OPTIMISATION OF EXTRACTION FROM *Tradescantia zebrina* LEAVES AND THE GASTROINTESTINAL STABILITY OF ITS PHYTOCHEMICALS AND BIOACTIVITIES

By

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ABSTRACT

OPTIMISATION OF EXTRACTION FROM *Tradescantia zebrina* LEAVES AND THE GASTROINTESTINAL STABILITY OF ITS PHYTOCHEMICALS AND BIOACTIVITIES

LIM WEY LOON

Tradescantia zebrina is a medicinal plant traditionally consumed by residents worldwide, which is extensively investigated for its phytochemical contents and bioactivities. However, the effects of gastrointestinal (GI) digestion on the potential health benefits are poorly understood. This study was conducted to optimise the extraction of phytochemical from *T. zebrina* leaves. The optimal extract was subjected to simulated GI digestion based on the INFOGEST 2.0 protocol. The current study discovered that sequential hybrid extractions were more effective than the individual methods like hot water extraction (HWE) and ultrasonic-assisted extraction (UAE). The "UAE for 20 min, followed by HWE for 15 min" extract was the optimal among all nine treatments, showing the highest total phenolic content (TPC) (8.114 ± 0.007 mg GAE per g dry extract) and total flavonoid content (TFC) (62.56 ± 0.29 mg QE per g dry extract). This extract also recorded the lowest EC_{50} in 1,1-diphenyl-2-picrylhydrazyl radical (DPPH•) (0.587 ± 0.000 mg/mL) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$) (1.069 ± 0.003 mg/mL) scavenging activities. Simulated GI digestion significantly reduced TPC and TFC, respectively, to 3.277 ± 0.005 mg GAE and 6.44 ± 0.22 mg QE per g dry extract. Simulated GI

digestion also declined the antioxidant and anti-inflammatory activities of the selected *T. zebrina* extract. The EC_{50} values of DPPH• and $ABTS^{•+}$ scavenging activity increased to 2.118 ± 0.042 and 1.585 ± 0.001 mg/mL, respectively. The EC_{50} values of hydrogen peroxide scavenging activity and inhibition of albumin denaturation increased, respectively, from 1.976 ± 0.006 to 3.658 ± 0.023 mg/mL and from 1.527 ± 0.005 to 2.918 ± 0.023 mg/mL. These changes could potentially proof the negative impacts of human GI digestion, signalling the necessity for strategies that enhance the bioaccessibility.

Keywords: Gastrointestinal digestion, *Tradescantia zebrina*, sequential hybrid extraction, phytochemical, antioxidant, anti-inflammatory

Subject Area: QD415 – QD436 Biochemistry

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LIST OF ABBREVIATIONS

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ABTS•$^{+}$	ABTS radical cation
BSA	Bovine serum albumin fraction V
CE	Catechin equivalent
CGE	Cyanidin-3-glucoside equivalent
DF	Dilution factor
DPPH•	1,1-diphenyl-2-picrylhydrazyl
ε	Molar absorptivity
EC_{50}	Half maximal effective concentration
Fe^{2+}	Ferrous ion
FRAP	Ferric reducing antioxidant power
FYP	Final year project
GAE	Gallic acid equivalent
GI	Gastrointestinal
H_2O_2	Hydrogen peroxide
HWE	Hot water extraction
IC_{50}	Half maximal inhibitory concentration
kHz	Kilohertz
µm	Micrometer
mM	Millimolar
MW	Molecular weight
nm	Nanometer
NO	Nitric oxide

PBS	Phosphate buffered saline
QE	Quercetin equivalent
RE	Rutin equivalent
rpm	Rotation per minute
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SPE	Solid phase extraction
SSF	Simulated salivary fluid
TAC	Total anthocyanin content
TE	Trolox equivalent
TFC	Total flavonoid content
TPC	Total phenolic content
TPTZ	2,4,6-tripyridyl-s-triazine
U	Unit of enzyme activity
UAE	Ultrasonic-assisted extraction
UV-VIS	Ultraviolet-visible
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

1.1 Foundational Information

Nowadays, development of foods or pharmaceutical products based on plants (fruits and vegetables) had gained focus in the aspect of improving life quality and life expectancy. Such plants are continuously used as effective, sustainable and safe origins of natural antioxidants. These antioxidants, particularly phenolics, marked a high contribution in combating many diseases, issues of aging and cancer (Yu *et al.*, 2021). In the Commelinaceae family, there are 37 genera and over 600 different species. Plants within this family are rich in compounds with various bioactivities (Tan *et al.*, 2014). Plants in this family generally have succulent stems and sheathing leaves (Chumroenphat and Saensouk, 2021). Among all, the spiderworts (*Tradescantia* sp.) are worth putting focus on. The second largest genus within the stated family is the genus *Tradescantia*, which was extensively used as traditional herbs for fragrance, taste and therapeutic properties. These traditional applications are likely to indicate that beneficial bioactive compounds are present (Butnariu *et al.*, 2022).

Tradescantia zebrina is a herbal plant native to Central America and Mexico. Its distribution covers many tropical and subtropical areas. This plant is characterised by leaves with a green colour on the top and purple undersides (Ramos-Arcos *et al.*, 2023). It grows well in varying types of soil. The leaves

from the plant are used in many regions like South America, China, Jamaica and Malaysia. Butnariu *et al.* (2022) and Feihrmann *et al.* (2024) said that the citizens of these countries mentioned usually soaked the leaves and boiled them in water before drinking it.

Figure 1.1: Both sides of *T. zebrina* leaves.
(Source: Photographs taken from samples purchased from local market in Kampar, Perak)

Hot water extraction (HWE) is frequently used to perform the extraction of polar substances from herbal materials and is considered safer for ingestion as compared to extraction using methanol (Saniya *et al.*, 2025). Water is the most polar solvent (polarity = 1.000) that dissolves a broad variety of substances. Generally, it is cheap and non-flammable (Abubakar and Haque, 2020). Decoction, as one example of HWE, applies the continuous heating of the sample-water mixture near the boiling point of water (90 to 100°C). It is only suitable for the diffusion of heat-stable compounds (Azwanida, 2015).

Ultrasonic-assisted extraction (UAE) of plant-based bioactive compounds has been broadly investigated. This is because UAE, in general, has a shorter extraction time compared to common techniques (Feihrmann *et al.*, 2024; Raspe *et al.*, 2021). In this extraction technique, the production of sound waves can generate a mechanical erosion that can break the cell walls, allowing a better penetration of solvent into the cell. This leads to an increased interaction between solvent and solute, which then causes an increase in the extraction efficiency (Raspe *et al.*, 2021).

1.2 Significance of the Research

While the phytochemical contents and various bioactivities of *T. zebrina* extracts were determined, literature is scarce regarding the effects of gastrointestinal digestion on various aspects of *T. zebrina* water extracts. The impact of gastrointestinal digestion on antioxidant capacities is yet undiscovered. The bioaccessibility of phytochemicals after digestion is also a key research gap.

Without the mentioned information on the impact of gastrointestinal digestion, the potential antioxidant and anti-inflammatory activities when consumed orally, would be an assumption, or even an overestimation. Furthermore, *in vitro* studies on stabilities of phytochemicals and bioactivities would lay a foundation towards future *in vivo* studies. Hence, this project is crucial to evaluate *T. zebrina*'s potential health benefits while the impact is to inform strategies to enhance bioaccessibility.

1.3 Objectives

i. To optimise the extraction of phytochemicals from *T. zebrina* leaves using hot water, sonication, and combined methods.
ii. To evaluate the stability of phytochemicals during simulated gastrointestinal digestion.
iii. To assess changes in antioxidant and anti-inflammatory activities of the extract before and after simulated gastrointestinal digestion.

CHAPTER 2

LITERATURE REVIEW

2.1 Antioxidants and Oxidative Stress

Reactive oxygen and nitrogen species, collectively known as RNOS, are understood as unstable species containing oxygen or nitrogen that react quickly with other molecules in the cells. RONS can be categorised as free radicals and non-free radicals, where nitric oxide and superoxide anion radical are examples of the former, while hydrogen peroxide and nitrogen dioxide are examples of the latter (Aranda-Rivera *et al.*, 2022). RONS are essential for normal biological functions in physiological systems when redox regulation, signalling, and sensing events are tightly regulated. However, overproduction of RONS can lead to the build-up of oxidative stress. Excess RONS induces cellular damage, especially affecting macromolecules like DNA and lipids. This overproduction will also result in destabilising redox signalling and control. It can progressively cause deleterious effects on gene expression and activates the apoptosis pathway (Masenga *et al.*, 2023).

Niki (2010) mentioned that antioxidant compounds can significantly delay or inhibit oxidation of the oxidisable molecules even at a lower concentration compared to the substrate molecule. Antioxidants may be grouped into three levels. The levels include preventing, scavenging, as well as repair and *de novo* antioxidants, as demonstrated in **Figure 2.1**. Preventing antioxidants, as the first

line defence, can prevent the formation of various RONS (Sachdev *et al.*, 2021). Scavenging antioxidants like phenolic compounds can act as the second line defence. Many enzymes function in the third line damage by means of repairing oxidative damage, waste clearing and reconstituting host function (Niki, 2010).

