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Faculty of Tropical Agrisciences



**Antioxidant activity of various fractions of
subcritical water extracts obtained from tea 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 various fractions of subcritical water extracts obtained from tea 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 24th April 2024

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Abstract

Oxidative stress is caused by an imbalance in accumulation and production of reactive oxygen species (ROS) in living cells and the biological systems ability to destroy the reactive products. Tea (*Camellia sinensis*) belongs to family *Theaceae* and is the most widely used beverage globally following only fresh water. This contains considerable amount of antioxidants, particularly the phenols that are commonly known to have the ability to lower the risk of the development of some serious diseases. Yerba mate (*Ilex paraguariensis*) is traditionally used tea-like beverage, and its leaves contain antioxidants while the extracts and isolated compounds from yerba mate showed pharmacological properties. The bioactive compounds are extracted from tea and tea-like plants to produce value added products by various extraction method in which different solvents are used. Previously, different antioxidant compounds from plant extracts were obtained by environmentally non-friendly, time taking, and inefficient methods. The green extraction techniques use less hazardous chemical, safer solvents, reduce time analysis, and prevent pollution. Subcritical water extraction (SWE) is a greener and faster method for the extraction of antioxidant compounds by using only water (solvent). In our study, antioxidant activity and total phenolic content (TPC) of different SWE fractions (1st 2min, 2nd 2min, 3rd 2min, 4th 2min, and 5th 2min; 1st 5min and 2nd 5min, and 10min) and water infusion (WI 10min) of green tea, black tea, and mate extracts were evaluated and compared. Their antioxidant activity was evaluated by DPPH and ORAC method while TPC was determined by Folin-Ciocalteu assay. Overall, in our results ORAC assay showed strongest antioxidant activity in *I. paraguariensis* – mate, SWE 3rd 2min fraction with the lowest IC₅₀ of 5.55 µg/mL among all cases. In *C. sinensis* – green tea, SWE 2nd 5min fraction showed strongest antioxidant activity with the IC₅₀ 9.73 µg/mL determined by DPPH method and highest TPC 778.53 mg GAE/g among tested species. This study proved that SWE is an effective method for the extraction of important bioactive compounds, but its extraction efficiency is affected by extraction time. Moreover, effectiveness depends on plant species, as the most active fractions differ between *C. sinensis* (SWE 2nd 5min fraction) and *I. paraguariensis* (SWE 3rd 2min fraction). Thus, the extraction parameters should be optimized according to evaluated species.

Key words: *Aquifoliaceae*, antioxidants, fractionation, non-conventional extraction method, tea plant

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List of abbreviations

•O ₂	superoxide radical
•OH	hydroxyl radical
¹ O ₂	singlet oxygen
Chl ³	chloroplast translational initiation factor 3
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EGCG	epigallocatechin-3-gallate
GAE	Gallic acid equivalents
GSH	Glutathione
H ₂ O ₂	hydrogen peroxide
H ₂ S	Hydrogen sulfide
HNO ₂	nitrous acid
IC ₅₀	Half-maximal inhibitory concentration
min	minute
N ₂ O ₄	dinitrogen tetroxide
NAC	N-acetylcysteine
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NO•	Nitric oxide
NO ₂ .	Nitric dioxide
ORAC	Oxygen radical absorbance capacity
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species

RS	Reactive species
RSS	Reactive Sulphur Species
SD	Standard deviation
SWE	Subcritical water extraction
TPC	Total phenolic content
WI	Water infusion

1. Introduction

Tea (*Camellia sinensis* L.) is an ancient crop belonging to the *Theaceae* family. This evergreen plant originates from south-eastern China and is widely distributed in tropical and subtropical countries of the world (Paiva et al. 2021). It is the most popular and oldest non-alcoholic beverage having a unique flavor with some health benefits (Xu et al. 2017). The global average consumption of this healthy drink is about 120 mL per day per person (Gardner et al. 2007).

The daily drinking of tea significantly reduces the body fat, risk of dying from chronic diseases, preventing the cancer, improves the insulin sensitivity and oral health, boost fertility, modify gut bacteria and lower the risk of neurodegenerative diseases due to the consumption of alcohol and tobacco (Liu et al. 2021). It contains substantial amounts of bioactive phenolic compounds such as catechins, theaflavins, flavonols and flavones (Monobe et al. 2015).

Antioxidants are compounds that prevent the formation of free radicals. Natural antioxidants are compounds derivatives of flavonoids, phenols, ascorbic acid, coumarin, hydroxycinnamic, dihydroflavone, tocopherol, and catechin that are commonly found in the fruits, grains, and vegetables (Dalimartha 1999). Degenerative diseases and cancer can be inhibited when antioxidants in the body acts as neutralizing free radicals (Poumorad et al. 2006). Natural products that can be used as antioxidants is green tea leaves and their antioxidant activity is associated with polyphenolic compounds especially flavonoid groups (Forester & Joshua 2011). Tea polyphenols are the effective antioxidants that can prevent and treat the diseases by scavenging free radicals and regulating the activity of different types of oxidases in the body. This is due to the active hydrogen ions that neutralizes the free radicals and other reactive oxygen species by scavenging the free radicals (Zuo et al. 2018).

There has been an increasing trend to extract the bioactive compounds from tea to produce value added products by using a variety of solvents for extraction. Cold brewing of tea has also gained popularity due to increased consumer acceptance. Water is a traditional solvent used to extract polyphenols from green and black teas (Perva-Uzunalic et al. 2006). The extraction efficiency of bioactive compounds is influenced by the polarity of the solvent and applied processing techniques for extraction (Zuo et al. 2002). Processing conditions such as tea to solvent ratio, particle size, agitation rate, and time/temperature have a significant effect

on the extraction of bioactive compounds (Rostami & Gharibzahedi 2017). The extraction of tea polyphenols and caffeine from green tea leaves were done by the microwave-assisted extraction method (Pan et al. 2003). Polyphenols and caffeine can be extracted using conventional and novel extraction methods (Farhoosh et al. 2007). Subcritical water is a newer and a greener method of extraction by keeping liquid water below its critical point and high pressure than the vapor pressure to keep the water in the liquid state . An increase in temperature improves the diffusion of the subcritical water, while the viscosity, dielectric constant, and surface tension decrease. Subcritical water extraction involves high extraction and modification efficiency with no residue, and is highly environmentally friendly technique (Basak & Annapure 2022).

2. Literature review

2.1. Oxidative stress

Reactive species (RS) include reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive sulphur species (RSS) and various free radicals having the potential to cause oxidative stress (Mittler 2002). Oxidative stress is a disruption of the redox equilibrium resulting from increased ROS production in the cell. This stress causes imbalance in antioxidants and oxidants that interrupts the redox signalling causing molecular damages (Sies 2018). Increased oxidative stress is a major causative factor of several life-threatening diseases, including neurodegenerative and cardiovascular diseases (Kasote et al. 2013).

2.1.1. Free radicals

Free radicals and oxygen derivatives are biological redox reactions by-products that are produced by aerobic metabolism in plants (Sharma et al. 2012). They get oxidized after combining with oxygen containing substances and steals surrounding electrons to harm the host entity (Wojtunik-Kulesza et al. 2016).

Free radicals are biochemical responses in the body which promotes cancer, ischemic heart disease, inflammation, diabetes, aging, atherosclerosis, immunosuppression, and neurodegenerative disorders (Li et al. 2015). They cause the degenerative diseases which affect neurons and nerve bundles. They play a role in regulation of various physiological functions, such as host defense, cellular signalling, regulation of gene expression of the human metabolic processes and immune system (Chen et al. 2017; Lushchak 2013). The free radical production become smaller and antioxidant capacity sufficiently maintain the redox homeostasis under normal physiological situations (Breusegem & Dat 2006).

2.1.2. Reactive oxygen species (ROS)

Stress whether biotic or abiotic, causes shift in metabolism resulting in a temporary ROS accumulation. ROS are often potent chemical messengers that trigger cellular stress responses (Choudhury et al. 2017; Farooq et al. 2019). They play an important role in signal transduction, in normal plant growth and can also induce cellular damage to prevent oxidative stress (Cuypers et al. 2016; Hu et al. 2020). They are mainly present in mitochondria, peroxisomes, chloroplasts, plasma membranes, endoplasmic reticulum, and apoplast (Sharma

et al. 2019). The ROS family mainly includes the free radicals such as $\bullet\text{O}_2$, $\bullet\text{OH}$ and non-radicals such as $^1\text{O}_2$ and H_2O_2 (Das & Roychoudhury 2014).

External factors such as diet, radiation, pollutants, and lifestyle can increase ROS production (Tripathi et al. 2022) that alter various cellular components such as oxidation of DNA and RNA, and biomolecules like lipid, pigments, carbohydrates, and protein leading to related signalling pathways (Bhattacharya et al. 2014; Gill & Tuteja 2010). ROS production can cause cancer, damage the liver and intestines, and even affect the human life span (Kim & Sieburth 2018).

2.1.3. Reactive nitrogen species (RNS)

RNS are important signalling molecules that regulates a wide range of phenomenon including solute transport, programmed cell death, abiotic and biotic stress response, growth and development in plants (Foyer & Noctor 2015). These species include radicals like $\text{NO}\bullet$ (nitric oxide) and NO_2 (Nitric dioxide), while non-radicals are HNO_2 (nitrous acid) and N_2O_4 (dinitrogen tetroxide) (Khan et al. 2014). These regulate several physiological processes including production, movement, differentiation, hypertrophy, cytoskeletal dynamics, and metabolism, while their excess availability causes their reaction with biomolecules (lipids, proteins, and nucleic acids) resulting in dysfunctional tissue by alter the functional and structural properties of target molecules. RNS are involved in the development of many cardiovascular diseases like hypertension, heart failure, atherosclerosis, and renal complications of diabetes mellitus (Griendling et al. 2016).

2.1.4. Reactive sulphur species (RSS)

RSS are redox-active sulphur containing molecules that can either oxidize or reduce biomolecules under physiological conditions (Gruhlke & Slusarenko 2012). These species include disulphide-*S*-oxides, sulfonic acids, and thiol radicals (Giles 2001). They have higher nucleophilicity and strong antioxidant activity (Ida et al. 2014). They interact with NO , ROS, and H_2S that can function in collaboration with these molecules in plants (Olson 2019). They are considered potent signalling molecules involved in the regulation of cell function. Under stress conditions, the antioxidant machinery maintains a balance in biosynthesis of reactive species and their transport to scavenge the whole metabolism for the survival of plant (Alvi et al. 2023).

2.2. Antioxidants

Antioxidants are the substances that contain pharmacologically active phytochemicals responsible to delay oxidation processes under some cellular pathological conditions (Ji et al. 2020). Exogenous application of antioxidants diminishes reactive oxygen signals and improve the plant growth under multiple stresses (Das & Roychoudhury 2014). Their exogenous application is a favourable method of disputing the harmful consequence of oxidative stress (Kasote et al. 2013). To avoid the interface between radicals and biological molecules, antioxidants should be close to the radical formation place competing the free radical for the biological substrate (Arora et al. 2002). Antioxidants can be produced in vivo e.g. reduced glutathione, superoxide dismutase etc. and can be taken as dietary antioxidants (Halliwell 2007).

