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Antioxidant activity of hot water infusions and subcritical water extracts from teas and tea-like plants

MASTER'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled “Antioxidant activity of hot water infusions and subcritical water extracts from teas and tea-like plants” independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague 21st April 2023

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Abstract

Oxidative stress and related non-communicable diseases are the No. 1 cause of death worldwide. It is generally said that such diseases concern mainly developed countries of the Western world, however, in recent years, they have become an emerging global problem with a growing trend in developing countries, which is often associated with the change of lifestyle and cultural norms. The main risks contributing to the spread of oxidative stress (unhealthy diet, low physical activity, smoking, alcohol misuse) and thus non-communicable diseases are preventable and can be managed on the individual level. Tea (*Camellia sinensis*) is the 2nd most popular beverage in the world and many other hot beverages made of different species (*Aspalathus linearis*, *Cyclopia intermedia*, *Hibiscus sabdariffa*) have also gained significant regional importance. They are cheap and available, and their consumption is associated with several health benefits, especially due to the high content of polyphenolic substances and other antioxidants. Nowadays we can find many food products with antioxidant content on the market. Plant extracts used for their manufacturing are often obtained by environmentally non-friendly, time-consuming, and inefficient methods. Subcritical water extraction (SWE) is known as a novel green method used for the extraction of natural products that can improve heat and mass transfer, thus increasing extraction yields and efficiency. In this study, antioxidant activity and Total phenolic content (TPC) of 5 teas and tea-like plant extracts obtained by water infusion (WI) and SWE were determined and compared. Extracts obtained by WI method showed stronger antioxidant activity and higher TPC than SWE extracts in all cases. Extract of *Camellia sinensis* – green tea obtained by WI was evaluated as the most promising one with the lowest IC_{50} of 17.22 µg/mL (DPPH) and 9.85 µg/mL (ORAC), and the highest TPC of 475.76 mg GAE/g. Even though SWE is considered a promising method for the extraction of plant material, the methodology is not uniform, especially in temperature and extraction time, which affect the extraction efficiency. For further research, it would be appropriate to optimize SWE conditions for teas and tea-like plants by collecting the extract in the form of separated fractions, or by shortening the extraction time, which may enhance the final antioxidant content.

Keywords: *Camellia sinensis*, DPPH, green extraction method, ORAC, total phenolic content, traditional beverage

Abstrakt

Oxidační stres a s ním spjaté civilizační choroby jsou nejčastější příčinou smrti na světě. Všeobecně je známo, že tato onemocnění postihují především rozvinuté země Západu, nicméně v posledních letech se stala globálním problémem s rostoucí tendencí v rozvojových zemích. To je způsobeno především změnou životního stylu a kulturních a společenských norem. Hlavním rizikům vzniku a šíření oxidačního stresu, a tedy i civilizačních chorob (nezdravá strava, nízká fyzická aktivita, kouření, nadměrná konzumace alkoholu) se dá předcházet a mohou být řešena na úrovni jedince. Čaj (*Camellia sinensis*) je druhý nejoblíbenější nápoj na světě, a i mnoho dalších horkých nápojů připravených z jiných druhů rostlin (*Aspalathus linearis*, *Cyclopia intermedia*, *Hibiscus sabdariffa*) nabylo značného regionálního významu. Jsou levné, dostupné a jejich konzumace je spjata s řadou zdravotních benefitů, především díky vysokému obsahu polyfenolů a dalších antioxidantů. Trhy v současné době nabízí velké množství výrobků s antioxidačními účinky. Rostlinné extrakty používané pro jejich výrobu jsou často získávány neekologickými, zdlouhavými a neefektivními extrakčními metodami. Subkritická vodní extrakce je známá jako nová inovativní metoda extrakce rostlinných materiálů, která umožňuje zvýšit přenos tepla i masy, čímž se zvyšují výnosy i efektivita. V této studii byla stanovena a porovnána antioxidační aktivita a celkový obsah fenolických látek u pěti čajů a čajových nápojů získaných tradiční přípravou a subkritickou vodní extrakcí. Extrakty získané tradiční přípravou prokázaly lepší antioxidační aktivitu i obsah fenolických látek než subkritické extrakty ve všech případech. Extrakty zeleného čaje získané tradiční metodou prokázaly nejslibnější výsledky s nejnižšími hodnotami IC_{50} – 17.22 µg/mL (DPPH), 9.85 µg/mL (ORAC), a zároveň nejvyšším obsahem fenolických látek – 475.76 mg GAE/g. Přestože je subkritická vodní extrakce považována za slibnou metodu extrakce rostlinného materiálu, její metodologie není jednotná, obzvláště v případě teploty a času extrakce, což ovlivňuje výslednou efektivitu. Pro další výzkum by bylo vhodné optimalizovat podmínky subkritické vodní extrakce pro čaje a čajové rostliny buď sběrem extraktů ve formě jednotlivých frakcí, nebo zkrácením doby extrakce, což by mohlo zvýšit celkový obsah antioxidantů.

Klíčová slova: *Camellia sinensis*, DPPH, ekologická metoda, fenolické látky, ORAC, tradiční nápoje

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List of abbreviations

$^{\bullet}O_2$	superoxide radical
$^{\bullet}OH$	hydroxyl radical
$^1O_2$	singlet oxygen
CVDs	cardiovascular diseases
DPPH	2,2-diphenyl-1-picrylhydrazyl
ET	electron transfer
FAO	Food and Agricultural Organization
F-C	Folin-Ciocalteu
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalents
H_2O_2	hydrogen peroxide
HAT	hydrogen atom transfer
O_3	ozone
ORAC	Oxygen radical absorbance capacity
RNS	Reactive Nitrogen Species
$ROO^{\bullet}$; $RO^{\bullet}$	peroxyl and alkoxyl radicals
ROS	Reactive Oxygen Species
SAE	Sinapic acid equivalents
SFE	Supercritical fluid extraction
SWE	Subcritical water extraction
TE	Trolox Equivalents
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic content
WHO	World Health Organization

1. Introduction

I became interested in teas quite a long time ago and my interest persists, perhaps also because I haven't quite come to the taste of another very popular hot drink – coffee. Generally, it is said that the human interest in tea and other hot beverages may initially have had a religious motive, however, these stimulating beverages have gained popularity at all levels of societies all around the world (van der Vossen & Wessel 2000). Tea, in other words, hot water infusion of *Camellia sinensis* (L.) Kuntze, is nowadays the second most popular drink in the world, but many other hot water infusions made of different species have also gained significant regional importance (van der Vossen & Wessel 2000; Ho et al. 2009).

Non-communicable diseases have become a global problem and their spread is associated with insufficient protection against oxidative stress (Halliwell & Gutteridge 2015). Recently, they have become more widespread in developing countries, where the lower social classes are particularly at risk (Ezzati et al. 2005; Islam et al. 2014). Oxidative stress and thus non-communicable diseases are preventable, and their spread depends on several factors, especially diet composition, physical activity, alcohol and tobacco use, but also genetic dispositions (Ezzati et al. 2005; Lawson et al. 2017). Teas and other hot beverages are cheap and available, and their consumption is associated with health benefits due to their high content of antioxidants, especially phenolic compounds (Ho et al. 2009; Shahidi & Ambigaipalan 2015).

Nowadays many nutraceutical, pharmaceutical, or cosmetic products with antioxidant content can be found on the market. Plant extracts used for their manufacturing are obtained by different methods, however, traditional extraction methods may often be environmentally non-friendly and time-consuming with low efficiency of extraction (Haghighi & Khajenoori 2013; Sulaiman 2013). Subcritical water extraction (SWE) is a novel green method suitable for the extraction of thermolabile natural products that can very efficiently improve heat and mass transfer, thus increasing yields and efficiency of the extraction. Extracts obtained by this method may have a very interesting chemical composition and could be used for the production of food supplements or pharmaceutical and other products (Zhang et al. 2020).

2. Literature review

2.1. Free radicals and oxidative stress

Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), in other words, free radicals, are produced naturally during metabolism processes and the human body regulates their quantity using endogenous antioxidant systems such as glutathione peroxidase, and superoxide dismutase (Stratil & Kubáň 2018). They are signalling molecules that regulate biological and physiological processes in the human body. Free radicals can have a positive impact on human health if they are produced physiologically. Examples of beneficial functions are inhibiting bacteria, parasites, and tumour cells, entry of sperm into the egg, or bone remodelling. However, the impact of free radicals is highly negative in most cases. Many reactive species possess one or more unpaired electrons and are highly reactive. Among them the most common are superoxide radical ($^{\bullet}O_2$), hydroxyl radical ($^{\bullet}OH$), carbonate radical, peroxyl and alkoxyl radicals, and nitric oxide radical. Biologically important non-radicals with oxidizing activity include peroxynitrite, hydrogen peroxide (H_2O_2), Singlet oxygen ($^1O_2$), and ozone (O_3) (Lipinski 2011; Cadet et al. 2012; Kehrer & Klotz 2015). If the amount of ROS and RNS in the body exceeds the bearing limit of antioxidant systems, it can cause changes in cell structure and function. Imbalances between ROS/RNS production and antioxidant scavenging cause damage to biomolecules (Stratil & Kubáň 2018; Pleńkowska et al. 2020).

