants entered the study centre (NuTraLab, Zieglergasse 6, 1060 Vienna), they were first asked to sit down and rest for 30 minutes. If possible, they were asked to avoid arriving by bicycle or on foot. All participants were required to follow an overnight fast on the night before the intervention day for at least 12 hours. During their rest, they answered the questions about the 24h-Recall. After 30 minutes of resting the first blood sampling was taken. Within 10 minutes after the first blood sampling, they received 250 mL of the intervention beverage. Followed by another two hours of resting. Subsequently, the second blood sampling was taken, and they were immediately guided through the 20-minute HIIT. Directly after completing the HIIT, the third blood sampling was taken, and the participant was asked to stay in the room so that the fourth and last blood sample could be taken exactly 15 minutes after the training session.

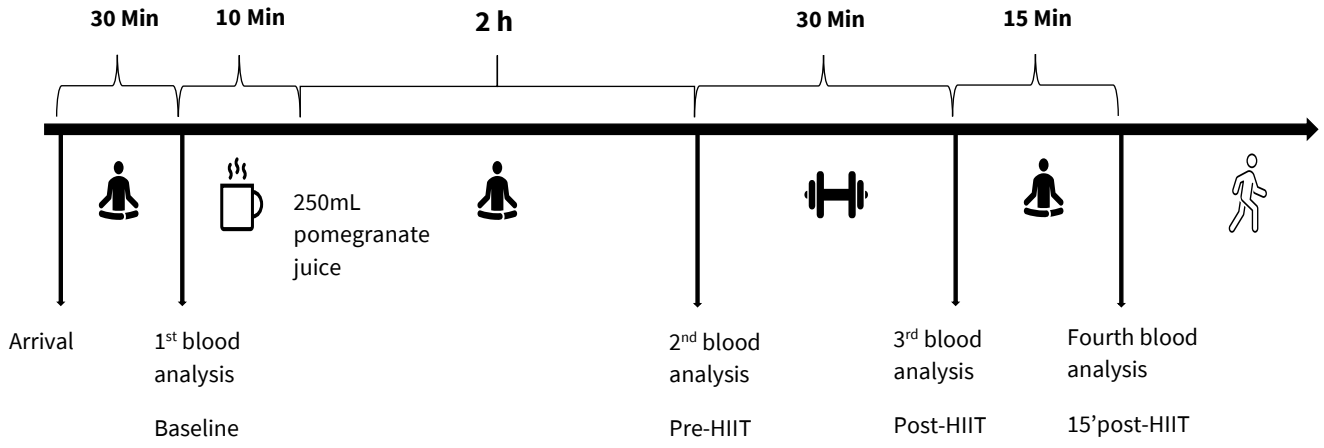

Figure 6: Schedule according to the study design

(Adapted from MSc. M. Gassner)

4.1.3 Beverage intervention

Each participant passed through seven intervention days. On each intervention day, one of seven intervention beverages with a volume of 250mL was administered prior to the strength training. For the administration of the pomegranate juice, exactly 250mL of pomegranate juice was measured with a measuring cup. The participant were advised to drink the beverage immediately after the first blood collection. To ensure the consumption of the entire glass's content, the glass was rinsed with water and the participant drank the remaining content as well. The pomegranate juice which was used in the study was free from additives and

contained 100% pure pomegranate juice from organic agriculture. All participants received the juice from the same manufacturer: dm Bio (dm – drogerie markt GmbH + Co. KG, Karlsruhe, Germany).

4.1.4 Strength training intervention

To ensure that the participants receive adequate training, a test session was conducted prior to the beginning of the seven intervention days. The exercises were explained, and the maximum weight was determined through the application of the five-repetition maximum test. The repetitions per exercise and the weights used were noted. A protocol of training (Appendix III) ensured a consistent training sequence with adequate weight for each intervention day and participant. In order to be able to assess the degree of stress during the exercises, the perceived stress was evaluated by using the BORG scale. The BORG Scale, or Rate of Perceived Exertion (RPE), is a method for measuring the subjective perceived intensity of a physical activity (Borg, 1982). To exclude training habituation effects to the training, the training weights were increased by 5 % compared to the previous training, every time a participant completed three times 20 repetitions (3 x 20) of an exercise.

4.1.5 Blood sampling

The blood samples were taken by specialists in the NuTraLab. All blood samples were centrifuged within 15 minutes after blood collection and were stored at -80°C until the performance of assay procedures. For each blood draw, 4 mL of plasma was collected in EDTA tubes (VACUETTE® Tube, Greiner). For the first blood draw on each intervention day, additional 4 mL of blood was collected in serum tubes (VACUETTE® Tube, Greiner). A total of about 20 mL of blood was taken from each subject on every intervention day. The adequate labelling of the EDTA tubes was done manually, as well as the aliquoting of the centrifuged plasma into 0.5 mL reaction vessels. For the performance of the assays which took place at the department of Nutritional Sciences, Josef-Holaubek-Platz 2 (UZAII), Vienna, the plasma samples were transported from the NuTraLab to the laboratory at UZAII using dry ice.

4.1.6 Raw data and Statistical analysis

The raw data obtained from the FRAP assay, were displayed using the program MARS Data Analysis Software Version 4.01 R4 (BMG Labtech). The raw data were tabulated in Microsoft

Excel® Version 2301. For further statistical analysis IBM SPSS Statistics 27.0 for Windows was used. A quality management sheet was created for the calculation of all FRAP values across all measurement days. In this, the mean values of all blanks were calculated and used again for further calculation of the FRAP values. The same goes for the Fe^{2+}-standard curve. In addition, the calculated FRAP values of each measurement day were baseline-corrected for the statistical analysis. For this purpose, the plasma values from time point 0 (baseline) were subtracted from all other time points (1 [pre-HIIT]; 2 [post-HIIT]; 3 [15'post-HIIT]) within this intervention day. For all statistical analysis, an alpha level of $p < 0.05$ was used. The Shapiro-Wilk test was used, verifying the data for normal distribution. Since none of the data (FRAP or ROS) is normally distributed, the Mauchly-test was used to define sphericity. The FRAP results showed violations of sphericity ($\varepsilon < 0.75$; $p = 0.001$) for this reason the Greenhouse-Geisser adjustment was used to correct the degrees of freedom downwards, otherwise there is an increased risk of type I errors. A repeated measures ANOVA (rmANOVA) with Greenhouse-Geisser correction was determined and followed by a Bonferroni-adjusted post-hoc test. The ROS results also showed violations of sphericity ($\varepsilon > 0.75$; $p = 0.001$). Since the epsilon is greater than 0.75, here the Huynh-Feldt correction was used. In addition, the Spearman correlation coefficient was determined as a measure of the relationship between the FRAP and ROS levels. The effect of time was determined using the t-Test by testing the effect of all intervention beverages before (baseline and pre-HIIT) and after (post-HIIT and 15'post-HIIT) exercise.

4.2 FRAP-Method

The FRAP assay is the main subject of this master's thesis. The procedure of the assay is explained below.

4.2.1 Reagents and technical equipment

A detailed list of all required reagents and used technical equipment is provided in Appendix IV.

4.2.2 Reagent preparation

Solution	*Concen-tration*	*Weigh-in*	*Laboratory equipment*	*Instruction*
Hydrochloric acid	0.5 mol / L			
		50 mL HCl (37%)	Volumetric flask	First, fill in Aqua dest. then the acid, store at 4 °C
		950 mL Aqua dest.		
Sodium hydroxide	0.5 mol / L			
		20 g NaOH	Magnetic stirrer	
		1000 mL Aqua dest.	Volumetric flask	
FRAP reagent				
			Falcon tubes	Store at 37 °C
Acetate buffer	300 mmol / L	3.1 g $C_2H_3NaO_2$	Volumetric flask	Adjust for pH 3.6
		16 mL CH_3COOH		Store at 4 °C
		1000 mL Aqua dest.		
TPTZ	10 mmol / L	585 µL HCl (25%)	Volumetric flask	Store at 4 °C
		100 mL Aqua dest.		
		0.312 g TPTZ		
Iron-(III)-chloride-hexahydrate	20 mmol / L	0.541 g $FeCl_3 *_6H_2O$	Volumetric flask	Store at 4 °C
		100mL Aqua dest.		
Standard solution				
Ferrous-(II)-sulphate-heptahydrate	2000 µmol / L	0.0556 g $FeSO_4*_7H_2O$	Volumetric flask	Produce fresh daily
		100 mL Aqua dest.		Store at 4 °C
Control solution	(Trolox solution)			
Trolox	200 µmol / L		1.5 mL Eppendorf tubes	Positive control
				produce fresh daily
				store at -20 °C
PBS	Phosphate-buffered saline (PBS)buffer			
		8.2 g NaCl	Volumetric flask	
		0.2 g KCl	Pasteur pipette	Adjust for pH 7.4 with HCl or NaOH
		1.2 g Na_2HPO_4		
		0.2 g KH_2PO_4		

Table 2: List of reagent preparation

(Adapted from Baron, 2021)

The preparation procedure according to Benzie and Devaki (2017) was as follows:

The listed reagents above were prepared before starting the FRAP assay. For pH adjustments of the PBS buffer, 0.5 M hydrochloric acid (HCl) and 0.5 M sodium hydroxide (NaOH) solutions were prepared.

0.5 M hydrochloric acid solution

For the 0.5 M hydrochloric acid solution 50 mL of hydrochloric acid (37%) was mixed with 950 mL distilled water in a volumetric flask.

0.5 M sodium hydroxide solution

For the preparation of 0.5 M sodium hydroxide solution, 20 g sodium hydroxide was weighed into a beaker and dissolved with 1000 mL distilled water while stirring on a magnetic stirrer.

Acetate buffer

The acetate buffer was prepared by weighing 3.1 g sodium acetate ($C_2H_3NaO_2$) in a small beaker. In the next step, it was transferred into a 1 Litre beaker by rinsing the small beaker with distilled water several times to avoid residues of sodium acetate in the small beaker. Subsequently, 16 mL of acetic acid (CH_3COOH) was added as well as the remaining distilled water. While stirring at the magnetic stirrer, the pH value was determined with a pH meter and adjusted by using either acetic acid or sodium hydroxide for a pH value of 3.6. After reaching a pH of 3.6, the solution was transferred into a volumetric flask.

2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ)

TPTZ was prepared by weighing 0.312 g of TPTZ and transferring it with distilled water into a 100 mL volumetric flask. 585 µl of HCl was added. Then the volumetric flask was filled up to the meniscus with distilled water.