2.2.1. Endogenous and exogenous antioxidants

Endogenous and exogenous antioxidants are a part of dietary supplement. Endogenous antioxidants are produced by the human body to prevent the formation of excess free radicals (Mironczuk-Chodakowska et al. 2018). The exogenous (i.e., dietary) antioxidants are mostly obtained from fruits, vegetables, flowers, mushrooms, cereals, drinks, and medicinal herbs (Deng et al. 2013; Li et al. 2016). Exogenous antioxidants contain a wide range of natural and synthetic substances such as vitamin C (in many fruits and vegetables), carotenoids (that the body converts them to the vitamin A for the maintenance of the epithelial retinal pigment in the eye), polyphenols (prevents lipid oxidation), and anthocyanins pigments present in red, blue, and purple fruits and vegetables (Rammohan et al. 2023). The consumption of exogenous antioxidants can increase the body protection and endogenous antioxidants aid in combating diseases (Carlsen et al. 2010).

2.2.2. Primary x secondary x tertiary antioxidants

Antioxidants can be classified into three groups on the basis of their mechanism:

Primary antioxidants function to terminate free radical by following these mechanisms. Firstly, they accept the free radicals to delay the initiation step. Secondly, they interact with peroxy radicals and convert them into stable compound to disrupt the propagation step. The addition of these antioxidants at the initial step of the auto-oxidation process is very useful. Primary antioxidants includes butylated hydroxy anisole, tertiary butylhydroquinone,

propyl gallate, butylated hydroxytoluene, tocopherols, flavonoids and carotenoids (Garg et al. 2022).

Secondary antioxidants are important preventive antioxidants that retard chain initiation and prevent auto-oxidation through hindering ROS production responsible for oxidation initiation, transition metals chelation, UV filtration, singlet oxygen deactivation, and inhibiting antioxidant enzyme cofactor. Moreover, they also act as oxygen scavengers and reducing agents which can decompose hydroperoxides into non-radical species. Secondary antioxidants are ascorbyl palmitate, citric acid, tartaric acid, ascorbic acid and lecithin (Garg et al. 2022).

Tertiary antioxidants are responsible for the repairing of oxidized molecules, and their function takes place (some enzymes of DNA, proteolytic enzymes, etc.) through dietary antioxidants (Liu 2021).

2.2.3. Antioxidant potential in humans

Antioxidant system in human body is responsible for the free radicals scavenging. However, excessive ROS and RNS can inhibit this due to the contact with cigarette smoking, radiation, alcohol, and environmental toxins (Li et al. 2015; Wang et al. 2016) that disturbs the oxidation and anti-oxidation balance resulting in diseases (Zhou et al. 2016). In biological system, DNA can be damaged by superoxide, hydroxyl, and nitric oxide radicals which cause the oxidation of lipid and proteins (Peng et al. 2014). Exogenous antioxidants intake increment would inhibit the initiation or propagation of oxidative chain reaction by acting as free radical scavengers, quenchers of singlet oxygen and reducing agents to lessen the damage caused under oxidative stress (Baiano & Nobile 2015).

In the human body, there is an efficient antioxidant defense system to maintain a balance between oxidative stress and free radical formation following the enzymatic and non-enzymatic antioxidant defenses (Carocho & Ferreira 2013). Antioxidants are involved in the prevention and cure of non-infectious diseases including cardiovascular, neurodegenerative diseases, inflammatory cancer and metabolic syndrome (Cassidy et al. 2020). The plant-derived antioxidants scavenge free radicals to reduce the damage of nerve cells and protect the nervous system under oxidative stress. Antioxidants i.e. polyphenols, vitamins, alkaloids, polysaccharides, and active peptides maintain the structure and function of neurons to extend their healthy condition (Del Rio 2015).

2.2.4. Plant-based antioxidants

Antioxidant potential of the plant extract depends on the phenolic compounds and flavonoids to reduce the oxidative stress (Jain et al. 2011). The activity and production of plant antioxidants are increased to prevent oxidative damage due to increased ROS accumulation in stress conditions. Major plant antioxidants are secondary metabolites of the shikimic acid pathway and phenyl-propanoid metabolism that includes phenolics, coumarins, tannins, chalcone and flavonoids (Sharma & Kumar 2011). It is said that two-thirds of the world's medicinal plant species have abundant antioxidant potential (Krishnaiah et al. 2011).

To prevent the harmful effects of free radicals, plants have effective complex enzymatic and non-enzymatic antioxidant defense system. Enzymatic systems include superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase whereas non-enzymatic systems include low molecular weight antioxidants (ascorbic acid, glutathione, proline, carotenoids, phenolic acids, flavonoids, etc.) and high molecular weight secondary metabolites such as tannins (Chanda et al. 2009). Ascorbate and glutathione are the major water-soluble antioxidant metabolites while polyphenols, flavonoids and terpenoids are secondary metabolites that detoxifies the ROS under various environmental stresses (Hashim et al. 2020; Nadarajah 2020).

Plants synthesize low molecular weight antioxidants in the chloroplast stroma and cytosol using NADPH as the electron donor (Alscher et al. 1997). These low molecular weight antioxidants interrelate with different cellular components to function as redox buffers, and effects the plant growth by controlling processes from cell division to senescence and death (Foyer 2005). The antioxidants and secondary metabolites production is affected by environmental factors that ultimately influence the plant's medicinal and nutritional importance (Lin et al. 2016; Scarano et al. 2018).

Allantoin is a nitrogen-rich compound produced in peroxisomes during purine breakdown that directly reacts with H_2O_2 (Lamberto et al. 2010). It is also called 5-ureidohydantoin or glyoxyldiureide that is a pharmacologically active (Fu et al. 2006; Tolun et al. 2010). It is significant in nitrogen metabolism for plant growth and development (Kim et al. 2009). It appears to have protective effects in plants through activation of antioxidant enzymes (Nourimand & Todd 2016). ROS play role in the formation of allantoin from uric acid during increased oxidative stress and is the major product of free radical-induced oxidation of uric acid as well as a biomarker of oxidative stress (Zitnanova et al. 2004).

Alkaloids are considered the strong antioxidants that have a complex structure; with more nitrogen atoms present in the heterocyclic structure, making easy to react with free radicals and reactive oxygen. Many naturally occurring plant derived alkaloids are used in the development of useful pharmaceutical products (Khadem & Marles 2012). The astragalus alkaloid and its derivatives are commonly present in food such as coffee, tea, and potatoes (Monteiro et al. 2016).

Amino acids are known to be antioxidant in oils and fats and are also naturally present in proteins (et al. 2003). They have important roles in mineral nutrition, signalling, and plants redox homeostasis modulation (Hildebrandt et al. 2015). In exogenous application, certain amino acids resist many stresses, such as water stress, heavy metals, and salinity in various plant species (De Queiroz et al. 2023). Sulphur containing amino acids can remove ROS and can reduce cell damage caused by oxidative stress. The antioxidant abilities of amino acids are useful in the food industry to prolong shelf-life of food and food products (Moskovitz 2005). Cysteine only has a thiol group among sulphur containing amino acids and is involved in oxidation-reduction reaction. It has antioxidant abilities such as metal chelating and free radical scavenging ability to quench singlet oxygen (Choe & Min 2009). The metal chelation activity of sulphur-containing amino acids could contribute to the excretion of toxic metal from the body by catalysing the hydroperoxides breakdown into free radicals (Decker et al. 2001; Flora et al. 2008).

Carotenoids are lipophilic antioxidants that are capable to detoxify various ROS and capture the lipid peroxy radical to protect the membrane. They react with lipid peroxy radical to form lipid hydroperoxide and carotenoid radical (Yachandra et al. 1996). They may capture ^3Chl , and $^1\text{O}_2$ to protect the photosynthetic machinery (Sharma et al. 2012). Their chemical structure is a polymer of 8 isoprene and an oxidized derivative, a precursor of vitamin A that are widely present in plant pigments. Its chemical structure contains several conjugated double bonds that maintains high chemical stability, inhibits lipid peroxidation, and scavenges free radicals (Kesse-Guyot et al. 2014). They are mostly found in fruits and vegetables, and act as antioxidants (Jomova & Valko 2013). They play role in photosynthesis and photoprotection and provide precursors for the formation of certain phytohormones and acts as signalling molecules in development of plant in responses to environmental stress (Nisar et al. 2015; Sun et al. 2018).

Glutathione (GSH) is a redox active molecule that is present in a reduced form and take part in biosynthetic pathways, antioxidant biochemistry, detoxification and redox homeostasis (Noctor et al. 2012). Reduced GSH is occurs naturally and have low molecular weight thiol tripeptide formed by glutamate, cysteine, and glycine (Gill et al. 2013). It has many functions, such as redox regulation, protection, growth and development, gene expression and protein activity, metabolism, the cell cycle and proliferation (Hasanuzzaman et al. 2017; Mhamdi et al. 2014). This also plays important role in regulation of sulphur transport, protein and nucleic acid synthesis, phytochelatin synthesis, xenobiotic purification, and genes expression responsible for stress (Bartoli et al. 2017). It is also used as coenzymes for some antioxidant enzymes and exogenous antioxidant application causes their accumulation (Hasanuzzaman et al. 2019).

N-acetylcysteine (NAC) is a precursor of GSH having antioxidant properties (Elbini Dhouib et al. 2016). It reduces the radicals such as nitrous dioxide and hypohalous acids due to its thiol group. NAC can also split intra-molecular and inter-molecular disulphide bonds in thiolate proteins to show its antioxidant effects by freeing thiols (Aldini et al. 2018). NAC increases the levels of GSH in cells and acts as a scavenger of oxidant species (Samuni et al. 2013). Their actions consist of restoring the antioxidant potential in cells by replacing the depletion of GSH by free radicals. NAC, as an anti-inflammatory compound, can control the release of cytokines in the immune proliferation early state (Omara et al. 1997).

Proline shows various activities in plants, such as an osmo-protectant for osmotic adjustment under salt, drought, and temperature stresses. It decreases the ROS production in thylakoids via quenching singlet oxygen and superoxide radicals. Proline cycle acts as a shuttle to transport redox couples from mitochondria to cytoplasm and back (Kavi Kishor et al. 2022). Proline, as an osmolyte is known as a strong antioxidant that is widely used as a non-enzymatic antioxidant to respond the harmful effects of different ROS species. It is synthesized using glutamic acid as a substrate, via a pyrroline 5-carboxylate intermediate. It is an active scavenger of OH^{\bullet} and $^1\text{O}_2$ that can hinder the harms (Verbruggen & Hermans 2008).