2.1.1. Oxidative damage of biomolecules

Oxidative stress can cause damage to biomolecules, tissues, and whole organs and result in various diseases (Stratil & Kubáň 2018). The most vulnerable biomolecules are DNA, lipids, and proteins (Wiseman 1996). If focused on the DNA, free radicals, most notably $^{\bullet}OH$, react with organic compounds by addition or abstraction. They add to double bonds of heterocyclic DNA bases and abstract a hydrogen atom from the methyl group of thymine and each of the carbon–hydrogen bonds of 2′-deoxyribose. Then, further reactions of such formed radicals of DNA bases and C-centred radicals of the sugar moiety result in a variety of final products (Evans et al. 2004). Overall, free radicals oxidation of the DNA cause changes in the DNA bases, single and double-strand breaks, DNA-protein cross-linking, and damage to the repair system. Lipids damage caused by peroxidation can occurs through 3 types of mechanisms. First, non-enzymatic non-radical oxidation, second, ROS-mediated oxidation, and third, enzymatic oxidation. Polyunsaturated fatty acids are susceptible to ROS oxidation due to the presence of multiple bonds in their structure. Various radicals ($^{\bullet}OH$, $ROO^{\bullet}$) cleave H atom of polyunsaturated fatty acids in the membrane structure, which starts the chain reaction. During the peroxidation, highly reactive aldehydes and hydroperoxides are produced as the end products and the changes in biochemical pathways can end up in disrupted fluidity, permeability, and biophysical properties of cell membranes. Proteins contain various functional groups (hydrocarbons, alcohols) and those containing unsaturated bonds and sulphur can undergo oxidation by free radicals. Their interaction with proteins can result in the inhibited function and activation of structural proteins. Free radicals can also cause direct damage to proteins, as well as the formation of highly reactive end products such as hydroperoxide (Kiran et al. 2023).

2.1.2. Consequences of oxidative stress

Oxidative stress consequences can include increased cell proliferation, adaptation of the cell by upregulation of the defence system, cell injury, cell senescence, or cell death. These changes are manifested in various pathological conditions ranging from autoimmune diseases to cardiomyopathies. Such diseases are also known as diseases of affluence, non-communicable diseases, lifestyle diseases, or Western diseases (Halliwell & Gutteridge 2015; Patel et al. 2018). It is believed that mainly the people who have a high-fat diet and inadequate physical exercise are the vulnerable ones, however, some individuals have genetic dispositions to be susceptible (Lawson et al. 2017). The most common diseases associated with oxidative stress are:

- Cardiovascular diseases (CVDs) such as atherosclerosis or hypertension
- Chronic kidney diseases
- Macular diseases
- Neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease
- Oncological diseases (Barnham et al. 2004; Liguori et al. 2018).

Non-communicable diseases now lead the world ranking of the cause of death. Ischemic heart disease is the most prevalent one, and dementia and diabetes are nowadays placed in the top 10 causes of death. It has been suggested that lifestyle changes at any time in life can improve cognitive, metabolic, and vascular health and therefore reduce the burden of non-communicable diseases (Poswal et al. 2019)

It is generally said that non-communicable diseases concern the developed countries of the Western world (Lawson et al. 2017). It has been found that the worst situation in high-income and upper-middle-income countries is in the lowest social classes, especially city dwellers, who consume many empty calories without sufficient antioxidants, fiber, high-quality fats, and proteins. These types of meals, also known as "the American diet", account for a greater and greater share of the diet, which leads to a reduced ability of the body to defend itself against free radicals (Ezzati et al. 2005; Al-Gubory 2017; Česká průmyslová zdravotní pojišťovna 2021). However, in recent years, non-communicable diseases have become an emerging global problem with a growing trend in developing countries. More than 80 % of CVDs and diabetes deaths, 90 % of chronic obstructive pulmonary disease deaths, and 66 % of all cancer deaths occur in developing countries. This transition from the main cause of death being infectious diseases to non-communicable diseases has been driven by several factors, often associated with the change in lifestyle and cultural norms. The move from traditional foods to processed ones, a decrease in physical activity, or an increased number of smoking women contributed the most (Islam et al. 2014).

The main risks contributing to non-communicable diseases (unhealthy diet, low physical activity, tobacco use, alcohol misuse) are preventable as they eventually progress in early life due to lifestyle aspects. The prevention of non-communicable diseases requires many strategies from several perspectives and on different levels, including large-scale (global, country, society) and individual levels. Large-scale strategies include national policies and plans, monitoring, improvement of budgetary allocations to support primary health care systems, opportunities for physical activities for all ages, an offer of healthy food in the workplace, and many others. From the individual point of view, the crucial step is following a healthy lifestyle (Budreviciute et al. 2020). The composition of the diet is an often-discussed aspect of a healthy lifestyle. According to the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), a healthy diet is defined as eating at least 400 g of fruits and vegetables per day, reducing the total fat intake to less than 30 % of total energy intake, reducing high sodium salt consumption and insufficient potassium intake, and reducing sugar intake by consumption of foods, but also drinks with additional sugars (WHO & FAO 2004). In the USA, regular soft drinks are the most popular beverage, followed by beer. Both milk and coffee consumption is currently less than half of their historical maxima and tea consumption remains constantly low. And precisely the increased consumption of beverages containing phenolics and other biologically active substances, such as tea, could help reduce the risk of diseases not only in the USA (Wolf et al. 2018; Shahidi & Ambigaipalan 2015).

2.2. Antioxidants

Antioxidants are chemical substances of natural or anthropogenic origin that may prevent, or delay cell damage caused by ROS and RNS and subsequently prevent the occurrence of oxidative stress. Antioxidants have been used for protection against oxidative stress by people since ancient times in the form of herbs, fruits, vegetables, spices, or beverages (Shahidi & Ambigaipalan 2015; Yadav et al. 2016). It has been proven that antioxidants have a positive impact on human health, ageing, life expectancy, and the evolution of many pathogens as well. Their beneficial function in human nutrition lies in the ability to neutralize ROS and RNS before they cause damage to body tissues (Yadav et al. 2016).

2.2.1. Antioxidants classification

Antioxidants can be classified into many categories depending on their origin, chemical structure, mechanism of action, and others. One of the major divisions is natural and synthetic antioxidants. Synthetic antioxidants are bioequivalents to their natural forms (chemically synthesized ascorbic acid or tocopherol), and they are being used in the form of dietary supplements. Naturally occurring antioxidants are nowadays isolated, fully characterized, and available for various applications to inhibit the adverse effects caused by free radicals. Those that are produced in the body (albumin, glutathione, ubiquinone) are endogenous antioxidants. They are either enzymatic or non-enzymatic and operate in different defence systems. Exogenous antioxidants are compounds derived from natural sources which are mostly plants we consume. These include carotenoids (α- and β-carotene, cryptoxanthin, lutein, lycopene, zeaxanthin), polyphenols (anthocyanins, flavanols, flavanones, flavones, flavonols, isoflavones, phenolic acids), trace elements (copper, iron, manganese, selenium, zinc), and vitamins (ascorbic acid, retinol, tocopherols). Since synthetic antioxidants pose a potential risk to the consumer in the form of contamination by chemical precursors, toxic solvents, and hazardous by-products, the scientific community is now focused on the research of effective, non-toxic, natural antioxidants, and their use in oxidative stress therapy (Flieger et al. 2021). If focused on the mechanism of action, 2 types of chemical substances play a role in antioxidant defence. They are either antioxidant nutrients or agents indirectly influencing antioxidant defence. Antioxidant nutrients (carotenoids, coenzyme Q, flavonoids, and others) directly protect body tissues by scavenging free radicals. Agents indirectly regulating oxidative stress are very diverse: minerals, proteins, vitamins A, B_3, B_6, and D, but also ethanol and polyunsaturated fatty acids. These operate in different metabolic processes. Minerals can be bounded to superoxide dismutase, they are part of enzymes, or their deficiency is linked to an increase in oxidative stress. Amino acids are responsible for the synthesis of antioxidant defence enzymes and metal-binding proteins. Vitamins are important in energy metabolism (B_3), and their deficiency is associated with an increase in oxidative stress. Last but not least is a total caloric intake, where the caloric restriction has been shown to reduce oxidative stress and improve antioxidant defences (Halliwell & Gutteridge 2015).

2.2.2. Antioxidants in the human diet

The richest source of antioxidants for human consumption are various plant species, but antioxidants are together with other biologically active compounds also found in actinomycetes, bacteria, cyanobacteria, fungi, and lichens. These can, compared to plants, grow very quickly under controlled conditions and they are a favourable source of antioxidants for nutraceuticals, and pharmaceuticals (Flieger et al. 2021). If focused on the human diet, rich sources of antioxidants are beverages (cocoa, coffee, beer, tea, red wine), fruits (apricots, citruses, papayas, and others), herbs (clove, ginger, rosemary, and others), and vegetables (carrots, lettuce, peas, tomatoes, and others). Antioxidants can be also found in nuts and vegetable oils that are rich in vitamin E, dark chocolate that contains flavonoids, and fish and meat that contain coenzyme Q10 (Kaliora et al. 2006;

Shahidi & Ambigaipalan 2015). Beverages such as green and black tea, coffee, and red wine should be considered a very prominent and attractive source of natural antioxidants since most of them are consumed on a daily basis all around the world, and they are cheap and available. Biologically active compounds present in beverages, especially simple phenols, show a wide range of antioxidant activities and are thought to exert protective effects against diseases such as cancer or CVDs (Shahidi & Ambigaipalan 2015). Tea, the second most widely consumed beverage in the world after water, is particularly rich in polyphenols and shows promising antioxidant activity (Shahidi & Ambigaipalan 2015; Al-Gubory 2017).

