CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences

Antioxidant Activity of Tea-like Plants Used in the Philippines

MASTER'S THESIS

Prague 2025

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Declaration

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In Prague 24th April 2025

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Bc. Jan Kotyk

Acknowledgements

I would like to sincerely thank to doc. Ing. Johana Rondevaldová, Ph.D. for her help during a difficult situation, when she found and offered me a thesis topic. I greatly appreciate her positive and motivating attitude, willingness to help, and her support gave me strength and encouragement to overcome obstacles and meet deadlines. I also extend my sincere thanks to Ing. Kateřina Berková, who guided me throughout the entire research process, always explained everything clearly, and was ready to advise and assist whenever needed. I would also like to thank the entire Faculty of Tropical AgriSciences for a great experience and the opportunity to confirm that transferring to this faculty for my master's studies was the right decision. Thanks also go to my fellow classmates for the great memories and friendly atmosphere that made study much easier. Last but not least, I express my deep gratitude to my family for their constant support and trust in my decisions, and to my partner, who stood by me and offered me constant reassurance and encouragement throughout all situations during my studies and while coping with challenging moments.

Abstract

This thesis examines the antioxidant activity and total phenolic content (TPC) of selected tea-like plants that are traditionally used in the Philippines, employing various extraction methods to evaluate their efficiency and potential as a natural source of bioactive compounds. The study primarily compares use of Subcritical Hot Water Extraction (SHWE), Hot Water Infusion (HWI), conventional extraction method Soxhlet, and analyses time fractions to determine the optimal conditions for maximizing antioxidant yields. Four plants species (*Blumea balsamifera*, *Clitoria ternatea*, *Ehretia microphylla*, *Lagerstroemia speciosa*) were selected based on their ethnobotanical relevance and reported properties. Extracts were evaluated for antioxidant capacity using both DPPH and ORAC assays, while TPC was quantified using the Folin-Ciocalteu method. Correlation between TPC and antioxidant activity was determined as very strong for both DPPH and ORAC, confirming phenolics as primary contributors. Results demonstrated the extraction efficiency and antioxidant activity varied significantly among species and extraction methods. SHWE generally yielded higher TPC and stronger antioxidant activity in *E. microphylla* and *B. balsamifera*, indicating its sustainability as a green and efficient extraction method. Notably, *E. microphylla* consistently exhibited the highest phenolic content (SHWE 1st 5 min – 870.30 mg GEA/g extract) and antioxidant activity (SHWE 2nd 5 min– 7.23 μg/mL (ORAC), SHWE 10 min – 7.82 μg/mL (DPPH)) across both assays, making it the most promising candidate to perform further research on. In contrast, *L. speciosa* showed better antioxidant activity in HWI extracts, suggesting species-specific responses to extraction approaches. Time fraction analysis revealed that shorter extraction times in SHWE and HWI can achieve comparable or superior antioxidant yield compared to prolonged Soxhlet extraction, highlighting the advantage of faster and environmentally friendly methods. This work underscores the importance of species-specific optimalization of extraction approaches to maximize recovery of bioactive compounds. The findings support that use of SHWE as a green, more sustainable, extraction alternative to the conventional ones, with potential of application in the development of natural antioxidants for food, pharmaceutical, and also cosmetic industries. Further research should focus on refining extraction conditions and exploring the bioavailability.

Key words: antioxidants, green extraction method, Southeast Asia, traditional beverages

Abstrakt

Tato diplomová práce zkoumá antioxidační aktivitu a celkový obsah fenolických látek (TPC) vybraných čajových rostlin, které jsou tradičně využívány na Filipínách, s využitím různých extrakčních metod za účelem zhodnocení jejich účinnosti a potenciálu jako přírodních zdrojů bioaktivních sloučenin. Studie primárně porovnává použití subkritické extrakce horkou vodou (SHWE), horkým nálevem (HWI) a konvenční extrakční metody Soxhlet a analyzuje časové frakce za účelem určení optimálních podmínek pro maximalizaci výtěžnosti antioxidantů. Byly vybrány čtyři rostlinné druhy (*Blumea balsamifera*, *Clitoria ternatea*, *Ehretia microphylla*, *Lagerstroemia speciosa*) na základě jejich etnobotanického významu a uváděných vlastností. Extrakty byly hodnoceny na antioxidační kapacitu pomocí DPPH a ORAC testů, zatímco TPC byl stanoven metodou Folin-Ciocalteu. Korelace mezi TPC byla stanovena jako velmi silná, jak pro DPPH, tak pro ORAC, což potvrzuje, že fenolické sloučeniny jsou hlavními přispěvateli k antioxidačnímu potenciálu. SHWE obecně vedla k vyššímu obsahu fenolických látek a silnější antioxidační aktivitě u *E. microphylla*va *B. balsamifera*, což naznačuje její udržitelnost jako zelené a účinné extrakční metody. Zejména *E. microphylla* vykazovala konzistentně nejvyšší obsah fenolických látek a antioxidační aktivitu (SHWE 2. 5 min– 7.23 μg/mL (ORAC), SHWE 10 min– 7.82 μg/mL (DPPH)) v obou testech, což z ní činí nejperspektivnější kandidátku pro další výzkum. Naproti tomu *L. speciosa* vykazovala vyšší antioxidační aktivitu v extraktech získaných HWI, což naznačuje druhově specifické reakce na extrakční přístupy. Analýza časových frakcí ukázala, že kratší extrakční časy u SHWE a HWI mohou dosáhnout srovnatelné nebo vyšší výtěžnosti antioxidantů než prodloužená Soxhletova extrakce, což zdůrazňuje výhodu rychlejších a šetrnějších metod. Tato práce zdůrazňuje význam druhově specifické optimalizace extrakčních přístupů pro maximalizaci zisku bioaktivních sloučenin. Zjištění podporují využití SHWE jako ekologičtější a udržitelnější alternativy ke konvenčním metodám s potenciálem využití při vývoji přírodních antioxidantů pro potravinářský, farmaceutický i kosmetický průmysl. Další výzkum by se měl zaměřit na zpřesnění extrakčních podmínek a zkoumání biologické dostupnosti

Klíčová slova: antioxidanty, Jihovýchodní Asie, tradiční nápoje, zelená extrakční metoda

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List of the abbreviations used in the thesis

•OH – hydroxyl radical

±SD – Standard Deviation

AAPH – 2,2‘-azobis(2-methylpropionamidine) dihydrochloride

ABTS – 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid))

AIDS – Acquired Immunodeficiency Syndrome

ASE – Accelerated Solvent Extraction

BHA – Butylated Hydroxyanisole

BHT – Butylated Hydroxytoulene

Cu^{+} – cuprous ion

Cu^{2+} - cupric cation

CUPRAC – Cupric Reducing Antioxidant Capacity

DNA – Deoxyribonucleic Acid

DPPH – 2,2-diphenyl-1-picrylhydrazyl

Fe^{2+} – ferrous ions

Fe^{3+} – ferric ions

FRAP – Ferric Reduction Antioxidation Power

GAE – Gallic Acid Equivalent

H_2O – water

HAT – Hydrogen Transfer Assay

HIV – Human Immunodeficiency Virus

HWI – Hot Water Infusion

IC_{50} – Half Maximal Inhibitory Concentration

K_2HPO_4 – dipotassium phosphate

KH_2PO_4 – monopotassium phosphate

MAE – Microwave-assisted extraction

Na_2CO_3 – sodium carbonate

NADPH – nicotinamide adenine dinucleotide phosphate

NCD – non-communicable diseases

NO• – nitric oxide

O_2- – superoxide anion

RNS – Reactive Nitrogen Species

ROS – Reactive Oxygen Species

SET – Single Electron Transfer

SFE – Supercritical Fluid Extraction

SHWE – Subcritical Hot Water Extraction

SOX – Soxhlet

TPC – Total Phenolic Content

UV – ultraviolet

WHO – World Health Organization

1. Introduction

Free radicals are highly reactive molecules that have unpaired electrons that can cause damage to cells, proteins, and DNA. This process is known as oxidative stress and is involved in various non-communicable diseases (NCDs), including cardiovascular diseases, cancer, and neurodegenerative disorders like Parkinson's and Alzheimer's. The reactivity of free radicals come from their ability to interact cellular components. That leads to series of cellular damage and causes the aging process, and the development diseases related to aging (Halliwell 2007). While free radicals normally play role in physiological processes like signalling cells and immune defence, excessive production overwhelms the antioxidant defence of the body. This is caused by environmental stressors like UV radiation, pollution, and toxins (Hermes-Lima 2004).

Antioxidants are molecules that protect cells from being damaged by free radicals by neutralizing them (Lobo et al. 2010). These protecting instruments can be classified as either endogenous (produced within the body) or exogenous (dietary source). For example, endogenous antioxidants, such as glutathione peroxidase and superoxide dismutase convert harmful reactive oxygen species into less reactive molecules. Whereas exogenous antioxidants (e.g. vitamin C, E, polyphenols, and carotenoids) directly scavenge free radicals or help to regenerate the antioxidant system of the body (Birben et al. 2012a). Thus, compounds with antioxidant properties can lower the risk of the development of NCDs.

Rich sources in our diet are mainly fruits, vegetables, but also various beverages especially tea or teas. Besides *Camelia sinensis* various number of plant species are used in traditional beverages worldwide. Many of these plants have not been tested yet, thus their antioxidant protentional is yet unknown. Testing new tea-like plants from areas like Philippines for their antioxidant properties is essential due to the rich biodiversity of local flora. That is great fundamental for discovering protentional novel unique antioxidants. They might surpass those found in traditional teas like green or black tea, enhancing disease prevention and health promotion. Furthermore, identifying these properties could also stimulate local agriculture, supporting economic development and sustainable practices. This corresponds with the increasing global demand for natural health products

and positions the Philippines as an arising leader in the natural tea market (Cabrera et al. 2006a; Khan & Mukhtar 2007a),

There are several methods to obtain antioxidants from plant samples. Green extraction methods, such as Subcritical Hot Water Extraction (SHWE), provide significant advantages over traditional solvent-based techniques, such as methanol extraction. Traditional solvent extraction is reliant on large volumes of potentially hazardous organic solvents, that pose risks to both environment and the human. In contrast, green methods utilize environmentally harmless solvents like supercritical H_2O. This reduces the usage of harmful solvents and aligns with eco-friendly sustainable practices (Chemat et al. 2012). Additionally, green techniques minimize generation of the waste and its associated environmental risks, enhancing their sustainability. Thanks to optimizing parameters such as temperature and pressure, selective extraction is enabled, reducing co-extraction of unwanted impurities (by-products) and the requirement for comprehensive purification measures (Herrero & Ibañez 2018). Energy efficiency is another notable advantage. While conventional methods often involve long processing times and notable energy consumption for solvent evaporation, SHWE and other green methods are designed to operation under controlled, more energy efficient conditions (Azmir et al. 2013). In view of above-mentioned fact, SHWE seems to be effective tool to obtain environmentally friendly extract with health beneficial properties.

I chose this topic due to my strong interest in discovering novel health-beneficial approaches through the food and beverages that we intake. My focus comes from concerns about the health of my close family and myself. I am aware of how every choice I make in terms of a diet and drink every day can significantly influence my overall quality of life. In nowadays environment, we are constantly surrounded by processed food, stress, air pollution, and UV radiation, which contribute to generation of free radicals in our bodies and can cause oxidative stress and development of NCDs. By deeper understanding of this topic, I would like to prevent my family and myself from excess oxidative stress and thus possible health issues. On top of it I seek greener approach on daily basis. Thus, I find the topic on green extraction methods of antioxidants from plants worthwhile. Current methods in any field need to be re-valuated and efforts should be made to discover new, greener approaches that ensure sustainable development without

causing significant negative impact on the environment, but also to identify traditional beverage plants and their antioxidant potential.