Thiamine is also known as vitamin B1 that is a co-enzyme with several nitrogen and sulphur-containing rings (Colinas & Fitzpatrick 2015). It acts as a chemical reluctant with ability of transporting 2 electrons and protons (Nga & Quang 2019). It might act as a potent antioxidant by scavenging free radicals to apoptotic inhibition (Vidhya et al. 2013). It can directly respond to ROS in the form of hydroxyl radicals and superoxide (Jung & Kim 2003). It exhibits suppressive effects on hydroperoxide generation in the auto-oxidation of linoleic acid (Okai et al. 2007). It plays a vital role in carbohydrate metabolism by participating in the pentose phosphate pathway and tricarboxylic acid cycle. It also increases antioxidant formation and NADPH levels (Hamano 1999). Thiamine diphosphate is a phosphate ester form of thiamine that exhibit coenzyme activity for oxidative decarboxylation of alpha-keto acids including pyruvate, alpha-ketoglutarate and branched chain amino acids (Cathcart & Thurnham 1998).

Vitamin C is also known as ascorbic acid, is one of the most well-known antioxidants, which has four hydroxyl functional groups, two of which are enol hydroxyl groups. It can be easily oxidized and dehydrogenated making it highly reductive and active antioxidant (Berger et al. 2003). It can easily donate electrons in enzymatic and non-enzymatic responses and is present in higher concentration (Das & Roychoudhury 2014). It is produced in aerobic metabolism, and then reacts rapidly with $O_2^{\cdot-}$, singlet oxygen and ozone (chemically), and H_2O_2 (enzymatically) through ascorbate peroxidase to nullify their poisonous effects. Their scavenging free radicals ability can reduce the degree of oxidative stress. It also helps to restore antioxidant pigments, carotenoids (carotenes and xanthophylls) and vitamin E (Ballaz & Rebec 2019).

Vitamin E (Tocopherols) are lipophilic antioxidants that are synthesized by photosynthetic organisms. These antioxidants defend lipids and other membrane constituents by trapping and reacting with 1O_2 in chloroplasts along with protecting the Photosystem II structure and performance (Sharma et al. 2012). It can regenerate lipid peroxy, alkyl and alkoxy radicals produced during the polyunsaturated fatty acids oxidation (Kohen & Nyska 2002). Plants also produce vitamin E that act as important lip soluble redox buffer systems. It is generally produced in chloroplasts, protoplasts and the membranes of cells. This is a basic singlet oxygen scavenger compound to provide defense against lipid peroxidation (Jaleel et al. 2009). It can become a reactive radical after combining with free radicals and it also functions as a prooxidant in the co-antioxidants absence (Carocho & Ferreira 2013). It strongly decreases

oxygen free radicals effect and can diminish the oxidative stress by avoiding the free radicals production (La Fata et al. 2017).

Phenols are the richest antioxidants in plants with excellent ability to capture oxidative free radicals (Jiang et al. 2016). They are a diverse group of secondary metabolites (flavonoids, tannins, hydroxycinnamate esters, lignin, etc.) in plant cells (Sakakibara et al. 2003). They scavenge free radicals by donating hydrogen atoms and electrons and metal cations chelation to rapidly stabilize phenol radical (Balasundram et al. 2006). They detoxify H₂O₂ after donating electrons to guaiacol-type peroxidases under stress conditions (Michalak 2006). Their potent radical scavenging ability provide protection against UV radiation. Moreover, they act as feeding deterrents for herbivores, enzyme inhibitors and also provides resistance to pathogens (Bennett & Wallsgrove 1994). They can be categorized into various groups:

- **Phenolic acids** are the phenolic compounds that have one carboxylic acid group. They are found in the variety of foods, i.e. seeds, fruits skins, and leaves of vegetables contain their high amount (Pereira et al. 2009). Phenolic acids retain much greater *in vitro* antioxidant activity as compared to well-known antioxidant vitamins (Tsao et al. 2004). They possess resonance stabilized structure and donates H-atom through radical scavenging mechanism. On the other hand, the known antioxidant activity of phenolic acids are; radical quenching *via* electron donation and singlet oxygen quenching (Kumar & Goel 2019).
- **Lignans** are secondary plant metabolites that exhibit diverse structures (Magoulas & Papaioannou 2014). Lignans consist of two propyl-benzene units coupled by a β , β' -bond (Pan et al. 2009) and belongs to the diphenolic compounds group (Suzuki & Umezawa 2007). They are found in many seeds, grains, and fruits. They have low concentrations in vegetables, but sesame and flax seeds contains them in higher amount (Landete 2012).
- **Stilbenes** are polyphenolic substances synthesized by plants, especially grapes, peanuts, rhubarb and berries to defend themselves from pathogens, bacterial and fungal growth (Ansari et al.2013). They have antioxidant, anti-proliferative and anti-inflammatory properties. They maintains the homeostatic conditions and reduce the inflammatory factors transcription (Al-Khayri et al. 2023). They consist of an ethylene moiety in the middle of two benzene rings and are have

carbon skeleton of 1,2-diphenylethylene (Nagumo et al.2019). They are involved in the protection and treatment of different diseases such as cancer owing to their cell death activation properties (Sirerol et al. 2016).They are capable of scavenging cellular-enzymatic antioxidant defense and decreases the production of intracellular reactive oxygen species (Frombaum et al.2012).

- **Flavonoids** are present in the leaves, flower, and pollen grains. They are involved in the pigmentation of flowers, fruits, and seeds. They also take part in plant fertility germination of pollen and pathogens defense. Flavonoids are secondary ROS scavenging system in plants that damage the photosynthetic machinery via the surplus excitation energy (Fini et al. 2011). They scavenge $^1\text{O}_2$ and relieve the harms caused to the outer chloroplastic membrane envelope (Agati et al. 2012). They improve the ability of antioxidants to combine with active oxygen and have lower redox potential than oxygen and superoxide radicals (Duthie & Crozier 2000). Their synthesis is initiated by UV stress, toxicity of heavy metals, and the conditions of low temperature and nutrient, attributed to their UV-absorbing, metal chelating and radical scavenging ability (Rivero et al. 2001; Winkel-Shirley 2002).

2.3. Tea and tea-like plants

Tea is the most widely used beverage globally following only fresh water (Soukand & Kalle 2012). The English term “tea” denotes the infusion made from the *Camellia sinensis* (L.) Kuntze leaves. It also refers to the wide variety of locally grown herbs used in different regions of the world for recreational tea. Recreational tea is a technical term for an infusion made from leaves or flowers of herbs. These beverages were already recognized in Europe prior the introduction of the oriental tea in 1606 by the Dutch East India Company there (Weisburger & Comer 2000).

The herbal teas are used in a food context and this use is considered a modern tendency that is related to the oriental teas introduction in Europe. Herbal teas were only used in a medicinal context, while those of herbal teas taken like a coffee after meals were modern and not common (Menendez et al. 2012). Different kinds of teas are prepared by using the leaves of the tea plant, depending upon the mode of processing demonstrating the high-quality tea with rich aroma and revitalizing flavour (Mondal 2014; Sang et al. 2011).

Teas of flowers or flowers mixed with other ingredients are commonly used in the traditional medicine with different names such as “*zhourat*” (Obon et al. 2014) or “*shai alwird*”. Other rose species and cultivars (Baser et al. 2013; Vinokur et al. 2006), jasmine, *Hibiscus sabdariffa* (Saeed et al. 2013), chamomile (Guzelmeric et al. 2017) and some species are also taken in the herbal teas preparation. Herbal mixtures are important in ethnopharmacological study and in folk medicine their significance is revealed by the effect of each individual ingredient (Gras et al. 2018).

Linden tea is one of the most popular medicinal plants in Serbia and in other Balkan countries (Pavlovic et al. 2020). Infusion made from linden flowers, *Tilia* spp. is considered the most well-known recreational teas but also it is often used in the common cold, flu inflammation, migraine, hysteria and hypertension treatment (Raal et al. 2013; Soukand et al. 2013). Linden tea acts as an ingredient in different cosmetic products due to its hydrating and astringent properties (Karioti et al. 2009s). This tea contains phenolic compounds mainly quercetin glycosides, kaempferol glycoside, procyanidins, and phenolic acids (Toker et al. 2001).

Another type of tea is Rooibos (*Aspalathus linearis* (Burm.f.) R.Dahlgren), this tea-like plant is a shrub from South Africa. It is safer than *C. sinensis* to take by pregnant and breastfeeding women because this tea is naturally caffeine free (Sharangi 2009). Rooibos tea is naturally slightly sweet with caramel, floral, honey and woody undertones (Koch et al. 2012). Its leaves and fine stems can be used to prepare the herbal tea in the ‘fermented’ red brown and ‘unfermented’ green traditional forms (Chen et al. 2013). Rooibos tea can also be extracted and dried to form powdered tea extract containing high number of antioxidants especially polyphenols (Fukasawa et al. 2009). It alleviates the allergies, asthma, dermatological conditions, and infantile colic (Joubert et al. 2008).

Hibiscus sabdariffa tea, also known as roselle tea or karkade, is other popular caffeine free herbal tea that is prepared from fresh or dried calyces of *H. sabdariffa* L. It is red in colour and tastes like berries (Qi et al. 2005). Calix can be also used for the preparation of cold and fermented drinks, wine, jam, sweets, ice cream, chocolates, flavouring agents, desserts and cakes (Bako et al. 2009; Bolade et al. 2009). This tea contains an enzyme inhibitor to block the amylase production and drinking a cup of hibiscus tea after meals can reduce the dietary carbohydrates absorption and help in weight loss (Da-Costa 2014). It is also used in lowering

the body temperature (Leung 1996), in treatment of coughs and sore throats, genital problems, and for the treatment of external wounds (Neuwinger 2000).

Chamomile tea and herbal extracts are prepared from dried flowers of *Matricaria* species (Astin et al. 2000), mainly *M. chamomile* L. Tea prepared by using dry flowers induces good sleep, prevent cold and regulates the sweat and intestines (Mckay & Blumberg 2006). The tea infusion is used as a wash or gargle for inflammation of the mucous membranes of the mouth and throat (Mazokopakis et al. 2005). A medicinal preparation of chamomile and other herbs produces calming and tranquillizing effects (Dai et al. 2023).

2.3.1. Water infusion

Tea and tea-like beverages are traditionally prepared as a hot water infusion. The time of extraction and temperature differ according to source of plant material. In *C. sinensis*, optimum temperature for black tea infusion preparation is 80-95°C, however for green tea it is 75 to 80°C, for oolong should be close to 80-85°C (or higher), in case of mate it is 70-80°C. Indeed, rooibos and chamomile express its full potential at 90°C (Harbourne et al. 2009) and time of extraction is 5-7 mins for rooibos, for green tea 1-2 mins and for black tea 2-3 mins while it is 2-3 mins for oolong and in case of chamomile 5-7 mins and is 3-5 mins for mate (Burnett 2021).