Even though antioxidants are thought to protect against oxidative stress, they can, under certain conditions (exposure to ionizing radiation, UV radiation, cigarette smoke), act as prooxidants (Sotler et al. 2019). Flavonoids show prooxidant activity that is directly proportional to the quantity of hydroxyl groups in a flavonoid molecule. These hydroxyl groups can, for example, significantly increase the production of hydroxyl radicals in the Fenton reaction. They can reduce copper (II) to copper (I) and thus they are enabling the formation of initiating free radicals. Some flavonoid-produced free radicals can inhibit mitochondrial respiration, and the presence of flavonoids with A- or B-ring pyrogallol configurations induces single-strand breakage of the DNA (Procházková et al. 2011). Another negative effect of antioxidants has been monitored in people who consume β-carotene. Even though several epidemiological studies have reported an association between β-carotene plasma level and reduced lung cancer risk, intervention studies in smokers have reported increased lung tumour rates after high, long-term β-carotene consumption (Goralczyk 2009). Despite the anti- or prooxidant properties of particular antioxidants depend on their concentration present, their beneficial effects prevail in most cases and the prooxidative ones can sometimes also be beneficial since a mild degree of oxidative stress leads to overall raised levels of antioxidant defences and cytoprotection (Procházková et al. 2011).

The most useful antioxidants in the diet in the terms of antioxidant defence are phenolic compounds, which are also one of the most abundant. Other highly abundant antioxidants are carotenoids, coenzyme Q, ergothioneine, histidine-containing dipeptides, and vitamins C and E (Halliwell & Gutteridge 2015).

Carotenoids are widely distributed fat-soluble pigments with several health benefits. There are almost 50 carotenoids with different mechanisms of action and antioxidant activity that protect cells and tissues from oxidative stress. They are very effective quenchers of $^1O_2$. Carotenoids provide enhancement of immune function, protect from sunburn reactions, delay the onset of various types of cancer and have many other functions such as the role of provitamin A. Although there are more than 700 carotenoids in nature, only about 40 of them are present in normal diets (α- and β-carotene, β-cryptoxanthin, lutein, lycopene, zeaxanthin). They can be found in several yellow-orange fruits and dark green leafy vegetables (Xavier & Pérez-Gálvez 2016). Carotenoids are also contained in teas with content ranging from 36 to 73 mg/100 g dry weight, however, they were found to be degraded to different extents during tea processing (Ravichandran 2002).

Coenzyme Q (also known as Ubiquinone, CoQ10) is a fat-soluble, vitamin-like benzoquinone that is present in all human cells. Coenzyme Q is essential for proper mitochondrial function, and it is also found in cell membranes and lipoproteins. *In vitro* ubiquinol (reduced form of coenzyme Q) can scavenge $RO^\bullet_2$, inhibit lipid peroxidation, and regenerate α-tocopherol from its radical. Unfortunately, *In vivo* antioxidant defence actions of coenzyme Q are uncertain and its beneficial functions in patients with diabetes and certain neurodegenerative diseases are limited. Rich dietary sources include both plant and animal tissues such as meat, fish, or plant oils. Lower concentrations can be found in dairy products, fruits, and vegetables. There are also novel forms of coenzyme Q in foods of different geographical origins such as juices, syrups, tea, and other beverages that allow the development of new pharmaceutical formulations (Pravst et al. 2010; Halliwell & Gutteridge 2015; Arenas-Jal et al. 2020).

Ergothioneine is a thio-histidine betaine amino acid possessing antioxidant activity. In our body, ergothioneine can resist H_2O_2-induced cell death, prevent Cu-induced oxidative damage to DNA, and thus protect body tissues against cardiovascular diseases, chronic inflammatory conditions, neuronal injuries, and ultraviolet radiation damages (Ey et al. 2007; Servillo et al. 2017; Borodina et al. 2020). Ergothioneine is synthesized by microbes, especially by fungi, but not by plants and animals. Plants can absorb ergothioneine via symbiosis of their roots and soil fungi and animals obtain it solely from dietary sources (Servillo et al. 2017). The richest sources are oyster mushrooms - *Pleurotus* spp. and shiitake mushrooms - *Lentinula edodes* (Ey et al. 2007; Borodina et al. 2020).

Flavonoids and other phenols exert a certain level of antioxidant activity. They inhibit lipid peroxidation, scavenge ferryl radical, $^{\bullet}OH$, $NO_2^{\bullet}$, and others (Halliwell & Gutteridge 2015). Generally, phenolic compounds are found in both edible and non-edible plants. In terms of diet, the greatest emphasis is placed on fruits, vegetables, herbs, and cereals, which contain the highest amount (El Gharras 2009). Plant polyphenols, especially flavonoids that are present in teas, wines, and other beverages are excellent antioxidants, and in the terms of hot water infusions, they are the most abundant ones. Green tea is rich in catechins, whereas black tea contains complex polymers called theaflavins and thearubigins. These are formed by the oxidation of catechins by the enzyme phenolase during black tea manufacturing. During the manufacturing of green tea, phenolase is inactivated by leaves steaming. Another excellent beverage source of phenolic antioxidants is red wine, which in addition to catechin also contains epicatechin and epigallocatechin gallate. If focused on other food categories, herbs and spices are also rich sources of antioxidants such as carnosic acid, rosmarinic acid, eugenol, and others (Halliwell & Gutteridge 2015).

Histidine-containing dipeptides are dipeptides composed of histidine that is attached to another amino acid. These include anserine, L-carnosine, and homocarnosine. Histidine-containing dipeptides can prevent tissues from Cu-induced oxidative damage and scavenge $RO^{\bullet}_2$ and $^{\bullet}O_2$ (Halliwell & Gutteridge 2015). The metal-binding affinity of carnosine has been proven to have antioxidant, membrane-stabilizing, and wound-healing effects. In addition to scavenging several free radicals, carnosine has also been shown to protect the DNA from ferric ascorbate-induced damage and the damage caused by γ-irradiation (Xie et al. 2013). Histidine-containing dipeptides are present in foods rich in proteins (Halliwell & Gutteridge 2015).

Vitamin C (ascorbic acid) scavenges $^{\bullet}O_2$, $^{\bullet}OH$, O_3, and other ROS, protects plasma lipids, membranes, and lipoproteins against peroxidation, and has other beneficial functions such as decreasing blood pressure in some patients. Plants and most animals can synthesize vitamin C in their body from glucose, but humans and some animals (primates, guinea pigs, fruit bats) lost this function during evolution, so they need to replenish vitamin C through their diet (Halliwell & Gutteridge 2015). In nature, there are many abundantly available sources of vitamin C such as citrus fruits, broccoli, kiwi, papaya, or strawberries. Unfortunately, vitamins are thermolabile and the content of vitamin C strikingly decreases in the heating and drying process. However, the beneficial effects of vitamins ingested from raw foods can be enhanced by the addition of antioxidants from tea such as epigallocatechin gallate (Lee et al. 1999; Intra & Kuo 2007; Devaki & Raveendran 2017).

Vitamin E is a fat-soluble vitamin essential for humans and animals. Term vitamin E is used for a group of tocopherols and tocotrienols. Among them, α-tocopherol has the highest biological activity and is the most abundant form of vitamin E in nature. Vitamin E is said to be one of the most important inhibitors of lipid peroxidation. In addition, vitamin E has other non-antioxidant roles in the metabolism such as gene expression and improved vascular function. Good dietary sources are cereal grains, green leafy vegetables, and vegetable oil, and are also contained in various teas. Furthermore, drying, and boiling processes can even increase the content of vitamin E in high-quality tea (Lee et al. 1999; Traber & Manor 2012; Halliwell & Gutteridge 2015).

2.2.3. Antioxidant extraction methods

Antioxidants are widely distributed in various species of different families across the plant kingdom, however, their concentrations differ significantly. Nowadays many products including dietary supplements, pharmaceutical products, cosmetic products, and food ingredients can be found on the market. The quality and amount of plant-derived substances related to antioxidant potential are strongly associated with the extraction methods (Sulaiman 2013). The goal of the antioxidant extraction methods is to obtain the maximum extraction yield of the compounds of interest, those that have better antioxidant activity, and, therefore, are capable of being more beneficial to human health. In the last few years, several studies focused on the composition and antioxidant properties of various plant species have been published, along with research on optimal extraction methods (Hidalgo & Almajano 2017).

Traditional extraction methods (liquid-liquid extraction, Soxhlet extraction) may often be time-consuming with low efficiency of extraction and furthermore, require large volumes of environmentally non-friendly organic solvents (Teo et al. 2010; Haghighi & Khajenoori 2013). In recent years, new, non-conventional, and environmentally friendly methods such as microwave, pressure-assisted extractions, and ultrasound have emerged (Hidalgo & Almajano 2017). The novel extraction techniques have the possibility of working in conditions with elevated pressures and/or temperatures. Novel extraction methods give the advantage of shorter extraction time and solvent consumption, and better extraction yields and reproducibility (Wang & Weller 2006).