2. Literature review

2.1. Free radicals and oxidative stress

Free radicals are highly reactive molecules characterized by the presence of unpaired electrons. That cause them unstable and prone to react with cellular component such as lipids, proteins, and DNA. This reactivity initiates a set of molecular events leading to cellular damage, termed oxidative stress (Halliwell 2007). Free radicals are commonly classified into reactive nitrogen species (RNS) and reactive oxygen species (ROS). Examples include nitric oxide (NO•), superoxide anion (O_2^-), and hydroxyl radicals (•OH). These are naturally produced by biological systems as by-product of regular metabolic processes, such as mitochondrial respiration and NADPH involving enzymatic reactions and cytochrome P450 (Valko et al. 2006). Exogenous sources, such as UV radiation, air pollution, exposure to certain toxins, smoking, and heavy alcohol consumption, can support the generation of free radicals. These external factors aggravate the oxidative burden on cells, specifically when the body's antioxidant defences are weakened (Hermes-Lima 2004). The production of ROS and RNS can be significantly increased by these environmental stressors, resulting into a pathogenical state that disbalance the delicate equilibrium between the free radicals and antioxidant defences (Sies 1997; Halliwell & Gutteridge 2015).

2.1.1. Oxidative stress of cells

Oxidative stress is a result of exceeded production of free radicals over the body's antioxidant defensive mechanism. This results in an imbalance that leads to molecular and cellular damage. Oxidative modification of lipids, nucleic acid, proteins, and disrupting cellular integrity and function are caused by this imbalance. Lipid peroxidation, a signature of oxidative stress, includes the oxidative degradation of polyunsaturated fatty acids within the lipid bilayer of cell membranes. This process degrades the fluidity of the cell membrane, increases permeability, and can result into cell destruction (Halliwell 2007).

Proteins are comparably affected by oxidative stress through the carbonyl groups formation, aggregation, and enzymatic inactivation, impairing cellular metabolic and

signalling pathways. Another significant consequence is DNA damage, along with oxidative stress including strand breaks, base modification, and cross-linking, for instance formation of mutagenic lesions like 8-hydroxy-2'-deoxyguanosine. These molecular successions can lead to genomic instability, mutations, and the trigger of carcinogenesis (Hermes-Lima 2004; Halliwell 2007).

The effect of oxidative stress that has influence beyond molecular damage, contributing to process of aging and the development of diseases. Neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, cardiovascular diseases, cancer, and diabetes are associated with chronic oxidative stress. The free radical theory highlighted its role in aging, where it proposes that accumulated oxidative damage over the time is a catalyst of the aging process and pathologies related to the age (Finkel & Holbrook 2000). Despite that oxidative stress is largely harmful, it also plays its role in normal physiological processes, such as immune responses and cellular signalling. Nevertheless, its pathological result underscore the importance of keeping a balance between free radicals and the antioxidant defence, both exogenous and endogenous. Dietary antioxidant intake to mitigate oxidative stress and the enhancement of antioxidant defences are essential for promoting cellular health and preventing disease outbreak (Birben et al. 2012b).

2.1.2. Non-communicable diseases

Non-communicable diseases (NCD), frankly called civilisation diseases, represent a global health issue, with chronic respiratory disorder, cancer, diabetes, heart disease, and mental health conditions that account for about 71 % of all deaths worldwide. This is equal to 41 million deaths each year (WHO 2021). When focused on particular continents, across most of Africa, communicable diseases like HIV/AIDS and malaria remain the main problem, however, NCDs such as diabetes and cardiovascular disorders are on their rise which is associated with urbanization and lifestyle changes (Gouda et al. 2019). Similar experience has Asia, dealing with transition of lifestyle, a surge in obesity, and hypertension to shifting diet followed by rapid industrialization (NCD Risk Factor Collaboration (NCD-RisC) 2019). In Europe, aging population is dealing with driving up rates of dementia and cancer, forcing healthcare system to come up with cost-effective prevention and telehealth (WHO 2021). North and South America continue to fight with

obesity, mental health disparities, and metabolic disorders characterized by both high-resource medical care and harsh disbalance (Freedman et al. 2017). In the Middle East, regions affected by conflict cope with infectious disease outbreaks beside rising conditions related to lifestyle (GBD 2015 Eastern Mediterranean Region Cardiovascular Disease Collaborators 2018). Meanwhile Oceania is challenging climate change and a higher prevalence of diabetes type 2 and heart diseases, especially in urban areas (Grant et al. 2010).

In general, urban living and socio-economic shifts lead to diet rich in energy, but poor in nutrients, followed by declining physical activity. These changes align with alcohol consumption, tobacco use, and pollutants that persist in our environment, all these elevate individual's susceptibility to NCDs (Mozaffarian 2016). Some targeted approaches, such as community-based nutrition programs and sugar taxes, prove to be promising tools for curbing obesity, but require financial and political support to achieve broader impact.

Another critical dimension of NCDs are mental health disorders, including anxiety, depression, and conditions worsened by psychosocial stressors (Patel et al. 2018; WHO 2021). Rapid sociocultural changes, covering migration, urban sprawl and global economic pressure, amplify these mental conditions. Telepsychiatry, digital platforms, and the integration of mental health services into primary care have drawn attention as a possible strategies for closing treatment gaps (Jefee-Bahloul 2014).

Environmental factors furthermore support the rise of NCDs. Air pollution has been associated with respiratory and cardiovascular problems, while the climate change supports shifting disease vectors, food insecurity, and extreme weather events (Romanello et al. 2021). On top of that, increased exposure to chemicals disrupting endocrine and other pollutants underline the connection between environmental quality and chronic disease outcome (GBD 2019 Risk Factors Collaborators 2020). These trends determine the complexity of tackling NCDs, requiring multi-sector engagement that incorporates community-based interventions, improvements, and regulatory actions in the health infrastructure.

The NDCs collectively have a significant impact on health trends globally. Addressing this challenge calls for coordinated effort throughout various levels, including increasing physical activity, promoting healthier diets, reducing alcohol consumption

followed by tobacco use, securing access to prevention screening and treatment, and forcing the social determination of health. The concurrent rise of NCDs and persistent infectious diseases underline the critical need for comprehensive health strategies that can adapt to diverse economic, cultural, and environmental conditions worldwide (WHO 2021).

2.2. Antioxidants

Antioxidants are compounds that reduce the harmful effects of oxidative stress by neutralizing free radicals and thus are effective tool to prevent NCDs occurrence (Carocho et al. 2018). They accomplish this by searching directly for reactive molecules or by bonding pro-oxidative metal ions, thus preventing the initiation or spread of oxidation reaction. Numerous plant-base food, such as vegetables, fruits, seeds, and nuts, but also beverages such as tea or coffee are rich in antioxidants. Human cells also produce certain antioxidant enzymes endogenously. The potentional health benefits of antioxidants have been tested across various chronic conditions, including certain cancers, cardiovascular diseases, and neurodegenerative disorders. Nevertheless, the precision of mechanisms of antioxidant action and the best intake levels are still topics of ongoing researches (Sies 2020a).

To evaluate antioxidant activity various methods exist to quantify the antioxidant capacity, where each rely on different reaction mechanisms. These are broadly categorized into hydrogen atom transfer assays (HAT), single electron transfer (SET) assays, and mixed-mode assays, where both mechanisms are incorporated (Apak et al. 2017). The choice of methods on factors such as the experimental conditions, the nature of the antioxidant, and the type of reactive species used for neutralisation. HAT assays measure the ability of the antioxidant to donate a hydrogen atom to stabilize the free radicals. One widely used HAT assay is the Oxygen Radical Absorbance Capacity (ORAC), where antioxidant effectiveness in scavenging peroxyl radicals is quantified. Fluorescence decay of a probe such as fluorescein is used to monitor the neutralisation of radicals (Ou et al. 2001a). Thy Hydroxyl Radical Antioxidant Capacity (HORAC) assay is another important method used. This method assesses the ability of antioxidants to scavenge radicals from the hydroxyl group, which is considered one of the most damaging reactive oxygen species (Prior et al. 2005). On the other hand, SET-based assays evaluate

an antioxidant's ability to donate an electron, which results in measurable colour change. The Ferric Reduction Antioxidant Power (FRAP) assay quantifies the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). The estimation of the total reducing power of the sample as a result is provided (Benzie & Strain 1996). A similar method, Cupric Reducing Antioxidant Capacity (CUPRAC), measures the copper reduction to Cu^{+} from Cu^{2+}, which produces a stable coloured complex (Apak et al. 2004). Specific assays integrate both HAT and SET mechanisms, which provides a more comprehensive evaluation of the antioxidant capacity. The ABTS Radical Cation Decolorization Assays uses $ABTS^{\bullet+}$ radical cation, which is reduced if antioxidants are present, which leads to a decrease in absorbance at 734 nm (Re et al. 1999). DPPH (2,2-diphenyl-1-picrylhydrazyl) assay evaluates the reduction of the stable DPPH• by antioxidants. This results in a measurable decrease in absorbance at 517 nm (Brand-Williams et al. 1995). As an indirect measurement, the Total Phenolic Content (TPC) can be done, where the Folin-Ciocalteu method, which involves a reagent containing molybdenum and tungsten ions, is performed (Folin & Ciocalteu 1927). Phenolic compounds in the sample do react to form a coloured complex. Since TPC provides a quantitative measure of these compounds, we can determine if the antioxidant activity, proven by other more accurate direct methods, was caused by the presence of phenolic compounds or might be caused by other compounds such as vitamins.

As no single assay fully represents the broad spectrum of mechanisms of antioxidants, multiple complementary methods are most often used to achieve a more complex understanding of an antioxidant potential. The results of these assays are crucial for application in food science, pharmacology, and most importantly medical research, where antioxidants play a key role in preventing diseases and promoting human health.

2.2.1. Types of antioxidants

Antioxidants are commonly grouped into two main categories: natural and synthetic. Natural antioxidants can be found in plants and other organisms, that provide defence against oxidative stress (Carocho et al. 2018). Numerous fruits, vegetables, herbs, nuts, and other species contain higher levels of phenolic compounds, carotenoids, and antioxidating vitamins. These molecules are not only able to preserve the strength of biological cells but may also deliver health benefits related to preventing or delaying

chronic diseases. Synthetic antioxidants, such as butylated hydroxyanisole, tert-butylhydroquinone, and butylated hydroxytoluene, have played an important role in prolonging the shelf life of food products and preventing rancidity. Yet current debates focus on the safety of long-term use and searching for alternative food preservatives (Carocho & Ferreira 2013). Consumers prefer products labelled as "natural" or either "clean" that initiated considerable research into developing novel sources of novel antioxidants, as well as safer synthetics with lower toxicology profiles (Carocho et al. 2018). There are another two groups that antioxidants might be put into. Endogenous antioxidants, also including enzymatic systems like superoxide dismutase and catalase, come from within the body to neutralise the reactive species that are produced by normal metabolism (Sies 2020b). Exogenous on the other hand derive from the external sources. Mostly comes from dietary intake such as fruits, vegetables, spices, herbs, and beverages such as tea.

In addition to mention classification above, vitamins and phenolic compounds represents two essential classes of exogenous antioxidants. Vitamins, including vitamin C (ascorbic acid), and E (tocopherols and tocotrienols), are recognized micronutrients for their redox properties and influence on many biochemical processes (Carr & Frei 1999). Vitamin C is soluble in water, which is relevant for collagen synthesis and iron absorption, while the E is lipophilic and often associated with stability of the membrane. Phenolic compounds such as phenolic acids, flavonoids, and tannins are all characterized by one or more hydroxyl groups on the aromatic rings (Shahidi & Ambigaipalan 2015). Both vitamins and phenolic compounds can show considerable chemical diversity which also vary in degrees of their bioavailability, reflecting the complexity of their roles in the biological systems (Krinsky & Johnson 2005).