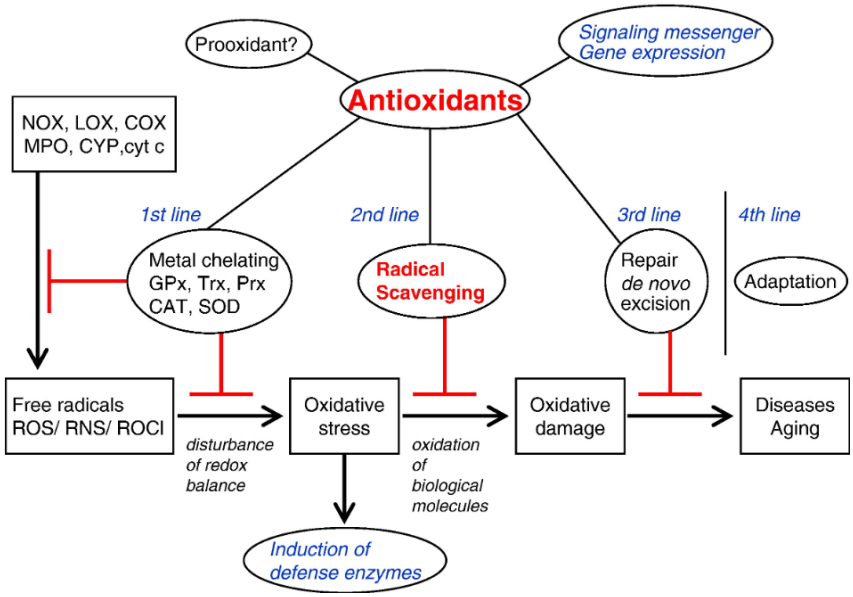

Figure 2.1: Defence network against oxidative stress.
(Source: Niki, 2010)

Conventionally, many assessments, including the DPPH radical (DPPH•) scavenging assay, ABTS radical cation ($ABTS^{\bullet+}$) scavenging assay and FRAP assay were employed in much research to study the antioxidant activity of various samples (inclusive of plant extracts) (Chai and Wong, 2012; Governa *et al.*, 2022; Wanyo *et al.*, 2024). By performing multiple assessments, different underlying mechanisms of the assays could possibly come together to prove the potential of a particular sample. For example, the principal mechanism in $ABTS^{\bullet+}$ scavenging activity and FRAP relies on electron transfer from potent antioxidants. Generally, $ABTS^{\bullet+}$ is a non-physiological radical while FRAP is

non-specific to antioxidants. RONS scavenging is the basic principle in both hydrogen peroxide (H_2O_2) and nitric oxide (NO) scavenging activity. Since RONS are present in the physiological systems of the human body, measuring the related activities would likely allow a better understanding of health benefits within a sample (Kotha *et al.*, 2022).

2.2 Anti-Inflammatory Activities

Inflammation is the body's basic immune system's reaction to pathogenic or microbial invasions, tissue or cell damage, harmful stimuli, or other irritants. It is mediated by a complex network of signalling pathways involving various transcription factors, cytokines, and other mediators (Nehme *et al.*, 2025). This immune response is usually regulated to serve as a protective mechanism (Baghalpour *et al.*, 2021). However, persistent inflammation would likely cause the development of chronic pathologies including cancers, neurodegenerative conditions, heart-related diseases, etc. To treat inflammation, aspirin and ibuprofen are two well-known examples of non-steroidal anti-inflammatory drugs (NSAIDs) (Liu *et al.*, 2018; Nehme *et al.*, 2025).

Many bioactive chemicals from plants are found possessing anti-inflammatory properties. Nehme *et al.* (2025) discovered that the *Quercus robur* extract showed anti-inflammatory proven via multiple *in vitro* studies. Liu *et al.* (2018) found that several different phenolic acids like danshensu, oresbiusin A, prolithospermic acid, lithospermic acid, salvianolic acid A, and salvianolic acid

C significantly decreased the production of different cytokines (TNF-α, IL-1β, and IL-6). Another research suggests that quercetin, a natural flavonoid present in multiple plant-based food, can inhibit monocyte adherence ability, which then suppresses expression of protein molecules like intercellular adhesion molecule-1 (ICAM-1) and matrix metalloprotease-9 (MMP-9). Without suppression, the activated inflammatory proteins would lead to the presence of an inflammation (Cheng *et al.*, 2019).

There are different methods, either *in vitro* or *in vivo*, which assist in the assessment of anti-inflammatory activities. The *in vivo* methods include inhibition of carrageenan-induced paw oedema (Baghalpour *et al.*, 2021) and assessment of levels of cytokines in rat paw (Fayez *et al.*, 2023). From another perspective, *in vitro* methods can also be applied. For instance, inhibition of haemolysis of erythrocyte membranes (Evelyn *et al*., 2020), inhibition of albumin denaturation (Evelyn *et al.*, 2020; Kpemissi *et al.*, 2012), nitric oxide (NO) scavenging activity (Chai *et al.*, 2014; Peiris *et al.*, 2025), and 15-lipoxygenase inhibitory activity (Alaba and Chichioco-Hernandez, 2014).

NO is one of the inflammation mediators produced by monocytes and macrophages. While NO performs a key role in the pathophysiology of inflammation, suppression of NO production can assist in regulating inflammation (Inkanuwat *et al.*, 2019). On the other hand, since the human body primarily comprises proteins, damage or denaturation of the tertiary and secondary structures will trigger an inflammatory response and cause various inflammatory diseases. This could explain how denaturation of albumin by

external stimuli, such as heat or chemicals, may lead to inflammation (Wulandari, Sunarsih and Musnaini, 2024). Therefore, measuring these two activities will give a hint to discovering the anti-inflammatory activity of a sample.

2.3 *Tradescantia zebrina*

2.3.1 Characteristics

T. zebrina is a herbal monocotyledonous plant native to Mexico and Central America, but its distribution covers many tropical and subtropical areas (Feihrmann *et al.*, 2024; Ramos-Arcos *et al.*, 2023). It grows well in varying types of soil, in forests and open woodlands This plant is succulent and easy to grow (Baghalpour *et al.*, 2021; Butnariu *et al.*, 2022; Feihrmann *et al.*, 2024). *T. zebrina*, known locally as the "Wandering Jew" is a perennial herb with trailing stems as well as fleshy and oval leaves (Baghalpour *et al.*, 2021). **Figure 2.2** demonstrates the appearance of *T. zebrina* leaves. This plant is characterised by leaves with a green colour on the top and purple undersides (Ramos-Arcos *et al.*, 2023).

Figure 2.2: Leaves of *T. zebrina*.
(Source: Photographs taken from samples purchased from local market in Kampar, Perak)

2.3.2 Traditional Uses

T. zebrina was used extensively as traditional Chinese medicine for diseases regarding renal system. According to Butnariu and the team (2022), the leaves are believed to have anti-inflammatory properties that can treat haemorrhoids and kidney infections in Jamaica. In the Caribbean, kidney and urinary problems can be relieved by consuming leaf extracts. Malaysians, on the other hand, consume a decoction of the plant to improve kidney function (Butnariu *et al.*, 2022; Luo, 2020).

Other than the uses above, extracts of *T. zebrina* are also consumed as a treatment of conjunctivitis in East Cuba (Bagahalpour *et al.*, 2021). In Jamaica, this plant was used to treat high blood pressure, cough and tuberculosis (Butnariu *et al.*, 2022). This plant is also used as a traditional remedy to treat colorectal cancer, possibly explained by the presence of antitumor compounds (Tan and Kwan, 2020). Since *T. zebrina* has promising antioxidant activity, it is used to make "*Matali*", a cold tonic drink in Mexico.

2.3.3 Phytochemical Compositions

Dash, Swe and Mathews (2017) reported phytochemical compounds like ecdysone, 3β,5α,6β-trihydroxy stigmastrol, β-sitosterol, and succinic acid from the organic extracts of *T. zebrina*. Alaba and Chichioco-Hernandez (2014) identified saponin, flavonoid and phenol in the methanolic extract. Meanwhile, Chunduri and Shah (2016) identified alkaloids, tannins, flavonoids and terpenoids in the methanolic extracts.

da Silva *et al.* (2022) compared the composition of different phenolics in the methanolic extract of dried and fresh leaves using HPLC-diode array detector (HPLC-DAD) as demonstrated in **Table 2.1**. It is notable that whether the leaves are dried or fresh brings effects to the extraction of certain phytochemicals like ferulic acid, chrysine, and rutin in the methanolic extracts.

Table 2.1: Phenolic compounds in methanolic extracts of *T. zebrina* (da Silva *et al.*, 2022).

Analyte	Concentration, µg/g (from dried leaves)	Concentration, µg/g (from fresh leaves)
Caffeic acid	182.25	296.41
Chlorogenic acid	nd	nd
Chrysine	115.90	nd
Ellagic acid	292.40	1252.03
Ferulic acid	136.80	nd
p-coumaric acid	64.85	59.92
Protocatechuic acid	84.33	35.74
Rutin	353.83	nd
Vanillin	42.36	17.72

nd: not determined.

Tatsuzawa *et al.* (2010) had identified the presence of a small amount of monodecaffeoylzebrinin and a large amount of zebrinin in the leaf of *T. zebrina*, as shown in **Figure 2.3**. The former is a rare anthocyanin that has been recognised in *T. zebrina* in the extraction using HCl-methanol. Zebrinin isolated from the plant was determined by Idaka *et al.* (1987) as 3-O-(6-O-(2,5-di-O-caffeyl-α-L-arabinofuranosyl)-β-D-glucopyranosyl)-7,3′-di-O-(6-O-caffeyl-β-D-glucopyranosyl)cyanidin. On the other hand, Stirton and Harborne (1980) identified the presence of cyanidin-3,7,3′-triglucoside acetylated with caffeic acid as the major pigment via paper chromatography isolation.

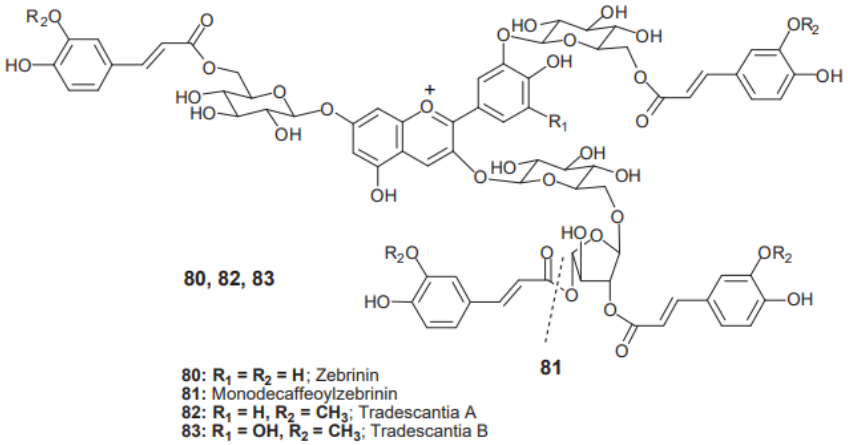

Figure 2.3: Structure of zebrinin and monodecaffeoylzebrinin isolated from the leaf extracts of *T. zebrina*.
(Source: Honda and Saito, 2002)

Feihrmann *et al.* (2024) discovered cyanidin-3-glucoside, annatto, cyanin, gallic acid, quercetin and chlorogenic acid in the aqueous extracts. When performing ultrasonic-assisted extraction (UAE) using aqueous solvent, the author identified malvidin-3,5-diglucoside and bixin in addition to those found in the aqueous extracts without ultrasound.