*Iron-(III)-chloride-hexahydrate (FeCl3 *6H2O)*

For the next solution, 0.541 g of Iron (III)-chloride-hexahydrate ($FeCl_3 *_6H_2O$) was dissolved in distilled water, transferred into a 100 mL volumetric flask, and filled with distilled water to the meniscus of the 100 mL volumetric flask.

Standard stock solution

The standard stock solution was prepared by weighing 0.0556 g of Ferrous-(II)-sulphate-heptahydrate ($FeSO_4*_7H_2O$) and adding it to a 100 mL volumetric flask filled up with distilled water.

Positive control

The positive control consists of the PBS buffer and Trolox. For the latter, 156.41 mg of 6-hydroxy-2,5,7,8-tetramethylchroman-carboxylic acid (Trolox) was diluted in 259 mL of PBS. This Trolox stock solution is stored at -20°C. For the Trolox control standards, the frozen Trolox was thawed and freshly diluted with PBS at the beginning of each measurement day.

Phosphate-buffered saline buffer (PBS)

To dilute the Trolox PBS is essential. For this 8.2 g sodium chloride (NaCl), 0.2 g potassium chloride (KCl), 1.2 g Di-sodium-hydrogen phosphate (Na_2HPO_4), and 0.2 g potassium dihydrogen phosphate (KH_2PO_4) were weighed up and poured into a 1 Litre beaker. 800 mL of distilled water were added and put onto a magnetic stirrer. While stirring, the pH value was measured with a pH meter and adjusted either with HCl or NaOH. When reaching a pH value of 7.4, the buffer was transferred into a 1L volumetric flask and filled up to 1 Litre with distilled water. These working solutions were made in advance of a FRAP assay implementation and stored continuously at 4 °C. For the use of the solutions, they were brought to room temperature previously. Furthermore, the pH meter was calibrated with calibration solutions before each use.

4.2.3 Devices and settings

The correct setting of the photometer is essential for measuring the prepared FRAP samples. The recommended wavelength according to Benzie and Strain (1996) is 593nm. Since the CLARIOstar^{plus} microplate reader was not able to precisely adjust this wavelength, the closest wavelength of 595nm was set. The measuring time was set at six minutes and eight seconds and the temperature of the microplate reader was adjusted to 37 °C, which is equivalent to human body temperature. A detailed description of the software settings can be found in Appendix V.

4.2.4 Sample preparation and execution of the FRAP assay

Standard series

For the quantification of the antioxidative potential of the plasma samples, a Fe^{2+}-standard series was prepared (Table 3). The aqueous solution of known ferrous concentrations had a range between 100 to 2000 µM per Litre. The standard series was freshly prepared on every measurement day. Using the known concentration of the standard series, the antioxidant

capacity of the plasma samples can be quantified. A FRAP-value of 100 µM / L can be equated with 100 µM / L Fe^{2+}-standard solution (Benzie and Devaki, 2017).

Standard	**Final concentration**	**Volume $FeSO_4*7H_2O$ (in µl)**	**Volume Aqua dest. (in µl)**
S7	2000 µM	500	0
S6	1500 µM	375	125
S5	1250 µM	312.5	187.5
S4	1000 µM	250	250
S3	500 µM	125	375
S2	250 µM	62.5	437.5
S1	100 µM	25	475

Table 3: Dilution series for the preparation of the ferrous standard series

FRAP reagent

The working FRAP reagent was prepared as shown in Table 2 above and for every new measuring day, a fresh FRAP reagent was mixed. After mixing, the FRAP reagent was immediately stored in a water bath at 37 °C.

Positive control

Trolox was used as a positive control for quality control purposes. For this, a Trolox-standard series was prepared (Table 4). Trolox is a water-soluble Vitamin E analogue (Benzie and Devaki, 2017). The dilution medium for Trolox is PBS and has been adjusted to pH 7.4 before every use. Until the desired pH value was reached, either the acid or the alkali was added by titration using a Pasteur pipette. After this, a PBS dilution series was prepared and served as a positive control. The known range of concentration was between 100 to 2000 µmol per Litre.

Trolox	Final concentration	Volume Trolox (in µl)	Volume PBS (in µl)
T7	2000 µM	400	100
T6	1500 µM	300	200
T5	1250 µM	250	250
T4	1000 µM	200	300
T3	500 µM	100	400
T2	250 µM	50	450
T1	100 µM	20	480

Table 4: Dilution series for the preparation of the Trolox series

Plasma samples

The ethylene-diamine-tetra-acetic-acid (EDTA)-plasma samples from all participants had been aliquoted to 100µL and stored at -80 °C. To prepare them for the measurement, they were first thawed in hand, briefly vortexed, then were kept on ice and covered with aluminium foil throughout the analysis to avoid exceeding 4 °C.

The application to a 96-well microtitre plate was carried out as follows: 10µL of Fe2+-standard solution, PBS-dilution, distilled water or EDTA-plasma was poured into each well with an electronic Eppendorf pipette. Followed by 30 µl distilled water and 300 mL FRAP reagent (see Table 5 below).

Pipetting in a well	Standard	Blank	control	sample
Fe^{2+} (100-2000 µmol / L)	10 µL	-	-	-
Trolox	-	-	10 µL	-
EDTA-Plasma	-	-	-	10 µL
Aqua dest.	30 µL	10 µL and 30 µL	30 µL	30 µL
FRAP reagent	300 µL	300 µL	300 µL	300 µL

Table 5: Pipetting scheme and volume of each reagent

The maximum volume of 350 µL of each well was almost reached. Immediately after pouring the FRAP reagent into the last well, the plate was put into the reader for measuring. It is important to start the measurement very quickly since the reaction mechanism of TPTZ and the substrate begins at first contact. Therefore, only two rows were determined per plate and

measurement.

Each standard, blank or plasma was determined in duplicates. The blank consisted of distilled water and was determined every 2nd measurement. During the measurement of plate one, two rows of plate 2 could be prepared (see Table 6 and Table 7) and vice versa. Additionally, on each plate, a plasma sample of known antioxidant capacity was measured as a control. This third control (besides the Fe^{2+}standard and Trolox) enables to check whether the conditions between the runs and days of measurement, are the same. For this purpose, the coefficient of variation (CV) was defined at < 5.5%.

PL1	**Standard plate**											
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	PL 1	PL 1	Blank	Blank	PL 31	PL 31	Blank	Blank	PL 61	PL 61
B	Fe2+ S 1	Fe2+ S 1	PL 2	PL 2	PL 17	PL 17	PL 32	PL 32	PL 47	PL 47	PL 62	PL 62
C	Fe2+ S 2	Fe2+ S 2	PL 3	PL 3	PL 18	PL 18	PL 33	PL 33	PL 48	PL 48	PL 63	PL 63
D	Fe2+ S 3	Fe2+ S 3	PL 4	PL 4	PL 19	PL 19	PL 34	PL 34	PL 49	PL 49	PL 64	PL 64
E	Fe2+ S 4	Fe2+ S 4	PL 5	PL 5	PL 20	PL 20	PL 35	PL 35	PL 50	PL 50	PL 65	PL 65
F	Fe2+ S 5	Fe2+ S 5	PL 6	PL 6	PL 21	PL 21	PL 36	PL 36	PL 51	PL 51	PL 66	PL 66
G	Fe2+ S 6	Fe2+ S 6	PL 7	PL 7	PL 22	PL 22	PL 37	PL 37	PL 52	PL 52	PL 67	PL 67
H	Fe2+ S 7	Fe2+ S 7	PL 8	PL 8	PL 23	PL 23	PL 38	PL 38	PL 53	PL 53	PL 68	PL 68

Table 6: Plate 1 – Standard Plate

PL2	Trolox plate											
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	PL 9	PL 9	Blank	Blank	PL 39	PL 39	Blank	Blank	PL 69	PL 69
B	T1	T1	PL 10	PL 10	PL 24	PL 24	PL 40	PL 40	PL 54	PL 54	PL 70	PL 70
C	T2	T2	PL 11	PL 11	PL 25	PL 25	PL 41	PL 41	PL 55	PL 55	PL 71	PL 71
D	T3	T3	PL 12	PL 12	PL 26	PL 26	PL 42	PL 42	PL 56	PL 56	PL 72	PL 72
E	T4	T4	PL 13	PL 13	PL 27	PL 27	PL 43	PL 43	PL 57	PL 57	PL 73	PL 73
F	T5	T5	PL 14	PL 14	PL 28	PL 28	PL 44	PL 44	PL 58	PL 58	PL 74	PL 74
G	T6	T6	PL 15	PL 15	PL 29	PL 29	PL 45	PL 45	PL 59	PL 59	PL 75	PL 75
H	T7	T7	PL 16	PL 16	PL 30	PL 30	PL 46	PL 46	PL 60	PL 60	PL 76	PL 76

Table 7: Plate 1 – Trolox Plate

4.2.5 Evaluation

As discussed in chapter 3, the FRAP assay uses the ability of antioxidants to reduce Fe^{3+} to Fe^{2+}. The redox system of TPTZ is characterised by the formation of the intense blue Fe^{2+}-complex, which is colourimetrically detected at its absorption maximum of 593nm (Benzie and Strain, 1996). Due to the limited technical possibilities of the photometer, the wavelength was set to 595nm. The microplate reader determined the changes in the absorption of plasma samples after six minutes and eight seconds. The resulting raw data values were divided by the obtained absorbance of the known standard series and then multiplied with the standard concentrations. The values of standard concentration were determined by the Fe^{2+}-standard straight-line equation (Klapp, 2021).

$\boldsymbol{Linear\ equation}$: $\bar{y} = b * \bar{x} + a$

$\bar{y}$ = *Arithmetic average of raw blank values of* Fe^{2+} *standards (S1 to S7)*

b = Slope

$\bar{x}$= *Arithmetic average of concentrations (µmol / L) of* Fe^{2+} *standards (S1 to S7)*

a = Intercept

y= Absorption values

x= Concentration values (µmol / L)

Intercept *= a =* $\bar{y} = b * \bar{x}$

$$\boldsymbol{FRAP\ value}\ \left(\mu\frac{\boldsymbol{mol}}{\boldsymbol{L}}\right)$$

$$= \left(\frac{\Delta\ Absorbance\ at\ 595nm\ of\ plasma\ samples}{\Delta\ Absorbance\ at\ 595nm\ of\ Fe^{2+}standard}\right) * Fe^{2+}standard\ concentration\ \left(\mu\frac{mol}{L}\right)$$

$$\boldsymbol{\Delta\ Absorbance\ at\ 595nm\ of\ plasma\ samples}$$

$$= Absorbance\ at\ 595nm\ of\ plasma\ samples - Absorbance\ at\ 595nm\ of\ Blank$$

$$\boldsymbol{\Delta\ Absorbance\ at\ 595nm\ of\ Fe^{2+}standard}$$

$$= Absorbance\ at\ 595nm\ of\ Fe^{2+}standard - Absorbance\ at\ 595nm\ of\ Blank$$

A few preliminary calculations are fundamental for these calculations above. The measured absorption values were named raw data. These raw data must be blankly corrected. Therefore, all measured absorptions of the blanks must be deduced from all other raw data. These new values (raw blank) are taken for further calculations. The arithmetic average was also necessary since the measurement was performed in duplicates. Both absorption values were averaged with the formula below.