C. sinensis is a good kind of mineral source. Mineral content in teas are variable that depends on the plant types, conditions of growth, and processing techniques while in infusion, transition rate of elements is influenced by time and temperature of water (Długaszek & Kaszczuk 2020; Pekal et al. 2012). In Turkey, traditional method is used in tea infusion followed by the teapot and kettle. The tea quality, the tea to water ratio, temperature and infusion time plays a significant role in consumer's choice (Cao et al. 2001). In tea infusions, there are different types of antioxidants comparable to the fat-soluble, water-soluble, and insoluble-bound fractions of tea that helps the people to select tea having strong antioxidant potential (Zhao et al. 2019).

Herbal infusions are prepared from dried parts of plants including roots, rootstocks, shoots, leaves, flowers, barks, fruits and seeds by frequently steeped in boiling water for being used in an infusion form (Guerrero et al. 2010). Herbal infusions are being used in traditional medicine and are popular global beverage (Poswal et al. 2019). Herbal infusions are an easily utilized form of the herbs especially for patients with swallowing problems.

Plant infusions provide a good distribution of active compounds in the intestine effectively. These infusions contain different polyphenol and flavonoid contents (Studzinska-Sroka et al. 2021). Hot water extraction techniques are used for the extraction of components of various herbs consumed through solution in form of infusion and decoction (Kaneria et al. 201). Decoction usually exhibits better extraction efficiency (Randjelovic et al. 2013) for the extraction of compounds strongly bound to the matrix while minerals of lesser interaction are extracted in infusion (Dias et al. 2015). The food consumed in form of aqueous extracts i.e. coffee, tea, and yerba mate the minerals and the matrix interaction effect their availability and solubility (Gharibzahedi & Jafari 2017).

2.3.2. *Camellia sinensis* (L.) Kuntze

The *Theaceae* family consists of 19 genera and 600 species. The economically important genus in this family is *Camellia* that contains about 120 species. These species were found in Cambodia, China, Northeast India, Indonesia, Southern Japan, South Korea, Laos, Malaysia, Myanmar, Nepal, Philippines, Thailand, and Vietnam (Wu et al. 2007).

Tea, *Camellia sinensis* (L.) Kuntze, is known as one of the basic drink worldwide, along with cocoa and coffee. It is widely consumed as a daily drink in China and in many countries. Chinese people start growing and using tea plants more than 3,000 years ago. Tea plants are being used to prevent and treat the different diseases since ancient times (Chan et al. 2011). It was grown in South Asia for first time and now is growing in Asia, Africa, and in different areas of the Middle East (Chopade et al. 2008). The tea cultivation in Turkey started along the Eastern black Sea Region in the early years of the Republic and mostly tea cultivation is centralizes on the Rise city of this country (Mendilcioglu 2000). Tea can be grown in fair temperature regions, acidic soils, and humid environmental conditions (Dufresne & Farnworth 2001). Two varieties of tea, green and black, are extensively consumed worldwide (Samanta 2022).

Tea is considered a non-alcoholic beverage that contain many active compounds including tea polysaccharides possesses excellent antioxidant potential (Yao et al. 2022). Antioxidant properties of tea plants are associated with presence of polyphenols, flavonoids and epigallocatechin gallate in tea leaves (Fernando & Soysa 2015). Tea polyphenols have been widely used as antioxidants in animal husbandry, to prevent cancer and regulation of lipid metabolism (Yan et al. 2020). Tea flavonoids reduces inflammation, prevents tooth decay, and has antimicrobial effects (Tariq et al. 2013). Different polyphenol content among tea varieties

might be influenced by several factors such as degree of ripeness, environmental factors, processing, and storage (Manach et al. 2004). Maturation of tea leaves also influence the polyphenols content and antioxidant activities due to chemical compounds transportation within the plant (Farhoosh et al. 2007). Total phenolic content in green tea is higher than black tea (Almajano et al. 2008). Tea also contains minerals and trace elements such as K, Mn, Cr, Ni, and Zn which are essential to human health. The regular consumption of tea may contribute to the daily dietary requirements of several elements and the large amount of potassium is beneficial for hypertensive patients (Fernandez et al. 2002).

Tea plant is an evergreen shrub with large number of branches. The leaves appear glossy dark green, elongate ovate, and roughly serrate, coriaceous, alternate, and short-petiolate. While young leaves appear silver because they bear downy hairs on the surface (Gruenwald 2007). The leaf blade is elliptic, and it has an obtuse end. Their leaves are resistant to very cold temperatures and garnet-brown to purple in colour (Panda 2016). Petals are obovate, free in two whorls, the outer whorls smaller than the inner, green patches present at the tip of outer petals, glabrous (Wu et al. 2007). Fruits are woody, sub-globose and seeds are rounded (Mahmood et al. 2010).

Depending on fermentation process, tea is categorized into three types. Green tea is unfermented form, a partially fermented oolong tea and fermented teas. Fermented teas must undergo a post harvested fermentation stage before drying and streaming. Fermentation of black tea is carried out by an oxidation process catalysed by polyphenol oxidase (Cabrera et al. 2006).

Tea extracts are widely used in the cosmetics industry, such as in face masks, face cleansers, facial toners, sun lotions, toothpastes, mouthwashes, shaving creams, aftershave lotion, deodorant, shampoos, and hair detangles (Koch et al. 2019). The anti-radical substances in tea extracts used in cosmetology are beneficial to human skin, such as polyphenols, flavonoids, catechins, and vitamin C (Katiyar et al. 2001). Green tea extracts enhance the activity and stability of skin-related enzymes (Hong et al. 2014). Green tea extracts are used as anti-obesity, anti-metabolic syndrome, and anti-diabetic treatments and to reduce insulin sensitivity. Green tea extracts are immune system modulators that reduce chronic cardiovascular inflammation and are antineoplastic agents. Catechins have antiviral activity and have regulatory approval for the treatment of genital warts (Coppock & Dziwenka 2016).



Figure 1. *Camellia sinensis* (L.) Kuntze (Kew Royal Botanical Gardens 2023)

2.3.3. *Camellia sinensis* var. *assamica*

Camellia sinensis var. *assamica* (Masters) or the Assam tea plant, is a member of *Theaceae* family belongs to Northeast India (Meegahakumbura et al. 2018; Parmar et al. 2012). It is known as “eating tea” or “chewing tea” (Kawakami et al. 1987). It is mainly produced in the mountainous areas of northern Thailand. It is an essential, popular, and important food for traditional religious ceremonies and funerals in certain areas (Gypmantasiri et al. 2001). Its consumption prevents from cardiovascular disorders, cancer, and diabetes (Khan & Mukhtar 2019; Singhal et al. 2017).

It is an evergreen perennial tree and exhibits high cross-pollinating behaviour (Olaniyi et al. 2014; Xia et al. 2020). It is considered a small plant that is 10-15 meters tall. The trunk of this tree equals the third of its height having a strong branching system. The leaves are either hairless or hairy that are dependent, shiny, thin, and pointed shape. The leaf blade is usually oval with 8-20 cm length and 3.5-7.5 cm width. Warm weather conditions suit it well (Marchand & Desharnais 2014). It has larger leaves and a semi arboreal habit (Carr 2018).

It is usually consumed as snack with or without salt (Tamang 2012). It is also consumed with other condiments such as roasted coconut, shredded ginger, peanuts, and coconut. Generally, it is chewed for about 10 minutes, but young people tend to swallow it (Mougne et al. 1982). Its active components are extensively used in skin care and skin treatment products (Rajbhar et al. 2015). In the cosmetic industry, tea extracts are used due to antioxidant activity,

anti-aging, anti-cellulite and photo-protective properties, and microcirculation (Koch et al. 2019).

C. sinensis var. *sinensis* (China tea) is the tea grown chiefly in China and Japan which has smaller leaves and more cold tolerance but grows less vigorously than *C. sinensis* var. *assamica* (Mast.). Green teas are always prepared from this. It is an evergreen and multi-stemmed shrub up to 3 m tall. Leaves are leathery, narrow, and less than 10 cm long, dark green with dull, flat surface and indistinct marginal veins (Schooler & Van der Vossen 2000).

C. sinensis (L.) Kuntze var. *lasiocalyx* (Watt) W. Wight has intermediate characteristics between China and assam teas (Schooler & Van der Vossen 2000).

2.3.4. *Ilex paraguariensis* A. St. Hil

Yerba mate (*Ilex paraguariensis* A. St. Hil) is a perennial tree belonging to the *Aquifoliaceae* family native of South America present in Uruguay, Paraguay, Argentina, and Brazil (Valduga et al. 2019). Mate tea is an herbal infusion prepared from the dried leaves of *I. paraguariensis* (Grigioni et al. 2004). It is considered one of the most widely consumed non-alcoholic beverage in South America (Small & Catling 2001). Its demand is also increasing due to its pharmacological properties and health benefits (Bracesco et al. 2011). It contains phenolic compounds such as caffeic acid, (Filip et al. 2001), xanthines, flavonoids, tannins, rutin and saponins (Lewinski et al. 2007). It also contains minerals (P, Fe, and Ca) and vitamins (C, B1, and B2) (Heinrichs & Malavolta 2001).

The name “yerba mate” originated in Spain. The word “yerba” means a drink made from the herb, while the word “mate” means drinking from a calabash mate gourd (Gawron-Gzella et al. 2021). Yerba mate was also known as ‘Jesuit tea’ or ‘Paraguayan tea’. This beverage has been consumed traditionally by Guarani indigenous people before the conquest of South America (Delacassa & Bandoni 2001). Its leaves and small branches after processing are used in the production of infusion drinks, such as chimarrao. Although the most consumed infusion is “mate” or “chimarrao” (hot beverage), but there are different forms of its consumption, such as tea, “terere” (cold beverage) and carbonated drinks (Lewinski et al. 2015).

I. paraguariensis is a subtropical dioecious evergreen tree that can reaches 18 m in height. The mate tree is a flower and fruit producing plant, flowering from October to November and producing fruit from March to June (Giberti 1994). This plant has monopodial

branching and rhythmic growth (Halle et al. 1978). Its rhythmic growth is expressed by the occurrence of two annual growth flushes forming portions of the two annual growth units, one in the spring and another in the autumn (Guedon et al. 2018; Matsunaga et al. 2014).

Yerba mate leaves have antioxidant (Filip et al. 2000), anti-obesity (Pittler & Ernst 2004), chemo-preventative (Filip et al. 2007), anti-diabetic, digestive improvement, and cardiovascular properties (Gorgen et al. 2005). It also shows hypo-cholesterol emic, hepato-protective, central nervous system stimulant and diuretic effect, and prevents oxidation of DNA and lipoprotein. It is used as an ingredient in the food or dietary supplement industries. Yerba mate tea is associated with both the prevention and the cause of some types of cancers (Heck et al. 2007).