Many researchers had investigated the phytochemical contents in various extracts of *T. zebrina*, generally for phenolic and flavonoid. **Table 2.2** below displays the evaluation of total phenolic content (TPC) and total flavonoid content (TFC) in previous research alongside with the reference. In the study done by Feihrmann *et al.* (2024), the total monomeric anthocyanins were successfully evaluated in the ultrasonic-assisted water extracts of *T. zebrina* leaf by the pH difference method. The authors found that total monomeric anthocyanins varied from 25 to 41 µg cyanidin-3-O-glucoside equivalent per g extract.

Table 2.2: Phytochemical contents in various extracts of *T. zebrina*.

Extract	TPC (mg GAE per g extract)	TFC	Reference
Aqueous	41.67 ± 0.12	13.12 ± 0.06 (mg CE per g extract)	Ramos-Arcos *et al.*, 2023
Aqueous (infusion)	47.2	21.1 ± 0.9 (mg CE per g extract)	Evelyn *et al.*, 2020
Hydroalcoholic	73.07 ± 0.78	24.34 ± 0.68 (mg CE per g extract)	Ramos-Arcos *et al.*, 2023
Ethanolic	76.88 ± 0.14 (µg GAE per g extract)	Not tested	Baghalpour *et al.*, 2021
Methanolic	33.5 ± 2.6	9.4 ± 1.1 (mg CE per g extract)	Cheah *et al.*, 2017
Methanolic	6.209 ± 0.397	0.576 ± 0.035 (mg RE per g extract)	Tan *et al.*, 2014
Maceration with methanol, followed by re-maceration with solvent exchange	67.68 ± 0.12	10.99 ± 0.08 (mg QE per g extract)	da Silva *et al.*, 2022

TPC: Total phenolic content, TFC: Total flavonoid content, GAE: Gallic acid equivalent, CE: Catechin equivalent, RE: Rutin equivalent, QE: Quercetin equivalent.

2.3.4 Antioxidant Activities

Various previous research has emphasised various antioxidant properties of *T. zebrina* leaves using different extraction methods and solvents. Most research focused on DPPH• and ABTS•$^{+}$ scavenging activity, and FRAP. Ramos-Arcos *et al.* (2023) performed two types of extraction (aqueous and hydroalcoholic) and three assays to analyse the antioxidant activities of the extracts. **Table 2.3** below demonstrates the comparison between the two extracts and the respective antioxidant activities. Whereas for the methanolic extracts, Tan *et al.* (2014) had determined that FRAP and DPPH• scavenging activity of the extract is 4.8 ± 0.3 mg gallic acid equivalent (GAE) per g dry extract and 906.5 ± 88.2 mg ascorbic acid equivalent (AAE) per 100 g dry extract, respectively. In the research by Cheah *et al.* (2017), 0.10 mg/mL extract can achieve a DPPH• scavenging activity of 18.10%.

Table 2.3: Antioxidant activities of aqueous and hydroalcoholic extracts of *T. zebrina* (Ramos-Arcos *et al.*, 2023).

Extract	**IC_{50} of DPPH• scavenging activity (µg/mL)**	**IC_{50} of ABTS•$^{+}$ scavenging activity (µg/mL)**	**FRAP (mM TE per g extract)**
Aqueous	1146.90 ± 92.53	1060.32±171.58	318.97 ± 0.37
Hydroalcoholic	831.09 ± 71.61	1033.67±149.91	353.92 ± 3.20

TE represents Trolox equivalent while IC_{50} represents half maximal inhibitory concentration.

2.3.5 Anti-Inflammatory Activities

Anti-inflammatory activities are usually determined via inhibition of oedema. Baghalpour *et al.* (2021) discovered that anti-inflammatory activity against carrageenan-induced paw oedema was recorded in the ethanolic extract of *T. zebrina* along with its hexane, ethyl acetate, and chloroform fractions. The activities were more significant in the ethyl acetate and chloroform fractions. Flavonoids could be the origin of the fractions' effectiveness. Research by Ramos-Arcos *et al.* (2023) found that the aqueous extracts can show oedema (induced using 12-O-tetradecanoylphorbol-13-acetate) inhibition of 42.16% (by mass differences comparing to untreated ear) at a dose of 1.6 mg/ear.

Evelyn *et al.* (2020) also assess the anti-inflammatory of various *T. zebrina* leaf extracts. They discovered that both 80% methanol extracts and aqueous extracts (by infusion) are able to inhibit the denaturation of egg albumin by heat. In this assay, the aqueous extracts can achieve a higher activity (85.46%) comparing to 80% methanol extracts (44.5%). However, when evaluated via inhibition of haemolysis of erythrocytes membranes, 80% methanol extracts show an activity of 29.42% while after infusion, the plant extract loses the activity. Alaba and Chichioco-Hernandez (2014) determined the presence of 15-lipoxygenase inhibitory activity (64.44%) as a possible indication of its anti-inflammatory activity.

2.4 Extraction

According to Ramos-Arcos *et al.* (2023) and Marrelli (2021), extraction is the preliminary step in the investigation of medicinal plants which aims to obtain the desired compounds from original materials. The efficiency of extraction largely depends on polarity of the solvent, temperature, pH, time of extraction and composition of sample (Ramos-Arcos *et al.*, 2023; Ouahabi *et al.*, 2023). Feihrmann *et al.* (2024) discovered that in ultrasonic-assisted extraction (UAE) of *T. zebrina*, temperature has a significant effect for extracting phenolic compounds and anthocyanins. An optimum temperature has a profound impact on softening the tissues to increase solubilisation and diffusion of phytochemicals. Ramos-Arcos *et al.* (2023) proved that the polarity of the solvent brought significant differences in the extraction yield of *T. zebrina*, where aqueous solvent outperformed hydroalcoholic solvent.

Extraction can be carried out using organic solvent such as methanol (Alaba and Chichioco-Hernandez, 2014; Cheah *et al.*, 2017; Tan *et al.*, 2014), ethanol (Baghalpour *et al.*, 2021; Felix-Sagaste *et al.*, 2023), etc. On the other hand, plant extracts can be prepared by boiling the materials in aqueous solutions such as deionised water (Feihrmann *et al.*, 2024; Felix-Sagaste *et al.*, 2023). Listed in **Table 2.4** are the different extraction parameters for performing hot water extraction.

Table 2.4: Hot water extraction parameters used in different studies.

Temperature (°C)	Duration	Sample to water ratio	Special procedure	References
90	10 min	9 g dry powder in 300 mL deionised water	Periodic shaking Unique filtration: Whatman No.1 filter paper, then Millipore equipment	(Ramos-Arcos *et al.*, 2023)
Boiled	10 min	1 g leaves in 10 mL water	Infusion	(Evelyn *et al.*, 2020)
Boiled	30 mins	5 g dry leaves in 500 mL Milli-Q water	Filtration through a 0.22 µm membrane	(Garcia-Varela *et al.*, 2015)

Comparing these extraction strategies, hot water extraction (HWE) is simple and non-toxic. Applying heat to boil the solution helps to extract thermally stable compounds such as phenolics and flavonoids at a higher efficiency. Studies also show that the application of boiling increases the extraction of phytochemicals and even functional antioxidants (Hng, 2017; Pagano *et al.*, 2021). HWE of culinary herbs is of special concern and is more reliable since the common kitchen processing like boiling can be mimicked using temperature around

100 °C (Pangsatabam and Nandhini, 2015; Yang *et al.*, 2007). Cheng, Xue and Yang (2023) and Leo *et al.* (2022) also recommended that polarity of water will be decreased by elevating the temperature in HWE.

An appropriate extraction duration is about 15 to 60 minutes. Jovanović *et al*. (2017) found that the total polyphenols yield from *Thymus serpyllum* was not affected after 15 minutes in HWE. In *Aloe vera* extracts, Elferjane *et al.* (2023) found that there is no effect of extraction time on $ABTS^{\bullet+}$ and DPPH• scavenging activity, but with an increase in FRAP up to 30 minutes of extraction. The absence of influence on TPC can be explained by the occurrence of extraction in two stages, which is an initial increase in the first 15 minutes followed by slow extraction in the following 45 minutes.

UAE is getting more concerns as a modern technique to boost the effectiveness of extraction of polyphenols, carotenoids, aromas and polysaccharides from plant matrices (Kumar, Srivastav and Sharanagat, 2021). This technique uses ultrasound (frequency > 20 kHz) to create cavitation bubbles. Cavitation is the main mechanism of UAE which leads to the fragmentation of cellular structure that causes solubilisation of targeted compounds to be extracted. By applying this unique mechanism, the duration of extraction could be shortened, and it can be appropriate to reduce the temperature (Milani *et al.*, 2020; Raspe *et al.*, 2021).

Feihrmann *et al.* (2024) had extensively studied the use of UAE of *T. zebrina* in aqueous solvent. When using the optimum conditions of performing UAE to perform a control experiment (without ultrasound), the phenolic contents and anthocyanin contents were both lower than that of the setting with ultrasound. On the other hand, the authors also discovered that a high temperature allowed a better extraction, but at remarkably high temperatures, it may impair cavitation and cause degradation of the compounds extracted. The positive relationship between temperature and extraction efficiency is also seen in Raspe *et al.* (2021) on stevia leaves.

According to Airouyuwa *et al.* (2024), to improve the efficiency of extraction at an industrial scale, a combination between extraction techniques can be applied. This combination may minimise shortcomings in individual extraction procedures by utilising synergistic effects and incorporating multiple mechanisms (Capaldi *et al.*, 2024). The sequential hybrid extraction technique is performed by sequentially employing two extraction techniques, where the first technique is seen as a pre-treatment for the subsequent method (Airouyuwa *et al.*, 2024).