Arithmetic average: $\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$

To quantify the error rate and to produce reproducible results the standard deviations and coefficients of variation were determined.

$$\boldsymbol{Standard\ deviation}:\ s = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}}$$

$$\boldsymbol{Coefficient\ of\ variation}\ (\%): CV(\%) = \frac{standard\ deviation}{arithmetic\ average} * 100 = \frac{s}{\bar{x}} * 100$$

$$\boldsymbol{Slope}\left(\frac{1}{\frac{\mu mol}{L}}\right) = b = \frac{\sum(x - \bar{x})(y - \bar{y})}{\sum(x - \bar{x})^2}$$

The calculation of the reduced Fe^{3+} to Fe^{2+} in all plasma samples and solutions can be estimated with the following calculation:

$$\boldsymbol{Concentration\ of\ Fe^{2+}equivalents}\ \left(\frac{\mu mol}{L}\right) = c\left(\frac{\mu mol}{L}\right) = \frac{raw\ blank - intercept}{Slope}$$

$$= \frac{raw\ blank - a}{b}$$

(Benzie and Devaki, 2017; and Klapp, 2021)

4.3 ROS-Measurement

Parallel to each blood sampling, capillary blood of each participant was collected to analyse the concentrations of ROS. The current state-of-the-art technique following the electron paramagnetic resonance (EPR) technology allows the direct measurement of ROS generation under *in vivo* conditions. The protocol for the determination of ROS was adapted from Zeng et al. (2021). The principle of EPR is based on the absorption of microwave radiation by free radicals and transition metal ions with unpaired electrons that are stimulated by an electromagnetic field (Dikalov et al. 2007). For measuring the ROS concentration, 10 µL of freshly drawn capillary blood was sampled and 10 µL of oxygen-sensitive label was added and vortexed. The spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) was diluted in Krebs-HEPES buffer. 20 µL of this buffer-spin probe solution was added subsequently to the blood-label solution. Releasing ROS in the capillary blood interacts with CMH to form the more stable CM*. The ROS formation rate was measured in µmol / min after transferring the 40 µL mixture into a capillary tube and the adequate setting of the e-scan, using the Bruker WinEPR Data Processing software (Bruker, Germany) (Zeng et al. 2021).

4.4 Folin-Ciocalteu-Method

The Folin-Ciocalteu Method according to Singleton et al. (1995) with slight adaptations was used to determine the total phenols in plant extracts. The principle is based on the reaction of FCR with polyphenols to form chromogens that can be detected colourimetrically at a wavelength of 750 nm. Gallic acid was used as a reference standard. Results are expressed as gallic acid equivalents (GAE). The pomegranate juice was diluted to 1:10.

5. Results

This chapter lists all results of the measurements performed.

5.1 Total polyphenol content

The pomegranate juice from dm Bio has a total polyphenol content of 3862.10 µg GAE / mL. Compared to the literature (see Table 10), this total phenol content is in the upper range. According to other authors, the range of total phenolics is very wide. Ozgen et al. (2008) gained values within 1245 to 2076 µg GAE/mL, whereas Mousavinejad et al. (2009) reported a range of total phenolics from 2380 to 9300 µg GAE / mL. Sources of error could be found in the FCR itself. However, the FCR is not optimised for polyphenols. All reducing compounds react with the FCR, especially glucose and fructose, leading to false high polyphenol levels (Sánchez-Rangel et al. 2013). In addition, the variation can be explained by the measurement of different cultivars. This was accompanied by the fact that the aril colour and peel colour as well as the origin influence the total phenolics values (Gözlekci et al. 2010).

The pomegranate juice used in this study from dm Bio shows the following nutritional values (Table 8):

Average nutritional values per 100g	
Energy	273 kJ/64 kcal
Fat Of which saturated fatty acids	< 0.5 g < 0.1 g
Carbohydrates Of which sugars	14 g 14 g
Fibre	1.1 g
Protein	< 0.5 g
Sodium	< 0.01 g

Table 8: nutritional values of the pomegranate juice from dm bio
(Adapted from dm Bio)

These values are comparable to other studies where the composition of the pomegranate juice was as follows (Table 9):

Reference	Average nutritional values of pomegranate juice	
Basu et al. 2009	Sugars	15.6 g / 100 g
	Fibres	2.1 g / 100 g
	Total polyphenols	875 mg / 100 g
Seeram et al. 2006	Total carbohydrates Of which sugars	14.6 g / 100 g 14.2 g / 100 g
	Sodium	0.01 g / 100 g
Trombold et al. 2011	Total carbohydrates	14.0 g / 100 g
	Tannins	1.979 mg / L
	Anthocyanins	384 mg / L
	Ellagic acid derivatives	121 mg / L
Rosenblat et al. 2006	Vitamin C	3 mg / 100mL

Table 9: Comparison of a selection of phytochemical compounds in different pomegranate juices

Table 10 shows a comparison of various values for the total polyphenol content of pomegranate juice in the existing literature.

Reference	Total polyphenols in µgGAE / mL in Pomegranate juice, measured with Folin-Ciocalteu Method	
Ozgen et al. 2008	Range within 1245 to 2076	Tested six cultivars
Gözlekci et al. 2010	Range within 784.4 to 1551.5	Tested four cultivars
Pagliarulo et al. 2016	Ranged within 2685 to 2721	-
Mousavinejad et al. 2009	Ranged within 2380 to 9300	Tested eight cultivars
Gil et al. 2000	Ranged within 1808 to 2566	Tested three different juices (from fresh arils; frozen arils; commercial juice)
Al Dujaili, 2016	Exactly 1685	-

Table 10: Comparison of the total phenol content according to literature

This leads to the assumption that the pomegranate juice used in this study has similar nutritional values to other commercial pomegranate juices.

5.2 Baseline Characteristics

In total, 20 healthy women, aged 20 to 37, were included in this study and successfully completed it. A body mass index (BMI) between 18.8 and 25 $kg^{*}m^{-2}$ and a body fat percentage > 20 % was determined.

Variable	Mean ± SD	MIN	MAX
Age (yr)	26,45 ± 4,06	20	37
Body mass (kg)	58,99 ± 6,82	46,25	70,75
Height (m)	1,7 ± 0,06	1,56	1,79
BMI (kg/m^2)	21,0 ± 1,88	18,78	24,95
Fat mass (%)	27,5 ± 3,86	21,8	34,6
LBM (%)	58,6 ± 16,44	34,42	77,90
SMM (kg)	19,3 ± 2,32	15,01	22,83
WC (m)	0,7 ± 0,07	0,58	0,87
phase angle (°)	5,1 ± 0,38	4,3	5,9

BMI: body mass index; LBM: lean body mass; SMM: skeletal muscle mass; WC: waist circumference

Table 11: Participants descriptive and anthropometric characteristics (n = 20)

5.3 Total antioxidant capacity of plasma (FRAP)

A repeated measures ANOVA (rmANOVA) with Greenhouse-Geisser correction determined mean FRAP levels with a statistical difference between measurements, $F(1.46, 194.24) = 152.55$, $p < 0.001$. In the event of this significant result, post-hoc test was used to determine the difference between the intervention beverages. Bonferroni-adjusted post-hoc analysis across all time points with comparison of all beverages revealed significantly ($p < 0.05$) lower FRAP values for the sugar-sweetened soft drink in comparison to the matcha drink (M_{Diff} = -44.02; 95%-CI[-78.69, -9.35]). Likewise, the comparison in pairs of the orange juice intervention and sugar-sweetened soft drink (M_{Diff} = -38.16; 95%-CI [-72.83, -3.49]), as well as the coffee intervention and the sugar-sweetened soft drink (M_{Diff} =-39.41; 95%-CI [-74.08, -4.73]), showed significant lower FRAP values for the sugar-sweetened soft drink intervention (Figure 7).

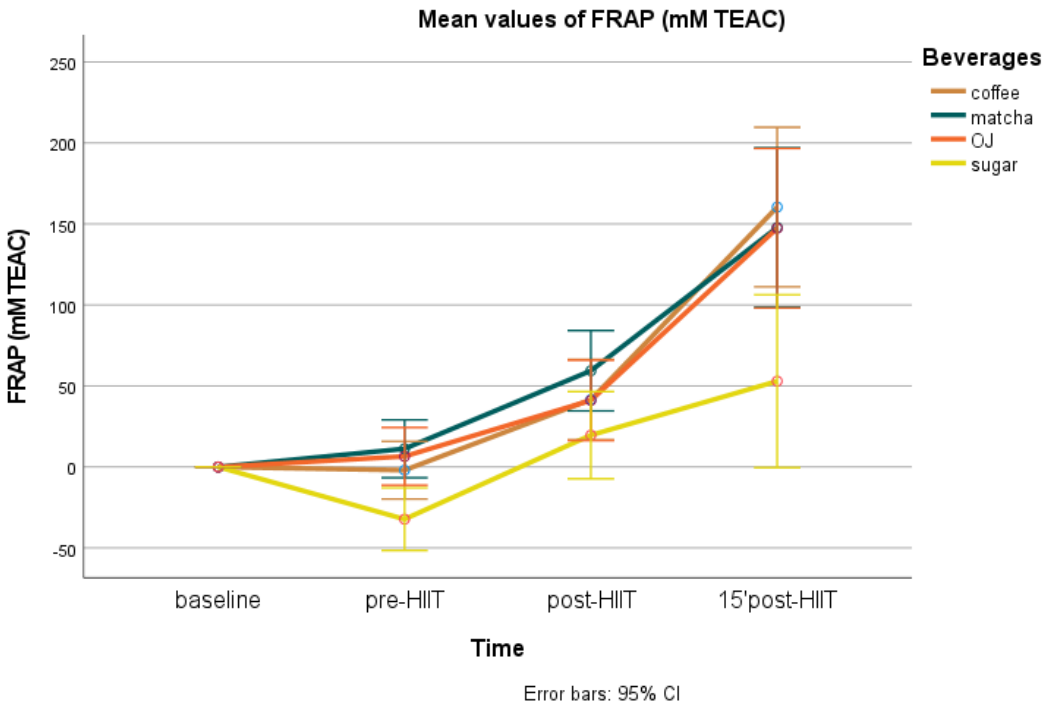

Figure 7: Error bar chart for significantly comparison of pairs.
Sugar-sweetened drink revealed significantly lower FRAP values compared to coffee, matcha, and OJ.