Although there is wide research on antioxidant extraction methods, high variability in results is still a major obstacle to reaching a global consensus. Factors influencing parameters on extraction yields and the antioxidant activity of extracts (extraction time, pressure, solvent, temperature) are very variable, as well as factors previous to extraction (cultivar, harvesting time, drying, storage). However, several researchers have highlighted the effectiveness of non-conventional extraction methods such as subcritical hot water extraction, accomplishing similar or even better results using these novel techniques (Teo et al. 2010; Hidalgo & Almajano 2017).

2.2.3.1. Subcritical water extraction

SWE is a type of pressure-assisted extraction, which has become a popular green method of extraction for different types of compounds in recent years. This method can be used for the extraction of substances present in botanical, food, and environmental samples, as well as in sample preparation in food safety analysis or environmental monitoring of soils and sediments. Sample matrices extracted by SWE include plants (58.4 %), food by-products (27.6 %), marine algae (9.2 %), and fungi (4.8 %). The range of natural products extracted is wide - alkaloids, essential oils, flavonoids, polyphenols, quinones, terpenes, and others. (Teo et al. 2010; Cheng et al. 2021). SWE is a very compelling method for extraction of heat-sensitive materials and therefore also antioxidants. Several researches show good results in the extraction of plant phenols, namely anthraquinones, flavonoids, lignins, and tannins (Liang & Fan 2013). If focused on the antioxidant activities of SWE extracts, there are significant correlations between the values obtained using different methods. Several studies also confirm the use of water as a solvent to be beneficial, as SWE extracts show better results than extracts using ethanol and methanol (Ko et al. 2020).

By the term “pressurized hot water” (also “near critical water”, “subcritical water”, and “superheated water”) we understand the area of the condensed phase of water between the boiling point and critical point of water (100–374 °C) (Teo et al. 2010; Haghighi & Khajenoori 2013). Water behaves differently from other solvents and has very unusual properties due to 2 strong hydrogen bonds in each molecule. In the temperature range of subcritical water, hydrogen bonds break and change their properties much more by increasing both temperature and pressure (Gbashi et al. 2016). Figure 1 shows the phase diagram of water with various states of water under different temperature and pressure conditions and highlights the conditions under which the water is in a subcritical state. (King & Gabriel 2002; Gbashi et al. 2016).

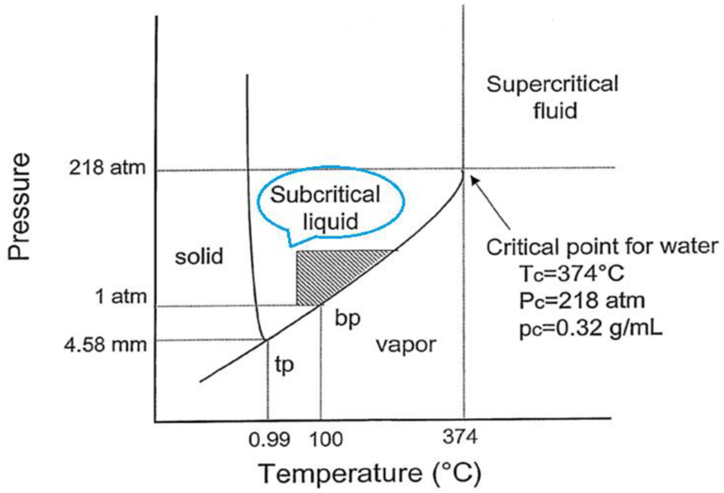

Figure 1: Phase diagram of water (King & Gabriel 2002)

The unique characteristic of subcritical water is its ability to dramatically decrease polarity with increasing temperature due to lowered dielectric constant of water. Subcritical water is then less polar, therefore can behave similarly to ethanol or methanol. Another positive aspect is that the pressure does not affect SWE efficiency if it is high enough to keep the water in a liquid state (Cheng et al. 2021).

The major advantages of SWE are the usage of water as a cheap, green, and readily available solvent; the ability to extract polar, moderately polar, low-polar and non-polar compounds separately; less expensive instrumentation; high efficiency and short time of extraction; and possibility of continuous operation. The major disadvantages are the need for moisture removal that may require additional procedures such as chemical dehydration, evaporation, or precipitation; at higher temperatures, thermal degradation may occur; and the equipment is not easy to clean. Despite some disadvantages, SWE is a promising extraction method in the food and pharmaceutical industries, and if we focus on environmental protection, it is a very advantageous method. Furthermore, SWE can very efficiently improve heat and mass transfer, thus increasing yields, and shortening the time of extraction. SWE also has a beneficial effect on the activity and structure of bioactive compounds. Based on the potential of SWE, future research should focus on the design of industrial equipment and large-scale operation. It is believed that further research would contribute to better understanding, advancement, and future use of plant extracts in the prevention and treatment of health problems (Zhang et al. 2020).

2.2.4. Methods of antioxidant activity evaluation

Antioxidant activity can be evaluated using a variety of assays with different mechanisms, including single electron transfer (ET), hydrogen atom transfer (HAT), metal chelation, reducing power, and others. Understanding of principles, advantages, and limitations of measurement assays is crucial for the proper selection of the methods for valid antioxidant activity evaluation of the samples. Methods and tools for antioxidant activity evaluation have advanced remarkably during the last few years. They vary in types of oxidation substrates, oxidants, reaction mechanisms and conditions, and results expression. Major groups of chemical assays for antioxidant activity evaluation are ROS scavenging methods, non-radical redox potential-based methods, and metal chelation capacity (Shahidi & Zhong 2015).

ROS scavenging methods are assays used for the measurement of ET or HAT from potential antioxidants to free radicals. Antioxidant activity is then expressed in their scavenging capacity against specific types of ROS using equivalents of reference antioxidants (ascorbic acid, Trolox), or as inhibition against ROS-mediated oxidation of the probe. The probe oxidation is measurable by several instrumentations including chemiluminescent, fluorometric, spectrophotometric, and others (Shahidi & Zhong 2015). Widely used ROS scavenging methods are:

1) 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, which is a very frequently used method based on a principle where the ability of a sample to neutralize DPPH radical by means of ET is measured.

2) Chemiluminescence assay that measures the H_2O_2 quenching capacity of the sample.
3) Oxygen radical absorbance capacity (ORAC), that measures the radical-chain breaking ability of the sample by monitoring the inhibition of $ROO^{\bullet}$ induced oxidation.
4) Trolox equivalent antioxidant capacity (TEAC) assay, which measures the ability of antioxidants to scavenge the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) radical.

(Floegel et al. 2011; Shahidi & Zhong 2015).

Non-radical redox potential-based methods are assays measuring electron-donating capability of the antioxidants that can not only scavenge free radicals but also reduce higher valent elements to their lower valence state. The reducing power of the antioxidants is a very important indicator of their efficacy and is measured through redox reaction with metal ions (cerium, chromium, copper, iron, and others). Reducing power can be measured by the method of Cyclic voltammetry and Metal chelation capacity, but the most used is the Ferric reducing antioxidant power (FRAP) assay measures the reduction of ferric ion complex into the ferrous complex. (Shahidi & Zhong 2015).

More detailed information on the antioxidant potential of the sample can be obtained from the evaluation of Total phenolic content (TPC), since phenolic compounds are largely responsible for the antioxidant activity. They have been proven as successful chemoprotective and therapeutic agents against several chronic diseases and polyphenols play a big role in the French paradox (low risk of coronary heart disease despite diets rich in saturated fat and cholesterol) due to their high share in the red wine that is consumed by the French population (Sánchez-Rangel et al. 2013; Halliwell & Gutteridge 2015). TPC is carried out by the Folin-Ciocalteu (F-C) assay. This assay is a colorimetric method based on ET between the F-C reagent and phenolic compounds under alkaline conditions (Shahidi & Zhong 2015).

2.3. Teas and tea-like plants

Teas and tea-like beverages prepared from various plant species have been popular for thousands of years and have become part of the daily life of people all over the world. The term tea is usually linked only to hot water infusion of *Camellia sinensis* (L.) Kuntze, which is considered one of the most important functional foods. In 1980, the term functional food was used for the first time in Japan (Hayat et al. 2015). Functional foods can be derived from both animal and plant sources and according to Hayat et al. (2015), this term refers to "any substance containing ingredients which in addition to fulfil basic nutrition requirements provides physiological benefits" (Hayat et al. 2015; Poswal et al. 2019). Preferences for tea consumption vary considerably all over the world and infusions of *Camellia sinensis* can be even consumed in the form of cold beverages or refreshment drinks (Venditti et al. 2010).

Teas are excellent sources of antioxidants and other micronutrients. Besides water, tea is the cheapest and the most popular beverage in the world. Drinking tea has been considered a health-promoting since ancient times and the leaves of *Camellia sinensis* have been used for medicinal purposes since time immemorial. Many research publications proven the health benefits of herbal teas in the prevention of cancer risk, diabetes, heart diseases, risks associated with the consumption of environmental contaminants, weight loss, and others (Khan & Mukhtar 2013; Poswal et al. 2019).