2.2.2. Source of antioxidants

Fruits, vegetables, nuts, and seeds often contains notable amounts of phenolic compounds, which include flavonoids, phenolic acids, stilbenes, and tannins (Manach et al. 2004). Onions, kale, and apples provide flavonols like a quercetin, while cocoa products and some teas are rich in flavan-3-ols (epicatechin, catechins). Phenolics acids like gallic and ferulic acid can be found in fruits, vegetables, and also cereals, while stilbenes such as resveratrol in grapes and red wine. Berries and cherries feature

anthocyanins, and carotenoids such as beta-carotene, lutein, and lycoepene, can be found in carrots, spinach, and tomatoes (Rodriguez-Amaya 2016). Vitamins also figure significantly among dietary antioxidants. Vitamin C is abundant in citruses, broccoli, and bell peppers, while Vitamin E can be found in nuts, plant oils, and seeds (Del Rio et al. 2013). These micronutrients help to preserve the structural integrity of cells and may assist to various physiological processes. Beyond the whole food, beverages enhance further intake of antioxidants. Green tea and coffee deliver chlorogenic acids, and catechins, while traditional drinks like yerba mate or kombucha contain unique phenolic compound that may support metabolic health (Chandrasekara & Shahidi 2018; Abaci et al. 2022). By diversifying one's diet that includes fruits, vegetables, seeds, nuts, and support it by beverages from tea or tea-like plants, the intake of bioactive compounds would be maximised.

2.2.3. Use of antioxidants in human life

Dietary supplements containing antioxidants become frequently introduced to mitigate oxidative stress that is linked to chronic diseases, inflammation, and aging. Nevertheless, the actual benefits can vary according to overall diet quality and factors or individuals metabolism (Tan et al. 2018). Some antioxidants may work the best when combined with synergistic compounds, such as Vitamin C enhancing Vitamin E regeneration, or polyphenols that work alongside carotenoids to provide superior antioxidant protection (Michalak 2022). Skincare formulas frequently include antioxidants to combat photoaging, pollution stressors, and oxidative damage from UC radiation. Vitamin C is used for its reducing hyperpigmentation, promoting collagen synthesis properties (Grether-Beck et al. 2014). It is often stabilized as a magnesium ascorbyl phosphate or ascorbyl palmitate to prevent degradation. Vitamin E protects lipid membranes from oxidation and its often used in conjunction with Vitamin C to regenerate its antioxidant capacity. Coenzyme Q10 (Ubiquinone) is a mitochondrial antioxidant which enhances production of cellular energy, reduces oxidative stress, and strengthens skin barrier (Vinardell & Mitjans 2015). Polyphenols derived from green tea, berries, grapes, and cocoa help mitigate inflammation, reinforce skin elasticity, and protect against DNA damage (Michalak 2022). Pigmented antioxidants such as Beta-Carotene, Lutein, and Lycopene neutralize singlet oxygen radicals, contribute to anti-aging effects, and enhance skin photoprotection (Budzianowska et al. 2025). Beyond direct application

on skin, functional beverages such as green tea, coffee, and yerba mate help maintain skin hydration, elasticity, and protection against oxidative stress. In addition to beauty and health benefits, antioxidants play a key role in stability of food, preventing oxidative degradation of essential vitamins, pigments, lipids, which help to maintain colour, flavour, and nutritional value while the shelf life is extended (Carocho et al. 2018). Tocopherols, Vitamin E derivates, used in preserving oils and nuts by preventing lipid oxidation. Vitamin C and its derivates helps with freshness in fruits and vegetables by preventing enzymatic browning and enhances the stability of beverages and cured meats. Catechins and flavonoids provide antimicrobial and antioxidant effects in meat, dairy, and beverages products (Awad et al. 2021). Chlorogenic acid, found in coffee and potatoes, prevents the rancidity of oil, reduces lipid oxidation, and acts as a natural preservative (Gutiérrez-Del-Río et al. 2021). Ferulic and gallic acid is used for their preservative effects, especially in sauces, bakery, and alcoholic beverages (Codina-Torrella et al. 2021). The increasing demand for natural antioxidants instead of synthetic stabilizers (such as BHT, BHA) emphasise consumer preference for cleaner and greener foods, preferring plant-derived alternatives (Nieto et al. 2023). The broad application of antioxidants in cosmetics, food technology, and healthcare underscores the immense biological and economic significance. Nevertheless, challenges with formulations remain as some antioxidants, such as Vitamin C, are unstable in formulation and require encapsulation or synergistic stabilizers (as ferulic acid in skincare). Optimizing concentrations to balance efficiency with safety in food, skincare, and health remains in ongoing studies as well (Pedro et al. 2022; Sezgin-Bayindir et al. 2023).

2.2.4. Extraction methods

Extraction techniques are key for obtaining antioxidants from plant-based sources. Significantly affecting bioactivity, yield, and purity of the final product. Key factors such as processing time, solvent selection, and temperature must be carefully optimized to prevent thermal or oxidative degradation of sensitive compounds (Zhang et al. 2018b). The choice of method depends on the properties of the source material and the intended application. Balancing yield and bioactivity are essential. Harsher conditions or extended extraction times can increase compound recovery but may also alter their structure or reduce its biological effectiveness. On the contrary, gentler extractions better preserve delicate flavonoids and phenolics but at cost of lower yields. Modern, more sustainable

methods aim to minimize use of solver and energy consumption while enhancing efficiency (Ivanova Stojcheva & Quintela 2022). The ideal extraction strategy must align with the material's characteristics and the target industry (e.g. functional foods, cosmetics, dietary supplements). Preciously adjusting extraction parameters ensures high-quality extracts that retain their bioactivity, meeting both industry requirements and consumer expectations (Zhang et al. 2024).

2.2.4.1. Conventional Extraction Methods

Conventional extraction methods, such as maceration, methanol extraction or hot infusion remain being widely used in obtaining antioxidant compound for their simplicity, general reliability, and cost-effectiveness (Zhang et al. 2018a).

Maceration is among the simplest methods, where dried and grinded plant material is submerged in a suitable solvent (e.g. ethanol, methanol, or water) at room temperature or gently elevated temperatures for several days. The mixture is then strained, extract I filtered and usually sealed in a container to reduce solvent loss. Even though maceration is often time-consuming method with lesser efficacy, optimized conditions can yield high levels of phenolics and anthocyanins as found in chokeberry extraction (Ćujić et al. 2016). Digestion is similar to maceration, but mild heating is require to enhance extraction efficiency when tougher materials are used. Typical temperatures are around 35-40 °C (up to 50 °C). Plant material is placed in a container with solvent, and the mixture is then kept under desired conditions for 30 min to 24 h with intermittent stir (Mondal et al. 2024). Infusion is described as a dilute solution of constituents of the plant material that soluble with ease. In this technique, the plant material is immersed in boiling solvent, particularly water, and left for 15 min. After this time the extract separated from the marc by filtration (Azwanida 2015). Percolation is widely used for obtaining fluid extracts, including tinctures, by passing a solvent (often ethyl alcohol) through a plant material drop by drop. The solvent becomes saturated with phytochemicals as is runs through the plant material downwards while fresh one is added from the top. The used plant material should be shredded but not overly fine, otherwise difficulties in separating particles from the solvent might appear. Slight pre-moistening of the sample can helps the cells to expand and facilitate diffusion of phytochemicals into the medium (Cittan et al. 2018). Once the plant material is placed in the percolator, solvent is poured from the top. The flow rate is regulated and should not be too rapid, to ensure adequate contact time, nor

too slow. The generally used flow rate should be sat to 5 mL per minute for 1 kg of plant material. Method where plant material is boiled in water for 15 to 60 min is called Decoction. This method is suitable for phytochemicals that remain stable even at elevated temperatures. Delicate parts such as flowers, leaves, roots typically require 15 minutes of boiling, while tender parts like branches or bark may require up to an hour. After boiling is done, the mixture is left to cool down, then its strained and diluted with cold water do get back the desired volume, followed by filtration. This process can yield high amount of target compounds, but it is not the right method for thermolabile compounds and may result into high production of unwanted byproducts (Perera et al. 2017). Soxhlet extraction is a continuous process where hot solvent passes repeatedly though a thimble containing a plant material. Originally this method was to determine fat in milk (Soxhlet 1879). The solvent heated in a flask below, vaporizes and travel upwards, condenses in cooling chamber, and drips back through the sample. This method has higher yields of extract when compared to maceration-based approaches, but even though this technique is quite efficient, the risk of degradation of thermolabile compounds persist (Chin et al. 2013).

The conventional extraction techniques face disadvantages including prolonged extraction time, substantial amount of solvents, or extraction steps. Significant amounts of thermolabile phytochemicals end up either decomposed or degraded during the prolonged exposure.

2.2.4.2. Non-conventional Extraction Methods

Non-conventional (or green) methods aim to protect human health and environment due to the lower solvent consumption, cut extraction times, and protect heat-sensitive compounds. Accelerated Solvent Extraction (ASE) has been gaining traction thanks to its low solvent requirement, relatively short processing time, and high yield. Its elevated temperature and pressure conditions enhance diffusion and reduce viscosity of the solvent, thus providing higher extraction efficiency. In ASE setup, the sample is placed in stain-less steel cell with inert packing material to avoid blockages. Solvent is then pumped into the sample, heated to desired temperature, and kept at elevated pressure for a given time. The extract is collected into vials. The cell is afterwards flushed with fresh solvent and nitrogen to ensure no leftovers (Richter et al. 1996). Another method is called Microwave-Assisted Extraction (MAE), or simply microwave extraction. This

method combines microwave and solvents to extract phytochemicals at frequencies between 300 MHz – 300 GHz. Thanks to targeting polar molecules, heat from microwaves work for both solvent and plant tissue, which accelerate the overall extraction process through dipolar rotations. The efficiency is bond to the solvent's dielectric constant, while lower viscosity aids ion dispersion and solvation (Bagade & Patil 2021). This technique preserves bioactive compounds by simplifying solvent diffusion into the sample, isolating solutes from their binding sites, and releasing them into the solvent. Optimized MAE proved to be efficient in enhancing antioxidant activity and total phenolic content in green tea (Taşkın & Aksoylu Özbek 2020). Another successful application was done where saponins from seeds, polyphenolic antioxidants from leaves, flavonoids from various plant parts, and sterols from dried mushrooms (Hu et al. 2021). Ultrasound-assisted extraction (UAE) is method where waves above the human hearing range (20 – 2000 kHz) are used. It is performed to enhance mass transfer by including acoustic cavitation, the formation, growth, and bubble collapse. This action increases both the surface area of a contact between plant material and the solvent and the permeability of cell walls with easier release of phytochemicals (Machado et al. 2019). Studies show that use of ultrasonic frequency can influence extraction outcomes, for example, a lower frequency of 40 kHz yielded more phenolics than 120 kHz in *Vitis vinifera L.* (González-Centeno et al. 2014). The efficiency is further supported by a study which reports considerably less time, just 30 min and much better yields compared to maceration method which took 120 min and performed lower yields of flavonoids from Lotus (Zhang et al. 2009). Another green-considered non-conventional method is Supercritical Fluid Extraction (SFE) which operates at conditions above a fluid's critical temperature (Tc) and critical pressure (Pc), where the liquid and gas phases become indistinguishable. This supercritical state enables the solvent to retain the solvating properties of a liquid but behaves more as a gas in terms of diffusivity and viscosity. That enables efficient extraction of high-value compounds at relatively low temperatures. Mostly used CO_2 becomes supercritical above 31.1°C and 7,380 kPa (Raja & Barron 2016). In practice, SFE dissolve target molecules as the supercritical solvent passes through a raw material. Before exiting the processor, the extract precipitates from the solution after controlled decrease in pressure (often followed with slight increase in temperature). This method is widely applicated in industry, such as extraction of sterol esters, glycerol-lipids, phospholipids from seeds at 80 MPa and 57°C (Belo et al. 2019), various phenolics,