A pairwise comparison of pomegranate juice with water but also with all other intervention beverages showed no significant differences between these beverages (see Table below).

Multiple Comparisons						
FRAP (mM TEAC) Bonferroni						
Beverage		Mean Difference	Std. Error	Sig.	95% Confidence Intervall	
					Lower Bound	Upper Bound
PJ	Coffee	-28,32	11,19	0,264	-62,99	6,35
	Matcha	-32,93	11,19	0,081	-67,60	1,74
	Milk	-1,68	11,19	1	-36,35	32,99
	OJ	-27,07	11,19	0,355	-61,75	7,60
	Sugar	11,09	11,19	1	-23,59	45,76
	Water	-7,10	11,19	1	-41,77	27,57
Sugar-sweetened soft drink	Coffee	-39,41*	11,19	0,012	-74,08	-4,73
	Matcha	-44,02*	11,19	0,003	-78,69	-9,35
	OJ	-38,16*	11,19	0,018	-72,83	-3,49
Based on observed means. The error term is Mean Square(Error) = 1252,892. *. The mean difference is significant at the .05 level.						

Table 12: Bonferroni-adjusted post-hoc test.

No significant different FRAP values for comparisons of pomegranate juice with coffee, matcha, milk, orange juice, sugar-sweetened soft drink, and water (ρ > 0.05). Sugar-sweetened soft drink showed significant lower FRAP values compared to coffee, matcha, and orange juice (ρ < 0.05).

A line graph below (Figure 8) is showing the increased levels of antioxidant capacity in plasma (FRAP [mM TEAC]) for all intervention beverages. Statistically significant differences between baseline, pre-HIIT, post-HIIT, and 15'post-HIIT could be found (see Table 14).

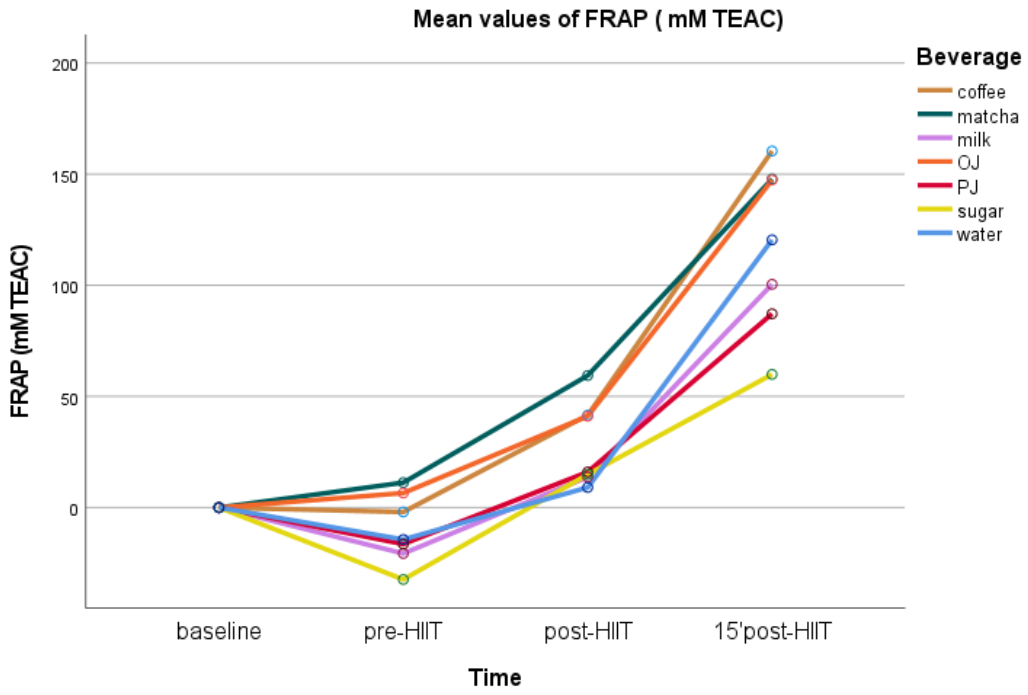

Figure 8: FRAP values (mM TEAC) of all intervention beverages.

Two split error bar charts give a more detailed picture for FRAP values, each with water (blue line) as the control beverage.

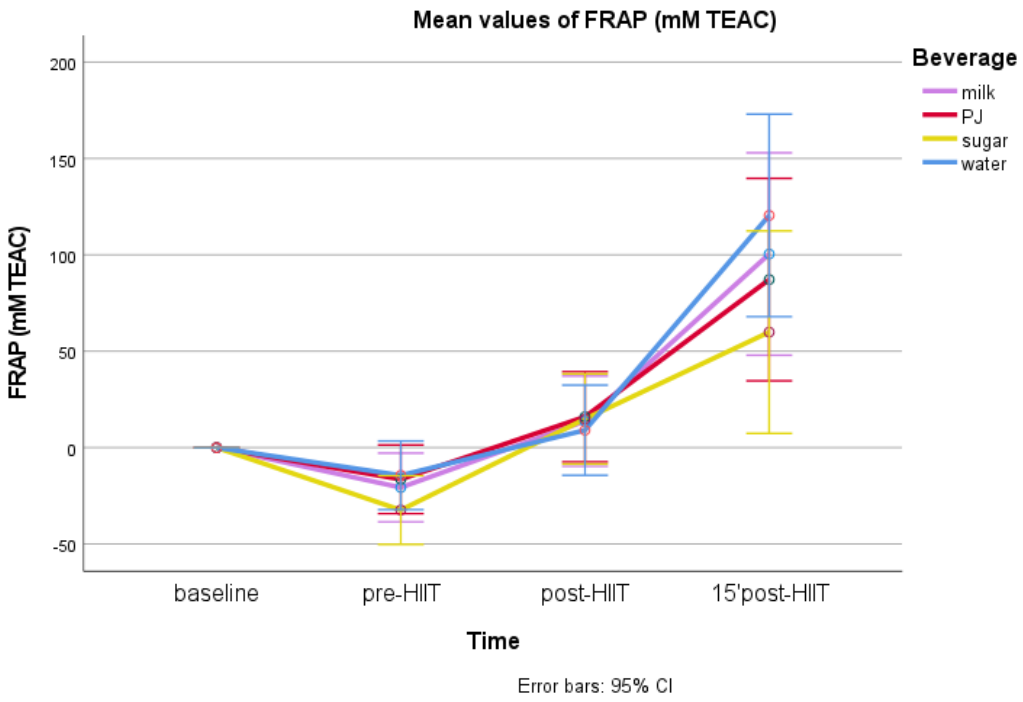

Figure 9: FRAP-Error bar chart of milk, pomegranate juice, and sugar-sweetened soft drink compared to water.

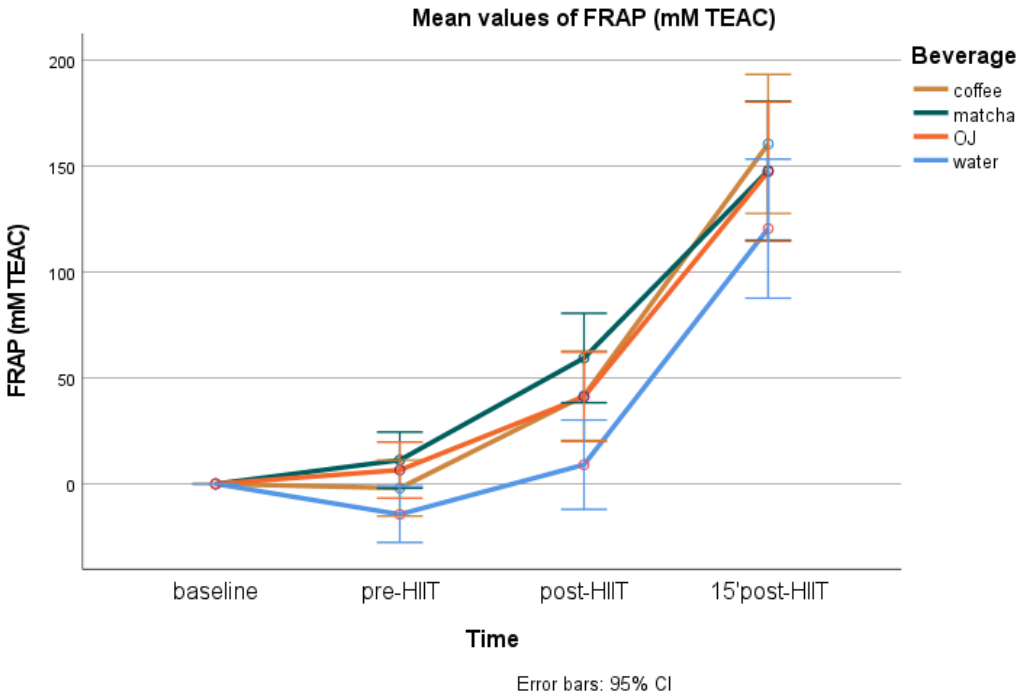

Figure 10: FRAP-Error bar chart of coffee, matcha, and orange juice compared to water.

Variable	Time Point	Water	PJ	Sugar sweetened soft drink	Milk	OJ	Matcha	Coffee
FRAP (mM/TEAC)	Baseline	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Pre-HIIT	-14,4 ± 27,8	-16.4 ± 24.8	-32.4 ± 66.3	-20.7 ± 24.9	6.5 ± 27.6	11.2 ± 20.3	-2.0 ± 39.7
	Post-HIIT	9.1 ± 41.0	16.0 ± 41.8	14.9 ± 80.6	13.7 ± 32,8	41.1 ± 48.1	59.4 ± 47.8	41.6 ± 51.4
	15' Post-HIIT	120.5 ± 69.0	87.2 ± 99.0	59.9 ± 196.4	100.5 ± 49.9	147.4 ± 82.9	147.8 ± 53.6	160.5 ± 84.9
Data are mean ± SD								

Table 13: FRAP values of all intervention beverages and all time points.