Combining UAE with microwave-assisted extraction can boost TPC in the *Abelmoschus sagittifolius* root powder (ADP) extracts compared to individual extractions. However, when ultrasonic-assisted extraction was performed first, both total phenolic and terpenoid content were higher than the one where microwave-assisted extraction is performed first (Vo *et al.*, 2023). Hence, it is notable that the sequence of extraction could be important to maximise the

extraction of phytochemicals. A study by Airouyuwa *et al.* (2024) compared the usage of individual, binary hybrid and ternary hybrid extraction techniques in the extraction of phenolics from date seeds. Almost all binary hybrid extractions were significantly better than their individual extractions. However, the effects of various ternary hybrid extractions were not consistent.

2.5 Simulated Gastrointestinal Digestion

2.5.1 Concepts

Simulated gastrointestinal (GI) digestion is an *in vitro* exposure of a sample to three succeeding digestive phases, which are oral, gastric and intestinal phases, with the details presented in **Figure 2.4**. There are several types of methods classified into static and dynamic models. Simulated GI digestion aims to mimic the conditions in the GI tract. (Brodkorb *et al.*, 2019). INFOGEST 2.0 protocol is an example of the static digestion model. In a static model, a constant proportion of samples to enzymes and salts is applied. The pH for each phase is also fixed at a constant value (Zhou, Tan and McClements, 2023). The main update from INFOGEST to INFOGEST 2.0 is that in the latter, all food samples should be subjected to an oral phase, and gastric lipase is recommended in the gastric phase (Dávila León *et al.*, 2024).

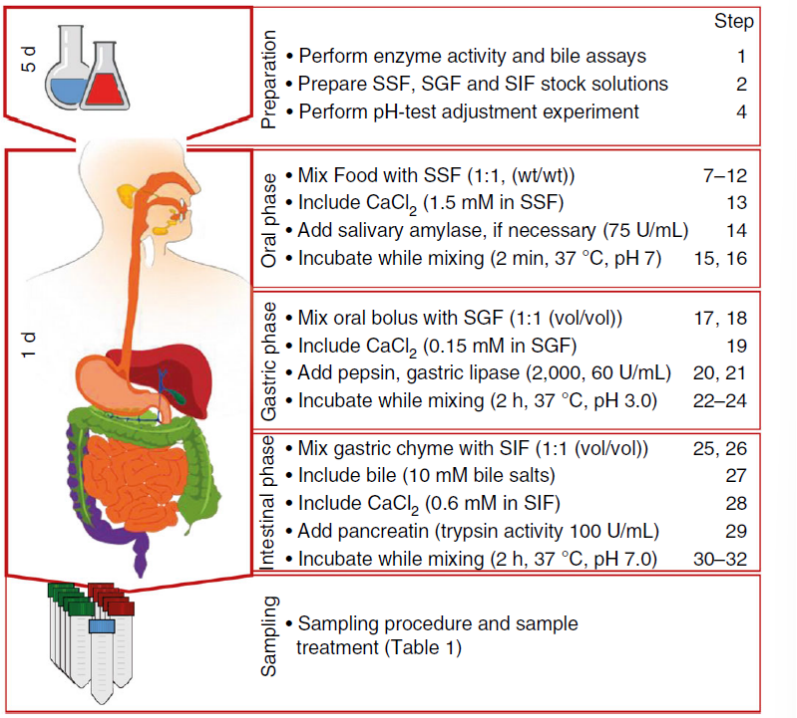

Figure 2.4: Flow diagram of the INFOGEST 2.0 protocol.
(Source: Brodkorb *et al.*, 2019)

A static model is relatively simpler as opposed to the relatively complex and expensive dynamic models. Static models are more reproducible and robust than the dynamic models (Brodkorb *et al.*, 2019). Zhou, Tan and McClements (2023) also mentioned that a static model standardises the conditions in each phase to allow better comparisons between different researchers or even different laboratories. Normally, using *in vivo* models to study digestion and absorption of foods is not experimentally and economically feasible (Qiao *et al.*, 2025). Hence, the development of *in vitro* models seems essential. However, static models cannot thoroughly mimic the actual complicated digestion that occurs in the human body. This makes static models unsuitable for detailed kinetic analysis (Brodkorb *et al.*, 2019).

2.5.2 Effect on Bioactivity of Plant Extracts

Bioaccessibility is the fraction or percentage of a compound released from its matrix after GI digestion. Bioactivity of bioactive compounds is subject to human GI conditions like physicochemical factors and biochemical factors. GI affects the availability of such compounds to be absorbed. On the other hand, the total bioactive compounds in food do not always represent the total available amount to be absorbed after GI digestion and metabolised by the human body. GI digestion in this study allows the analysis of phenolic compounds' stability (or bioaccessibility) during digestion (Brodkorb *et al.*, 2019; Odriozola-Serrano *et al.*, 2023; Ośko, Nasierowska and Grembecka, 2024; Peña-Vázquez, 2022; Sęczyk and Kołodziej, 2024; Wanyo *et al.*, 2024).

Many researchers had used the INFOGEST 2.0 protocol to inspect how GI digestion affects various bioactivities of plant extracts. Some studies discovered that simulated GI digestion will cause a decrease in various phytochemical contents. For instance, Ma *et al.* (2024) discovered that prior to successive phases of GI digestion, both TPC and TFC of *Lophatherum gracile* leaf extracts had dropped. Further comparisons between "only pH treated" and "digested" samples suggest that changes in phytochemical contents were mainly influenced by pH. In purple rice bran extracts, TPC was found to drop about 28% while TFC was found to drop about 25 to 28% after the intestinal phase of GI digestion (Wanyo *et al.*, 2024).

The effect of GI digestion was also extensively studied on the antioxidant activities of various plant extracts. In *Camellia sinesis* (L.) Kuntze leaf extracts, the evaluated DPPH• scavenging activity was reduced by GI digestion. GI digestion significantly increased the IC_{50} values of different tea leaf extracts from as low as 2.3-fold in green tea extracts to 9.6-fold in Oolong tea extracts (Governa *et al.*, 2022). According to Wanyo *et al.* (2024), the antioxidant activity (as evaluated by the FRAP assay) in Hom Nil and Riceberry extracts showed a reduction of 19.98% and 24.00%, respectively, after the intestinal phase of GI digestion.

However, jumping into conclusion that GI digestion would only cause negative effects is inappropriate. Several studies found that GI digestion may improve certain bioactivities. Un *et al.* (2022) discovered that after three successive phases of GI digestion, the α-amylase inhibitory activity increased in two of the three chromatography fractions of the *Sargassum* spp. hexane extract. The authors also found that the same activity of palmitic acid had increased by about 63.53% after completing the intestinal phase.

In the chestnut shells' extracts, Pinto *et al.* (2024) identified that hydroxyhippuric acid, dihydroxyphenylpropionic acid and hydroxybenzaldehyde were present in the digesta of gastric phase and intestinal phase, which was not found in the undigested extract. During GI digestion, certain phenolic compounds were biotransformed into other metabolites, which explains the presence of compounds listed above in the post-digested extract. The authors also pointed out that 45 compounds were identified in the

undigested extract, but only 24 compounds were identified after the intestinal phase, indicating that some compounds were degraded during simulated GI digestion.

2.6 Research Gap

Currently, antioxidant activity, TPC and TFC were well quantified in different extracts of *T. zebrina*. However, the effects of GI digestion on antioxidant or anti-inflammatory activities are yet undiscovered. The bioaccessibility of phytochemicals after GI digestion is also a key research gap. Many extracts of different species show declined bioactivities after GI digestion. Yet, there are still examples which show improved activity after the treatment.

Without any discoveries on the impact of GI digestion, the potential benefits such as antioxidant and anti-inflammatory activities when consumed orally, would be an assumption, or even an overestimation. Therefore, addressing these gaps is important to confirm whether the extracts retain activities upon consumption, thus providing possible contributions to its medicinal uses. Moreover, *in vitro* studies on bioaccessibility of phytochemicals and changes in bioactivities would lay a foundation towards the more physiologically applicable *in vivo* studies.

CHAPTER 3

MATERIALS AND METHODS

3.1 Instruments and Chemicals

The instruments and chemicals used in this project were provided by Department of Chemical Science, Faculty of Science in Universiti Tunku Abdul Rahman. The instruments and chemicals are listed in **Tables 3.1** and **3.2**, respectively, with their manufacturers.

Table 3.1: List of instruments used with their manufacturers.

Instrument	**Manufacturer**
Analytical balance	Sartorius / CP224S
Freeze-dryer	LABOGENE / ScanVac
Hot plate	Stuart
Oven	BINDER / FD115
pH meter	EUTECH
Refrigerated centrifuge	Dynamica / Velocity14R
Refrigerator	LG
Rotary evaporator	Heidolph
Shaking incubator	N-BIOTEK / NB-205
Ultrasonic bath	Bransonic / 5510E-DTH
UV-VIS spectrophotometer	ThermoFisher / Genesys 10S
Vortex mixer	Science Lab Asia Sdn. Bhd. / VM-300
Water bath	Memmert WB-22
Weighing balance	BEL S1502

Table 3.2: List of chemicals used with their manufacturers.