vitamins, carotenoids, and anthocyanins from sweet cherry leaves at 30 MPa (Zhang et al. 2020a), and alkaloids from seeds at 27 MPa (Rosas-Quina & Mejía-Nova 2021). Furthermore, Enzyme-assisted extraction (EAE) method enhances recovery of phytochemicals from plant material bound within lignin or any other polysaccharides matrices, where the solvent extraction alone is not sufficient. Cell walls are hydrolysed by adding enzymes like pectinase, cellulase, or amylase, releasing previously inaccessible compounds (Calderón-Oliver & Ponce-Alquicira 2021). There are two primary approaches, Enzyme-assisted aqueous extraction (EAAE) and Enzyme-assisted cold pressing (EACP). Both break down plant cell structures to increase extraction efficiency. EAE proved to successfully release non-extractable polyphenols from sweet cherry at 55°C, phenolics from citrus pees at 20 – 60°C, lycopene from tomato peels at 30°C flavonoids from grapefruit peels at 50°C, anthocyanins from Cacahuacintle Maize, and carotenoids from sunflower petals at 40°C (Zuorro et al. 2011; Fernandez-Aulis et al. 2019; Zarate Vilet et al. 2020; Domínguez-Rodríguez et al. 2021; Wang et al. 2023). EAE works even better when it is combined with other techniques. For example, combining enzymes with microwave processing enhanced phenolic extraction from olive pomace at higher temperatures supported by faster heating compared to conventional water-based methods (Macedo et al. 2021). Further, enzymatic treatment followed by ultrasound produced more pectin from sisal waster then in methods where no enzymes were used (Yang et al. 2018). Finally, Pressurized Hot Water Extraction (SHWE), method that utilizes water at elevated pressure (approximately 5MPa) and temperature between 100°C and 250°C to retain in a liquid state beyond the normal boiling point (Curren & King 2001). These conditions make water less polar and can dissolve both polar and non-polar phytochemicals, making it a great alternative to conventional extraction methods. SHWE propose several advantages, such as high-quality extracts, shorter extraction times, and use of water as a cost-effective, environmental friendly solvent. Numerous compounds have been successfully extracting with SHWE method. These compounds include, antioxidants, phenolics, avoparcine, and saponins (Curren & King 2001; Ju & Howard 2003), as well as phenolic compounds followed by vitamin C from *Moringa olifera* leaves at 91°C and 100°C (Nuapia et al. 2020). Notable amount of cannabinoids have been recovered from *Cannabis sativa* seeds at 100°C using SHWE and at 145°C for *in situ* decarboxylation under hot pressurized water conditions (Nuapia et al. 2021). In case the temperature of the water is controlled bellow its critical point of 374°C, the polarity of

the solvent decreases, which enables the dissolution of a broader range of phytochemicals. For example, both polar and non-polar antioxidant, and steviol glycosides, were extracted in efficient amount from *Stevia rebaudiana* leaves at 160°C, yielding even higher concentrations than in those at 100°C (Bursać Kovačević et al. 2018). On the other hand, some compounds may degrade at elevated temperatures, as observed in *Thymus vulgaris*, where the temperature at 200°C reduced both antioxidant activity and polyphenol content (Vergara-Salinas et al. 2012). Furthermore, a study showed that adding natural deep eutectic solvents as co-solvents can even boost extraction yields, thus highly enhancing phenolic recovery from grape pomace at 100°C (Loarce et al. 2020). SHWE emerges a high-efficacy and sustainable method for extracting bioactive compounds from various plant materials. However, the precise extraction conditions significantly influence extract quality and yields, since different phytochemicals exhibit varying degrees of solubility and thermal stability, which may sometimes conflict with one another.

2.3. Tea and tea-like plants

Tea is worldwide known beverage that everyone has drunk, is about to or will drink. It is one of the most important functional foods. That is term first time introduced in 1980 in Japan (Hayat et al. 2015a). Such foods are defined as "Any substance containing ingredients which in addition to fulfil basic nutrition requirements provides physiological benefits.". Teas come in various forms depending on the region of consumption. Tea holds the second place in consumption among all beverages (Hayat et al. 2015a).

Leaves from *Camelia sinensis* has paved its way for more than 50 centuries. The plant itself was originated from the Southeast Asia but it is cultivated in more than 30 countries nowadays. Average of 3 billion kilograms of tea is produced and consumed every year. Most of the production is black tea (78 %), then green tea (20 %), and at last oolong tea (2 %). Each tea is preferably consumed in different part of the world. Black tea is usually consumed in the Western countries, while green tea in Asian, and oolong in southern China where it is also produced by partial fermentation (Cabrera et al. 2006b; Khan & Mukhtar 2007b; Butt & Sultan 2009; Hayat et al. 2015b). These three types of tea differ in the manufacturing process. Green tea fresh leaves are rapidly steamed or either pan-fried to inactivate polyphenol oxidase, thus preventing fermentation. Black and

oolong teas are firstly withered which reduces leaf moisture < 50 %, concentrating polyphenols (PPs). Leaves are then rolled and crushed, which initiates fermentation, during which catechins convert to polymeric compounds (e.g. theaflavins and thearubigins) lowering the amount of catechin. Oolong halts the oxidation sooner, making it roughly half as fermented as black tea. When fermentation extends, PPs decrease and increase caffeine. So black tea contains about 2 – 3 times more caffeine when compared with green tea (Adak & Gabar 2011). *C. sinensis* has many health benefits. This is mainly caused by the antioxidative property of tea PPs, which entrap the ROS. For example, epigallocatechin-3-gallate (EGCG) is the most effective tea catechin that react with most of ROS. *In vitro* research indicates that both green and black teas can inhibit Cu^{2+}, an induced lipoprotein oxidation. That suggest a protective role against arthrosclerosis and other cardiovascular diseases. Clinical trial has also shown that the plasma antioxidant capacity of healthy adults increases within 30 – 60 minutes after a single dose of tea. Another investigation confirmed that repeated intake of tea its encapsulated extracts for 1 to 4 weeks can reduce oxidative markers (Khan & Mukhtar 2007c). Tea should be name only to beverage that is made of *C. sinensis*, such as green, black, or oolong tea. Any other beverages are made of tea-like plants.

Aspalathus linearis or so-called Rooibos is another widely used plant in production of Rooibos tea. *Aspalathus* is from family *Fabaceae*, and its endemic to South Africa. It is an erect, decumbent or ascending shrub up to 2 m tall. Young branches are often of reddish colour. Grean leaves are linear-piniform, 15 – 60 mm long and 0.4 to 1 mm thick. Flowers solitary or in racemes on tips of the branch. Petals are pale to bright yellow or could be partly violet (Dahlgren 1988). This beverage became increasingly popular thanks to its unique taste, versatility, and most importantly the reputation of a healthy drink. This reputation comes from claims of its antioxidant activity and its physiological and therapeutic advantages (Breiter et al. 2011). It has been established that Rooibos tea contains substances which can mimic superoxide dismutase, in its antioxidant activity (Yoshikawa et al. 1990). One of these antioxidants is aspalathin. This compound has been only found in *A. linearis*, thus making the plant's composition unique (Koeppen & ROUX 1965).

Ilex paraguariensis A. St.-Hill from the family *Aquifoliaceae* is evergreen tree that is native to Paraguay, Uruguay, Brazil, and Argentina (Heck & De Mejia 2007). It

can grow up to 18 meters but more likely to be 4 – 8 meters tall. Hard leaves are 8 cm long of olive-green colour with darker tops, they are obovate with wedge-shaped base. The tree blooms from October to November. Silky white flowers, usually with four petals, are gathered in inflorescences at the top of the branches. Fruits are red or reddish-brown berries (5 – 8 mm in diameter) and ripe from March to June (Heck & de Mejia 2007). First harvest of leaves is possible after 3 – 5 years after planting the plant. Subsequent harvests take place every 3 years. The leaves and twigs undergo a couple of processing steps, such as roasting, drying, and ripening. The process determines the properties of the prepared infusion, including the taste and content of bioactive compounds (Heck & de Mejia 2007; Gawron-Gzella et al. 2021).

All plants mentioned above were originated or traditionally consumed in either South America, South Africa, or Asia. Quite wide use of herbs and other plants is connected with Europe. There is slightly difference in plants used in preparation of beverages in each region of Europe. In a study, done to determine locally mostly used plants in beverage preparation, top 10 species were picked which have been used at least in three countries. In all off the interviewed countries *Origanum vulgare* (aerial parts), *Achillea millefolium* (inflorescence, aerial parts, and roots), *Mentha longifolia* (aerial parts), and *Tilia cordata* (bark and complete inflorescence) were traditionally used. Then the plants differed for Southern, Central, and Eastern Europe. For the Southern, *Thymus pulegioides* and *Matricaria chamimolla* were typicall. In the Central Europe, *Rosa canina*, *Hypericum perforatum*, *Vaccinium myrtillus*, *Mentha pulegium*, and *Sambucis nigra* dominated. At last, Eastern Europe used more species such as *Thymus serpyllum*, *Rubus ideaus*, *Vaccinium vitis-idaea*, and *Prunus cerasus* (Sõukand et al. 2013). It could be said that most frequent families mentioned in the study are *Lamiaceae*, *Rosaceae*, and *Asteraceae*. All these plants are traditionally prepared by hot infusion to be consumed either as recreational beverage or in times of cold or any other illness for their health-beneficial properties.

The traditionally used plants in various places in the world give us an insight of what are the potentional novel species to be observed to obtain information on their properties and their health benefits. Some species are yet to be found and described but there are so many species that we are aware of but there have not been tested for their proparties yet. This brings us to SE Asia, the Philippines.

2.3.1. The Philippines

The Philippines (Fig. 1) is situated in SE Asia, on the eastern edge of the Asiatic Mediterranean. It is bordered by the South China Sea from the west, the Pacific Ocean from the east, the Sulu and Celebes Seas from the south, and the Bashi Channel from the north. Manila is the national capital. It serves as its principal port of entry, while Cebu, a south-located city, is the second largest international gateway. Even though Manila is the capital city, Quezon City is the most populated located just next to the capital. Around 113 million people live on this archipelago, of which are is estimated to be about 300 000 km^2 (Tan 2015).

Figure 1: Physical Map of the Philippines (1993)

There are more than 7600 of islands, but there are still new found. The Philippines has about 50 volcanoes, of which more than 10 are active. The most important rivers are Cagayan, Agno, Mindanao and Agusan (Holden et al. 2017). To mention, the most unique waterway lies underground, which emerges directly into the ocean at Puerto Princesa Subterranean River National Park which is listed on the UNESCO World Heritage list since 1999. The climate is tropical and strongly monsoonal. Generally, rain-bearing winds blow from the SW from May to October, and drier ones from the NE from November to February. Most of the vegetation is indigenous and closely mirrors that of Malaysia, with coastal areas (such as mangrove swamps) largely sharing the same flora as similar regions in the Malay Archipelago. Himalayan elements can be found in the mountains of northern Luzon, while some Australian species are found at various elevations. The islands are home to thousands of ferns, and flowering plants, including orchid species, some of which are exceptionally rare (Perez et al. 1999). The Philippines are a global biodiversity hotspot, that emphasises its high endemism (Myers et al. 2000). Fragmented habitats, rapid use of land, and overexploitation are cited as critical drivers of biodiversity loss (Barnosky et al. 2012)

2.3.2. Tea-like plants from the Philippines

Based on the information mentioned above, the Philippines, as a recognized biodiversity hotspot, offers a rich source of underexplored phytochemicals. Their distinct niches and microclimates can result in yields of compounds with potentially high antioxidant capacities. Discovering these novel bioactive compounds could contribute to the development of functional foods. Moreover, investigating local plants promotes ethnopharmacological knowledge and supports conservation efforts.