Figure 2. *Ilex paraguariensis* A.St.-Hil. (Kew Royal Botanical Gardens 2023)

2.4. Extraction methods

Antioxidants from plants can be extracted by different extraction methods. The extraction of polyphenols and other functional compounds from different parts of tea is carried out using different solvents (e.g. water, water-ethanol, ethanol, methanol, acetone, ethyl acetate, and acetonitrile) that play role in antioxidant activities. Solvent that has higher polarity (aqueous methanol) is more efficient to scavenge free radicals than less polar solvent (methanol and hot water) (Turkmen et al. 2006). The choice of solvent must consider several factors including selectivity, ability to extract, toxicity, ease of evaporation and prices (Harborne

1998). Ethanol is frequently considered as an efficient solvent to extract polyphenols (Koffi et al. 2010). Moreover, the particle size of the raw materials, the solvent-to-solid ratio, the extraction temperature, and the extraction duration also affect the extraction efficiency.

The extraction of natural products processes through the following stages:

- (1) The solvent penetrates the solid matrix
- (2) The solute dissolves in the solvents
- (3) The solute is diffused out of the solid matrix
- (4) The extracted solutes are collected (Li et al. 2014; Zhou et al. 2012).

Several conventional techniques, such as boiling, heating, Soxhlet, and cold extraction are used to extract bioactive ingredients. However, these procedures are unsuitable for achieving high yields and biological activities due to the long extraction times of cold brewing and the high temperatures in other heating methods (Raghunath et al. 2023). These techniques are also vulnerable to degradation of heat-labile compounds, high solvent and energy consumption, and the formation of toxic residues (Banerjee et al. 2017). Many efforts have been carried out to replace conventional extraction techniques with innovative technologies (Raghunath et al. 2023).

The green extraction techniques, also known as non-conventional extraction methods, possess advantages such as the use of less hazardous chemical synthesis, safer solvents, use of renewable feedstock, reduce derivatives and time analysis, prevent degradation, and pollution prevention (Azmir et al. 2013). The emerging extraction processes include faster extraction rate, more effective energy use, increased mass and heat transfer and a reduction in the number of processing steps (Jacotet-Navarro et al. 2016). Recently, subcritical, superheated, or pressurized hot water has become of great interest as an alternative solvent for extraction of natural active compounds (Herrero et al. 200).

2.4.1. Microwave extraction (MAE)

This technique was first used in the 1980s and it is one of the most popular extraction methods today. Microwaves are used for the extraction of phytoconstituents from herbal sample (Bagade & Patil 2021). This technique combines microwave and regular solvent used for the extraction of polyphenols from tea. Microwaves heat up the solvents and plant tissues in the extraction process for increasing the kinetics of extraction (Al-hatim et al. 2022). This

extraction method is more effective in the recovery of high-quality phenols and flavonoids from plant matrix in a shorter time (Chan et al. 2011; Desai et al. 2010). This method is being affected by different extraction parameters i.e. irradiation time, temperature, microwave power level, feed to solvent ratio, and solvent concentration (when a mixture of solvent is used) (Raut et al. 2015). This technique has several advantages, including shorter extraction time, less solvent, higher extraction rate, and cheaper cost (Al-hatim et al. 2022).

2.4.2. Subcritical Water Extraction

Subcritical water extraction (SWE) is also referred to as accelerated solvent extraction, pressurized fluid extraction, pressurized hot solvent extraction, high-pressure solvent extraction and subcritical solvent extraction. SWE is a faster method, which uses a pressurized liquid kept below its critical point (374°C for water) and above its boiling point (100°C for water). These conditions allow fluids to remain in a liquid state due to the applied pressure and it creates low polar water with equivalent to organic solvents at ambient temperature (Shimizu et al. 2019; Zhang & Wolf 2019). Due to conditions of pressure and temperature physicochemical properties of solvent are changed. For instance, mass transfer rates are enhanced, while at the same time, solvent surface tension and viscosity are decreased, and solubility of analytes is increased that allows the solvent to penetrate deeper into the solid matrix being extracted. As a result, significantly higher extraction yields are obtained. Therefore, SWE is a faster extraction processes with less consumption of solvent for the sample preparation (Alvarez-Rivera et al. 2020). This technique facilitates rapid extraction without the loss or changes of the chemical integrity of thermolabile compounds (Essien et al. 2020).

In this technique, water acts as a solvent and catalyst to convert biomass into valuable products (Abdelmoez et al. 2007). The properties of subcritical water can be adjusted by temperature. When the temperature of water increases, its hydrogen bonding breaks with decreasing dielectric constant and polarity (Mazaheri et al. 2010) resulting in increased concentration of hydrogen ion (Abdelmoez & Yoshida 2006). This allows selective extractions such as extracting polar compounds at lower temperatures and less polar ingredients at higher temperatures (Cheng et al. 2021). Combining subcritical water with an organic solvent such as ethanol and methanol improve the yield, extraction time and solubility of compounds (Kwon & Chung 2015). Another advantage of this technique is that water is non-toxic, and no liquid waste disposal is required after extraction (Mazaheri et al. 2010). Extraction time is the main

factor to increase the antioxidant extraction yield (Li et al. 2020). Phenolic compounds could be efficiently achieved in a very short time (Zullaikah et al. 2015).

This technology is used for the extraction of active compounds from different biomass materials with low cost, mild operating conditions, short process times, and environmental sustainability. This technique is used in the pharmaceutical, environmental, and food fields for the extraction of nutritional compounds and organic contaminants. It is also useful for the extraction of organic acids, amino acids, proteins, fatty acids, oils, and it can be applied in wastewater (Sun et al. 2012). This method is suitable for the extraction of seasoning herbs, vegetables, fruits, food by-products, algae, shrubs, tea leaves, grains, and seeds. Many natural products extracted through this method includes alkaloids, carbohydrates, flavonoids, glycosides, lignans, polyphenolics, quinones, steroids and terpenes (Cheng et al. 2021).

SWE process is also often used for the separation of hemicellulose and other undesirable compounds from biomass in the cellulosic industry (Ruiz et al. 2021). During this process, lingo-cellulosic biomass is immersed in hot water at high pressure. This results in pentose recovery with high efficiency after enzymatic hydrolysis and dissolution rate of 4-22% for cellulose and 35-60% for lignin (Alvira et al. 2010). The available polysaccharides in extracted wood chips can be hydrolysed to convert them into simpler sugars that can be fermented to produce ethyl alcohol (bioethanol). Due to current climate policy, this method is used in the production of bioethanol using new raw materials such as hemp can increase access to high quality alternative fuel (Leszczynski & Roman 2023).

3. Hypothesis

Tea and tea-like plants are a rich source of antioxidants and bioactive compounds (polyphenols and catechins) that can scavenge free radicals. Subcritical water extraction (SWE) is a novel method used for extraction of natural active compounds with low extraction time, higher extract quality and lower operational cost. SWE is an effective method for extracting valuable bioactive compounds but, the main operating parameters i.e. temperature and time (mainly in our study) affect final yield and antioxidant activity of extracts.

Aims of the Thesis

The aim of this thesis was to evaluate and compare the antioxidant activity and TPC of different fractions of SWE extracts and WI of green tea, black tea and mate. Specific objectives are:

- To evaluate the effect of time of extraction on yield of phenolic compounds and antioxidant activity.
- To determine antioxidant activity of obtained extracts by various methods; 2,2-diphenyl-1-picrylhydrazyl radical assay (DPPH) and Oxygen radical absorbance capacity assay (ORAC) were used.
- To determine content of polyphenolic substances in obtained extracts by using TPC assay.
- To compare results of SWE extracts with extracts obtained by classic infusion method.

4. Methods

4.1. Plant material

Plant materials used for extraction of bioactive compounds were bought from commercial sources on the Czech market from the company Herbs Life Sokolov (*Camellia sinensis* – black and green tea) and Salvia Paradise (*Ilex paraguariensis* - mate) due to their availability. The places of their origin listed by suppliers were China for black and green tea, and Paraguay for mate.

4.2. Preparation of Extracts

Plant materials were grounded to mild powder by using an electric mill GM 100 (Retsch, Germany) prior the extraction.

4.2.1. Subcritical water extracts

The fine powder of each plant sample weighing 7.5 g was placed into the extraction vessel SFE Helix (Applied Separations, USA). The sample was subsequently extracted for 1st 2min, 2nd 2min, 3rd 2min, 4th 2min and 5th 2 min by keeping the water temperature 120°C and extraction vessel pressure 50 Bar. Then again fine powder weighing 7.5 g of each plant sample was taken and placed into the extraction vessel and extracted the sample for 1st 5min and 2nd 5min. At last, fine powder of each plant sample weighing 7.5 g was taken and placed into the extraction vessel and extracted the sample for whole 10minutes. After collecting the final extract, all the fractions were concentrated by rotary evaporator R-200 (Buchi, Switzerland) at 50°C and vacuum, until the lowest possible yield was obtained from dry sample. The dry material was weighed and stored at -20°C for further use. The extract yields of samples are presented in Table 1.

4.2.2. Water infusion

The fine powder weighing 7.5 g was poured into 1 L boiling water. Brewing was done for 10mins and the membrane vacuum pump KNF Laboport (KNF Neuberger GmbH, Germany) was used to filter the infusion. Rotary evaporator R-200 (Büchi, Switzerland) was used to make the final extract concentrated at 50°C and vacuum to obtain lowest possible yield from dry sample. Then, the dry residue after weighing was stored at -20°C for use. The extract yields are presented in Table 1.

Table 1. Extraction yield (%)

Species, Family	Used part	SWE			WI	Source
		2mins fractions	5mins fractions	10mins fractions	10mins fractions	
Green tea (<i>Camellia sinensis</i> , <i>Theaceae</i>)	Leaves	19.52%	22.89%	21.82%	31.6%	HerbsLife Sokolov
Black tea (<i>Camellia sinensis</i> , <i>Theaceae</i>)	Leaves	8.82%	9.92%	8.49%	32.54%	HerbsLife Sokolov
Mate (<i>Ilex paraguariensis</i> , <i>Aquifoliaceae</i>)	Leaves	23.24%	23.52%	25.08%	40.22%	Salvia Paradise

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

4.3. Chemicals

Methanol and Folin-Ciocalteu reagent were bought from Penta (Prague, Czech Republic), and inorganic salts (K_2HPO_4 , KH_2HPO_4 and Na_2CO_3) were bought from Lach-Ner (Neratovice, Czech Republic). 2,2'-azobis (2-methylpropionamide) dihydrochloride (AAPH), (\pm)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, and fluorescein sodium salt were bought from Sigma-Aldrich (Prague, Czech Republic).

4.4. Evaluation of Antioxidant activity

4.4.1. DPPH

The Sharma and Bhat (2009) method was used to evaluate the antioxidant activity of the extracts on the bases of their ability to inhibit DPPH radical. Stock solutions of Trolox and samples were prepared in methanol at the concentration 512 $\mu\text{g/mL}$. For the two-fold serial dilution of each sample the automatized pipetting platform Freedom EVO 100 (Tecan, Mannedorf, Switzerland) was used in 96-well microtiter plates. Then, 25 μL of 1 mM DPPH in methanol and 75 μL of methanol were pipetted into each well. The final concentrations of each sample and Trolox in the microtiter plate was 0.125–256 $\mu\text{g/mL}$. Incubation of the plates was performed for 30 min at room temperature in the dark. After that, a Multimode Reader Cytation 3 (BioTek Instruments, Winooski, VT, USA) was used for the measurement of the absorbance spectrophotometrically at 517 nm. Experiments were performed in triplicate as three independent tests and their results were calculated as a mean value of half maximal inhibitory concentration with standard deviation ($IC_{50} \pm SD$) in $\mu\text{g/mL}$.