Mean values for total antioxidant capacity through all intervention beverages. No statistical differences between the intervention beverages could be found (ρ > 0.05), except for the comparison of sugar-sweetened soft drink with matcha, orange juice, and coffee described within the Bonferroni adjusted post-hoc test.

Paired samples tests through all intervention beverages determined significant effects of time points. Pre-HIIT FRAP values were significantly lower (-9.72 mM TEAC) than baseline FRAP values (ρ < 0.05). On the other hand, post-HIIT and 15'post-HIIT FRAP values were significantly higher (+27.97 and +117.69 mM TEAC) than baseline FRAP values (ρ < 0.05). Supporting the increasing trend seen in Figure 8. A detailed t-Test for all intervention beverages was made, comparing the following time points as pairs:

Baseline X Post-HIIT
Baseline X 15'post-HIIT
Pre-HIIT X Post-HIIT
Pre-HIIT X 15'post-HIIT

For orange juice, matcha, and coffee all paired sample tests of time points are significantly different (ρ < 0.05). Results for water, pomegranate juice, and milk showed three significant different pairs, which were baseline X 15'post-HIIT, pre-HIIT X post-HIIT, and pre-HIIT X 15'post-HIIT (ρ < 0.05). For the sugar-sweetened soft drink, only pre-HIIT X post-HIIT revealed

significant differences between time points ($\rho < 0.05$). Table 14 below is showing these data, with significant results highlighted in bold.

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Water	FRAP_baseline x FRAP_post-HIIT	-9,06	41,48	9,28	-28,47	10,36	-0,976	19	0,341
	FRAP_baseline x FRAP_15post-HIIT	-120,49	68,97	15,42	-152,76	-88,21	-7,813	19	**0,000**
	FRAP_pre-HIIT x FRAP_post-HIIT	-23,42	25,81	5,77	-35,51	-11,34	-4,058	19	**0,001**
	FRAP_pre-HIIT x FRAP_15post-HIIT	-134,85	60,91	13,62	-163,36	-106,34	-9,901	19	**0,000**
PJ	FRAP_baseline x FRAP_post-HIIT	-16,01	41,83	9,35	-35,59	3,56	-1,712	19	0,103
	FRAP_baseline x FRAP_15post-HIIT	-87,20	99,04	22,15	-133,55	-40,85	-3,938	19	**0,001**
	FRAP_pre-HIIT x FRAP_post-HIIT	-32,44	42,37	9,47	-52,27	-12,61	-3,424	19	**0,003**
	FRAP_pre-HIIT x FRAP_15post-HIIT	-103,63	96,06	21,48	-148,59	-58,67	-4,825	19	**0,000**
OJ	**FRAP_baseline x FRAP_post-HIIT**	-41,12	48,13	10,76	-63,64	-18,59	-3,821	19	**0,001**
	FRAP_baseline x FRAP_15post-HIIT	-147,43	82,90	18,54	-186,23	-108,63	-7,953	19	**0,000**
	FRAP_pre-HIIT x FRAP_post-HIIT	-34,58	33,14	7,41	-50,09	-19,07	-4,667	19	**0,000**
	FRAP_pre-HIIT x FRAP_15post-HIIT	-140,89	64,91	14,52	-171,27	-110,51	-9,707	19	**0,000**
Matcha	**FRAP_baseline x FRAP_post-HIIT**	-59,42	47,82	10,69	-81,80	-37,04	-5,557	19	**0,000**
	FRAP_baseline x FRAP_15post-HIIT	-147,85	53,57	11,98	-172,92	-122,78	-12,343	19	**0,000**
	FRAP_pre-HIIT x FRAP_post-HIIT	-48,19	47,08	10,53	-70,22	-26,15	-4,577	19	**0,000**
	FRAP_pre-HIIT x FRAP_15post-HIIT	-136,61	48,79	10,91	-159,45	-113,78	-12,521	19	**0,000**
Coffee	**FRAP_baseline x FRAP_post-HIIT**	-41,59	51,44	11,50	-65,66	-17,51	-3,616	19	**0,002**
	FRAP_baseline x FRAP_15post-HIIT	-160,46	84,90	18,98	-200,19	-120,72	-8,452	19	**0,000**
	FRAP_pre-HIIT x FRAP_post-HIIT	-43,57	31,45	7,03	-58,29	-28,86	-6,197	19	**0,000**
	FRAP_pre-HIIT x FRAP_15post-HIIT	-162,44	68,70	15,36	-194,60	-130,29	-10,574	19	**0,000**

Milk	**FRAP_baseline x FRAP_post-HIIT**	-13,72	32,77	7,33	-29,06	1,62	-1,872	19	0,077
	FRAP_baseline x FRAP_15post-HIIT	-100,46	49,95	11,17	-123,84	-77,09	-8,995	19	**0,000**
	FRAP_pre-HIIT x FRAP_post-HIIT	-34,39	30,49	6,82	-48,66	-20,12	-5,045	19	**0,000**
	FRAP_pre-HIIT x FRAP_15post-HIIT	-121,14	51,42	11,50	-145,20	-97,07	-10,534	19	**0,000**
Sugar	FRAP_baseline x FRAP_post-HIIT	-14,86	80,62	18,03	-52,60	22,87	-0,825	19	0,420
	FRAP_baseline x FRAP_15post-HIIT	-59,94	196,38	43,91	-151,85	31,97	-1,365	19	0,188
	FRAP_pre-HIIT x FRAP_post-HIIT	-47,23	36,77	8,22	-64,44	-30,02	-5,745	19	**0,000**
	FRAP_pre-HIIT x FRAP_15post-HIIT	-92,30	210,76	47,13	-190,94	6,33	-1,959	19	0,065

Table 14: Paired sample test for each intervention beverage.

Comparison of FRAP values before and after HIIT.

5.4 ROS production

A rmANOVA with Huynh-Feldt correction was used to demonstrate that there was a statistically significant difference in mean ROS levels between measurements, $F(3.0, 369.86) = 5.11$, $p < 0.002$ (Figure 11). Pairwise comparisons of ROS levels across all intervention beverages revealed significantly higher baseline ROS levels compared to pre-HIIT ROS levels (+0.09 µM / min, $\rho < 0.05$). In addition, significantly higher post-HIIT ROS levels compared to pre-HIIT ROS levels could be proven (+ 0.08 µM / min, $\rho < 0.05$). Comparisons between baseline and 15'post-HIIT showed significantly lower ROS levels for 15'post-HIIT (-0,07 µM / min, $\rho < 0.05$).

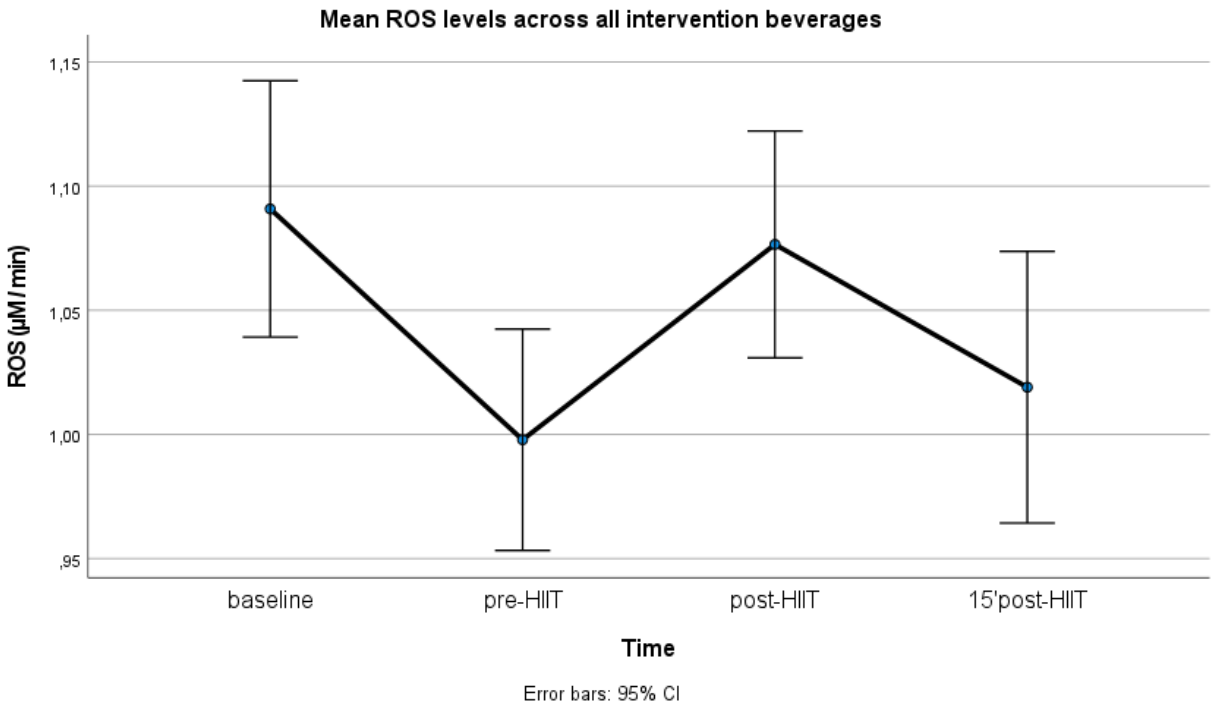

Figure 11: ROS levels across all intervention beverages.

Baseline ROS levels averaged 1.09 (± 0.3) µM / min at the first blood draw across all participants, which can be interpreted as uninfluenced status quo levels. This is in accordance with the expected physiological levels of ROS in healthy subjects reported in the literature, ranging from 0.69 to 0.90 µM / min (Zeng et al. 2020) and 1.84 to 2.04 µM / min (Mrakic-Sposta et al. 2014). Pairwise comparisons for separate consideration of water and pomegranate juice revealed no significant differences between both beverages (Figure 12). Water and pomegranate juice also individually showed increasing ROS levels from pre-HIIT to post-HIIT (+0.11 µM / min and +0.10 µM / min, $\rho < 0.05$). Both beverages also revealed significant lower 15'post-HIIT ROS levels compared to baseline (-0.08 µM / min for water and -0.16 µM / min for pomegranate juice, $\rho < 0.05$). Comparisons between baseline and pre-HIIT ROS levels showed no significant differences when considering water consumption and pomegranate juice consumption separately.