There is a wide range of traditional beverages consumed in the Philippines. Fermentation has a long tradition and one of the common beverages is fermented Tapuy which is an alcoholic sweet-acid beverage produced by saccharification and fermentation of glutinous white rice (Dela Rosa & Medina 2021). Lambanog is distilled palm liquor, which is either made of coconut, sugar or nipa palm. But there are some Lambanog-related deaths caused by methanol contamination (Caleja-Ballesteros & and Ballesteros 2025). Bignay red wine made of bignay tree is made of its berries (Ocampo 2006). Ginger

tea is another traditional beverage, locally called Salabat, served sliced and mostly sweetened with honey. Batangas coffee is made of beans of *Coffea liberica* that is grown in the Philippines, in province Batangas (Lagman & Carmen 2023). Among above mentioned plants, there are also species that are used in the preparation of hot beverages are mentioned in next subchapters.

2.3.2.1. Blumea balsamifera

Blumea balsamifera (L.) DC., also known as sambong (Fig. 2) is a perennial herb from the family *Asteraceae*, whose leaves are traditionally used to prepare hot tea-like beverages in Southeast Asia. More specifically, the hot beverage is usually prepared to treat Calcium Oxalate Stones in the Philippines (Montealegre & Leon 2016). This herb or subshrub rises to 1 – 3 meters in height. The upper internodes are covered by dense tawny nonglanduar hairs. Wide ovoid to oblong-lanceolate leaves are 22 – 25 cm long and 8-10 cm wide, they have an attenuated base, 3 – 5 pairs of narrow linear appendages, with the above part having pubescent and silky-villous from beneath and 10 – 15 pairs of lateral veins. Upper leaves (7 – 12 cm long and 1.5 – 3.5 cm wide) are oblong-lanceolate or ovate-lanceolate. They bear an acuminate apex, a slightly acuminate base, and either no petiole or just a short one with 1 – 3 pairs of linear appendages. Highly branched leafy panicles, where capitula occur, have yellow, densely pubescent peduncle and a campanulate involucre. Flowers are yellow, with a high number of female parts, a honeycomb-like receptacle, and a tubular corolla. The cylindrical achenes have five edges, red-brown rough hairy pappus, and dense pubescence. *Blumea* flowers almost all year-round and typically inhabits forest edges, riverbeds, valleys, or grasslands around 600 – 1000 m. a. s. l.. Besides Philippines, it also grows in India, Pakistan, Indo-Chinese Peninsula, Malaysia, Indonesia, Myanmar, and China (Y 1983).

Figure 2: *Blumea balsamifera* (L.) DC. (Berková, 2022), habitus (A) and close up photo of the top of the branch baring flowers with a scale (B)

2.3.2.2. Clitoria ternatea

Clitoria ternatea L. commonly known as a Butterfly pea is a perennial leguminous twiner from the family *Fabaceae*. This plant (Fig. 3) found its way to people worldwide. This is due to the rich blue colour of the prepared drink. Thus, becoming a trendy beverage with an uncommon appearance but a regular taste. Twining fine stems can grow from 0.5 to 3 m long. Pinnate leaves have 5 – 7 elliptic to lanceolate leaflets, and they are 3 – 5 cm long with shortly pubescent underneath. Solitary flowers with deep blue to blue mauve and very short pedicellate are 4 – 5 cm long. Pods are linear, flat, beaked, 6 – 12 cm long and 0.7 – 1.2 cm wide. They are slightly pubescent and bear up to 10 seeds. These seeds are brown, olive green, or black, often mottled, and up to 7 mm long and 4 mm wide (Bogdan 1977).

Figure 3 : *Clitoria ternatea* L. (Berková, 2022), plant habitus (A), detail of a flower bud (B)

C. ternatea is a very bioactive plant and is used in various diseases as a folklore medicine (Al-Snafi 2016). Diuretic use of roots and cathartic use of seeds (Evans 2009). It has been widely used in traditional medicine, particularly in Ayurveda. The roots, leaves, and seeds have long been used as a brain tonic and are believed to promote memory and intelligence (Mukherjee et al. 2007). For the treatment of earache, warm leaf juice is mixed with a common salt and applied around the area. Leaves are also used for swollen joints. Seeds are used as mildly laxative, anthelmintic, and purgative. Then used for throat, skin diseases, and eye infections. Root juice can be used in the nose to fight migraine (Gomez & Kalamani 2003; Mukherjee et al. 2007). The methanol extract derived from the aerial parts was evaluated for its effects on seizures in mice using two models, pentylenetetrazol-induced seizures and maximum electroshock-induced seizures. Orally given at a dose of 100 mg/kg, the extract significantly delayed the onset of convulsions and prolonged the duration before tonic hind limb extensions occurred in MES-induced seizures (Sethiya et al. 2009). Flower extracts of *C. ternatea* have been used in Thailand as a component in skin care for their anti-oxidative activity. Aqueous extracts performed better anti-oxidant activity than ethanol ones (Kamkaen & Wilkinson 2009). Leaf extract showed stronger antibacterial activity than extract from roots. The powerful activity of leaves was found against *E. coli* and *V. cholera*, known for dysentery

causes, and *S. aureus*, an agent of fever. Quercetin may contribute to this leaf extract activity (Mazumder et al. 2008).

2.3.2.3. Ehretia microphylla

Ehretia microphylla Lam. (notable synonym *Carmona microphylla* (Lam.) G.Don.) is a shrub (Fig. 4) 1 – 3 m tall from the family *Boraginaceae*. The common name for *E. microphylla* is Tsaang gubat or the Philippines tea tree, where it is also traditionally consumed as tea substitute (Eclarin & Nas 2023). It is an evergreen shrub native to India, Ryukyu Islands of Japan, Taiwan, Guandong and Hainan China, Indonesia, Papua New Guinea, Sri Lanka, Solomon Islands, Indochina, Malaysia, and also the Philippines (Raterta et al. 2014). The shrub can reach from 1.5 to 4 meters and is highly branched. The bark, up to 3 mm thick, is divaricated and brown. Small rounded and obovate leaves are around 1 – 25 cm long and 4 – 8 mm thick with lobed apex and short bristly hairs. The white flowers are axillary, solitary or in pairs on a slender and hairy peduncle (Ghorbani et al. 2006). Orange to scarlet drupe fruits have a globose shape and a diameter of 4 mm. The endocarps are hard with one-celled pyrene. Brown roots are cylinder-shaped with approximately 10 – 40 mm in diameter (Mageswari & Karpagam 2015).

Figure 4: *Ehretia microphylla* Lam. (Berková, 2022), detailed branch with a scale (A), habitus of the plant at the place of collection (B)

E. microphylla has been recognized as a scientifically validated plant of medicinal value by the Department of Health in the Philippines ("Directory of Herbs | PITAHC" n.d.). *E. microphylla* has been used to treat diarrhea, inflammation, gastrointestinal, spasm, and biliary colic in the Philippines (Mageswari & Karpagam 2015). In Indian traditional medicine, the leaves were used as a relief from stomachache, fever, cough, and constitutional syphilis. To be more specific, the roots were used especially in Southern India to counter the effects of phytotoxins and syphilis (Padua 1977). The leaves were also used as a decoction to treat again cough, and stomachache, but also diarrhea with a bloody discharge and dysentery (Amudha & Rani 2014). To improve women fertility the leaves were used (Ignacimuthu et al. 2006), while roots performed anti-venom ability (Amudha & Rani 2014).

2.3.2.4. Lagerstroemia speciosa

Lagerstroemia speciosa (L.) Martyn is a medium to large-scaled evergreen tree (Fig. 5) with hight up to 25 m. *L. speciosa,* commonly known as Banaba (Stohs et al. 2012) is from the family *Lythtraceae*. Traditionally is prepared as a hot herbal folkloric beverage in the Philippines (Solidum 2014). The leaves are oppositely arranged, with a leather texture and an oblong to ovate shape. They are smooth, 10 – 20 cm long and 5 – 7.5 cm wide, with a short petiole. The flowers grow in large terminal panicles, regular in shape and varying in colour from pink to purple. Flowers range in size from 5 to 7.5 cm in width. Green calyx, ribbed tube featuring six leathery sepal lobes and six lilacs to purple petals with wavy margins, where each is up to 3.5 cm long and attached to sepals via short claws. The stamens are numerous, with purple filament and yellow-golden anthers. Simple pistils, consisting of a long purple style that extends up to 5 cm, stigma is dark green, and superior ovary. The seeds are winged (Castro 2006).

Figure 5: ***Lagerstroemia speciosa*** **(L.) Martyn (**Berková, 2022), plant habitus of the plant at the place of collection (A) and detailed top of the branch with flowers and scale (B)

The plant has been traditionally used for the treatment of diabetes mellitus and weight loss. Its leaves, bark, and flower have been utilized as a purgative. A decoction or infusion of the leaves has been employed to treat bladder and inflammation of kidneys, dysuria, and other urinary dysfunctions, as well as to lower cholesterol levels, control diabetes, and manage hypertension. Poultices from the leaves have been applied to alleviate malaria, headaches, and cracked heels by direct application to the areas affected. Moreover, a decoction of the bark has been used in addressing gastrointestinal disturbance, haematuria, depression, and stomach-aches. Narcotic properties have been found in the seeds (Vardhana 2008). Flower extract showed to have hypoglycemic effect when examined on mice. The extract (400 mg/kg dose) reduced blood sugar level by 56.12 % after three hours of administration of glucose solution (Sharmin et al. 2018). The essential oil from the LS fruits possessed cytotoxicity of value 1.701 µg/mL for IC_{50} in brine shrimp toxicity assay (Oloyede et al. 2011).

3. Aims of the Thesis

The main aim of the thesis was to evaluate the potential of the SHWE method, which was considered a green one, for the recovery of bioactive compounds from tea-like plants, with a focus on antioxidant activity. The research was motivated by the fact that while common plants like *Camellia sinensis*, *Aspalathus linearis*, and *Ilex paraguariensis*, as sources of antioxidants, had been extensively studied, many other tropical plants traditionally use for the preparation of hot beverages remained unexplored in terms of their bioactive potential. Thus, the study contributed to the advancement of sustainable green extraction methods while expanding the knowledge on the antioxidant properties of the traditional lesser-known tropical plants. This research was based on the hypothesis of SHWE being able to provide a higher yield of bioactive compounds, and that these extracts would demonstrate superior antioxidant potential when compared to extracts obtained using conventional methods.

The specific objectives were:

a) Selection of plant species traditionally used for the preparation of the hot beverages in the Philippines
b) Application of various extraction methods (conventional – Soxhlet and HWI, vs. modern – SHWE) and its fractions on selected plants
c) Determination of antioxidant activity by two different assays (DPPH and ORAC) and TPC
d) Calculation of correlation between antioxidant activity and TPC

4. Methods

This study focused on three indigenous and one introduced Filipino tea-like plants, which were extracted using three different methods: SHWE, traditional HWI, and Soxhlet extraction. Additionally, different time fractions of extractions performed were analyzed to determine the effect of the duration of the extraction on the yield of bioactive compounds. The antioxidant activity of the obtained extracts was evaluated and also compared using two different methods, the DPPH radical scavenging assay, and the ORAC assay. Moreover, in the view of the fact that phenolic compounds were considered strong antioxidants, the TPC was thus determined. Furthermore, the correlation between TPC and antioxidant activity was assessed, providing insight into the effectiveness of different extraction techniques in preserving and maximizing the yield of bioactive compounds.