Chemical	Manufacturer
1,1-diphenyl-2-picrylhydrazyl (DPPH)	ALDRICH
2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)	Tokyo Chemical Industry (TCI)
2,4,6-tripyridyl-s-triazine (TPTZ)	MERCK
Aluminium chloride hexahydrate	Asia Pacific Specialty (APS)
Ammonium hydrogen carbonate	Fisher Scientific
Ascorbic acid	ChemSol
Bile extract porcine	SIGMA Life Science
Bovine serum albumin fraction V	MERCK
Calcium chloride dihydrate	Fisher Scientific
Ethanol	Fisher Scientific
Ferrous sulphate heptahydrate	MERCK
Folin-Ciocalteu reagent	Chemiz
Gallic acid	MERCK
Glacial acetic acid	RCI Labscan
Hydrochloric acid	Synerlab laboratory reagent
Hydrogen peroxide	R&M Chemicals
Iron (III) chloride hexahydrate	Supelco
Magnesium chloride hexahydrate	Fisher Scientific
Methanol	EMSURE®

Table 3.2: (continued)

Chemical	Manufacturer
N-(1-naphthyl)ethylenediamine dihydrochloride	ALDRICH
Phosphate buffered saline (PBS) tablet, pH 7.3 ± 0.2	OXOID
Phosphoric acid	ALDRICH
Porcine pancreatin	SIGMA Life Science
Porcine pepsin	ChemSolv
Potassium chloride	R&M
Potassium dihydrogen phosphate	SYSTERM
Quercetin hydrate	ARCOS ORGANICS
Sodium acetate	SYSTERM
Sodium carbonate decahydrate	HmbG chemicals
Sodium chloride	Fisher Scientific
Sodium hydrogen carbonate	Rdeh
Sodium hydroxide	MERCK
Sodium nitrite	QReC™
Sodium nitroprusside	MERCK
Sulphanilamide	ARCOS ORGANICS

3.2 Experimental Design

The overview of the experimental approaches for this study is summarised in the following:

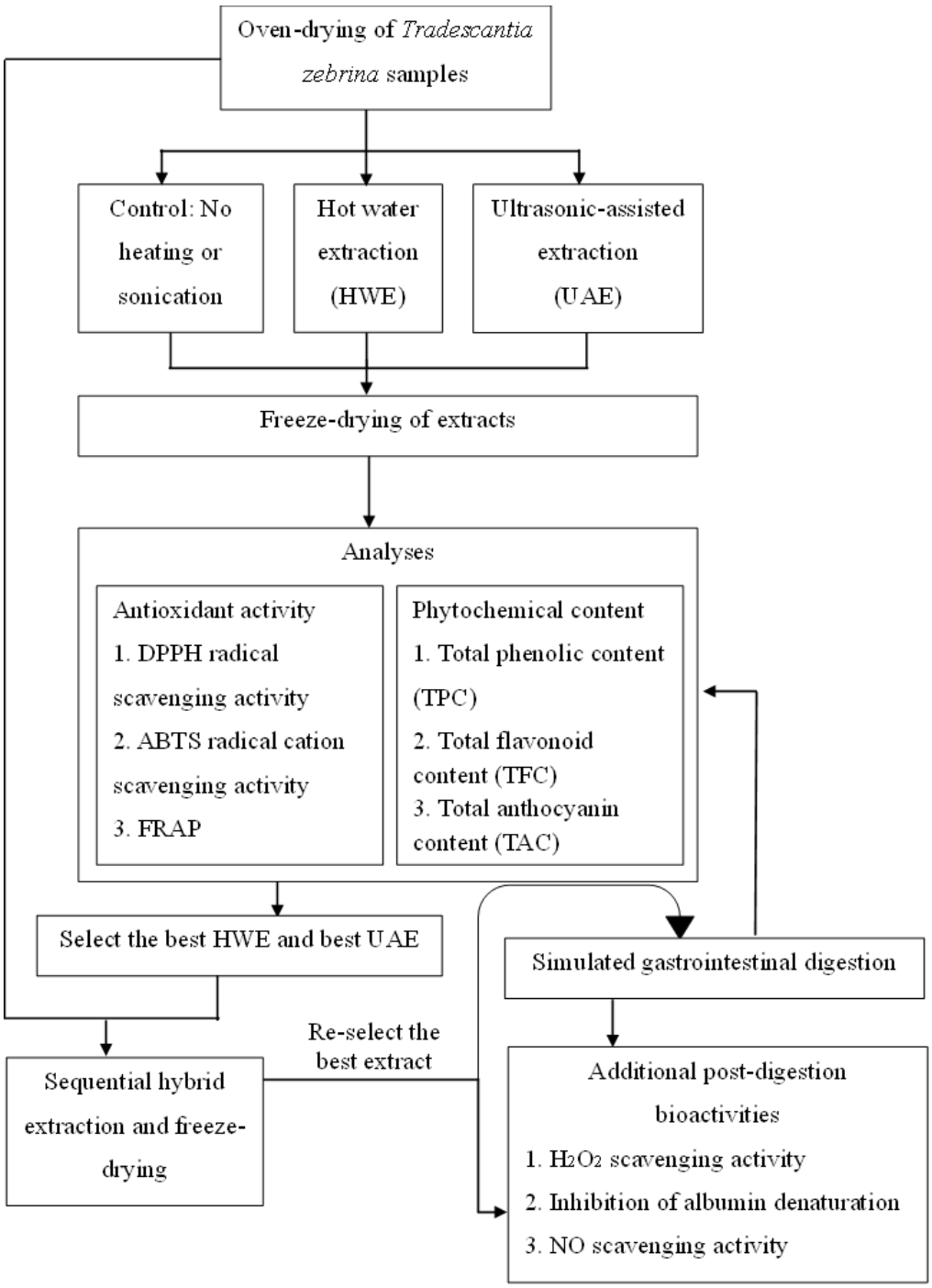

Figure 3.1: Summary of the experimental approaches for this research.

3.3 Sample Materials and Oven Drying

Fresh leaves of *Tradescantia zebrina* were purchased from a local morning market at Kampar, Perak on 8 March 2025. The leaves were oven-dried at 50°C to a constant weight. Dried leaves were powdered and kept in chilled condition until use.

Figure 3.2: Oven-dried leaves of *T. zebrina* after 48 hours at 50°C.

3.4 Extraction

A “No heating or sonication” treatment was performed by mixing 4 g of leaf powder with deionised water in a 3 g:100 mL ratio, which was subsequently centrifuged at 10,000 rpm for 15 minutes. Next, the supernatant was freeze-dried to obtain the extract powder, which was stored at -20°C for further uses.

3.4.1 Hot Water Extraction

Hot water extraction (HWE) was performed according to Ramos-Arcos *et al.* (2023) with slight modifications. Extraction was performed by mixing 4 g of leaf powder with deionised water in a 3 g:100 mL ratio, respectively. The mixture was then incubated in a 90℃-water bath with periodic shaking for either 15, 30, or 60 minutes, respectively. Next, the mixture was centrifuged at 10,000 rpm for 15 minutes. The supernatant was freeze-dried to obtain the extract powder, which was stored at -20℃ for further uses.

3.4.2 Ultrasonic-Assisted Extraction

Ultrasonic-assisted extraction (UAE) was performed according to Feihrmann *et al.* (2024) with slight modifications. Extraction was performed by mixing 4 g of leaf powder with deionised water in a 3 g:100 mL ratio, respectively. The mixture was then incubated in an ultrasonic bath at 60℃ and 42 kHz for either 5, 10, or 20 minutes, respectively. Next, the mixture was centrifuged at 10,000 rpm for 15 minutes. The supernatant was freeze-dried to obtain the extract powder, which was stored at -20℃ for further uses.

3.4.3 Hot Water – Ultrasonic-Assisted Sequential Hybrid Extraction

To investigate the effect of combining two extraction methods on various bioactivities, a sequential hybrid extraction was performed as described by Airouyuwa *et al.* (2024). Briefly, the extraction strategies included:

(i) HWE for 15 minutes, followed by UAE for 20 minutes.

(ii) UAE for 20 minutes, followed by HWE for 15 minutes.

The extraction mixtures were subjected to centrifugation and the resultant supernatants were freeze-dried as described in **Section 3.4.1**

3.5 Yield Determination

The yield of all extracts was calculated by applying the formula below and was expressed in percentage (%).

$$\text{Extraction yield (\%)} = \frac{\text{Mass of freeze-dried extract (g)}}{\text{Mass of leaf powder used for extraction (g)}} \times 100\%$$

3.6 Phytochemical Content

3.6.1 Total Phenolic Content

Total phenolic content (TPC) was determined using a Folin-Ciocalteu colorimetric method according to Chai and Wong (2012). Sample (200 µL), 800 µL of deionised water and 100 µL Folin-Ciocalteu reagent was first incubated for 3 minutes at room temperature. Subsequently, 300 µL of 20% (w/v) sodium carbonate decahydrate was added and the mixture was incubated at room temperature for 120 minutes. Absorbance at 765 nm was recorded. Each sample was accompanied by a simultaneous control reaction in which Folin-Ciocalteu reagent was replaced with water. TPC was expressed as mg gallic acid equivalent (GAE) per g dry extract, which was calculated from a calibration curve prepared using 0 to 100 mg/L gallic acid.

3.6.2 Total Flavonoid Content

Total flavonoid content (TFC) was determined through a colorimetric assay using aluminium chloride according to Chai and Wong (2012). Two hundred µL sample was added to 150 µL of 5% (w/v) sodium nitrite and the mixture was incubated for 6 minutes at room temperature. Next, 150 µL 10% (w/v) of aluminium chloride hexahydrate was added following an incubation for another 6 minutes at room temperature. Then, 800 µL of 10% (w/v) sodium hydroxide solution was added and the mixture was allowed to stand at room temperature for 15 minutes. Absorbance was measured at 510 nm. Each sample was accompanied by a simultaneous control reaction in which aluminium chloride hexahydrate was replaced with water. TFC was expressed as mg quercetin equivalent (QE) per g dry extract, which was calculated from a calibration curve prepared using 0 to 500 µg/mL quercetin (in 80% ethanol).

3.6.3 Total Anthocyanin Content

Total anthocyanin content (TAC) was determined through a pH differentiation method according to Giusti and Wrolstad (2001). Two hundred µL sample was added to 800 µL of 25 mM potassium chloride-hydrochloric acid buffer (pH 1.0). Another 200 µL of extract was introduced into 800 µL of 400 mM sodium acetate-acetic acid buffer (pH 4.5) simultaneously. The reaction mixtures were incubated in dark for 15 minutes at room temperature. Absorbance of each treatment was taken at 510 nm and at 700 nm. Water was used as blank. The corrective absorbance of the diluted sample was calculated as follows:

$$\text{Corrective absorbance } (A_c) = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}$$

where A_c represents corrective absorbance, A_{510} and A_{700} represent absorbances at 510 nm and 700 nm, respectively. TAC was calculated directly by using A_c calculated beforehand using the formula:

$$TAC\ (mgL^{-1}) = \frac{A_c \times MW \times DF \times 1000}{\varepsilon \times 1}$$

where MW is the molecular weight of cyanidin-3-glucoside (equivalent to 449.2 g/mol); DF is the dilution factor of the sample; ε is the molar absorptivity of cyanidin-3-glucoside (equivalent to 26900 $M^{-1}cm^{-1}$). Finally, TAC was presented as μg cyanidin-3-glucoside equivalent (CGE) per g dry extract.