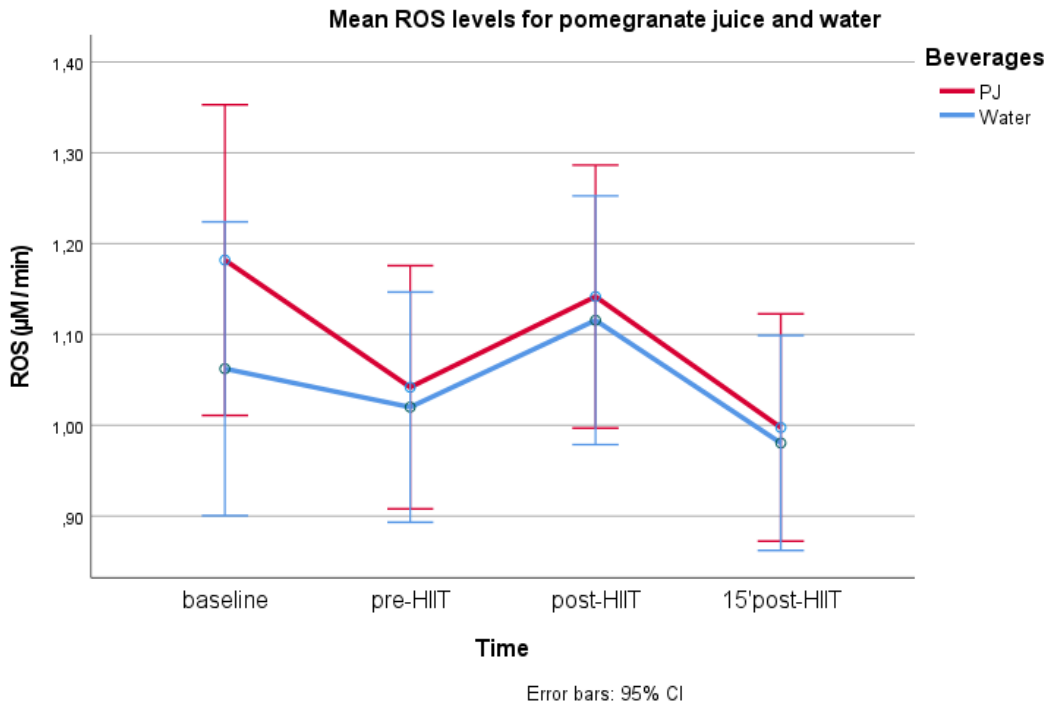

Figure 12: ROS levels for water and pomegranate juice.

In the calculations of the ROS data, all ROS values were not baseline corrected since a few times baseline measurement of ROS was not possible due to technical failures of the e-scan and therefore a baseline correction was not possible.

5.5 Correlation

Spearman correlation for ROS and FRAP levels after consumption of pomegranate juice showed no significant correlation between these parameters.

6 Discussion

Results of this underlying master's thesis showed that an acute consumption of pomegranate juice and other beverages have an influence on plasma FRAP levels over time. This supports the hypothesis that ingestion of a juice rich in polyphenolic antioxidant compounds would increase FRAP levels. Statistical analysis showed no significant difference in the antioxidant capacity (FRAP) of water intervention in comparison to pomegranate juice. This indicates a possible involvement of other radical scavengers than polyphenols, such as UA or bilirubin as endogenous antioxidants, displaying the body's first line defence. UA and bilirubin, both metabolic end products and non-enzymatic, endogenous antioxidants are powerful antioxidants in the organism. UA is an aqueous antioxidant known to be the most abundant

antioxidant in humans, contributing to two-thirds of all plasma free radical scavenging capacity (Waring et al. 2002). The end-product of purine metabolism, UA, increases with intense physical exercise and diffuses into the muscle cell for protection against free radicals and scavenges $O_2^{\bullet-}$. Most importantly, UA is able to form stable complexes with Fe^{3+} and therefore leads to inhibition of Fe^{3+} catalysed Vitamin C oxidation and lipid peroxidation (Finaud et al. 2006). It can be assumed that UA also reacts with the iron in the FRAP assay and thus leads to FRAP values similarly high for water and pomegranate juice, which was contrary to the expectation. If the UA concentration is known, it can easily be removed by calculation (Benzie and Devaki, 1997). The byproduct of haeme catabolism, bilirubin, and other bile pigments such as biliverdin act as further potent radical scavengers by transferring electrons to free radicals (Finaud et al. 2006). It is proven that bilirubin can reduce the mutagenic effects of ROS (Bulmer et al. 2008). UA and bilirubin are also determined within the FRAP assay, but not separately. Therefore, a single determination of UA and bilirubin, could provide more detailed information about the exact contribution to the antioxidant capacity of polyphenol rich beverages.

6.1 Intervention duration

Studies which demonstrated the beneficial health effects of pomegranate juice performed the interventions of consuming a polyphenol-rich beverage for a longer period. The research for other clinical trials in humans showed periods from 10 days, one month, two months, to three months (Sumner et al. 2005, and Viuda-Martos 2010) and one, or three years (Rosenblat et al. 2006), as described in chapter 2.3. Other exercise-associated studies examining the positive effects of pomegranate juice on exercise-related oxidative damage, had a significantly higher frequency of consuming the beverage. Ammar et al. (2017) provided 250 mL of freshly squeezed pomegranate juice three times per day for the 48 hours post-training period and 500 mL of freshly squeezed pomegranate juice 60 minutes before the training session. As a result, it was shown, that the pomegranate juice group had significantly lower post-exercise MDA levels and significantly higher rates of increase in enzymatic and non-enzymatic antioxidant responses immediately three minutes after exercise compared to the placebo group (Ammar et al. 2017). Al Dujaili et al. (2016) administered 500 mL of pomegranate juice per day for one week (seven days) and thus achieved a significant reduction in systolic and diastolic blood pressure, as well

as a significant reduction in MDA levels in the pomegranate juice group in comparison to the placebo group. Fuster-Muños et al. (2016) designed a study with 200 mL pomegranate juice per day for five weeks. Results showed a significant reduction in MDA levels for the pomegranate juice group in comparison to the control group. Further measurements revealed significantly increased levels of PC in the control group whereas in the pomegranate juice group PC levels remained constant (Fuster-Muños et al. 2016). Koivisto et al. (2019) observed increased levels of FRAP in elite endurance athletes after doubling the intake of polyphenol-rich food for three weeks. Lorzadeh et al. (2022) summarized 21 RCTs in a meta-analysis from 2022. A high heterogeneity among the studies with different doses, sources of pomegranate juice or -extracts, and subjects (with or without type 2 diabetes, coronary heart diseases, postmenopausal, obese, or overweight, etc.) makes it hard to compare the effects of pomegranate on markers of oxidative stress (Lorzadeh et al. 2022). The meta-analysis concluded that pomegranate consumption led to no significant effects or changes in FRAP levels (Lorzadeh et al. 2022), which is not in agreement with the results found in the present study. Although it could not be shown that there is a difference between intervention beverages, it can be assumed that a longer period of time would have yielded more significant data for the underlying study, as the human organism has numerous defence and adaptation mechanisms (chapter 1 Introduction), which is why it can be assumed that a single intervention does not immediately throw the body out of balance. Nevertheless, as suspected, a significant time effect could be observed. Increased FRAP values through all time points could be proven (see chapter 5. Results). It remains unclear what exactly causes the elevated FRAP levels, because all beverages showed the increasing trend and the FRAP assay does not distinguish between exogenous (non-enzymatic) and endogenous, but non-enzymatic, antioxidants. Contrary to the hypothesis, pomegranate juice did not have a prominent effect on FRAP levels compared to other beverages, especially water. Matcha, coffee, and OJ were more noticeable, which is why they are briefly discussed below.

6.2 Matcha

Statistical analysis showed significantly higher FRAP levels for matcha intervention compared to sugar-sweetened soft drink intervention. This is in accordance with the study from Panza et al. (2008) who showed the positive effects of seven-day green tea consumption on oxidative

stress markers in weight-trained men. Catechins are the major polyphenols found in green tea and are efficient natural antioxidants, able to increase the post-exercise concentration of FRAP levels (Panza et al. 2008). Matcha, consumed as a powder from whole leaves of *Camellia sinensis,* is considered to have the highest antioxidant potential compared to other green tea types. The high content of polyphenols, rutin, and vitamin C seem to have significant effects on elevating plasma FRAP levels. (Jakubczyk et al. 2020).

6.3 Orange Juice

Statistical analysis showed trending (but not significant) higher FRAP values for OJ, compared to pomegranate juice and significant higher FRAP values compared to sugar-sweetened soft drink. OJ is a fruit juice, high in phenolic compounds, especially flavonoids. The flavanone glycosides hesperidin, naringin, and narirutin are a subgroup of flavonoids and are mainly found in the solid parts of the fruit (Rangel-Huerta et al. 2015). This working group showed that OJ consumption in obese and overweight participants was able to improve the enzymatic antioxidant defence system, especially through a decreased CAT activity (Rangel-Huerta et al. 2015). Additionally, Dourado et al. (2015) could prove that OJ has a greater effect than pomegranate juice on the antioxidant capacity in plasma.

6.4 Coffee

Statistical analysis was able to show, that acute coffee consumption leads to significant higher plasma FRAP levels than consumption of a sugar-sweetened soft drink. Total antioxidant capacity of brewed coffee is comparable to those of red wine or green tea, likewise as pomegranate juice (Brezová et al. 2008). Detailed investigations on the two major coffee compounds, caffeine and caffeic acid, showed a noteworthy scavenging activity for the strong radical OH* (Brezová et al. 2008). The randomized controlled trial by Agudelo-Ochoa et (2016) resulted in a significantly increased antioxidant capacity in plasma after drinking 400 mL coffee per day in an eight-week period (Agudelo-Ochoa et al. 2016), which is in accordance with this underlying study.