It is believed that tea leaves were first brewed and consumed as a beverage in China, however, historians are debating whether it is a myth. In 780, Lu Yu wrote literary work called The Classic of Tea that included sections on the pleasure of tea drinking, the preparation of tea, tea varieties, tea utensils, and tea history. In the 15th century, tea spread from China to Japan and the Japanese created the Tea Ceremony, which elevated tea drinking from a social pastime to an aesthetic cult. Later, tea was introduced from Asia to Europe, Africa, and America. Each country has developed its own customs of tea blending, brewing, and serving. In England, for example, several traditions have developed around the concept of afternoon tea, known as a late afternoon meal after lunch and before dinner (Jolife 2003). In the Cape region, South Africa, the tradition of drinking tea, introduced by early European settlers in the 17th century, has stimulated the use of numerous indigenous species as a tea substitute. Some of those species remain poorly known (e.g *Leysera gnaphalodes*), however, honeybush (*Cyclopia intermedia*) and rooibos (*Aspalathus linearis*) are today well-known species worldwide (Van Wyk & Gorelik 2017).

2.3.1. Hot water infusions

Hot water infusions are being prepared from wide a range of plant species and traditionally consumed all over the world (Hayat et al. 2015). Those infusions made from species other than *Camellia sinensis* or their mixture can be also called herbal teas or teas (Venditti et al. 2010; Hayat et al. 2015; Poswal et al. 2019).

There are large differences in tea preparation and consumption. Variations include temperature, quality, and volume of used water, different tea vessels, time of infusion, whether the tea bag is stirred or dunked, removed or left, and also if there are some additional flavourings such as sugar or milk. Studies focusing on polyphenols and caffeine content also suggested that whether the tea was in a bag or loose-leaf form was an important factor (Keenan et al. 2011). Although many consumers prefer to use loose-leaf tea, the more popular way of making tea is by using teabags, especially in Western countries. The differences between the two are said to be negligible, however, some studies report, for example, differences in caffeine content. This can be affected by many factors including tea particle size, teabag paper type, teabag dimensions, and agitation (Astill et al. 2001). Another important aspect is the amount of tea used per serving which usually varies from 1.5 to 3 g (Richelle et al. 2001; Brzoňová 2016). However, one of the most important factors is the brew time. According to consumer studies, they vary from 30 s to more than 5 min and the average brew time is fewer than 2 min. Packet instructions and recommendations vary usually from 1 to 5 min, depending on the tea type (Keenan et al. 2011). Tea temperatures and brew time influence the total content of biologically active substances (catechins, phenolic acids, and others). The study of Komes et al. (2010) focused on green tea showed that for powdered tea, the best results were obtained by only brewing for 3 min, whereas in bagged tea it was 15 min and in loose-leaf tea even 30 min. Considering the extraction dynamics of different tea forms, the advantage of powdered tea is undoubtedly a larger specific area. The same study also suggested that the best combination for the extraction of bioactive compounds of green tea are higher water temperature and short extraction time, as well as lower water temperature and longer extraction time. Opposite conditions (high temperature & time or low temperature & time) had a negative impact on the total catechin and total phenolic acids content (Komes et al. 2010). The temperature of water used for tea making is, together with brew time, the most crucial parameter affecting polyphenol content and antioxidant capacity.

Recommended water temperature varies in different teas. Green teas, unlike black teas, are traditionally poured with water at temperatures lower than the boiling point. The reason is the bitter and astringent taste caused by catechins, which is undesirable for consumers and arises if the tea is poured with too hot water (Astill et al. 2001; Lantano et al. 2015). From a nutritional point of view, the reason is that if the water is above 90 °C, polyphenolic compounds are destroyed, so the recommended temperatures for green tea are between 70 and 80 °C (Kowalska et al. 2021; Yu et al. 2021). The optimal temperature for white tea is 80 to 90 °C and for black tea 95 to 100 °C (Brzoňová 2016).

2.3.2. *Aspalathus linearis* (Burm.f.) R.Dahlgren

Aspalathus linearis (Figure 2) from the Fabaceae family, the source of rooibos tea, is an endemic South African fynbos species (Joubert & de Beer 2011). It is erect to spreading shrub or shrublet up to 2 m high with reddish branches, with green, needle-like leaves. Leaves are 1.5-6 cm long and 0.1 cm thick, have no stipules and may be densely clustered. The flowers are yellow, solitary or arranged in dense groups at the tips of branches, and bloom in spring to early summer. The fruit is a small lanceolate pod usually containing 1 or 2 seeds (Govender 2007).

Figure 2: ***Aspalathus linearis*** **(Burm.f.) R.Dahlgren** (Erasmus 2018)

The natural habitat of *A. linearis* is the winter rainfall area in Cape Floristic Region, South Africa. This area is characterized by cold wet winters and hot dry summers with annual rainfall of 300-350 mm. The species is not reported to be endangered but several factors are causing a decrease in areas with natural populations. High demand for rooibos tea, both nationally and internationally, started an increase in the conversion of land into plantations (Govender 2007; Joubert & de Beer 2011).

The rooibos tea popularity spread in the 20th century when Benjamin Ginsberg, a Clanwilliam merchant, obtained the tea from the Khoi and started marketing it in 1904. In 1930 the agricultural value of rooibos was recognised and growing participation of together with increased demand expanded the area under cultivation to 36,000 ha, mostly in the area of Clanwilliam. The utilisation of rooibos also moved beyond the production of value-added products such as beverages, cosmetics, and nutraceuticals (Joubert & de Beer 2011).

Herbal tea made from *A. linearis* is caffeine-free, contains a low amount of tannins, and is considered health-promoting due to significant antioxidant activity (Joubert & de Beer 2011). Rooibos provides promising health benefits against cardiac pathologies, diabetes, obesity, skin disorders, and others (Maarman & Lecour 2022). Furthermore, rooibos is a globally available product with promising dietary support during the COVID-19 pandemic. Rooibos contains a unique mixture of antioxidants including aspalathin that can be found only in rooibos, but also isoorientin, isovitexin, nothofagin, orientin, quercetin, rutin, and vitexin. These can significantly contribute to mitigating some negative symptoms and reducing lung damage caused by the hormone angiotensin II in COVID-19 patients (Sheik & Marnewick 2021).

2.3.3. *Camellia sinensis* (L.) Kuntze

Camellia sinensis (Figure 3) or *Thea sinensis* is a species from Camelliaceae (Theaceae) family. This species is known worldwide as a tea plant. It is an evergreen, branched shrub up to 3 meters high (var. *sinensis*) or a 10 to 15 m tall tree (var. *assamica*). When cultivated, the tea plant is pruned to 1 to 1.5 m to support branching and spreading shrubs. The tea plant has a strong taproot and many lateral roots which create a dense mat in the top 50 to 75 cm of the soil. Leaves are alternate with short petiole and lanceolate to obovate blade. In var. *sinensis* the leaves are usually leathery and narrow, dark green, not longer than 10 cm, and with indistinct marginal veins. In var. *assamica* the leaves are thinner, wider, and longer (15 to 20 cm). The colour is lighter and marginal veins are more distinct. Flowers are axillary, arranged singly or in clusters. Fruit is a subglobose capsule (Schoorel & van der Vossen 2000).

Figure 3: *Camellia sinensis* (L.) Kuntze (Kew Royal Botanical Gardens 2022)

It is presumed that the centre of origin is the area of the Irrawadi river in northern Burma and the natural habitat is the lower montane forest from south-western China to north-eastern India (Schoorel & van der Vossen 2000).

The highest quality tea is obtained by only harvesting the twigs with bud and 2 or 3 leaves. These are rich in caffeine, var. *sinensis* contains 3.5 % caffeine and var. *assamica* even 4.5 %. On average, tea is harvested 4 times a year (flushes), but the number of harvests depends on the habitat conditions. Harvest is done manually, using different types of scissors, or by combined harvester that can replace the work of 30-50 tea pickers (Hušák & Valíček 2002). Based on harvesting and processing methods, there are 4 major tea types of *C. sinensis* – black, green, oolong, and white (Keenan et al. 2011). Their chemical composition is mainly influenced by drying and fermentation conditions. Green tea is a non-fermented product, as well as white tea, made of young leaves that are withered, steamed, or pan-fried, dried, and graded. Fermentation of the leaves by the natural enzyme activities is prevented by pan frying. Production of black tea is done by first oxidizing the leaves in air, which turns the leaves into dark brown colour, and the flavour is intensified. Then, leaves can be left as such or heated, dried, and graded. Black tea accounts for 85 % of world tea production. Oolong tea is a partially fermented product, sometimes called the intermediate between green and black tea. The fourth tea type made of *C. sinensis* is a white tea that is prepared from downy buds. These buds have a typical silvery colour and are consumed either separately in the form of white tea or they are represented 3–4 % in tea blends of black or green tea (Hušák & Valíček 2002; Khan & Mukhtar 2013).

The terms tea, tay, or cha are used for beverages obtained by infusing the leaves of *C. sinensis* in hot water. It has highly refreshing and stimulating properties due to the high content of a specific polyphenolic substance – alkaloid caffeine (Schoorel & van der Vossen 2000). It is the second most widely consumed beverage in the world and its consumption is associated with health benefits due to its high antioxidant content lowering the risk of various diseases such as cancer, coronary heart disease, and stroke (Ho et al. 2009). Beneficial polyphenolic components present in tea are especially catechins. These are found in green tea, whose catechin profile closely resembles that originally present in the leaves at harvest due to gentle processing. Black tea, unlike green one, is a product that has undergone a fermentation process in which oxidative enzymes naturally occurring in tea leaves oxidize catechin monomers to generate a complex mixture of polyphenol-derived products such as theaflavins, and thearubigins (Shahidi & Ambigaipalan 2015). These substances are both commercially and scientifically important due to their high biological activity such as anti-cancer, anti-diabetes, anti-inflammatory, and anti-obesity (Ho et al. 2009). The antioxidant activity of different teas has been shown in the following order: green > oolong > black (Shahidi & Ambigaipalan 2015).