4.1. Plant samples

Plants were selected based on the literature review focused on plants traditionally used in the preparation of hot beverages in the Philippines. In this study, four plant species have been evaluated, namely *Blumea balsamifera* (L.) DC., *Clitora ternata* (L.), *Ehretia microphylla* Lam., and *Lagerstroemia speciosa* (L.) Martyn. All plant materials were collected at the campus of Visayas State University in Baybay City on the island of Leyte in the Philippines in 2022. Fresh plant material was airdried under shade conditions for several days and stored in textile bags in dark at the room temperature until further use.

4.2. Extract preparation and methods

Each airdried plant material was prepared by grinding to mild powder using an electric mill GM 100 (Retsch, Germany). For each method, 7.5 g of the sample powder was used and extracted using either water or methanol, based on the method.

4.2.1. Subcritical Water Extraction

The mild powder was placed on top of the glass wool and was also covered with it before placement in the cylinder vessel, SFE Helix (Applied Separation, USA). Three

different time fractions were performed. The total 10 min extraction, 1st and 2nd 5 min extractions. The sample underwent a water temperature of 120 °C and a pressure of the vessel of 50 Bar. The collected samples were then evaporated under controlled conditions in rotary evaporator R-200 (Büchi, Switzerland) at vacuum and 50 °C until the sample was dried out. After weighing the residue, samples were stored at -20 °C. Yields of the samples are presented in Table 1.

4.2.2. Hot Water Infusion

The plant powder was placed in tea bags and poured with boiling water in amounts depending on the time fraction. Again, three fractions were performed: the total 10 min and the first and second 5 min of infusion. In case of a total 10 min, 1 L of boiling water was poured into the flask with the 7.5 g of the sample in the textile beg. In case of the 1st 5 min, 500 mL of boiling water was poured into the first flask with the bag of sample. When the 1st 5 min came to an end, the bag with the sample was transferred into new flask and poured with another 500 mL of boiling water. After the extracts were filtered and then evaporated under controlled conditions in rotary evaporator R-200 (Büchi, Switzerland) at vacuum and 50 °C until the sample was dried out. After weighing the residue, samples were stored at -20 °C. The yields of the samples are presented in Table 1.

4.2.3. Soxhlet

In Soxhlet, methanol instead of water was used as a solvent. The Soxhlet extractor was used, and the solvent was heated to 80 °C. Since the first condensation drop appeared, the four-hour extraction was counted down. The collected samples were then evaporated under controlled conditions in rotary evaporator R-200 (Büchi, Switzerland) at vacuum and 35 °C until the sample was dried out. After weighing the residue, samples were stored at -20 °C. The yields of the samples are presented in Table 1.

4.3. Chemicals Used

(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2‘-azobis(2-methylpropionamidine) dihydrochloride (AAPH), fluorescein sodium salt, and gallic acid were acquired from

Sigma-Aldrich (Prague, Czech Republic). Methanol and Folin-Ciocalteu reagent were sourced from Penta (Prague, Czech Republic), and inorganic salts (KH_2PO_4, K_2HPO_4, Na_2CO_3) from Lach-Ner (Neratovice, Czech Republic).

4.4. Evaluation of Antioxidant Activity

Antioxidants from plants are assessed using biological and chemical assays to determine compounds that are able to neutralize free radicals and combat oxidative stress. The DPPH assay measures hydrogen donation to DPPH radicals, shown by a colour change (Sharma & Bhat 2009). The ORAC assay quantifies the scavenging of peroxyl radicals using fluorescence as an indicator (Cao & Prior 1999). Total phenolic content (TPC) is another widely used as indirect method. Phenolic compounds contribute significantly to antioxidant activity (Singleton & Rossi 1965).

4.4.1. DPPH

The antioxidant activity of the extracts was evaluated using the method described by Sharma and Bhat (2009), based on their capacity to scavenge DPPH radicals. Stock solutions of both Trolox (control) and the sample were prepared in methanol at a concentration of 512 μg/mL. A two-fold serial dilution of each sample was performed using the automated pipetting platform Freedom EVO 100 (Tecan, Männedorf, Switzerland) in 96-well microtiter plates. Subsequently, 75 μL of methanol and 25 μL of 1 mM DPPH solution in methanol were added to each well. The resulting concentration range of Trolox and the samples in the plate was from 0.125 to 256 μg/mL. The plates were incubated under dark conditions at room temperature for 30 minutes. The absorbance was then measured at 517 nm using a Multimode Reader Cytation 3 (BioTek, Instruments, Winooski, VT, USA). All experiments were conducted in three independent experiments, each performed in triplicate. Results were expressed as the mean of half-maximal inhibitory concentration (IC_{50}) values with a standard deviation (±SD), in μg/mL.

4.4.2. ORAC

The antioxidant capacity of the samples to protect fluorescein from oxidative degradation induced by AAPH radicals was assessed using the ORAC assay, following the protocol described by Ou et al. (2001). Stock solutions of 153 mM AAPH and 48 nM fluorescein were prepared in 75 mM phosphate buffer (pH 7.0). Two-fold serial dilutions of each extract were prepared in 96-well black absorbance microtiter plates using the automated pipetting platform Freedom EVO 100 (Tecan, Männedorf, Switzerland). Following dilution, 150 µL of fluorescein solution was added to each well and the plate was incubated at 37 °C for 90 min. The final concentration of the extract ranged from 1 to 256 µg/mL. To ensure thermal mass stability, all outer wells of the microtiter plates were filled with 200 µL of distilled water. Each plate included two types of blanks: fluorescein with AAPH in buffer (Blank 1), and fluorescein in buffer only (Blank 2). For positive control, Trolox was used. Fluorescence was measured using Multimode Reader Cytation 3 (BioTek, Instruments, Winooski, VT, USA) at an excitation wavelength of 485 nm. The assay was conducted in three independent experiments, each performed in triplicate, and the results were expressed as the mean IC_{50} values with a ±SD, in µg/mL.

4.4.3. TPC

The TPC of the tested extracts was determined using the method of Singleton and Rossi (1965) and adapted to a 96-well microtiter plate format. The stock solution of each sample was prepared at a concentration of 32 µg/mL, and 100 µL of 12 % (w/v) sodium carbonate (Na_2CO_3) were added to each well. The plate was incubated for 2 hours at 37 °C in the dark. Absorbance was measured at 700 nm suing a Multimode Reader Cytation 3 (BioTek, Instruments, Winooski, VT, USA). A standard calibration curve was constructed using gallic acid at eight concentrations levelled (0.25, 0.5, 1, 2, 4, 8, 16, and 32 µg/mL). All experiments were carried out in three independent experiments, each performed in triplicate. The results were expressed as the mean value of gallic acid equivalents (mg GAE/g extract) and ±SD.

4.4.4. Statistical Analysis

Linear correlation coefficient (r) between antioxidant assay results (DPPH and ORAC) and TPC were calculated using the Pearson product-moment correlation in order

to assess whether phenolic compounds contribute to the antioxidant activity of the tested plant extracts. The strength of the correlations was interpreted according to the classification proposed by Evans (1996), where the absolute value of r indicates the following: very weak (r = 0 – 0.19), weak (r = 0.20 – 0.39), moderate (r = 0.40 – 0.59), strong (r = 0.60 – 0.79), and very strong (r = 0.80 – 1.00) correlation. For each sample, statistical comparisons of the extraction methods and the time fractions and their effects on antioxidant activity and TPC were performed using Microsoft Excel (Microsoft 365 MSO, Microsoft Corporation, Redmond, USA). Statistical significance was determined using Student's t-test, with differences considered significant at $p < 0.05$.

5. Results

As a part of testing, extraction yields were also determined (Table 1). The highest yield 37.6 % was obtained from *C. ternatea* from the 1st 5 min of HWI, followed by 36.0 % from 10 min HWI. The least was extracted from *L. speciosa* with 1.6 % from 2nd 5 min of SHWE. Results showed that the highest yields were mostly from HWI extraction with the exception of *E. microphylla* where 10 min from SHWE were also high (18.9 %).

Antioxidant activity and TPC were compared between all extracts obtained by three different extraction methods (SHWE, HWI, and Soxhlet), and their fractions, and the results are presented in Table 2. Obtained results revealed considerable differences in antioxidant potential both between plant species and across extraction methods and durations. Among all tested samples, *E. microphylla* performed the strongest DPPH scavenging activity, with the best value in the SHWE group. The IC_{50} values decreased with the extended extraction time: 10.48 µg/mL (1st 5 min). 9.22 µg/mL (2nd 5 min), and the lowest value of 7.82 µg/m at 10 min, being the most active. In the case of HWI, the values grew with the extraction time, 8.18 µg/mL (1st 5 min), 9.20 µg/mL (2nd 5 min), and 9.72 µg/mL (10 min). Consistent high antioxidant activity for this species across aqueous-based extraction methods was indicated from these data when compared to Soxhlet extract, which showed a reduced activity and an IC_{50} of 34.92 µg/mL. *B. balsamifera* extracts also exhibited substantial antioxidant activity. SHWE extract IC_{50} values were 16.56 µg/mL (1st 5 min), 17.26 µg/mL (2nd 5 min), and 19.64 µg/mL (10 min). Similar activity was performed in HWI extract with the highest activity of 10 min extract with IC_{50} of 13.00 µg/mL, followed by 16.23 µg/mL (1st 5 min) and 18.95 µg/mL (2nd 5 min). Soxhlet resulted in an IC_{50} of 21.80 µg/mL, placing this method within a comparable but slightly less active range. *L. speciosa* presented much broader variability in DPPH activity depending on the extraction method. The weakest activity performed extracts obtained from SHWE with values 189.97 µg/mL (1st 5 min), 136.08 µg/mL (2nd 5 min), and 171.88 µg/mL (10 min). In contrast, HWI extracts were significantly more active, with values of 13.48 µg/mL (1st 5 min), the best value of 11.97 µg/mL (2nd 5 min), and 29.62 µg/mL (10 min). Soxhlet yielded moderate IC_{50} of 22.03 µg/mL, positioning it between the SHWE and HWI in terms of the extract activity. The plant with the lowest antioxidant activity in the case of the DPPH assay demonstrated *C. ternatea*. SHWE

values of IC_{50} were 254.22 µg/mL (1st 5 min), and >256 µg/mL (2nd 5 min and 10 min). HWI with values 254.68 µg/mL (10 min) and >256 µg/mL (both 1st and 2nd 5 min), and Soxhlet extract of 255.47 µg/mL. Those values were close to the upper detection limit and indicated minimal variation in DPPH activity regardless of extraction time and type. The controlling Trolox was determined as an average of three independent tests with IC_{50} of 7.41 µg/mL.