6.5 ROS levels

The main findings of this master's thesis were that the FRAP levels across time points (baseline, pre-HIIT, post-HIIT, 15'post-HIIT) showed significantly increased FRAP values after consumption of the respective intervention beverages. Moreover, ROS levels for all intervention beverages showed significant differences between time points. Pairwise comparisons revealed significantly higher baseline ROS levels compared to pre-HIIT ROS levels (+0.09 µM / min), indicating the sufficient relaxation phase of the study protocol. To minimize participants' stress levels prior to the first and second blood draw, the study protocol was designed to calm down the participants as much as possible. After arriving, they rested for 30 minutes, then the first blood draw was taken, followed by two hours rest until the second blood draw. Furthermore, the HIIT was designed to initiate exercise-induced oxidative stress in participants, which could be proven by the evaluation of ROS levels from pre-HIIT to post-HIIT. On average, the pre-HIIT ROS levels increased by approximately 0.08 µM / min to post-HIIT ROS levels. These results suggest that the HIIT in general had an effect on the participants' ROS production, which agrees with the literature, reporting increased generation of ROS due to exercise (Sen, 1995 and Skenderi et al. 2008). Determinations of the ROS levels after consumption of water and pomegranate juice did not have showed differences between both beverages but demonstrated the increasing effect from pre-HIIT to post-HIIT (+0.10 µM / min) and the lower 15'post-HIIT levels compared to baseline (-0.16 µM / min) after pomegranate juice consumption. This is adding further support to the hypothesis that the antioxidants in pomegranate juice are able to diminish ROS production. Although the bioavailability of polyphenols is known to be relatively low (see 1.5 Phytochemicals), pomegranate juice appears to have a positive effect on the scavenging of ROS. However, as the effect of water is nearly the same, it is debatable whether pomegranate juice would have such an outstanding role on reducing ROS production or quenching of almost existing ROS. Consideration must be given to the enzymatic antioxidants, which may be more effective than dietary antioxidants in quenching ROS. This aspect seems to be even more relevant when considering that the spearman correlation was not significant for FRAP and ROS values after pomegranate juice consumption.

6.6 Further limitations

The participant's diet before an intervention day might influence the ROS and FRAP levels on the day of blood collection. Participants were not given a list with information about foods rich in antioxidants that are better avoided the day before. An influence of the diet before the intervention day, for example by vitamin A, E, and C, can therefore not be excluded. Further potential limitation can be seen in the gender of the study group. Science is aware of gender-related differences in oxidative stress due to female hormonal fluctuations. The primary female sex hormone estradiol-17β is known to have an effective antioxidant capacity (Ruiz-Larrea et al. 2000). In the underlying study, estradiol-17β was determined and should also be further investigated for correlations with the other oxidative stress markers. Presumably, the timing of the menstrual cycle has an influence on athletic performance on the one hand and on total antioxidant capacity and ROS on the other. For this, it would be important to know the individual length of the subject's cycle, as well as the exact day. Hormonal determination is considered to be the most accurate option, but both estradiol-17β and progesterone must be determined (Janse de Jonge, 2003). As part of the overall research project "Acute impact of different beverages on exercise induced oxidative stress and inflammation", estradiol-17β and the diet of the previous day will be also considered.

7 Summary

In the present work, the FRAP analysis was used to determine the total antioxidant capacity in the blood plasma of healthy women, using the CLARIO star^{plus} photometric microplate reader. The FRAP assay is a simple and rapid test method, based on the iron reduction ability of antioxidant substances in blood plasma. This work aimed to investigate whether pomegranate juice in combination with HIIT influences the antioxidant capacity in healthy women. HIIT was used as a stressor to influence women's ROS scores. After statistical evaluation using SPSS, it could be shown that HIIT was able to increase the ROS values in women. As expected, the pomegranate juice intervention obtained an increase in the total antioxidant capacity in plasma across the four measurement times. This leads to the conclusion that ROS released by the HIIT can be partly intercepted by the antioxidants contained in pomegranate juice. But not only consumption of pomegranate juice revealed such significant results. For all intervention beverages, excepted the sugar-sweetened soft drink, significantly increased FRAP values could be determined. A correlation with ROS values could not be established. The study thus supported the hypothesis that pomegranate juice had a positive influence on plasma FRAP values, but it also showed that water and other beverages rich in polyphenols appeared to have an influence. Pomegranate juice might be a powerful and healthy polyphenol-rich fruit juice, but since the mechanism behind antioxidant capacity in plasma is not fully understood yet, it is not possible to attribute the positive properties solely to polyphenols in pomegranate juice. However, since many other research groups reported positive effects, which also strengthens the thesis for pomegranates being a 'superfood', it can be assumed that pomegranate juice has health-promoting effects when consuming the juice in a higher dose and for a longer period. Given that pomegranate juice has no significant adverse effects on the organism and contributed to elevated plasma FRAP levels in this study, it can be incorporated into a varied and balanced diet and promotes a healthy lifestyle.

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Attachment

Appendix I

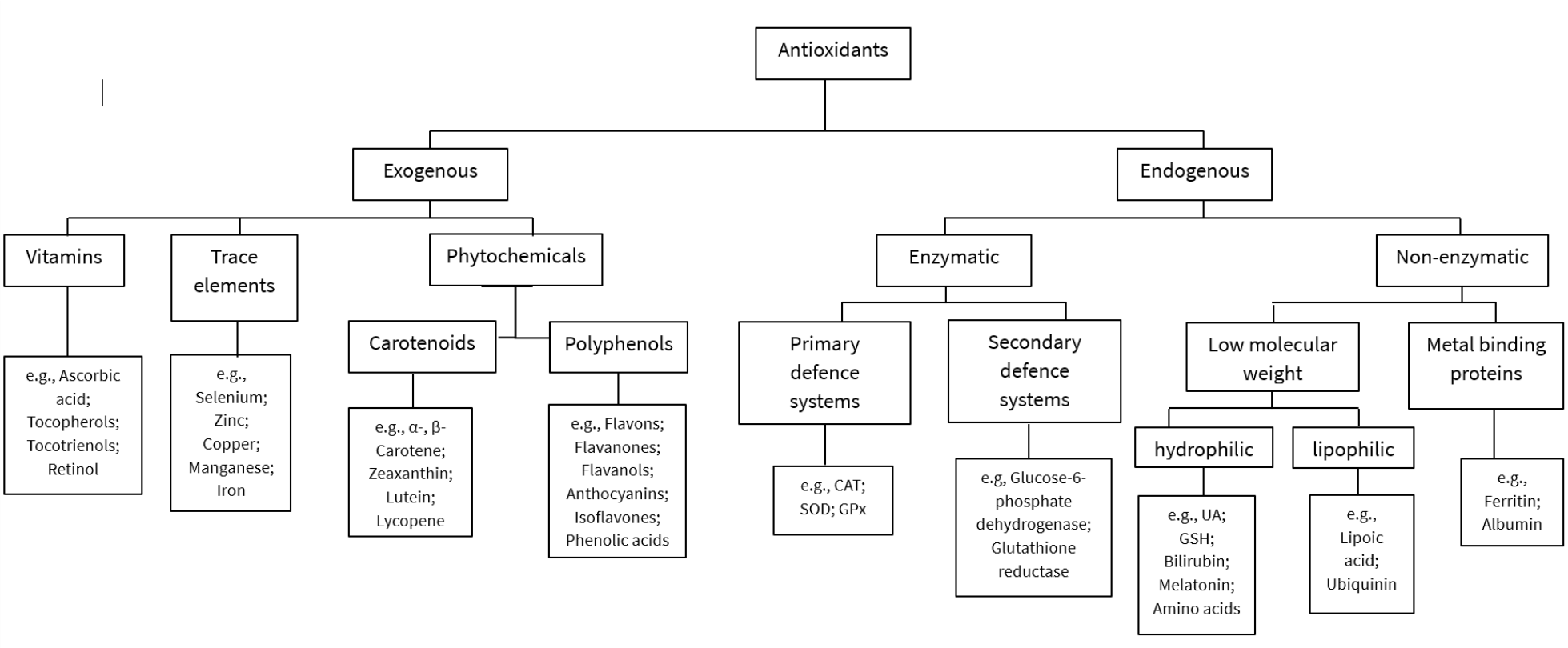

Figure 13: Classification of antioxidants.

*(*Adapted from Flieger et al. 2021*)*

Appendix II

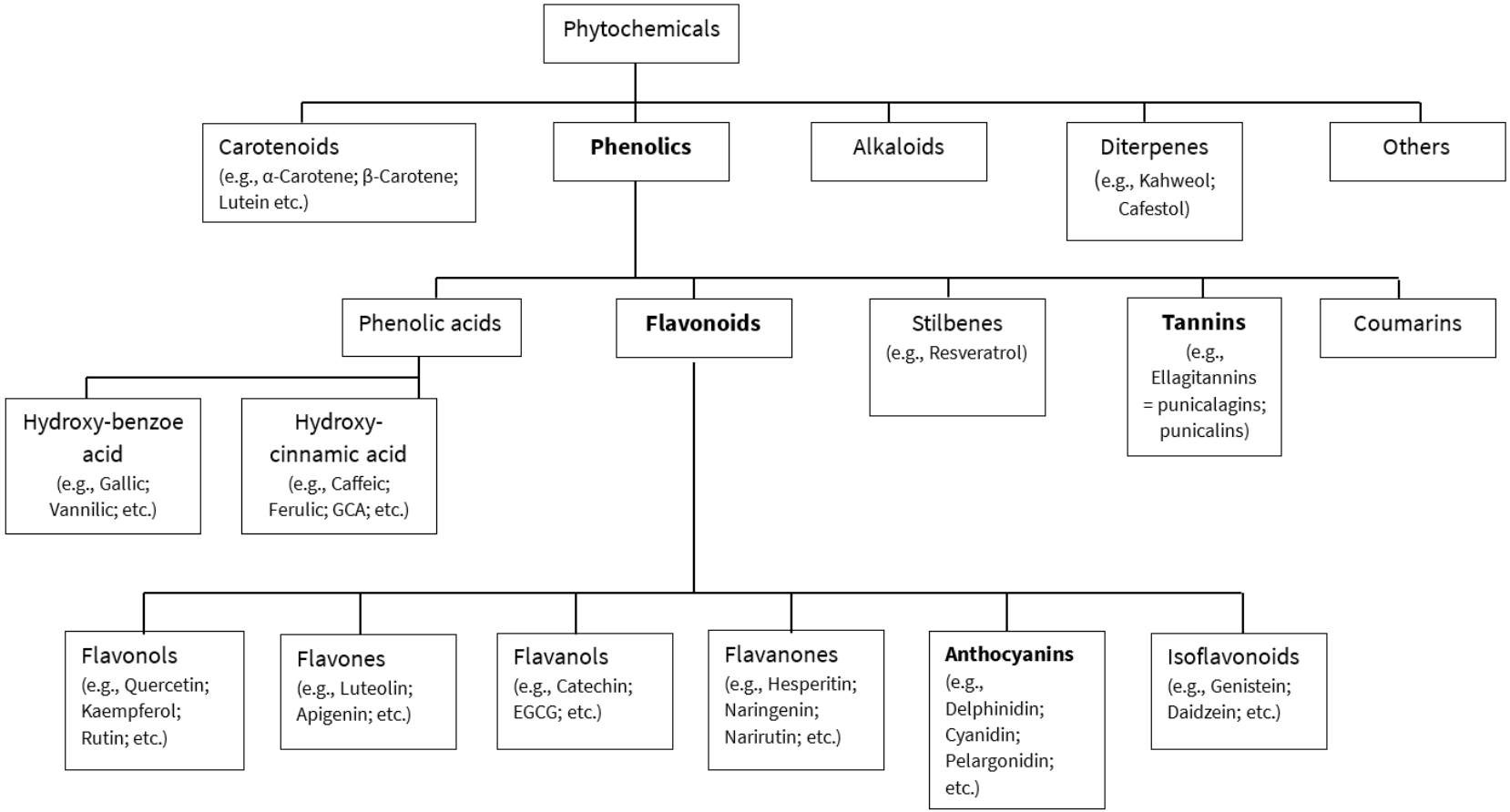

Figure 14: Schematic overview of phytochemicals.