4.4.2. ORAC

The method of Ou et al. (2001) was used to determine the ability of samples to protect fluorescein from oxidative degradation by AAPH radical using ORAC assay. Firstly, stock solutions of 48 nM fluorescein and 153 mM AAPH radical were prepared in 75 mM phosphate buffer (pH 7). For the two-fold serial dilution of each extract, phosphate buffer was prepared in 96-well black absorbance microtiter plates using automatized pipetting platform Freedom EVO 100 (Tecan, Mannedorf, Switzerland). Then, 150 μL of fluorescein was added into each well for

incubation at 37°C for 10 mins. Consequently, the application of 25 µL of AAPH and ORAC buffer in three wells (control) started the reaction and the plates were placed into the incubator for 1.5 hours at 37°C. The 32 to 0.125 µg/mL concentrations were used for this sample. Each microtiter plate was filled with 200 µL of distilled water in the outer wells to enhance the thermal mass stability. Fluorescein with AAPH in phosphate buffer (blank 1) and fluorescein in buffer (blank 2) were part of each plate while using the Trolox as a positive control. Finally, the radical inhibition results were recorded using Multimode Reader Cytation 3 (BioTek Instruments, Winooski, VT, USA) at 485 nm wavelength. Experiments were carried out in triplicate as three independent tests whereas their results were calculated as a mean value of $IC_{50} \pm SD$ in µg/mL.

4.4.3. TPC

The method of Singleton et al. (1999) was used for the determination of TPC in the extracts that was performed in the 96-well microtiter plates. Stock solutions (32 µg/mL concentration) of samples were prepared and pipetted out the 100 µL of each sample into the plate in triplicates. Then, 75 µL of 12% Na₂CO₃ and 25 µL of Folin-Ciocalteu reagent were mixed in the sample to start the reaction. The incubation of plate was done for 2 hours at 37°C in the dark. For the measurement of absorbance, Multimode Reader Cytation 3 (BioTek Instruments, Winooski, VT, USA) was used at 700 nm. The standard calibration curve was created by using 8 levels of gallic acid concentration (0.25, 0.5, 1, 2, 4, 8, 16, 32 µg/mL). Experiments were carried out in triplicate as three independent tests whereas their results were described 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 evaluated by using Pearson product moment correlation to find that the phenolic compounds are responsible for the antioxidant activity in studied plants. The correlation degree was found according to the principle of Evans (1996), where the correlation is evaluated based on absolute value of r 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).

The statistical analysis for the comparison of different SWE fractions (1st 2min, 2nd 2min, 3rd 2min, 4th 2min, and 5th 2min; 1st 5min and 2nd 5min, and 10min) and Water infusion (10min),

their effect on antioxidant activity and TPC was performed by Microsoft Excel (Microsoft 365 MSO, Microsoft Corporation, Redmont, USA) using Student's T-test for each sample. The differences were considered to be statistically significant at $p < 0.05$.

5. Results

The confirmation of antioxidant activity by more than one method can provide complete evaluation of total antioxidant capacity in individual plants. Thus, the antioxidant activity of plant extracts is determined by two methods differing based on mode of action, the DPPH representing electron atom transfer reaction and ORAC representing a hydrogen atom transfer reaction (Prior et al. 2005). In view of the fact that phenolic compounds especially phenolic acids and flavonoids are considered the most important plant constituents responsible for antioxidant activity (Chaves et al. 2020), TPC is also evaluated. In this study, antioxidant activity and TPC were measured for both types of extracts (WI and SWE) while their results were expressed as IC_{50} ($\mu\text{g}/\text{mL}$) for DPPH and ORAC, and as mg of gallic acid equivalent per g of extract (mg GAE/g) for TPC, including $\pm\text{SD}$.

5.1. ORAC

In *C. sinensis* – green tea, the strongest antioxidant activity (IC_{50} 6.50 $\mu\text{g}/\text{mL}$) was obtained in SWE 2nd 5min fraction. The strong antioxidant activities were obtained also in SWE 10min, 3rd 2min, 4th 2min and 5th 2min fractions with IC_{50} in range from 7.44 to 8.54 $\mu\text{g}/\text{mL}$ for ORAC assay. The weaker antioxidant activity was obtained in SWE 1st 2min, 2nd 2min, and 1st 5min fractions having IC_{50} in range from 9.47 to 11.89 $\mu\text{g}/\text{mL}$. The antioxidant activity was also weak in WI 10min (IC_{50} 9.56 $\mu\text{g}/\text{mL}$).

Similarly, for *C. sinensis* – black tea, the strongest antioxidant activity was determined in SWE 2nd 5min fraction having IC_{50} 6.43 $\mu\text{g}/\text{mL}$. Other strong antioxidant activity was observed in same fractions as for green tea - in SWE 10min, 3rd 2min, 4th 2min, and 5th 2min fractions with IC_{50} in range from 7.03 to 8.89 $\mu\text{g}/\text{mL}$. On the other hand, the antioxidant activity was weakest in SWE 1st 2min fraction (16.93 $\mu\text{g}/\text{mL}$), and weak in 1st 5min (14.62 $\mu\text{g}/\text{mL}$) and 2nd 2min fractions (11.29 $\mu\text{g}/\text{mL}$). In WI 10min, the antioxidant activity was also weak with IC_{50} 12.01 $\mu\text{g}/\text{mL}$.

From above mentioned results it is evident that extraction time has substantial effect on antioxidant activity of *C. sinensis*. Specifically, 2nd 5min fraction was the most active in both cases (black tea and green tea). Moreover, SWE 10min, 3rd 2min, 4th 2min, and 5th 2min fractions were with strong activity again in both, black tea, and green tea. Similarly, the lowest antioxidant effect

was determined for 1st 2min fraction, 2nd 2min fraction, and 1st 5min fraction. Furthermore, WI extracts was weak in both cases.

In *I. paraguariensis*– mate, the antioxidant activity was strongest in 3rd 2min and 5th 2min fractions having IC₅₀ (5.55 µg/mL and 5.97 µg/mL). Other strong antioxidant activity was determined in 4th 2min and 2nd 5min fractions (IC₅₀ 6.46 µg/mL and 6.05 µg/mL, respectively). Weak antioxidant activity was obtained in 1st 2min, 2nd 2min, 1st 5min and 10min fractions with IC₅₀ in range from 7.51 to 7.97 µg/mL. The weakest activity was also obtained in WI 10min with IC₅₀ 8.14 µg/mL. All the results of ORAC assay are summarized in Table 2.

The effect of fractions on antioxidant potential was evaluated by student T-test, and the statistically significant differences (p<0.05) were determined in many cases (results are part of Table 2):

In both cases, green tea and black tea, between 5min fractions (1st 5min and 2nd 5 min);

In green tea between:

- SWE 1st 2min and 4th, 5th 2min fractions, and 2nd 5min fractions;
- SWE 2nd 2min, and 1st 5min and 2nd 5min fractions;
- SWE 4th 2min and 1st 5min fractions;
- SWE 5th 2min and 1st 5min fractions;
- SWE 1st 2min, 2nd 2min, 1st 5min with 10min fractions;
- SWE 1st 5min, 2nd 5min and 10min with WI 10min;

In black tea between:

- SWE 1st 2min and 2nd, 3rd 4th, 5th 2min, and 2nd 5min fractions;
- SWE 2nd 2min and 3rd 2min, 5th 2min, and 2nd 5min fractions;
- SWE 3rd, 4th, 5th 2min and 1st 5min fractions;
- SWE 1st 2min, 2nd 2min, 1st 5min, 2nd 5min and 10min fractions;
- SWE 1st, 3rd, 5th 2min, 2nd 5min and WI 10min;
- SWE 10 min and WI 10min ;

In mate between:

- SWE 1st and 2nd 2min, 3rd and 5th 2min, and 2nd 5min fractions;
- SWE 3rd 2min and 1st 5min fractions;
- SWE 5th 2min and 1st 5min fractions;
- SWE 3rd, 5th 2min, and 2nd 5min and 10min fractions;
- SWE 1st, 3rd and 5th 2min, 2nd 5min and WI 10min;

Table 2. Antioxidant activity of different fractions of subcritical water extraction and water infusion of tea and tea-like plants by ORAC method.

Samples, Family	Fractions	IC ₅₀ (µg/ml) ±SD*
Green tea (<i>Camellia sinensis</i> , <i>Theaceae</i>)	SWE 1 st 2min	11.48±1.0 ^{ABCI}
	SWE 2 nd 2min	9.47±0.5 ^{DEJ}
	SWE 3 rd 2min	8.54±1.5
	SWE 4 th 2min	8.20±1.0 ^{AF}
	SWE 5 th 2min	8.48±1.1 ^{BG}
	SWE 1 st 5min	11.89±0.8 ^{DFGHKL}
	SWE 2 nd 5min	6.50±0.4 ^{CEHM}
	SWE 10min	7.44±0.4 ^{IJKN}
	WI 10min	9.56±0.7 ^{LMN}
Black tea (<i>Camellia sinensis</i> , <i>Theaceae</i>)	SWE 1 st 2min	16.93±0.7 ^{ABCDEMR}
	SWE 2 nd 2min	11.29±1.4 ^{AFGHN}
	SWE 3 rd 2min	7.94±0.8 ^{BFIS}
	SWE 4 th 2min	8.89±1.3 ^{CJ}
	SWE 5 th 2min	7.03±1.3 ^{DGKPT}
	SWE 1 st 5min	14.62±1.6 ^{I-L, O}
	SWE 2 nd 5min	6.43±0.8 ^{EHLQU}
	SWE 10min	8.37±0.1 ^{M-Q, V}
WI 10min	12.01±1.9 ^{R-V}	
Mate (<i>Ilex paraguariensis</i> , <i>Aquifoliaceae</i>)	SWE 1 st 2min	7.77±0.1 ^{ABCM}
	SWE 2 nd 2min	7.51±0.5 ^{DEF}
	SWE 3 rd 2min	5.55±0.5 ^{ADGJN}
	SWE 4 th 2min	6.46±1.0
	SWE 5 th 2min	5.97±0.07 ^{BEHKO}
	SWE 1 st 5min	7.89±0.1 ^{GHI}
	SWE 2 nd 5min	6.05±0.4 ^{CFILP}
	SWE 10min	7.97±0.06 ^{JKL}
WI 10min	8.14±0.1 ^{MNOP}	
Trolox		12.39±2.24

Notes: SWE- subcritical water extraction; WI- water infusion; *- same letters means the values are significantly different (p<0.05); IC₅₀- half maximal inhibitory concentration; SD- standard deviation

5.2. DPPH

DPPH assay showed that in *C. sinensis* – green tea, the strongest antioxidant activity was found in SWE 2nd 5min fraction (IC₅₀ 9.73 µg/mL). The other strong antioxidant activity was obtained in 2nd, 3rd, 4th, 5th 2min and 10min fractions having IC₅₀ in range from 12.04 to 15.70 µg/mL. The antioxidant activity was also quite strong in WI 10min (IC₅₀ 13.88 µg/mL). The weakest antioxidant activity was determined in 1st 2min and 1st 5min fractions (IC₅₀ 21.98 µg/mL and 21.34 µg/mL).