ORAC assay, as another method to determine antioxidant activity, showed a similar trend as DPPH. Among all the samples, *E. microphylla* exhibited the most vigorous ORAC activity, with consistently low IC_{50} values across extraction types and time fractions. SHWE extracts yielded minimal variation within time. The values were 7.90 µg/mL (1st 5 min), the strongest 7.23 µg/mL (2nd 5 min), and 7.24 µg/mL (10 min). Similarly, HWI showed only slight differences, with values 8.42 µg/mL (1st 5 min), 8.49 µg/mL (2nd 5 min), and 10.30 µg/mL (10 min). The Soxhlet extract of this species exhibited reduced activity with a higher IC_{50} of 29.86 µg/mL. *B. balsamifera* also demonstrated strong antioxidant activity with slight variability across methods and time fractions. SHWE extracts had values of 13.77 µg/mL (1st 5 min), 12.13 µg/mL (2nd 5 min), and 17.22 µg/mL. HWI values were a bit better than those from SHWE and Soxhlet, with the best value of 10.49 µg/mL from the 1st 5 min, then 12.90 µg/mL (2nd 5 min), and 13.02 µg/mL (10 min). Soxhlet extract reached a higher IC_{50} of 19.86 µg/mL. In the case of *L. speciosa,* results showed a different trend from the other three tested plants. SHWE extracts demonstrated the weakest antioxidant activity with IC_{50} values of 22.29 µg/mL (1st 5 min), 30.678 µg/mL (2nd 5 min), and 39.14 µg/mL (10 min). HWI extractions resulted in improved activity at shorter times, 17.98 µg/mL for 1st 5 min and 17.95 µg/mL for the 2nd 5 min, but 10 min extract. The Soxhlet method yielded an intermediate IC_{50} value of 20.55 µg/mL. As in the DPPH assay, *C. ternatea* performed the weakest activity among tested plants. SHWE values obtained were as follows: 63.32 µg/mL (1st 5 min), 64.74 µg/mL (2nd 5 min), and 54.09 µg/mL (10 min). HWI extracts had the best values out of the three methods. 10 min extract performed the best antioxidant activity with IC_{50} of 48.75 µg/mL, followed by 58.75 µg/mL (1st 5 min) and 60.84 µg/mL (2nd 5 min). The results showed that in both methods, with the longer extraction times of 10 min, the antioxidant activity was enhanced, when compared to shorter extraction times. Soxhlet then showed again the highest value in the dataset at 70.22 µg/mL.

E. microphylla consistently exhibited the highest TPC values among all tested plants across SHWE and HWI methods, while *B. balsamifera* had the higher values in Soxhlet. *E. microphylla* SHWE extract showed the highest recorded value (870.30 mg GAE/g) for the 1st 5 min, followed by the 10 min extract (837.88 mg GAE/g) and 2nd 5 min (800.11 mg GAE/g). HWI similarly yielded elevated values: 773.40 mg GAE/g (1st 5 min), 713.10 mg GAE/g (2nd 5 min), and 781.21 mg GAE/g (10 min). In contrast, the Soxhlet extract resulted in just 248.69 mg GAE/g, which is a significant reduction compared to both SHWE and HWI. This again underscored the lower efficiency of Soxhlet in this context. For *B. balsamifera*, the TPC was consistently high. SHWE yielded 575.30 mg GAE/g (1st 5 min), 670 mg GAE/g (2nd 5 min), and 568.09 mg GAE/g (10 min), with the highest value from the second 5 min. HWI performed similarly high TPC yields of 651.06 mg GAE/g (10 min), 653.24 mg GAE/g (1st 5 min), and 611.91 (2nd 5 min). Soxhlet values, again lower than both SHWE and HWI with 400.66 mg GAE/g. Comparable results were performed by *L. speciosa* and its HWI extracts reaching 840.181 mg GAE/g (1st 5 min), 762.18 mg GAE/g (2nd 5 min), and 533.15 mg GAE/g (10 min). SHWE yielded lower values of 216.59 mg GAE/g (1st 5 min), 265.87 mg GAE/g (2nd 5 min), and 254.69 mg GAE/g (10 min). Soxhlet resulted in 270.12 mg GAE/g, which is comparable to SHWE. *C. ternatea* performed values of the lowest TPC. SHWE produced 176.60 mg GAE/g (1st 5 min), 139.81 mg GAE/g (2nd 5 min), and 164.45 mg GAE/g (10 min). HWI resulted in slightly lower values: 115.39 mg GAE/g (1st 5 min), 134.61 mg GAE/g (2nd 5 min), and 129.78 mg GAE/g (10 min). Values of Soxhlet samples were the lowest with 127.49 mg GAE/g.

To determine the correlation between the antioxidant activity and the TPC, the Pearson product-moment correlation was performed. Correlations were found to be very strong, both between DPPH and TPC ($r = 0.932$), and ORAC and TPC ($r = 0.882$), with regard to $p < 0.05$.

Indications of significant differences between methods and their extraction times were calculated using the Student's T-test (with $p < 0.05$). There were a couple of significant differences, mostly addressing strong differences between the results of Soxhlet samples with both SHWE and HWI methods and their time fraction in most of the plants significant difference. This was a case of *E. microphylla* where in both DPPH and ORAC testing were both SHWE and HWI and their fractions were significantly

different from Soxhlet extractions. *B. balsamifera* showed only two significant differences, first between 1st 5 min of SHWE and 10 min of HWI (DPPH) and second between 10 min of SHWE and 1st 5 min of HWI (ORAC). Significant differences were abundant in *L. speciosa*. Differences in DPPH were found between 1st 5 min of SHWE and all HWI fractions; 2nd 5 min of SHWE and all HWI fractions; the 10 min of SHWE and all HWI fractions; and between 10 min and both 1st and 2nd 5 min of HWI. In the case of ORAC, differences appeared between 2nd 5 min and 10 min of SHWE; 1st 5 min of SHWE and both 1st and 2nd 5 min of HWI; 2nd 5 min of SHWE and 1st and 2nd 5 min of HWI; 10 min and both 1st and 2nd 5 min of HWI; and between 10 min of SHWE and Soxhlet. *C. ternatea* exhibited significant ORAC differences between Soxhlet extraction and nearly all fractions obtained by SHWE and HWI methods, with the exception of the 10 min SHWE extract. All significant differences can be found in Table 2. Even though SHWE is considered a green and effective method, our results did not support this hypothesis in all the plants due to different nature of plant's compounds.

Results

Table 1: Extraction Yields (%)

Scientific name and Family	Common name	Plant part used	SHWE			HWI			SOX
			1st 5 min	2nd 5 min	10 min	1st 5 min	2nd 5 min	10 min	4 h
Blumea balsamifera (L.) DC. *Asteraceae*	sambong	leaves	11.0 %	5.1 %	12.2 %	14.9 %	3.6 %	16.8 %	7.3 %
Clitoria ternatea L. *Fabaceae*	Butterfly pea	flowers	14.1 %	11.4 %	2.1 %	37.6 %	9.4 %	36.0 %	32.4 %
Ehretia microphylla Lam. *Boraginaceae*	Tsaang gubat Philippines tea tree	leaves	6.6 %	4.8 %	18.9 %	17.6 %	6.0 %	21.0 %	9.1 %
Lagerstroemia speciosa (L.) Martyn *Lythraceae*	Banaba	leaves	5.2 %	1.6 %	5.4 %	15.2 %	3.4 %	25.4 %	4.4 %

Notes: SHWE – Subcritical Hot Water Extraction; SOX – Soxhlet; HWI – Hot Water Infusion

Table 2: Antioxidant Activity (IC_{50} µg/mL)

Scientific name	DPPH*						
	SHWE			HWI			SOX
	1st 5 min	2nd 5 min	10 min	1st 5 min	2nd 5 min	10 min	4 h
Blumea balsamifera (L.) DC.	16.56 ±11.93^{F}	17.26 ±4.63	19.64 ±5.33	16.23 ±3.11	18.95 ±4.53	16.48 ±3.27	21.80 ±3.16
Clitoria ternatea L.	254.21 ±2.52	>256	>256	>256	>256	254.68 ±1.870	255.47 ±0.74
Ehretia microphylla Lam.	10.48 ±1.89^{G}	9.22 ±1.19^{G}	7.82 ±1.10^{G}	8.18 ± 0.13^{FG}	9.20 ±0.95^{G}	9.72 ±0.57^{G}	34.92 ±2.45^{ABCDEF}
Lagerstroemia speciosa (L.) Martyn	189.97 ±32.26^{DEFG}	136.08 ±16.92^{DEFG}	171.88 ±17.22^{DEFG}	13.48 ±1.48^{ABCFG}	11.87 ±2.76^{ABCFG}	29.62 ±2.07^{ABCDEG}	22.03 ±0.88^{ABCDEF}
	ORAC**						
	SHWE			HWI			SOX
	1st 5 min	2nd 5 min	10 min	1st 5 min	2nd 5 min	10 min	4 h
Blumea balsamifera (L.) DC.	13.77 ±2.03	12.13 ±1.79	17.22 ±2.22^{K}	10.49 ±1.36^{J}	12.90 ±3.10	13.02 ±2.89	19.86 ±8.28
Clitoria ternatea L.	63.32 ±0.46^{N}	64.74 ±13.84^{N}	54.09 ±15.36	58,75 ±4.61^{N}	60.84 ±2.95^{N}	48.75 ±9.44^{N}	19.86 ±8.28^{HIKLM}
Ehretia microphylla Lam.	7.90 ±0.44^{N}	7.23 ±0.84^{N}	7.24 ±0.84^{N}	8.42 ±1.39^{N}	8.49 ±1.16^{N}	10.30 ±2.56^{N}	39.27 ±4.23^{HIJKLM}
Lagerstroemia speciosa (L.) Martyn	33.29 ±7.16^{KL}	30.68 ±1.47^{JKL}	39.14 ±2.18^{IKLN}	17.98 ±0.63^{HIM}	17.95 ±0.27^{HIM}	31.84 ±5.96^{KL}	20.55 ±3.75^{J}

Notes: SHWE – Subcritical Hot Water Extraction; SOX – Soxhlet; HWI – Hot Water Infusion. Letters at the top index mean that which method and its fraction it is significantly different to are as following:

DPPH	ORAC
A – 1st 5 min of SHWE	H – 1st 5 min of SHWE
B – 2nd 5 min of SHWE	I – 2nd 5 min of SHWE
C – 10 min of SHWE	J – 10 min of SHWE
D – 1st 5 min of HWI	K – 1st 5 min of HWI
E – 2nd 5 min of HWI	L – 2nd 5 min of HWI
F – 10 min of HWI	M – 10 min of HWI
G – Soxhlet	N – Soxhlet

* positive control Trolox value: 7.41±0.77 μg/mL

** positive control Trolox value: 12.35±2.32 μg/mL

Table 3: Total Phenolic Content (mg GAE/g extract)

Scientific name	SHWE			HWI			SOX
	1st 5 min	2nd 5 min	10 min	1st 5 min	2nd 5 min	10 min	4 h
Blumea balsamifera (L.) DC.	575.30 ±88.60	670.85 ±37.08^{G}	568.09 ±68.87^{G}	653.24 ±50.16^{G}	611.91 ±42.49^{G}	651.06 ±82.81^{G}	400.66 ±47.32^{BCDEF}
Clitoria ternatea L.	176.60 ±5.50^{DEG}	139.81 ±14.28	164.45 ±36.80	115.39 ±6.92^{AE}	134.61 ±4.14^{AD}	129.78 ±19.47	127.49 ±5.04^{A}
Ehretia microphylla Lam.	870.30 ±154.40^{G}	800.12 ±107.13^{G}	837.88 ±216.04^{G}	773.40 ±106.75^{G}	713.10 ±110.03^{G}	781.21 ±106.04^{G}	248.69 ±77.90^{ABCDEF}
Lagerstroemia speciosa (L.) Martyn	216.59 ±42.65^{DEF}	265.87 ±25.89^{DEF}	254.69 ±67.02^{DEF}	840.18 ±188.59^{ABCG}	762.18 ±94.58^{ABCG}	533.15 ±115.78^{ABCG}	270.12 ±22.04^{DEF}

Notes: SHWE – Subcritical Hot Water Extraction; SOX – Soxhlet; HWI – Hot Water Infusion. Letters at the top index mean that which method and its fraction it is significantly different to are as following:

A – 1st 5 min of SHWE

B – 2nd 5 min of SHWE

C – 10 min of SHWE

D – 1st 5 min of HWI

E – 2nd 5 min of HWI

F – 10 min of HWI

G – Soxhlet

6. Discussion

The antioxidant potential of well-known *C. sinensis* (tea) has been extensively investigated, with numerous studies confirming its rich content of polyphenols, particularly catechins, which are responsible for its well-documented health benefits (Mukhtar & Ahmad 2000; Moore et al. 2009; Bawono et al. 2023). However, as the demand for sustainable and diverse sources of antioxidants grows, there is increasing pressure to explore alternative, less-studied tea-like plants that may also serve as significant dietary sources of antioxidants. These plants are often used in traditional medicine or consumed as herbal teas in specific cultural contexts. They may offer comparable or complementary bioactivate compounds while contributing to biodiversity, sustainable sourcing, and regional phototherapeutic knowledge (Shahrajabian et al. 2023). In this study, four plants – *B. balsamifera*, *C. ternatea*, *E. microphylla*, and *L. speciosa* – were evaluated for their *in vitro* antioxidant activity using the DPPH and ORAC assays and some of them were done for the first time. Because phenolic compounds are key antioxidants, TPC was measured using the Folin-Ciocalteu assay as an indirect indicator of antioxidant activity (Singleton & Rossi 1965). Limited research has been done on these plants using just conventional extraction methods with organic solvents comparing to each other or to one aqueous extract. In this study two aqueous extracts against each other and Soxhlet extract were tested for the first time in all plants.