3.7 Determination of Bioactivities

3.7.1 1,1-Diphenyl-2-picrylhydrazyl Radical Scavenging Activity

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH•) scavenging activity was determined according to Chai and Wong (2012), with slight modifications. Five hundred μL sample (varying concentrations) was mixed with 500 μL of 0.10 mM DPPH (in pure ethanol). The reaction mixture was incubated in dark for 30 minutes at room temperature. Absorbance at 517 nm was measured. DPPH• was replaced by pure ethanol as blank for each sample. DPPH• scavenging activity of the sample was calculated as shown in the formula:

$$\text{DPPH} \bullet \text{ scavenging activity } (\%) = (1 - \frac{A_{sample}}{A_{control}}) \times 100\%$$

where $A_{control}$ is the absorbance of control reaction in the absence of sample (sample was replaced by its solvent) and A_{sample} is the absorbance of reaction in the presence of sample. The positive control used for this assay was quercetin

(in 80% ethanol). Results were expressed as EC_{50}, which is defined as the concentration of sample required to achieve 50% of DPPH• scavenging activity.

3.7.2 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) Radical Cation Scavenging Activity

2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation ($ABTS^{\bullet+}$) scavenging activity of sample was determined according to Chai and Wong (2012), with slight modifications. $ABTS^{\bullet+}$ stock solution (7 mM) was prepared by mixing 8 mg/mL ABTS solution and 1.32 mg/mL potassium persulfate solution in a one-to-one ratio. An $ABTS^{\bullet+}$ working solution was prepared by diluting the stock solution with phosphate buffered saline (PBS) (pH 7.3) to reach an absorbance of 0.700 ± 0.005. Sample (100 µL) was added to 1000 µL of $ABTS^{\bullet+}$ working solution. The reaction mixture was kept in dark at room temperature for 10 minutes. Absorbance was measured at 734 nm. $ABTS^{\bullet+}$ working solution was replaced by PBS as blank for each sample. $ABTS^{\bullet+}$ scavenging activity of the sample was calculated as shown in the formula:

$$\text{ABTS}^{\bullet+}\ \text{scavenging activity}\ (\%) = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100\%$$

where $A_{control}$ is the absorbance of control reaction in the absence of sample (sample was replaced by its solvent) and A_{sample} is the absorbance of reaction in the presence of sample. The positive control used for this assay was quercetin (in 80% ethanol). Results were expressed as EC_{50}, which is defined as the concentration of sample required to achieve 50% of $ABTS^{\bullet+}$ scavenging activity.

3.7.3 Ferric Reducing Antioxidant Power

Ferric reducing antioxidant power (FRAP) was determined according to Chai and Wong (2012). FRAP reagent was prepared fresh by mixing acetate buffer (300 mM, pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) and 20 mM iron (III) chloride hexahydrate in a 10:1:1 (volume to volume to volume) ratio and pre-warmed at 37℃ before use. Sample (200 µL) was mixed with 1200 µL of FRAP reagent and incubated at 37℃ for 5 minutes. Absorbance was measured at 593 nm. A blank was prepared by replacing sample by its solvent. FRAP value was expressed as µmol Fe^{2+} equivalents per g dry extract, which was calculated from a calibration curve established using 0 to 0.40 mM ferrous sulphate heptahydrate.

3.7.4 Hydrogen Peroxide Scavenging Activity

Hydrogen peroxide (H_2O_2) scavenging activity was determined according to Ruch, Cheng and Klaunig (2012), with slight modifications. Sample (200 µL) was added to 600 µL 40 mM H_2O_2 (in PBS). The reaction mixture was kept in dark at room temperature for 10 minutes. Absorbance was measured at 230 nm. H_2O_2 was replaced by PBS as blank for each sample. H_2O_2 scavenging activity of the sample was calculated as shown in the formula:

$$\mathrm{H_2O_2}\text{ scavenging activity (\%)} = (1 - \frac{A_{sample}}{A_{control}}) \times 100\%$$

where $A_{control}$ is the absorbance of control reaction in the absence of sample (sample was replaced by its solvent) and A_{sample} is the absorbance of reaction in the presence of sample. The positive control used for this assay was gallic acid (in pure ethanol). Results were expressed as EC_{50}, which is defined as the concentration of sample required to achieve 50% of H_2O_2 scavenging activity.

3.7.5 Nitric Oxide Scavenging Activity

Nitric oxide (NO) scavenging activity was determined according to Chai *et al*. (2014), with slight modifications. Sample (1200 µL) was added to 300 µL of 5 mM sodium nitroprusside in phosphate buffered saline (PBS) (pH 7.3). The reaction mixture was kept at room temperature under light source for 150 minutes. Then, 600 µL of reaction mixture was added to 600 µL of Griess reagent (1% w/v sulphanilamide and 0.1% w/v N-(1-naphthyl)ethylenediamine dihydrochloride in 5% v/v phosphoric acid). The reaction mixture was kept in dark for 10 minutes. Absorbance was measured at 546 nm. Griess reagent was replaced by deionised water as blank for each sample. NO scavenging activity of the sample was calculated as shown in the formula:

$$\text{NO scavenging activity (\%)} = (1 - \frac{A_{sample}}{A_{control}}) \times 100\%$$

where $A_{control}$ is the absorbance of control reaction in the absence of sample (sample was replaced by its solvent) and A_{sample} is the absorbance of reaction in the presence of sample. The positive control used for this assay was ascorbic acid (in deionised water). Results were expressed as EC_{50}, which is defined as the concentration of sample required to achieve 50% of NO scavenging activity.

3.7.6 Inhibition of Albumin Denaturation

Inhibition of albumin denaturation was determined according to Kpemissi *et al.* (2012), with slight modifications. Twenty-five µL sample was added to 225 µL 5% w/v bovine serum albumin fraction V (BSA) in phosphate buffered saline (PBS) (pH 7.3). The reaction mixture was incubated at 37°C for 15 minutes. Then, the reaction mixture was incubated at 70°C for 5 minutes. Absorbance was measured at 660 nm after adding 500 µL of PBS. BSA was replaced by PBS as

blank for each sample. Percentage inhibition on albumin denaturation of the sample was calculated as shown in the formula:

$$\text{Inhibition of albumin denaturation (\%)} = (1 - \frac{A_{sample}}{A_{control}}) \times 100\%$$

where $A_{control}$ is the absorbance of control reaction in the absence of sample (sample was replaced by its solvent) and A_{sample} is the absorbance of reaction in the presence of sample. The positive control used for this assay was quercetin (in 80% ethanol). Results were expressed as EC_{50}, which is defined as the concentration of sample required to achieve 50% of inhibition of albumin denaturation.

3.8 Simulated Gastrointestinal Digestion

The simulated gastrointestinal (GI) digestion was performed based on the INFOGEST 2.0 protocol by Brodkorb *et al.* (2019) with slight modifications (Ferreira-Santos *et al.*, 2024; Jaouhari *et al.*, 2025). GI digestion was performed on the "UAE for 20 min, followed by HWE for 15 min" extract. A "GI Blank" was also prepared by replacing the extract with deionised water (Jaouhari *et al.*, 2025).

3.8.1 Preparation of Simulated Digestive Fluids

Simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared in 1.25 × (to be diluted to 1 × during GI digestion) referring to the INFOGEST 2.0 protocol. The compositions of each of the fluids are shown in Appendices A.

3.8.2 Preparation of Sample and Oral Phase

Fifty mg/mL of the selected extract was prepared using deionised water as the solvent (Ferreira-Santos *et al.*, 2024). Five mL of the prepared sample was added. Subsequently, 1.25 × SSF (4 mL) was introduced to reach a final concentration of 1 ×. Next, 0.3 M $CaCl_2•2H_2O$ (25 µL) was added to achieve a final concentration of 1.5 mM in the reaction mixture. Water (975 µL) was added to reach the target volume (10 mL), which is double of the initial volume of the sample used. The reaction mixture was incubated in a shaking incubator at 37℃ and 125 rpm for 2 minutes.

3.8.3 Gastric Phase

The reaction mixture from the oral phase was subjected to the gastric phase. Eight mL 1.25 × SGF was added to achieve a final concentration of 1 ×. The pH of the reaction mixture was adjusted to 3.0 using 5 M HCl. Next, 0.3 M $CaCl_2•2H_2O$ (5 µL) was added to achieve a final concentration of 0.15 mM in the reaction mixture. Five hundred µL porcine pepsin (40,000 U/mL) was added to achieve 2,000 U/mL in the final volume. Water was added to reach the target volume (20 mL), which is double of the volume from oral phase. The reaction mixture was incubated in a shaking incubator at 37℃ and 125 rpm for 2 hours.

3.8.4 Intestinal Phase

The reaction mixture from the gastric phase was subjected to the intestinal phase. Twelve mL 1.25 × SIF was added to achieve a final concentration of 1 ×. The pH of the reaction mixture was adjusted to 7.0 using 5 M NaOH. Then, 0.3 M $CaCl_2•2H_2O$ (40 µL) was added to achieve a final concentration of 0.6 mM in

the reaction mixture. Next, 2.5 mL porcine pancreatin (800 U/mL) was added to achieve 100 U/mL in the final volume. Following, 1.5 mL porcine bile extract (133.3 mM) was added to achieve 10 mM in the final mixture. Water was added to reach the target volume (40 mL), which is double of the volume from gastric phase. The reaction mixture was incubated in a shaking incubator at 37℃ and 125 rpm for 2 hours.

3.8.5 Post-Digestion Treatment

The reaction mixture was incubated at 100℃ for 5 minutes to stop the reaction by enzymes in pancreatin. Following, the mixture was freeze-dried to obtain samples in powder forms, which was stored at -20℃ for further uses.