2.3.4. *Cyclopia* spp.

Cyclopia genus (Fabaceae family) is a group of 23 legume species endemic to the fynbos vegetation in South Africa. The raw material is used to produce Cape herbal beverage called honeybush tea, whose popularity as a healthy beverage is continuously growing. The species of the *Cyclopia* genus are both chemically and morphologically similar and most of them have been used to produce honeybush tea in rural areas. They are sprouting or non-sprouting woody shrubs with showy yellow flowers, yellowish twigs, trifoliate leaves, and brown pods. A major phenolic constituent present in *Cyclopia* spp. is mangiferin, a xanthonoid with numerous pharmacological uses including gastroprotective effects (Stepanova et al. 2012).

In South Africa, 3 major species of *Cyclopia* are used to produce tea – *Cyclopia genistoides* (L.) R.Br. (honeybush tea), *Cyclopia intermedia* E. Mey. (berg tea, Kouga berg tea), and *Cyclopia subternata* Vogel (vlei tea). Small quantities of honeybush tea have been also produced from *Cyclopia maculata* (Andrews) Kies, *Cyclopia meyeriana* Walp., and *Cyclopia sessiliflora* Eckl. & Zeyh. Traditionally, the tea is made during the flowering season in spring, as it is believed that the flowers give the tea a honey aroma and taste (Stepanova et al. 2012). The most studied species is *Cyclopia intermedia* (Figure 4) which constitutes the bulk of exports (Windvogel 2020).

Figure 4: *Cyclopia intermedia* E.Mey. (Hurt 2022)

Honeybush tea, similar to rooibos, is caffeine free. The species has been traditionally used for medicinal purposes such as an expectorant, to promote appetite, as a restorative, for the treatment of pulmonary tuberculosis, and as a health drink for digestive and stomach ailments. Furthermore, recent research shows the phytoestrogenic potential of Cyclopia due to its phenolic composition that is coupled with stimulating milk production and alleviating menopausal symptoms (Joubert & de Beer 2011; Louw et al. 2013).

2.3.5. *Hibiscus sabdariffa* L.

Hibiscus sabdariffa (Figure 5), also known as Jamaica sorrel, karkade, roselle, or sour tea is a species from the family Malvaceae, cultivated in tropical Asia and America, but mostly in Africa (Egypt, Sudan) where it originates. Roselle grows naturally in dry, subtropical, mountain climates (Guardiola & Mach 2014). It is an annual to perennial plant with an erect, mostly branched stem that grows up to 4.5 m high. Leaves are heart-shaped or lobed. Flower calyces are pink to red coloured when mature and after flowering they become fleshy with a sour taste. They are produced all year round and contain about 30 % of acids, especially citric acid, hibiscus acid, malic acid, and tartaric acid. Fruit is an ovoid, obtuse capsule containing 30-40 small seeds (Hušák & Valíček 2002).

Figure 5: ***Hibiscus sabdariffa*** **L.** (Useful Tropical Plant Database 2022)

Among acids, other chemical substances of higher interest present in roselle are anthocyanins that cause very rich red pigmentation of calyces' aqueous extracts, flavonoids (gossypetin, hibiscin), phytosterols, pectin, and polysaccharides. The plant is rich in iron, magnesium, and potassium, and some vitamins (vitamins B3, B6, and C) can be also found in appreciable amounts (Shruthi & Ramachandra 2019). Roselle is an important multipurpose plant grown for fiber, medicinal purposes, as an ornamental plant, or as a food crop. In traditional medicine, roselle is used in the treatment of heart ailments, hypertension, hyperlipidemia, leukaemia, and type 2 diabetes. Roselle calyces are reported to be antiseptic, antimicrobial, aphrodisiac, astringent, laxative, and sedative properties. Fresh calyx, the outer whorl of the flower, can be eaten raw in salads, or cooked and used as a flavouring agent in caked, jellies, soups, pickles, puddings, etc. Young leaves and stems are used as a vegetable. Roasted seeds are consumed as a snack and yield about 17 % of edible oil, however, the oil might contain toxic compounds (Hušák & Valíček 2002; Ifie et al. 2016; Shruthi & Ramachandra 2019; Useful Tropical Plant Database 2022).

Functional beverages like tea blends or soft drinks made of roselle are nowadays marketed around the world (Ifie et al. 2016). In developing countries, the production of roselle calyces is of great importance since it is a very important source of income for rural communities. In Sudan, roselle is a major crop of export, and in the western part of the country it even occupies the second largest area of cultivation after pearl millet. According to FAO, roselle from Sudan is ranked as of the best quality, however poor quality of packaging and distribution prevents further expansion into the world market (Shruthi & Ramachandra 2019).

3. Aims of the Thesis

The aim of this thesis was to determine and compare the antioxidant activity of subcritical hot water extracts and water infusions of 5 different teas and tea-like plants. Partial goals were the evaluation of antioxidant activity by using 2,2-diphenyl-1-picrylhydrazyl radical assay (DPPH) and Oxygen radical absorbance capacity assay (ORAC). Since polyphenolic substances are the most important antioxidants in teas and tea-like plants, their content was determined by using Total phenolic content (TPC) assay.

Hypothesis:

Teas and tea-like plants are known worldwide for their promising antioxidant activity and high content of biologically active compounds. Subcritical hot water extraction is a novel extraction method that can very efficiently improve heat and mass transfer, thus increasing yields and efficiency of the extraction. Therefore, extracts obtained by subcritical water extraction were presumed to possess better antioxidant activity and TPC than traditionally prepared hot water infusions due to the possible higher content of phytochemicals acting as antioxidants.

4. Methods

4.1. Plant material

Based on current availability, 5 teas and tea-like plants were purchased from commercial sources on the Czech market from the company Herbs Life Sokolov (*Aspalathus linearis*, *Camellia sinensis* – black and green tea) and Salvia Paradise (*Cyclopia intermedia* and *Hibiscus sabdariffa*). Suppliers list the places of origin of these teas and tea-like plants, namely South Africa for rooibos and honeybush, China for black and green tea, and Nigeria for roselle. All the samples were grounded to mild powder using an electric mill GM 100 (Retsch, Germany) and used for the extraction.

4.2. Extracts preparation

4.2.1. Subcritical water extracts

The fine powder was weighed to 15 grams and placed into the extraction vessel SFE Helix (Applied Separations, USA). Subsequently, the sample was extracted for 20 min under the conditions of a water temperature of 120 °C and a pressure of the extraction vessel of 50 Bar. After the collection of the final extract, the extract was concentrated by rotary evaporator R-200 (Büchi, Switzerland) at vacuum and 50 °C, until the sample was dry with the lowest possible yield. The dry residue was weighed and stored at -20 °C until use. Extract yields of the samples are presented in Table 1.

4.2.2. Water infusions

The fine powder was weighed to 7.5 grams and poured with 1 L of boiling water. Brewing was carried out for 20 min and the infusion was then filtered using a membrane vacuum pump KNF Laboport (KNF Neuberger GmbH, Germany). The final extract was concentrated by rotary evaporator R-200 (Büchi, Switzerland) at vacuum and 50 °C, until the sample was dry with the lowest possible yield. The dry residue was weighed and stored at -20 °C until use. Extract yields of the samples are presented in Table 1.

Table 1: Extraction yields

Species	Family	Common name	Used part	Extracts yields (%)		Source/company
				WI	**SWE**	
Aspalathus linearis (Burm.f.) R.Dahlgren	Fabaceae	rooibos	leaves, shoots	10.76%	12.00%	HerbsLife Sokolov
Camellia sinensis (L.) Kuntze	Camelliaceae	black tea	leaves	30.76%	20.76%	HerbsLife Sokolov
Camellia sinensis (L.) Kuntze	Camelliaceae	green tea	leaves	30.76%	20.76%	HerbsLife Sokolov
Cyclopia intermedia E. Mey.	Fabaceae	honeybush	leaves, shoots	22.67%	5.33%	Salvia Paradise
Hibiscus sabdariffa L.	Malvaceae	roselle	flowers	62.70%	26.70%	Salvia Paradise

Notes: SWE - subcritical water extraction; WI - water infusion.

4.3. Chemicals

2,2’-azobis(2-methylpropionamidine) dihydrochloride (AAPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, and fluorescein sodium salt were purchased from Sigma-Aldrich (Prague, Czech Republic). Folin-Ciocalteu reagent and methanol were purchased from Penta (Prague, Czech Republic), and inorganic salts (K_2HPO_4, KH_2HPO_4, NA_2CO_3) were purchased from Lach-Ner (Neratovice, Czech Republic).