*(*Adapted from Hwang et al. 2019*)*

Appendix III

Setting the weight for the participants

Exercise session 1:

- Warm up is identical to the study training (see below)
- Explain the correct movement execution in detail one time
- Three easy low weight sets of 40 seconds per exercise and 10 seconds rest between each exercise
- Rest between sets of 1 minute each (ask for BORG scale)

Exercise session 2: 5 RM test

- Ideally on an empty stomach after blood collection
- Warm up identical to the study training (see below)
- No circuit training but station training
- Step by step, each exercise is carried out individually until it is exhausted -until only 5 to 10 clean repetitions are possible (the last repetition should be from the execution may no longer be correctly)
- Rest 1 minute between each set
- Several sets of rows
- Multiple sets of chest presses
- Several sets of lat pulldowns
- Multiple sets of leg presses

Training on study day:

Warm up: mobilization part

- 10x circling wrists
- 10x circling forearms from the outside to the inside
- 10x circling forearms from the inside to the outside
- 10x shoulder circles small circles forwards; 10x shoulder circles small circles backwards
- 10x shoulder circles big circles forwards; 10x shoulder circles big circles backwards
- 10x one-legged hip circles outward on the left; 10x one-legged hip circles inward on the left

- 10x one-legged hip circles outward on the right; 10x one-legged hip circles inward on the right
- 10x one-legged knee circles left clockwise; 10x one-legged knee circles left anti-clockwise
- 10x one-legged knee circles right clockwise; 10x one-legged knee circles right anti-clockwise
- 10x circle left ankle (standing) clockwise; 10x circle left ankle (standing) anti-clockwise
- 10x circle right ankle (standing) clockwise; 10x circle right ankle (standing) anti-clockwise
- 10x pelvic circles to the left (two-legged stand); 10x pelvic circles to the right (two-legged stand)
- 20x jumping jacks
- 20x high knees run
- 10 reps side plank (with bent knees) on the left
- 10 reps side plank (with bent knees) on the right

---------- 10 minutes over ----------

Warm up: 50% of the given training weight and 40 second per exercise.

order: **rowing; chest press; leg curls; lat pulldown; leg press** (for every session)
goal: 20 repetitions

----------1 minute rest----------

Session 1:

- Training weight according to plan
- 40 second load
- 10 second rest between exercises (immediately changing to the next machine)
- The trainer counts out loud and notes the values; target: 20 repetitions per exercise
- BORG scale should be around 12-13 (if lower -> more repetitions! If higher-> participant is allowed to downshift)

---------- 1 Minute rest + ask BORG scale ----------

Session 2:

- Training weight according to plan
- 40 second load; target: 20 repetitions per exercise
- 10 second rest between exercises (immediately changing to the next machine)
- BORG scale should be around 14-15

---------- 1 Minute rest + ask BORG scale ----------

Session 3:

- Training weight according to plan
- 40 second load; target: 20 repetitions per exercise
- 10 second rest between exercises (immediately changing to the next machine)
- Participant should be motivated to give everything again in each exercise; possibly increasing the weight
- Go immediately to the fourth blood collection, where the participant is asked to lie down on the couch

Exercise execution instructions:

Rowing machine:

- Starting position: upright sitting position; buttocks are in contact with the seat; spine in neutral position; the shoulders are pulled slightly downwards and backwards (stable position of the shoulder girdle); hands grip the handles slightly below shoulder height
- Execution: arms are bent against the resistance; the elbows are pulled backwards; the shoulder blades are pulled back and down; then the arms slowly straightened again; look straight; squeeze shoulder blades together (when pulling); pull handle to solar plexus

Chest press machine:

- Starting position: grab the lower handle; the top handle should be at sternum level; upright sitting position; buttocks are in contact with the seat; the spine is located in neutral position; the shoulders are slightly pulled back and down; the hands are on the handles positioned slightly below shoulder height; as a starting position the elbows should be positioned at least at shoulder height or pulled back

- Execution: the arms are stretched against the resistance and then slowly bent again; the shoulders remain stable throughout the movement in starting position to use the chest muscles optimally; looking straight ahead; pressing the neck and back straight against the back seat

Leg curl machine:

- Starting position: middle wheel (knee should be at mark 0 ideally; 1 would also be ok); back rests against the backrest; leg stretched forward approximately 90 degrees; 2nd pad rests on the shin; the buttocks are in contact with the seat and the spine is in a neutral position
- Execution: bend the leg as much as possible (move to the floor, at least 90 degree angle)

Lat pulldown machine:

- Starting position: upright sitting position; buttocks are in contact with the seat; the spine is in neutral position; the shoulders are slightly pulled back and down; the hands are positioned shoulder width apart in an overhead grip on the lat pulldown bar in front of the body
- Execution: the arms are bent against the resistance; the elbows are pulled backwards; the shoulders remain in a neutral or back position respectively during the exercise to optimally claim the back muscles (latissimus dorsi = target muscle of this exercise); always pull the bar all the way up to your breastbone; the movement is controlled by the elbows

Leg press:

- Starting position: ankle, knee and hip joint in one line; back and buttocks are in contact with the seat or backrest; setting level 9 (there should be at least a 90 degree angle in knee joint; position feet on plate (toes point slightly outwards)
- Execution: push leg extensions and body away from the plate; exhale and tighten the abdominal muscles.

Appendix IV

Reagents	chemical formula	Supplier	Product number
Sodium acetate	$C_2H_3NaO_2$	Sigma-Aldrich, Germany	1003143621
Acetic acid 99-100%	CH_3COOH	Sigma Aldrich, Germany	K54113455 206
2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ)	$C_{18}\ H_{12}\ N_6$	Sigma Aldrich, Switzerland	102265472
Iron-(III)-chloride-hexahydrate	$FeCl_3\ *_6H_2O$	Sigma Aldrich, Germany	102290944
Iron-(II)-sulphate-heptahydrate	$FeSO_4*_7H_2O$	Sigma Aldrich, Germany	102246530
Sodium chloride	NaCl	Merck KGaA, Germany	1.06404.1000
Potassium chloride	KCl	Sigma Aldrich, Germany	SZBF0020V
di-sodium-hydrogen-phosphate	Na_2HPO_4	Merck KGaA, Germany	102036121
Potassium-phosphate-monobasic	KH_2PO_4	Sigma Aldrich, Germany	1003064794
6-hydroxy-2,5,7,8-tetramethylchroman-carboxylic acid (Trolox)	$C_{14}H_{18}O_4$	Sigma Aldrich, Germany	56510
Hydrochloric acid 0,5M	HCl	Own production. Production date: 06.10.2020	

Table 15: List of required reagents.

Technical equipment	Company	Product number
CLARIO star^{plus}	BMG Labtech	-
Microtiter plates (96-w. BRANDplates -F- pureGRade -clear-	Brand GmbH + Co KG	REF: 781602 LOT: 661142
Multipette Xplorer – electronic single channel pipette Xplorer 5 – 100µL	eppendorf®	P43266J
Multipette E3 – electronic single channel pipette 1µL – 50mL	eppendorf®	L90886L
Manual pipettes - FINNIPETTE 100 – 1000µL	FINNIPETTE®	DH23696
Manual pipettes – FINNIPETTE 2 – 10mL	FINNIPETTE®	DH45055
Manual pipettes – PROLINE 1-5mL	BIOHIT	10173059
Manual pipettes – PROLINE 5 - 50µL	BIOHIT	4066358
Pipette tips 200mL	Starlab	Cat. No. S1111-0006 Lot: J128242M
Combitips advanced 5mL	eppendorf®	L205285M
Combitips advanced 1.0mL	eppendorf®	L2069020
Pasteur pipette 7mL non-sterile, graduated up to 3mL	VWR	2030806
Pasteur pipette 5.0mL non-sterile	VWR	06172017
Water bath with thermometer	GFL	-
pH meter	Metrohm	827 pH lab
Magnetic / heat stirrer	Heidolph	MR 3001-K
Vortex, Reax top	Heidolph	-
LaboStar pure water system UV	Evoqua Water Technologies	354900
Microcentrifuge tubes, 1.5mL, with cap, natural, PP	Ratiolab	5615000
50mL centrifuge tube with screw cap, skirted bottom, loose, sterile	starlab	E1450-0400
Precision balance, Dual Balance, Max. 81g/220g	METTLER TOLEDO	XSE205
Balance	Sartorius	-

Table 16: Technical equipment for FRAP assay.

Appendix V

Devices and settings for FRAP assay.

Basic parameters:

- Test name: FRAP
- Microplate: Greiner 96-F BOTTOM
- General settings:
    - Position delay: 0.5 sec
    - Number of kinetic windows: 1
- Kinetic window: 1 (measure absorbance change at intervals of 36 sec, 10 times, for a total time of 6 min and eight seconds)
    - Number of cycles: 10
    - Measurement start time: 1 sec
    - Numbers of flashes per well and cycle: 20
    - Cycle time: 36
    - Total measurement time: 6 min 08 sec
- Filter settings:
    - No of multichromatics: 1
    - Exitation filter: A 540
    - Emission filter: empty
    - Gain: 1591
- Well scanning: none
- Pause before plate reading: 360 sec

Wavelength settings:

- Discrete wavelength: 540 and 595

Layout:

- Content: S…Standard; C…Control; B...Blank; X…Samples
- Replicates:
    - Number: 2
    - Horizontal

Concentrations, Volume, Shaking:

- Concentration: enter manual

- Shaking options:
    - Mode: orbital
    - Frequency: 300 rpm
    - Additional shaking: shake before plate reading
    - Shaking time: 5 sec

Temperature control:

- 37°C …incubation on

Measure:

- Plate identification
- Gain adjustment: automatic
- Sample IDs: sample number or standard

Declaration of Originality

I declare that I have authored this thesis independently, that I have not used other than the declared resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

…… ……

Date Schlosser, Laura