In this study, Soxhlet extract of *B. balsamifera* performed better than the methanol extract used by Lie-Fen et al. (2005), where DPPH IC_{50} was 74 μg/mL. Additionally, Dai et al. (2023) reported even higher IC_{50} values of 105.1 μg/mL. In contrast, our extract demonstrated a significantly lower IC_{50} value of 21.8 μg/mL. This variation may be attributed to differences in the plant material's collection location or source. While our material was collected in the Philippines, Lie-Fen et al. (2005) used plant material from Taiwan. Seasonal variations and leaf maturity can also influence antioxidant activity, as immature leaves tend to exhibit better antioxidant activity, with higher amounts of phenolic compounds and flavonoids than mature leaves (Jirakitticharoen et al. 2022). Additionally, the season of collection can affect the properties of the plant, with essential oil obtained from leaves collected in December performing better than those collected in

other months (Wang & Zhang 2020). These variations underscore the importance of consistent and standardized collection practices in obtaining reliable and reproducible results. *C. ternatea* exhibited DPPH IC_{50} values similar to those reported by Lakshan et al. (2019), with values ranging from 254.21 to >256 μg/mL. In their study, the water extract of *C. ternatea* reached an IC_{50} values of 241.84 μg/mL. In contrast, the research conducted by Phrueksanan et al. (2014) reported a much higher DPPH IC_{50} value of 470 μg/mL. This discrepancy could be attributed to the prolonged extraction time of 2 h under boiling conditions, which may have resulted in the degradation of thermolabile compounds. It is important to note that the duration of the extraction process, in addition to temperature, can play a significant role in efficacy of the extract. Our results, which are more in line with the study by Lakshan et al. (2019) suggest that extracting *C. ternatea* at lower temperature for shorter time might be a more efficient approach to preserving antioxidant activity. This comparison highlights the need for optimalization of extraction conditions to maximize the retention of bioactive compounds. One issue that should be taken into consideration when assessing the antioxidant activity of these plant extracts is the presence of naturally occurring pigments, which can interfere with the DPPH assay. Pigments can overlap with the DPPH absorbance spectrum, leading to unreliable IC_{50} values. As highlighted by Yeo & Shahidi (2019), this interference can result in an underestimation of antioxidant activity. They proposed using Electron Paramagnetic Resonance as an alternative method to eliminate this interference, as DPPH values were found to be underestimated by 16.1 % due to pigmentation interference. This highlights the importance of carefully selecting the assay method and considering potentional sources of interference when interpreting results. This could be also supported by our ORAC results that proved that *C. ternatea* exhibit antioxidant activity not that much worse in comparison to other tested plants as exhibited in DPPH testing. For *E. microphylla*, there are no publications that could directly compare our results with other studies. This plant, which is known in Europe as a decorative bonsai, has a history of being consumed as a beverage in the Philippines, where it is also known as the “Philippine tea tree”. In the view of the fact that this plant showed strong antioxidant activity in present study, this plant deserves further investigation for its potential health benefits and its possible introduction into the broader market as a functional beverage. In the case of *L. speciosa*, DPPH IC_{50} value of 22.03 μg/mL from Soxhlet extraction was slightly higher than the value of 12.5 ppm (approximately 15.82 μg/mL) reported by Rezqiyana & Rijai

(2024). Although our result was marginally worse, it still indicates antioxidant potential in this plant.

When comparing the SHWE method to other extraction techniques, it is important to consider several factors before determining its suitability as a green and better-yielding method. The harsh condition required for SHWE extraction (120 °C and 50 Bar) can influence the yield and composition of the extracts, depending on the nature of the compounds being extracted (Zhang et al. 2020b; Pedras et al. 2020). For both *B. balsamifera* and *E. microphylla*, there were almost no significant differences between SHWE and HWI in terms of antioxidant activity and TPC, suggesting that the bioactive compounds responsible for these properties were not thermolabile. As a result, they could be efficiently extracted at elevated temperatures, with the SHWE even yielding better results compared to HWI in some cases. This finding aligns with previous studies, such as the work by Kumar et al. (2011) on Seabuckthorn, where DPPH activity increased with temperature, peaking at 150 °C (353.43 mg Trolox eq./g), before decreasing at 200 °C. Similarly, Essien et al. (2020) found that SHWE extracts of kānuka performed better than conventional extraction methods, with the optimal temperature and extraction time being 170 °C for 20 min. However, prolonged exposure to high temperature led to degradation of the extract properties. Therefore, every plant starts to have bioactive compounds thermally degraded at different temperature and time. This is a case of our *L. speciosa*, that in contrast performed significant difference between SHWE and other two methods.

The IC_{50} values obtained for SHWE ranged from 136.08 to 189.97 µg/mL (DPPH) and 30.68 to 39.14 µg/mL (ORAC), indicating a relatively lower antioxidant activity compared to other methods. In contrast, hot water infusion (HWI) demonstrated significantly stronger antioxidant effects, with IC_{50} values ranging from 11.87 to 29.62 µg/mL (DPPH) and 17.98 to 31.84 µg/mL (ORAC). Soxhlet extraction produced intermediate results, with an IC_{50} of 20.55 µg/mL. These findings suggest that HWI may better preserve or extract antioxidant compounds under the tested conditions, possibly due to milder extraction parameters that minimize degradation. This interpretation is consistent with previous reports showing that higher extraction temperatures and longer durations in subcritical water extraction can lead to degradation of phenolic compounds and a reduction in antioxidant activity (Yu et al. 2015). The total phenolic content (TPC) results further support this, as HWI yielded the highest phenolic concentration (840.18

mg GAE/g extract), while SHWE showed the lowest (216.59 mg GAE/g extract for the first 5 minutes), and Soxhlet extraction again performed slightly better than SHWE, with 270.12 mg GAE/g extract. Overall, these results highlight the importance of selecting an appropriate extraction method, as both the yield and antioxidant potential can be significantly influenced by extraction parameters and the specific plant material involved as in research of Khuwijitjaru et al. (2014) where 10 phenolic compounds after extraction completely disappeared.

When comparing the results of tested plants with commonly consumed species such as *Camellia sinensis*, *Ilex paraguariensis*, and *Aspalathus linearis*, it becomes evident that the antioxidant activity of selected species was comparable, and in some cases even superior. Among the tested species, *E. microphylla* emerged as the most promising. It demonstrated superior antioxidant activity in the ORAC assay, outperforming the SHWE fractions of green tea (*C. sinensis*) reported by Walayat (2024). Specifically, the ORAC IC_{50} values of *E. microphylla* were 7.90 µg/mL (1st 5 min SHWE) and 7.24 µg/mL (10 min SHWE), compared to green tea's corresponding values of 11.89 µg/mL and 7.44 µg/mL, respectively. In DPPH testing, *E. microphylla* again surpassed the green tea fractions: 21.33 µg/mL (1st 5 min SHWE), 9.73 µg/mL (2nd 5 min SHWE), 15.70 µg/mL (10 min SHWE), and 13.89 µg/mL (10 min HWI), while the IC_{50} values for *E. microphylla* were 10.48 µg/mL (1st 5 min SHWE), 9.22 µg/mL (2nd 5 min SHWE), 7.82 µg/mL (10 min SHWE), and 9.72 µg/mL (10 min HWI). When compared to black tea, *E. microphylla* outperformed it in all DPPH-based evaluations and in nearly all ORAC tests. The only exception was the 2nd 5 min SHWE fraction of black tea, which reached an IC_{50} of 6.43 µg/mL—slightly better than *E. microphylla*'s 7.23 µg/mL. Berková's (2023) research also supported that *E. microphylla* exhibited superior antioxidant activity compared to *C. sinensis* (green tea – DPPH of SHWE 19.80 µg/mL, HWI 17.22 µg/mL), surpassing black tea, *A. linearis*, *Cyclopia intermedia*, and *Hibiscus sabdariffa*. However, it is important to consider that Berková used an extraction time of 20 minutes, which may have influenced the results compared to this study with different extraction durations.

Based on results these plants could be a great novel source of antioxidants. Some of them already have found their way to broader public from all over the world, such as *C. ternatea* and its products of wide purpose. *C. ternatea* is sold as a herbal tea for its

beautiful colour, but also in form of herbal tincture or extracts, that promise health benefits. *B. balsamifera* can be mostly found at Asian market where it is sold as a herbal tea but also as herbal drug in form of tablets to treat kidney stones in the Philippines ("Sambong Tablet" n.d.). *E. microphylla* can be found in human diet just in Asia, where it is sold as a herbal tea, while it is more known as a bonsai tree sold all over the world. *L. speciosa* is sold to broader public in form of food supplements and herbal tea.

7. Conclusion

In this thesis, the antioxidant activity and TPC of different extracting methods (SHWE, HWI, and Soxhlet) and their time fractions (1st 5 min, 2nd 5 min, 10 min, 4 h – Soxhlet) were tested to determine if the SHWE method, considered as a greener approach in obtaining bioactive compounds, proves its efficiency in comparison to the other two conventional methods. The results indicated that the efficacy of each method was highly species dependent. While SHWE and HWI generally led to higher yield and improved antioxidant activity compared to Soxhlet extraction, the optimal method varied considerably among the tested plants.

E. microphylla consistently performed strong antioxidant activity in both DPPH and ORAC assays, with SHWE often producing the highest TPC values. Moreover, it surpassed common tea (*C. sinensis*). *B. balsamifera* also showed substantial antioxidant activity, with HWI extracts performing comparably to SHWE. In contrast *C. ternatea* generally exhibited the lowest antioxidant activity, potentially due to interfering pigments in the DPPH assay. Notably *L. speciosa* displayed a distinct trend: HWI extracts showed promising antioxidant activity and high TPC, but SHWE extracts demonstrated the weakest activity, suggesting that SHWE may degrade antioxidant compounds or TPC in this species.

The extraction method and duration significantly influenced not only the quantity of extracted phenolic compounds but also their biological activity. Strong positive correlations between TPC and both DPPH ($r = 0.932$) and ORAC ($r = 0.882$) support the role of phenolic compounds as a key contributor to antioxidant potential. Given that HWI often achieved comparable results to SHWE, particularly for *B. balsamifera* and certain fractions of *E. microphylla*, the simpler, more energy-efficient HWI method presents a compelling alternative. Further research should optimize these parameters for each plant, consider potential application of these extracts in various industries, taking into account factors such as sustainable sources and regional phytotherapeutic knowledge, The study confirms the potential of SHWE as a green extraction method, while highlighting the importance of species-specific optimization.

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