4.4. Antioxidant activity evaluation

4.4.1. DPPH

The method of Sharma and Bhat (2009) was used for the evaluation of the antioxidant activity of the extracts based on their ability to inhibit DPPH radical. Stock solutions of Trolox and samples were prepared at the concentration 512 μg/mL in methanol. Two-fold serial dilution of each sample was performed via the automatized pipetting platform Freedom EVO 100 (Tecan, Mannedorf, Switzerland) in 96-well microtiter plates. Subsequently, 75 μL of methanol and 25 μL of 1 mM DPPH in methanol were pipetted into each well. The final range of concentrations of each sample and Trolox in the microtiter plate was 0.125–256 μg/mL. Incubation of the plates was carried out for 30 min in the dark at room temperature. Afterwards, the absorbance was measured spectrophotometrically at 517 nm using a Multimode Reader Cytation 3 (BioTek Instruments, Winooski, VT, USA). Experiments were performed as three independent tests, each performed in triplicate, and results were expressed as a mean value of IC_{50} (half maximal inhibitory concentration) with standard deviation (±SD) in μg/mL.

4.4.2. ORAC

The ability of samples to protect fluorescein against oxidative degradation by AAPH radical using ORAC assay was determined based on the method of Ou et al. (2001). Initially, stock solutions of 153 mM AAPH radical and 48nM fluorescein were prepared in 75 mM phosphate buffer (pH 7). Two-fold serial dilution of each extract in phosphate buffer was prepared in 96-well black absorbance microtiter plates using automatized pipetting platform Freedom EVO 100 (Tecan, Mannedorf, Switzerland). Thereafter, 150 µL of fluorescein was added into each well and incubated for 10 min at 37 °C. Subsequently, the application of 25 µL of AAPH started the reaction and the plates were placed into the incubator for 90 min at 37 °C. The resulting concentrations ranged from 1 to 256 µg/mL. Repeated measurements showed that the concentration of 256 µg/mL was too high for rooibos, and therefore a concentration in the range of 32 to 0.125 µg/mL was used for this sample. Each microtiter plate was filled with 200 mL of distilled water in all the outer wells to improve thermal mass stability. Fluorescein with AAPH in phosphate buffer (blank 1) and fluorescein in buffer (blank 2) were part of each plate, and Trolox was used as a positive control. Eventually, the results of radical inhibition were measured using Multimode Reader Cytation 3 (BioTek Instruments, Winooski, VT, USA) at a wavelength of 485 nm. Experiments were performed as three independent tests, each performed in triplicate, and results were expressed as a mean value of IC_{50} ±SD in µg/mL.

4.4.3. TPC

The previously described method by Singleton et al. (1999) was used for the estimation of TPC in the tested extracts. The method was performed in the 96-well microtiter plates. Stock solutions of samples were prepared at the concentration of 32 µg/mL. and the amount of 100 µL of each sample was pipetted into the plate in triplicates. Subsequently, 25 µL of Folin-Ciocalteu reagent and 75 µL of 12 % Na_2CO_3 were mixed with the sample to initiate the reaction. The plate was then incubated for 2 hours at 37 °C in the dark. Absorbance was measured using Multimode Reader Cytation 3 (BioTek Instruments, Winooski, VT, USA) at 700 nm. The standard calibration curve was created using 8 concentration levels of gallic acid (0.25, 0.5, 1, 2, 4, 8, 16, 32 µg/mL). Experiments were performed as three independent tests, each performed in triplicate and the results were expressed as a mean value of gallic acid equivalents (mg GAE/g extract).

4.4.4. Statistical analysis

Linear correlation coefficients (r) between antioxidant assays (DPPH, ORAC) and TPC were determined using Pearson product moment correlation to estimate if the phenolic compounds are responsible for the antioxidant activity of tested plants. The correlation degree was estimated based on the principle by Evans (1996), where based on the absolute value of r, the correlation is evaluated as: very weak ($r = 0$-0.19), weak ($r = 0.20$–0.39), moderate ($r = 0.40$–0.59), strong ($r = 0.60$–0.79), very strong (0.80-1).

For each sample, statistical analysis for comparison of extraction methods and their effect on antioxidant activity and TPC was performed using Microsoft Excel (Microsoft 365 MSO, Microsoft Corporation, Redmont, USA), using Student's T-test. The differences were considered statistically significant when $p < 0.05$.

5. Results

Antioxidant activity and TPC were compared for both types of extracts (WI, SWE). Results listed in Table 2 were expressed as half maximal inhibitory concentration IC_{50} (µg/mL) for DPPH and ORAC, and as milligrams of gallic acid equivalent per gram of extract (mg GAE/g) for TPC. The strongest antioxidant activity for DPPH was measured in WI of *Camellia sinensis* – green tea (IC_{50} 17.22 µg/mL), whereas the IC_{50} of SWE extract was 19.80 µg/mL. Strong antioxidant activities were also obtained in *C. sinensis* – black tea, and *Aspalathus linearis* (rooibos). The IC_{50} of *C. sinensis* – black tea was 28.57 µg/mL (WI) and 46.35 µg/mL (SWE), and *A. linearis* possessed IC_{50} of 32.27 µg/mL (WI) and 40.80 µg/mL (SWE). Species *Cyclopia intermedia* (honeybush) possessed weaker antioxidant activity than both tea types of *C. sinensis* and *A. linearis*, again with WI (63.67 µg/mL) having stronger antioxidant activity than SWE (79.17 µg/mL). The weakest antioxidant activity was obtained in *Hibiscus sabdariffa* (roselle) with IC_{50} of 223.17 µg/mL (WI) and 247.63 µg/mL (SWE). Trolox was determined as an average value of 3 independent tests with IC_{50} 10.17 µg/mL. Although results are comparable in most cases, the study showed weaker IC_{50} of SWE extracts than WI extracts in all tests performed.

ORAC assay showed the same trend of WI having stronger IC_{50} than SWE. *C. sinensis* – green tea showed the strongest antioxidant activity of IC_{50} 9.85 µg/mL for WI and 11.19 µg/mL for SWE. Surprisingly, both WI and SWE extracts of *A. linearis* showed lower IC_{50} values (10.88 µg/mL and 12.12 µg/mL, respectively) than *C. sinensis* – black tea (14.14µg/mL and 15.47 µg/mL, respectively). Species *C. intermedia* showed IC_{50} of 13.85 µg/mL for WI and 19.45 µg/mL for SWE. The weakest antioxidant activity for ORAC assay was measured in *H. sabdariffa* with IC_{50} 67.49 µg/mL for WI and 83.44 µg/mL for SWE. Trolox was determined as an average value of 3 independent tests with IC_{50} 13.76 µg/mL.

The highest TPC was identified in *C. sinensis* – green tea, again with WI (475.76 mg GAE/g) having worthier results than SWE (339.88 mg GAE/g). *A. linearis* showed a high TPC of 343.61 mg/g GAE for SWE, which is even higher than in *C. sinensis* – green tea. However, if we compare SWE and WI of *A. linearis*, WI again showed higher TPC with 357.31 mg GAE/g. *C. sinensis* – black tea showed higher TPC than *C. intermedia*, but their results were surprisingly not much different, although for DPPH and ORAC, *C. sinensis* - black tea showed significantly stronger antioxidant activity. The TPC of *C. sinensis* – black tea was 363.70 mg GAE/g (WI) and 270.07 mg GAE/g (SWE), whereas in *C. intermedia* TPC was 342.23 mg GAE/g (WI) and 263.34 mg GAE/g (SWE). Species *H. sabdariffa* showed the lowest TPC with 25.41 mg GAE/g (WI) and 19.62 (SWE).

The Pearson product moment correlation was performed to determine the correlation between antioxidant activity and TPC. It has been found that there are very strong correlations between DPPH and TPC ($r = 0.81$), and ORAC and TPC ($r = 0.97$; both significant at $p < 0.05$).

The Student's T-test indicated that in most of the cases, there are no significant differences ($p > 0.05$) in the antioxidant activity of the WI and SWE extracts of studied samples. Only for *C. intermedia* the results obtained for ORAC were evaluated as significantly different ($p=0.0007$).

Table 2: Antioxidant activity and total phenolic content of SWE and hot water infusions of teas and tea-like plants

Species	**Family**	**Common name**	**DPPH IC$_{50}$(µg/mL)**		**ORAC IC$_{50}$(µg/mL)**		**TPC (mg GAE/g)**	
			WI	SWE	WI	SWE	WI	SWE
Aspalathus linearis	Fabaceae	rooibos	32.27±3.9	40.80±5.4	10.88±1.3	12.20±1.6	357.31±69.4	343.61±52.5
Camellia sinensis	Camelliaceae	black tea	28.57±5.0	46.35±1.5	14.14±1.3	15.47±0.6	363.70±77.2	270.07±33.5
Camellia sinensis	Camelliaceae	green tea	17.22±3.1	19.80±2.2	9.85±0.5	11.19±0.9	475.76±80.0	339.88±40.6
Cyclopia intermedia	Fabaceae	honeybush	63.67±11.7	79.17±6.5	13.85±0.7*	19.45±0.5*	342.23±80.0	263.34±67.7
Hibiscus sabdariffa	Malvaceae	roselle	223.17±0.7	247.63±14.4	67.49±0.6	83.44±5.3	25.41±13.5	19.62±8.9
Trolox			10.17±0.2		13.76±1.7		none	

Notes: SWE - subcritical water extraction; WI - water infusion; * - means are significantly different ($p<0.05$).