For *C. sinensis* – black tea, the strongest antioxidant activity was determined in SWE 2nd 5min fraction (IC₅₀ 16.23 µg/mL). While other strong antioxidant activity was obtained in 2nd 2min, 3rd 2min, 4th 2min, 5th 2min and 10min fractions having IC₅₀ in range from 18.68 to 24.11 µg/mL. In WI 10min, the antioxidant activity was also strong having IC₅₀ 20.618 µg/mL. The weakest antioxidant activity was obtained in SWE 1st 2min fraction (IC₅₀ 40.94 µg/mL) and the weak in 1st 5min fraction (IC₅₀ 28.11 µg/mL).

It is evident from our results that antioxidant activity of *C. sinensis* is substantially affected by extraction time. Specifically, 2nd 5min fraction was the most active in both cases i.e. black tea and green tea. Moreover, SWE 2nd 2min, 3rd 2min, 4th 2min, 5th 2min and 10min fractions have strong activity in both cases, black tea, and green tea. Similarly, the lowest antioxidant effect was determined for 1st 2min and 1st 5min fractions. Although in DPPH assay WI extracts possessed quite strong activity, some SWE fractions showed better results, confirming hypothesis, that SWE and fractionation is effective method for extraction of bioactive compounds.

In *I. paraguariensis* – mate, the strongest antioxidant activity was found in SWE 4th 2min fraction with IC₅₀ 29.16 µg/mL. Other strong antioxidant activity was obtained in SWE 1st 5min, 2nd 2min, 3rd 2min and 10min fractions having IC₅₀ in range from 29.30 to 30.81 µg/mL. On the other hand, the weakest antioxidant activity was determined in 1st 2min fraction (IC₅₀ 33.79 µg/mL). Other weak antioxidant activity was determined in 2nd 5min, 5th 2min fractions, and WI 10 min (31.22-31.90 µg/mL). All the results of DPPH assay are presented in Table 3.

The effect of fraction on antioxidant potential was also evaluated by student T-test, and the statistically significant differences (p<0.05) were determined in many cases (these results are a part of Table 3):

In both cases, green tea and black tea, between 5min fractions (1st 5min and 2nd 5 min);

In green tea between:

- SWE 2nd 2min and 3rd 2min, 4th 2min, 1st and 2nd 5min fractions;
- SWE 3rd, 4th, 5th 2min, and 1st 5min fractions;
- SWE 3rd 2min, 4th 2min and 2nd 5min fractions;
- SWE 3rd 2min, 4th 2min, 1st 5min, 2nd 5min and 10min fractions;

In black tea between:

- SWE 1st 2min and 2nd, 3rd 4th, 5th 2min, 1st and 2nd 5min fraction
- SWE 3rd, 4th, 5th 2min and 1st 5min fractions;
- SWE 3rd 2min and 2nd 5min fractions;
- SWE 1st 2min, 4th 2min and 10min fractions;
- SWE 1st 2min, 1st 5min, 2nd 5min and WI 10min;

In mate between:

- SWE 5th 2min and SWE 10min fractions;

Table 3. Antioxidant activity of different fractions of subcritical water extraction and water infusion of tea and tea-like plants by DPPH method.

Samples, Family	Fractions	IC₅₀(µg/mL) ±SD*
Green tea (<i>Camellia sinensis</i> , <i>Theaceae</i>)	SWE 1 st 2min	21.99±2.5
	SWE 2 nd 2min	15.05±0.3 ^{ABCD}
	SWE 3 rd 2min	12.04±0.8 ^{AEFK}
	SWE 4 th 2min	12.73±0.3 ^{BGHL}
	SWE 5 th 2min	14.07±0.9 ^I
	SWE 1 st 5min	21.33±1.2 ^{CEGIJM}
	SWE 2 nd 5min	9.73±0.6 ^{DFHJN}
	SWE 10min	15.70±0.5 ^{KLMN}
	WI 10min	13.89±1.2
Black tea (<i>Camellia sinensis</i> , <i>Theaceae</i>)	SWE 1 st 2min	40.94±5.3 ^{A-F, LN}
	SWE 2 nd 2min	24.12±4.1 ^A
	SWE 3 rd 2min	21.27±1.5 ^{BGH}
	SWE 4 th 2min	18.68±1.3 ^{CIM}
	SWE 5 th 2min	18.92±1.9 ^{DJ}
	SWE 1 st 5min	28.12±2.1 ^{EGIJKO}
	SWE 2 nd 5min	16.23±1.5 ^{FHKP}
	SWE 10min	23.67±1.8 ^{LM}
	WI 10min	20.62±0.8 ^{NOP}
Mate (<i>Ilex paraguariensis</i> , <i>Aquifoliaceae</i>)	SWE 1 st 2min	33.79±3.3
	SWE 2 nd 2min	29.89±0.8
	SWE 3 rd 2min	30.82±2.6
	SWE 4 th 2min	29.16±2.3
	SWE 5 th 2min	31.90±0.8 ^A
	SWE 1 st 5min	29.97±1.1
	SWE 2 nd 5min	31.22±1.5
	SWE 10min	29.30±0.7 ^A
	WI 10min	31.60±3.2
Trolox		10.35±0.8

Notes: SWE- subcritical water extraction; WI- water infusion; *-same letters means the values are significantly different (p<0.05); IC₅₀-half maximal inhibitory concentration; SD- standard deviation

5.3. TPC

For *C. sinensis* – green tea, the highest TPC was determined in SWE 2nd 5min fraction (778.53 mg GAE/g). This was high in SWE 3rd 2min, 4th 2min, and 5th 2min fractions ranging from 654.92 to 688.66 mg GAE/g. TPC was lowest in SWE 1st 5min and 1st 2 min fraction as 454.40 and 482.83 mg GAE/g respectively and low in 2nd 2min and 10min fractions in range from 543.29 to 573.02 mg GAE/g. For WI 10min, the obtained TPC was also low 568.64 mg GAE/g.

For *C. sinensis* – black tea, the highest TPC was obtained in SWE 2nd 5min fraction (650.55 mg GAE/g). High TPC was obtained in 3rd, 4th, 5th 2min fractions in range from 524.05 to 574.33 mg GAE/g. For WI 10min, the TPC was low 453.47 mg GAE/g. The lowest TPC was obtained in SWE 1st 2min (283.82 mg GAE/g) and low TPC was also obtained in 2nd 2 min, 1st 5min and 10 min fractions in range from 372.76 to 414.788 mg GAE/g).

It is revealed from above mentioned results that extraction time substantially affect the total phenolic content of *C. sinensis*. Specifically, SWE 2nd 5min fraction showed highest TPC in both cases (black tea and green tea). Moreover, SWE 3rd 2min, 4th 2min, and 5th 2min fractions showed high TPC again in both cases i.e. black tea and green tea. Similarly, the low TPC was determined for 2nd 2min and 10min fraction in both green tea and black tea. Furthermore, WI extracts were weak in both cases.

In *I. paraguariensis*– mate, the obtained TPC was highest in SWE 4th 2min fraction (543.31 mg GAE/g). The high TPC was found in 2nd 5min, 3rd 2min, and 5th 2min fractions ranging from 521.51 to 540.31 mg GAE/g. However, TPC was lowest in 10min fraction (448.79 mg GAE/g) whereas it was low in 1st 2min, 2nd 2min and 1st 5min fractions in range from 459.08 to 482.36 mg GAE/g. In WI 10min, the TPC was low (492.11 mg GAE/g). The results of TPC are presented in Table 4.

The effect of fractions on antioxidant potential was also evaluated by student T-test, and the statistically significant differences ($p < 0.05$) were determined in many cases (these results are also described in Table 4):

In both cases, green tea and black tea, between 5min fractions (1st 5min and 2nd 5 min);

In green tea between:

- SWE 1st 2min and 2nd 2min, 3rd 2min, and 2nd 5min fractions;
- SWE 2nd 2min, and 4th 2min, 1st 5min and 2nd 5min fractions;
- SWE 3rd 2min, and 1st 5min, 2nd 5min and 10min fractions;
- SWE 4th 2min, and 1st 5min, and 10min fractions;
- SWE 5th 2min and 1st 5min fractions;
- SWE 1st 5min, and 2nd 5min and WI 10min;
- SWE 2nd 5min, and 10min and WI 10min;

In black tea between:

- SWE 1st 2min, and 2nd, 3rd and 4th 2min, 1st and 2nd 5min, 10min and WI 10min;
- SWE 2nd 2min, and 3rd, 4th 2min, 5th 2min and 2nd 5min fractions;
- SWE 3rd 2min, and 1st 5min, 2nd 5min, 10min and WI 10min;
- SWE 4th 2min, and 1st 5min, 2nd 5min and 10min fractions;
- SWE 5th 2min, and 1st 5min, 10min and WI 10min;
- SWE 1st 5min, and 2nd 5min and WI 10min;
- SWE 2nd 5min, and 10min and WI 10min;

In mate, results were statistically evaluated but they were not significantly different.