3.8.6 C18 Solid-Phase Extraction

Samples were purified by performing C18 solid-phase extraction (SPE) to remove the interfering GI components. “GI Sample” and “GI Blank” were reconstituted in water to achieve a concentration of 10 mg/mL and were filtered using 0.45 μm membrane filter. First, conditioning of each Phenomenex Strata C18-E 500 mg/6 mL cartridge was done with 6 mL pure methanol. The cartridge was subsequently equilibrated using 6 mL deionised water according to the manufacturer’s user manual. Two mL of the filtered samples were loaded to the cartridge. Six mL of 5% (v/v) methanol were used for washing. The flow-through was discarded. Next, 6 mL of 70% (v/v) methanol were used for elution. The eluted solutions were collected in a glass vial and were subjected to rotary evaporation (vacuum set at 337 mbar while water bath temperature set at 40℃) to remove methanol in the eluted sample. The residual water was removed by

freeze-drying. Both “GI sample” and “GI Blank” were reconstituted in water for assays on phytochemical contents and various activities.

3.9 Statistical Analysis

Assays were carried out in three replicates. Data were expressed as mean ± standard error. One way ANOVA and Tukey’s HSD multiple comparison tests were carried out using StatsKingdom (statskingdom.com) to determine the significance of differences between means at a significance level of $p < 0.05$.

CHAPTER 4

RESULTS

4.1 Extraction Yield

Table 4.1 demonstrates the yield of extractions, which varied between each treatment. Extraction yield was calculated based on 4 g of leaf powder. Yield data presented were based on one replicate. The "HWE for 15 min, followed by UAE for 20 min" showed the highest yield while the "HWE for 60 min" gave the lowest yield. Among three variations in HWE, "HWE for 30 min" had the highest yield whereas for UAE, "UAE for 20 min" recorded the highest yield.

Table 4.1: Yield of various aqueous extractions of *T. zebrina*.

Extract		Mass of extract (g)	Extraction yield (%)
HWE	15 min	0.7892	19.73
	30 min	0.8143	20.36
	60 min	0.6176	15.44
UAE	5 min	0.7338	18.35
	10 min	0.7215	18.04
	20 min	0.8242	20.60
No heating or sonication		0.7418	18.54
HWE for 15 min, followed by UAE for 20 min		0.8885	22.21
UAE for 20 min, followed by HWE for 15 min		0.8282	20.71

HWE: hot water extraction, UAE: ultrasonic-assisted extraction.

4.2 Phytochemical Contents of Various Extracts

4.2.1 Total Phenolic Content

The total phenolic content (TPC) of *Tradescantia zebrina* extracts varied significantly among all nine treatments, as determined by one way ANOVA test at a significance level of $p < 0.05$. **Table 4.2** demonstrated the TPCs for each treatment, which were calculated from a standard curve established using gallic acid, as in **Figure 4.1**. The "UAE for 20 min, followed by HWE for 15 min" extract exhibited the highest TPC, significantly outperforming other extractions. The "No heating or sonication" extract showed the lowest TPC, which was 24.70% lower than the extract with the highest TPC. Among three variations in HWE, "HWE for 15 min" shows the highest TPC, which was significantly higher by 1.04- and 1.13-fold than the "HWE for 30 min" and "HWE for 60 min", respectively. On the other hand, "UAE for 20 min" gave the highest TPC (with significant differences) among three variations in UAE.

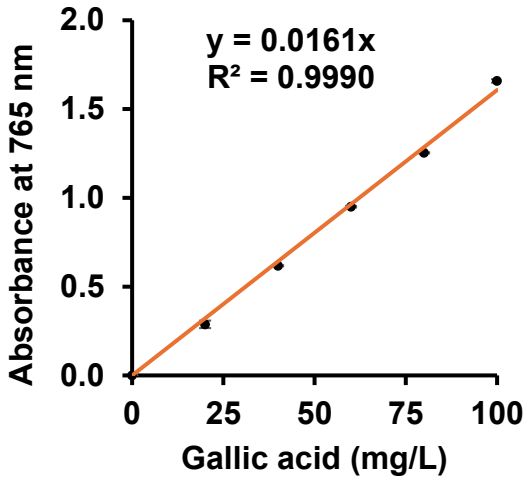

Figure 4.1: Standard curve of TPC constructed using gallic acid. Data are presented as mean ± standard error (n = 3).

Table 4.2: Total phenolic content of *T. zebrina* leaf extracts.

Extract		TPC (mg GAE per g dry extract)
HWE	15 min	7.075 ± 0.013^{a}
	30 min	6.776 ± 0.007^{b}
	60 min	6.246 ± 0.005^{c}
UAE	5 min	6.315 ± 0.004^{d}
	10 min	$6.331 \pm 0.009^{d,e}$
	20 min	6.476 ± 0.010^{f}
No heating or sonication		6.110 ± 0.014^{g}
HWE for 15 min, followed by UAE for 20 min		7.203 ± 0.009^{h}
UAE for 20 min, followed by HWE for 15 min		8.114 ± 0.007^{i}

HWE: hot water extraction, UAE: ultrasonic-assisted extraction, TPC: total phenolic content, GAE: gallic acid equivalent. Data are presented as mean ± standard error (n = 3). Different superscript letters at the values within the same column indicate significant differences ($p < 0.05$), as determined by Tukey's HSD multiple comparison test.

4.2.2 Total Flavonoid Content

The total flavonoid content (TFC) of *T. zebrina* extracts varied significantly among all nine treatments, as determined by one way ANOVA test at a significance level of $p < 0.05$. **Table 4.3** demonstrated the TFCs for each treatment, which were calculated from a standard curve established using quercetin in **Figure 4.2**. The "UAE for 20 min, followed by HWE for 15 min" and "HWE for 15 min" extract showed the highest TFC, outperforming other extractions. The "no heating or sonication" extract continued to exhibit the lowest TFC, which was significantly lower than all other extractions. The TFC of this extract was 62.54% lower than the "HWE for 15 min" extract. Trends within individual extraction methods were similar to those in TPC, where "HWE for 15 min" and "UAE for 20 min" demonstrated the highest TFC among three variations, respectively, in HWE and in UAE.

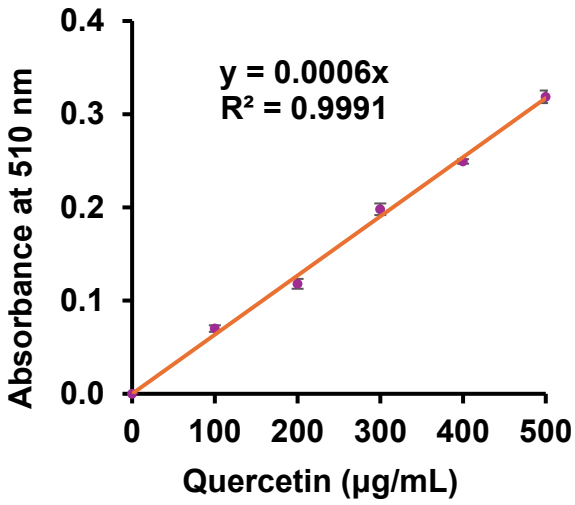

Figure 4.2: Standard curve of TFC constructed using quercetin. Data are presented as mean ± standard error (n = 3).

Table 4.3: Total flavonoid content of *T. zebrina* leaf extracts.

Extract		TFC (mg QE per g dry extract)
HWE	15 min	63.78 ± 0.97^{a}
	30 min	50.00 ± 0.19^{b}
	60 min	46.22 ± 0.99^{c}
UAE	5 min	33.33 ± 0.51^{d}
	10 min	$35.22 \pm 0.48^{d,e}$
	20 min	$43.44 \pm 0.29^{c,f}$
No heating or sonication		23.89 ± 0.59^{g}
HWE for 15 min, followed by UAE for 20 min		59.00 ± 0.36^{h}
UAE for 20 min, followed by HWE for 15 min		$62.56 \pm 0.29^{a,i}$

HWE: hot water extraction, UAE: ultrasonic-assisted extraction, TFC: total flavonoid content, QE: quercetin equivalent. Data are presented as mean ± standard error (n = 3). Different superscript letters at the values within the same column indicate significant differences ($p < 0.05$), as determined by Tukey's HSD multiple comparison test.

4.2.3 Total Anthocyanin Content

"HWE for 30 min", "HWE for 60 min", and "No heating or sonication" extracts showed undetectable TAC. The TAC of *T. zebrina* extracts varied significantly among six treatments, as determined by one way ANOVA test at a significance level of $p < 0.05$. **Table 4.4** showed the TACs for each treatment. All UAE treatments possessed detectable TAC, with "UAE for 5 min" extract showing the highest TAC, which was 1.54-fold greater than the other two treatments.

Table 4.4: Total anthocyanin content of *T. zebrina* leaf extracts.

Extract		TAC (mg CGE per g dry extract)
HWE	15 min	13.92 ± 2.78^{a}
	30 min	n.d.
	60 min	n.d.
UAE	5 min	55.66 ± 2.78^{b}
	10 min	36.18 ± 5.57^{c}
	20 min	$36.18 \pm 2.78^{c,d}$
No heating or sonication		n.d.
HWE for 15 min, followed by UAE for 20 min		$33.40 \pm 0.00^{c,d,e}$
UAE for 20 min, followed by HWE for 15 min		$16.70 \pm 0.00^{a,f}$

HWE: hot water extraction, UAE: ultrasonic-assisted extraction, TAC: total anthocyanin content, CGE: cyanidin-3-glucoside equivalent, n.d. undetectable. Data are presented as mean ± standard error (n = 3). Different superscript letters at the values within the same column indicate significant differences ($p < 0.05$), as determined by Tukey's HSD multiple comparison test.

4.3 Antioxidant Activities of Various Extracts

4.3.1 DPPH Radical Scavenging Activity

All extracts demonstrated concentration-dependent DPPH• scavenging activity. **Figure 4.3** compares the EC_{50} among all nine treatments, which varied significantly as determined by one way ANOVA test at a significance level of $p < 0.05$. The "UAE for 20 min, followed by HWE for 15 min" extract exhibited the strongest activity with a significantly lowest EC_{50} value when compared to other extracts. The "No heating or sonication" extract showed the weakest activity, which had a 1.83-fold higher EC_{50} value than the extract with the strongest activity. The EC_{50} for the positive control (quercetin) used was 4.928 ± 0.034 µg/mL.