6. Discussion

Teas and tea-like plants, especially *Camellia sinensis*, which is one of the most popular beverages in the world, show significant levels of antioxidant activity (Ho et al. 2009), which can be measured by various methods and the credibility and complexity of the results increase if more methods of evaluation are used. In this study, the *in vitro* antioxidant activity of SWE extracts and WI of 5 teas and tea-like plants was determined by DPPH and ORAC assays. In addition, since phenolic substances, especially flavonoids and phenolic acids are considered to be the most effective antioxidants, TPC was determined by the Folin-Ciocalteu assay (Tabart et al. 2009). Plant extracts of these species have been tested for antioxidant activity and TPC many times and the results of such studies correlate with the results of this study in the evaluation of individual species, among which *C. sinensis* – green tea clearly predominates and is followed by *Aspalathus linearis* (rooibos) and *C. sinensis* – black tea (Lee et al. 2002; Erickson 2003). If focused on WI of teas and tea-like plants, several studies agreed with our DPPH and ORAC results such as Lee & Jang (2004) who set IC_{50} of DPPH assay in *A. linearis* on 33.1 µg/mL (our result was 32.27 µg/mL), or study of Malongane et at. (2022) who set IC_{50} of *C. intermedia* on 54.98 µg/mL, while our result was 63.67 µg/mL. ORAC tests, usually stated in different units in scientific papers, confirmed the expected trend that green tea has the highest antioxidant activity among the teas tested and is followed by black tea. Carloni et al. (2013) set ORAC of *C. sinensis* – green tea on 61 mM Trolox Eqivalents (TE) and *C. sinensis* – black tea on 32 mM TE. A study of Pekal et al. (2012) determined TPC of black and green tea to be 278.6–419.6 mg GAE/g dry matter and 448.7– 513.4 mg GAE/g dry matter, respectively. The same study also confirmed a significant correlation between TPC and cupric ion reducing antioxidant capacity, indicating that phenolic compounds are responsible for the antioxidant activity of *C. sinensis*. The findings of this study again correspond with our results, where TPC was determined to be 363.70 mg GAE/g (black tea) and 475.76 mg GAE/g (green tea), and at the same time, there was a significant correlation between TPC and antioxidant activity assays (DPPH, ORAC).

To the best of our knowledge, apart from *H. sabdariffa*, all the species (in the form traditionally used to make hot beverages) prepared by SWE have been analysed for their antioxidant activity for the first time here. Only a few studies comparing SWE extracts to hot water extracts exist, however, none of them are focused on teas or tea-like plants. The study of Hassasroudsari et al. (2009) compared antioxidant activity and TPC of canola meat in SWE extracts, hot water extracts and ethanolic extracts. SWE was carried out under the conditions of 110 and 160 °C for 30 min, while hot water extraction was carried out under the temperature of 80 °C for 30 min, and ethanolic extracts were obtained using a reflux system and 95 % ethanol for 30 min. The highest yields were obtained in SWE at 160 °C (0.45–0.48 g/g meal) followed by hot water extracts (0.19–0.21 g/g meal), SWE at 110 °C (0.20 g/g meal), and ethanolic extracts (0.14–0.15 g/g meal). In our study, this trend was observed only in the extract of *A. linearis*, where the yield of SWE extract was higher (12.00 % of dry weight) than in the case of WI (10.76 % of dry weight). All the other species possessed higher yields in WI extracts, which may again be a consequence of non-optimized extraction parameters. Interestingly, Hassasroudsari et al. (2009) determined higher TPC in ethanolic extracts with 52.2–70.9 mg sinapic acid equivalent (SAE)/g extract, followed by SWE at 110 °C (35.1–41.4 mg SAE/g), SWE at 160 °C (31.8–36.2 mg SAE/g), and hot water extraction (28.0–30.4 mg SAE/g). The authors attribute the loss of phenolics to degradation at high temperatures. Although they showed higher TPC in extracts obtained at lower temperatures, SWE extracts still showed stronger antioxidant potential than hot water extracts, which confirms our predicted hypothesis, but our results deviate from this pattern. Another study by Rizkiyah et al. (2022) focused on the SWE extraction of *H. sabdariffa* showed that high temperatures are favourable for achieving maximum concentrations of phenolic compounds, flavonoids, and anthocyanins. The highest TPC was obtained under the conditions of 120 °C, 8.75 MPa, 4.89 mL/min, and time of extraction 5 min, whereas under the of 100 °C the TPC was lower. However, in our study, the TPC of *H. sabdariffa* SWE (120 °C) was lower than in WI (100 °C). This phenomenon was observed even for all samples tested in this thesis. Our results indicate that the higher temperature affects the amount of the phenolic compounds that are generally considered thermolabile. However, a study of Antony & Farid (2022) declared that the level of thermal degradation may vary for specific or sub-groups of phenolic acids. Moreover, high extraction temperatures can support the formation of new compounds known as Maillard reaction products that may also influence the extracted polyphenols and TPC. A key role is played by the selection of the starting material for extraction, i.e., the plant matrix, and the influence of the extraction conditions.

The results of this thesis may also be influenced by the different conditions under which the extracts were prepared. WI extracts were prepared based on the traditional preparation methods of pouring hot water and subsequent steeping. According to consumer studies, teas and other hot infusions are prepared at different temperatures (70 to 100 °C) and steeping times (30 s to 5 min) and may also differ in the amount of tea used per serving, varying from 1.5 to 3 g (Keenan et al. 2011; Brzoňová 2016). For the uniformity of the experiment, all WI extracts were prepared with boiling water regardless of species and steeped for 20 min at room temperature with the aim of ensuring conditions as similar as possible to classical homemade preparation. However, during the 20 min steeping, the temperature was not constant but decreased. On the other hand, SWE extracts were prepared at a constant temperature of 120 °C, pressure 50 bar, and extraction time 20 min, meaning that the possible degradation of thermolabile natural products was higher than in WI extracts.

SWE is a novel method suitable for the extraction of natural products, however, the methodology is not globally uniform, and studies differ significantly in temperature, pressure and/or extraction time variances (Hiep et al. 2020; Essien et al. 2020). In this study, extracts of teas and tea-like beverages obtained by 2 types of extraction methods were compared. The results showed that SWE extracts possess weaker antioxidant activity and TPC than WI extracts. This phenomenon can be the result of several factors such as thermal degradation or non-optimized extraction time. The study by Hiep et al. (2020) focused on the optimization of subcritical water extraction of epigallocatechin gallate from *C. sinensis* showed, that the concentration of catechins was highest under the conditions of 100 °C and extraction time of 5 min. The efficiency of epigallocatechin gallate extraction slightly decreased under temperatures between 100–120 °C and decreased rapidly when the temperature was higher than 140 °C. The same study also confirmed a significant influence of extraction time on the resulting epigallocatechin gallate content, where in the case of optimization of the extraction duration, 3 time periods were used (1, 5 and 9 min), and the best results were obtained with extraction of only 1 min. Losses of catechins content during longer extractions were attributed to dimerization, oxidation, and polymerization reaction. The study of Ko et al. (2014) on optimization of subcritical water extraction of flavanols from green tea showed similar trends of shorter extraction (5 min) having better yields than longer extractions (10 and 15 min). This may have affected the results of SWE extracts, as the extraction was carried out under conditions of 120 °C and a duration of 20 min. Another study by Essien et al. (2020) mentioned that SWE is a method with high selectivity resulting in extract fractions with diverse compositions and biological activity. This may also have affected the composition and thus antioxidant activity and TPC of the SWE extracts, because the extracts used in this study were collected as a whole and not as individual fractions.

7. Conclusions

In this study, antioxidant activity and TPC of extracts of 5 teas and tea-like plants obtained by 2 different extraction methods (WI, SWE) were determined and compared. Extracts obtained by the WI method showed stronger antioxidant activity and higher TPC than SWE extracts in all cases. Extract of *Camellia sinensis* – green tea obtained by WI was evaluated as the most promising one the with lowest IC_{50} of 17.22 µg/mL (DPPH) and 9.85 µg/mL (ORAC). The same species also possessed the highest TPC of 475.76 mg GAE/g, indicating that phenolic compounds are responsible for the antioxidant activity of tested species. Apart from *H. sabdariffa*, antioxidant activity and TPC of SWE extracts of teas and tea-like plants have been determined for the first time in this thesis and the most promising results were obtained in *Camellia sinensis* – green tea, where the IC_{50} was set at 19.80 µg/mL (DPPH) and 11.19 µg/mL (ORAC), and TPC was set at 339.88 mg GAE/g. Even though SWE is considered a novel and promising method for the extraction of plant material, our study did not confirm the hypothesis, that the yield of phenolics and thus antioxidant potential will be higher. It is clear from recent papers that the methodology is not uniform, especially in temperature and extraction time, which strongly affect the extraction efficiency. Thus, it is important to optimize SWE conditions for teas and tea-like plants, e.g., to set the optimum temperature to obtain high-yield extracts, to collect the extract in the form of separated fractions, or to shorten the extraction time. Such an optimized method can be further used to obtain high-nutritious food supplements, food/beverage ingredients, or nutraceuticals and thus help to fight against oxidative stress and resulting non-communicable diseases. In addition to being a promising tool for obtaining nutrient-rich products, SWE is also an environmentally friendly and sustainable method, thus offering a promising future in the extraction of plant materials.

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