Table 4. Total phenolic content of different fractions of subcritical water extraction and water infusion of tea and tea-like plants

Samples, Family	Fractions	IC ₅₀ (µg/mL) ±SD*
Green tea (<i>Camellia sinensis</i> , <i>Theaceae</i>)	SWE 1 st 2min	482.83±10.3 ^{A-C}
	SWE 2 nd 2min	573.02±6.9 ^{D-F}
	SWE 3 rd 2min	654.92±9.2 ^{A, G-I}
	SWE 4 th 2min	688.67±39.9 ^{BDJK}
	SWE 5 th 2min	683.39±31.5 ^L
	SWE 1 st 5min	454.40±21.2 ^{EGJLMN}
	SWE 2 nd 5min	778.53±6.8 ^{CFHMO}
	SWE 10min	543.29±5.4 ^{IKOP}
	WI 10min	568.64±7.4 ^{NP}
Black tea (<i>Camellia sinensis</i> , <i>Theaceae</i>)	SWE 1 st 2min	283.82±15.4 ^{A-G}
	SWE 2 nd 2min	402.17±4.2 ^{AHIJK}
	SWE 3 rd 2min	524.06±7.5 ^{BHL-O}
	SWE 4 th 2min	528.52±13.5 ^{CIPQR}
	SWE 5 th 2min	574.34±7.6 ^{JSTU}
	SWE 1 st 5min	372.76±7.6 ^{DLPSVW}
	SWE 2 nd 5min	650.55±8.3 ^{EKMQVXY}
	SWE 10min	414.79±13.4 ^{FNRTX}
	WI 10min	453.47±5.1 ^{GOUWY}
Mate (<i>Ilex paraguariensis</i> , <i>Aquifoliaceae</i>)	SWE 1 st 2min	459.08±3.6
	SWE 2 nd 2min	481.16±4.6
	SWE 3 rd 2min	530.37±5.6
	SWE 4 th 2min	543.31±59.6
	SWE 5 th 2min	521.51±7.6
	SWE 1 st 5min	482.36±4.7
	SWE 2 nd 5min	540.31±42.5
	SWE 10min	448.79±6.1
	WI 10min	492.11±15.1
Trolox		None

Notes: SWE- subcritical water extraction; WI- water infusion; *-same letters means the values are significantly different (p<0.05); IC₅₀- half maximal inhibitory concentration; SD- standard deviation

5.4. Pearson product moment correlation

The Pearson product moment correlation was performed to determine the correlation between antioxidant activity and TPC. It has been found that in green tea, there are strong correlations between DPPH and TPC ($r = 0.72$) and very strong correlations between ORAC and TPC ($r = 0.81$; both significantly different at $p < 0.05$). In case of black tea, there is very strong correlations between DPPH and TPC ($r = 0.96$), ORAC and TPC ($r = 0.91$), both are significantly different at ($p < 0.05$). In mate, a very weak correlations were found between DPPH and TPC ($r = 0.09$), while correlation was very strong between ORAC and TPC ($r = 0.83$) which are significantly different at $p < 0.05$.

Overall, strong correlations were found between DPPH and TPC ($r = 0.75$) while correlations were moderate between ORAC and TPC ($r = 0.53$) both are significantly different at ($p < 0.05$).

6. Discussion

C. sinensis is the most common, popular, and widely used drink all over the world which is a potential source of antioxidant (Chan et al. 2007; Roshanak et al. 2016). Their antioxidant activity can be measured by various methods while the credibility and complexity of the results increases with various methods of evaluation (Maslov et al. 2022). In this study, the *in vitro* antioxidant activity of SWE extracts and WI of green tea, black tea and mate was determined by DPPH and ORAC assays. Moreover, phenolic substances especially catechins, flavonoids and phenolic acids are the most effective antioxidants (Kumar & Pandey 2013) which were quantified by the Folin-Ciocalteu assay.

The ORAC assay measures free-radical damage to a fluorescent probe by changing its fluorescence intensity. The change of fluorescence intensity indicates the degree of free-radical damage. The ability of antioxidants to prevent free-radical damage is stated as the degree of protection against the change of probe fluorescence in the ORAC assay (Huang et al. 2002). The antioxidant activity is evaluated by DPPH radical uptake method because it is simple and easy method that uses a small amount of sample (Hanani et al. 2005). Moreover, this method does not need a substrate as free radicals are directly available to replace the substrate. DPPH molecule is a free radical molecule in the presence of electrons delocalization around the molecule (Permana et al. 2003). IC_{50} value states the antioxidant concentration ($\mu\text{g/mL}$) inhibiting 50% of free radicals. The smaller the IC_{50} value means the antioxidant activity become stronger (Indarti et al. 2019).

The differences among different types of teas are due to differences in the plant variety, growth conditions and processing methods that produce variations in the chemical compositions of products. Different methods of manufacturing are also responsible for the difference in the chemical compositions of teas (Kyle et al. 2007; Yao et al. 2006). By focusing on WI and SWE of teas and tea-like plants, several studies agreed with our DPPH and ORAC results such as Unachukwu et al. (2010) investigated that green tea with IC_{50} 23.26 $\mu\text{g/mL}$ exhibit higher antioxidant activity than white tea that has fewer non-catechin antioxidants. As described by Indarti et al. (2019) that ethanolic extract (by using 96% ethanol) having the IC_{50} value 9.017 $\mu\text{g/mL}$ in green tea due to higher total phenolic and flavonoid content contains polar and semipolar compounds. These results can correlate with our results WI 10min in which green tea showed

weaker antioxidant activity with IC_{50} (9.56 $\mu\text{g/mL}$). Saito et al. (2007) found strong antioxidant activity of green teas with IC_{50} values ranges from 8.33 to 10.10 $\mu\text{g/mL}$ extracted through the sonication method (water/acetone solvent) that efficiently extract epigallocatechin gallate and epicatechin gallate. In our results, the strong antioxidant activities were obtained in SWE 10min, 3rd 2min, 4th 2min and 5th 2min fractions with IC_{50} in range from 7.44 to 8.54 $\mu\text{g/mL}$ due to difference in extraction time. Previously studies on green teas showed that the antioxidant capacity and the total polyphenols content in tea extracts correlate with extraction time (Armoskaite et al. 2011; Cheong et al. 2005). Baba et al. (2016) reported that the prolonged extraction time decreases the antioxidant capacity and polyphenols of green tea due to the thermal degradation of the antioxidant components.

In our results, green tea showed highest TPC 778.534 mg GAE/g as compared to black tea and mate. The total phenolic content in green tea was reported as higher than in black tea also by Almajano et al. (2008). Turkmen et al. (2006) demonstrated that in black tea, polyphenol contents were determined by ferrous tartrate method ranged from 2.1 to 131.9 mg GAE/g and Folin-Ciocalteu method from 1.8 to 99.8 mg GAE/g (in our results from 283.823 to 650.554 mg GAE/g) by using 50% dimethylformamide solvent. In case of mate, polyphenol content ranged from 3.6 to 132.5 mg/g by ferrous tartrate method and from 2.6 to 120.4 mg GAE/g (in our results from 448.79 to 543.308 mg GAE/g) by Folin-Ciocalteu method by using (50% acetone solvent) because solvent with different polarity significantly affect the polyphenol content and antioxidant activity (Goli et al. 200).

SWE is a new technique used for the extraction of less-polar compounds that uses only water for short extraction time. Subcritical water is maintained in a liquid state under high pressure at a temperature between 100 and 374°C. Water at a higher temperature weakens the hydrogen bonds and makes subcritical water more similar to less-polar organic solvents by increasing the solubility of less polar phenolics compounds (Ayala et al. 2001; Teo et al. 2010). Combining subcritical water with an organic solvent such as ethanol and methanol has also been used to improve the yield, extraction time and solubility of compounds (Kwon & Chung 2015; Pronyk & Mazza 2009).

In present study, teas and tea-like plant extracts obtained by two extraction methods were compared. In SWE, different fractions were obtained by following different extraction time and these fractions were compared while in WI only one fraction was followed. It is important to optimise the extraction time and temperature to minimise energy cost of the process (Spigno & De Faveri 2007). The effect of extraction time on the polyphenolic content of green tea extracts reveals that with increasing the extraction time from 20 to 40 min, the polyphenolic content in the extract significantly increased from 120.79 to 137.59 GAE/g and 116.59 to 131.37 GAE/g respectively, but further increasing the extraction time up to 120 min decreased the polyphenols (Randhret et al. 2008). Our results had similar trend, that after 5 minutes of extraction, TPC was highest, however, in all ten-minute TPC was lower while highest polyphenolic content was obtained in SWE 2nd 5min fractions (778.54 mg GAE/g and 650.554 mg GAE/g) but it was decreased in 10min fractions (543.29 mg GAE/g and 414.79 mg GAE/g) in both green and black tea. Silva et al. (2007) reported that extended extraction time causes the decomposition and structural destruction of the phenolic compounds even some phenolic compounds can be denatured at high extraction temperature. The studies of Zielinski et al. (2016) optimized the extraction process of phenolic compounds in tea, and optimum conditions were 10min extraction time at 66°C by using 30% ethanol solution.

The study of Perva-Uzunalić et al. (2006) observed the tendency of catechins to degrade during prolonged extraction time in green tea and found highest extraction efficiency at 80°C for 20-30 min of extraction. In another study, the highest TPC (47.5 mg GAE/g) was obtained at the optimum conditions of 60°C extraction temperature and 33min extraction time using ethanol. In study of Kim et al. (2016) the optimization of the TPC, antioxidant activity and EGCG of green tea leaves was evaluated by following extraction times (3-15 min) and extraction temperatures (10-70°C) at different concentrations of ethanol. While the maximum antioxidant activity was obtained at 70°C for 15 min in ethanol.

7. Conclusions

In our study, antioxidant activity and TPC of different SWE fractions (1st 2min, 2nd 2min, 3rd 2min, 4th 2min, and 5th 2min; 1st 5min and 2nd 5min, and 10min) and water infusion (WI 10min) of green tea, black tea, and mate extracts were compared and statistically compared through student's T-test. SWE is an efficient extraction method that does not use an organic solvent to extract phenolic content and can provide extracts having high biological activities by preventing any toxicity solvents. Our results showed that in *C. sinensis* – green tea, SWE 2nd 5min fractions showed the strongest antioxidant activity evaluated by ORAC assay (IC₅₀ 6.50 µg/mL), DPPH assay (IC₅₀ 9.73 µg/mL) and highest TPC evaluated by Folin-Ciocalteu assay (778.53 mg GAE/g). Similarly, in *C. sinensis* – black tea, SWE 2nd 5min fractions the strongest antioxidant activity IC₅₀ 6.43 µg/mL was evaluated with ORAC assay, IC₅₀ 16.23 µg/mL with DPPH assay and highest TPC 650.55 mg GAE/g by Folin-Ciocalteu assay. On the other hand, in case of mate, the strongest antioxidant activity having IC₅₀ 5.55 µg/mL was evaluated by ORAC assay in SWE 3rd 2min fractions, and by DPPH assay strongest antioxidant activity was evaluated in SWE 4th 2min having IC₅₀ 29.16 µg/mL and highest TPC 543.30 mg GAE/g by Folin-Ciocalteu assay in SWE 4th 2min fractions. However, in case of WI antioxidant activity and TPC was weaker in many cases as compared to SWE. Different fractions also showed significant difference ($p < 0.05$) with each other evaluated by student T-test. The Pearson product moment correlation showed a strong correlation between DPPH and TPC ($r = 0.75$).

It can be seen from our studies that the antioxidant activity and TPC yield is affected by different extraction time. As SWE 2nd 5min fraction showed strongest antioxidant activity and TPC in green and black tea. While SWE 1st 2min, 1st 5min in green tea and in black tea showed weakest antioxidant activity and TPC. In mate, SWE 3rd 2min and 4th 2min showed strongest whereas SWE 1st 2min, 10min and WI 10min showed weakest antioxidant activity and TPC. Temperature is also the key factor in SWE due to which different reactions may occur leading to differences in the chemical composition of final extract thus affecting the overall bioactivity. SWE is also an environmentally eco-friendly, faster, and cost-effective extraction technique used in different fields such as foods, pharmaceutical and cosmetics in addition to be a promising method for fractionation.

8. References

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