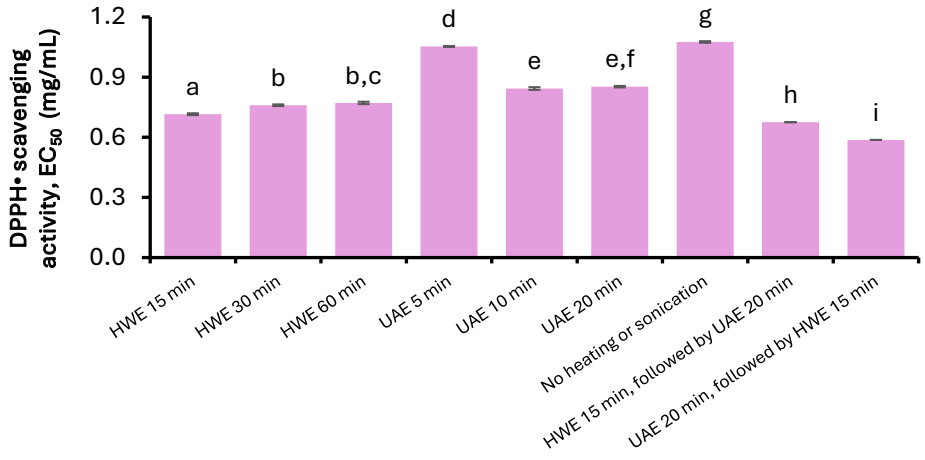

Figure 4.3: DPPH• scavenging activity of *T. zebrina* leaf extracts, expressed as EC_{50} values. HWE: hot water extraction, UAE: ultrasonic-assisted extraction. Each bar represents mean ± standard error (n=3). Different superscript letters indicate significant differences ($p < 0.05$), as determined by Tukey's HSD multiple comparison test.

4.3.2 ABTS Radical Cation Scavenging Activity

All extracts demonstrated concentration-dependent ABTS•$^{+}$ scavenging activity. **Figure 4.4** compares the EC_{50} among all nine treatments, which varied significantly as determined by one way ANOVA test at a significance level of $p < 0.05$. Similarly, the "UAE for 20 min, followed by HWE for 15 min" extract exhibited the strongest activity with a significantly lowest EC_{50} value when compared to other extracts. The "No heating or sonication" extract showed the weakest activity, which had a 1.65-fold higher EC_{50} value than the extract with the strongest activity. The extract with the weakest activity has a significantly higher EC_{50} value as compared to other extracts except for "UAE for 5 min". The EC_{50} for the positive control (quercetin) used was 5.550 ± 0.036 µg/mL.

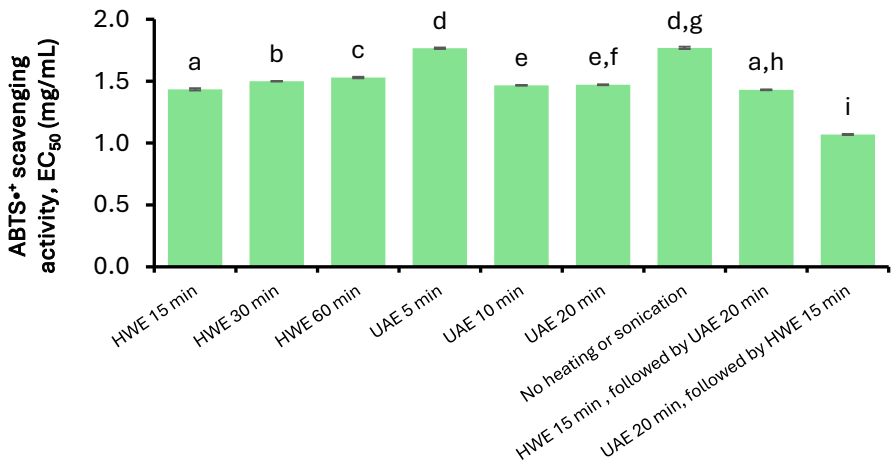

Figure 4.4: ABTS•$^{+}$ scavenging activity of *T. zebrina* leaf extracts, expressed as EC_{50} values. HWE: hot water extraction, UAE: ultrasonic-assisted extraction. Each bar represents mean ± standard error (n=3). Different superscript letters indicate significant differences ($p < 0.05$), as determined by Tukey's HSD multiple comparison test.

4.3.3 Ferric Reducing Antioxidant Power

Figure 4.5 compares the ferric reducing antioxidant power (FRAP) values among all nine treatments, which varied significantly as determined by one way ANOVA test at a significance level of $p < 0.05$. Unlike the trend in DPPH• and ABTS•$^{+}$ scavenging activity, the "HWE for 15 min, followed by UAE for 20 min" showed the highest FRAP value, which was significantly better than other extracts except for the other variety of sequential hybrid extraction. The "No heating or sonication" extract once again showed the weakest activity, with its FRAP value significantly lower than other extracts. Both "HWE for 15 min, followed by UAE for 20 min" and "UAE for 20 min, followed by HWE for 15 min" extracts were 1.94-fold stronger (higher FRAP values) than the "No heating or sonication".

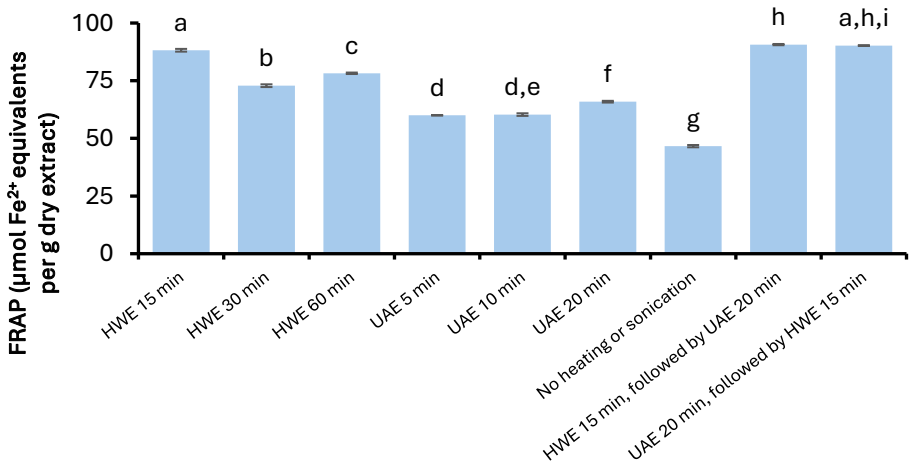

Figure 4.5: FRAP of *T. zebrina* leaf extracts. HWE: hot water extraction, UAE: ultrasonic-assisted extraction. Each bar represents mean ± standard error (n=3). Different superscript letters indicate significant differences ($p < 0.05$), as determined by Tukey's HSD multiple comparison test.

4.4 Effect of Simulated Gastrointestinal Digestion on Phytochemical Contents

The extract with the overall highest activity among the nine treatments was the "UAE for 20 min, followed by HWE 15 min" extract. Only this extract was investigated for stability of phytochemicals during simulated gastrointestinal (GI) digestion. **Table 4.5** demonstrates the phytochemical contents before and after simulated GI digestion on the selected extract, as well as the "GI Blank". The TPC, TFC, and TAC, respectively, of all treatments were significantly different as determined by one way ANOVA test at a significance level of $p < 0.05$. TPC and TFC significantly reduced after simulated GI digestion while the change in TAC was not significant. The TPC, TFC, and TAC of "Pre-GI" treatment were 2.48-, 9.71-, and 1.5-fold higher than the "GI Sample", respectively.

Table 4.5: Effects of simulated gastrointestinal digestion on total phenolic content, total flavonoid content, and total anthocyanin content.

Treatment	TPC (mg GAE per g sample)	TFC (mg QE per g sample)	TAC (µg CGE per g sample)
Pre-GI	8.114 ± 0.007^{a}	62.56 ± 0.29^{a}	16.70 ± 0.00^{a}
GI Sample	3.277 ± 0.005^{b}	6.44 ± 0.22^{b}	$11.13 \pm 2.78^{a,b}$
GI Blank	1.710 ± 0.005^{c}	3.44 ± 0.59^{c}	0.00 ± 0.00^{c}

GI: gastrointestinal digestion, TPC: total phenolic content, TFC: total flavonoid content, TAC: total anthocyanin content, GAE: gallic acid equivalent, QE: quercetin equivalent, CGE: cyanidin-3-glucoside equivalent. Data are presented as mean ± standard error (n = 3). Different superscript letters at the values within the same column indicate significant differences ($p < 0.05$), as determined by Tukey's HSD multiple comparison test.

4.5 Effect of Simulated Gastrointestinal Digestion on Antioxidant and Anti-Inflammatory Activities

As aforementioned in **Section 4.4**, similarly, only the "UAE for 20 min, followed by HWE for 15 min" was further analysed for changes in antioxidant and anti-inflammatory activities before and after simulated GI digestion. **Figure 4.6** illustrates the graphs of various bioactivities of "Pre-GI", "GI Sample", and "GI Blank" treatments. All treatments demonstrated concentration-dependent bioactivities. In most of the illustrated activities (**Figure 4.6A**, **B**, **D**, **E**), a lower concentration of "Pre-GI" treatment was required to reach 50% of bioactivities as compared to "GI Sample" treatment, hence having a higher activity. While a linear trendline was established for each of the bioactivities (**Figure 4. 6A**, **B**, **D**, **E**) of "GI Sample", the "GI Blank" treatment did not achieve at least 50% of activities within the same range of concentration used for "GI Sample". As presented in **Figure 4.6C**, the FRAP values of different treatments varied significantly as determined by one way ANOVA test at a significance level of $p < 0.05$. FRAP significantly reduced after simulated GI digestion, with the FRAP value of "Pre-GI" treatment was 2.92-fold higher than that of the "GI Sample".

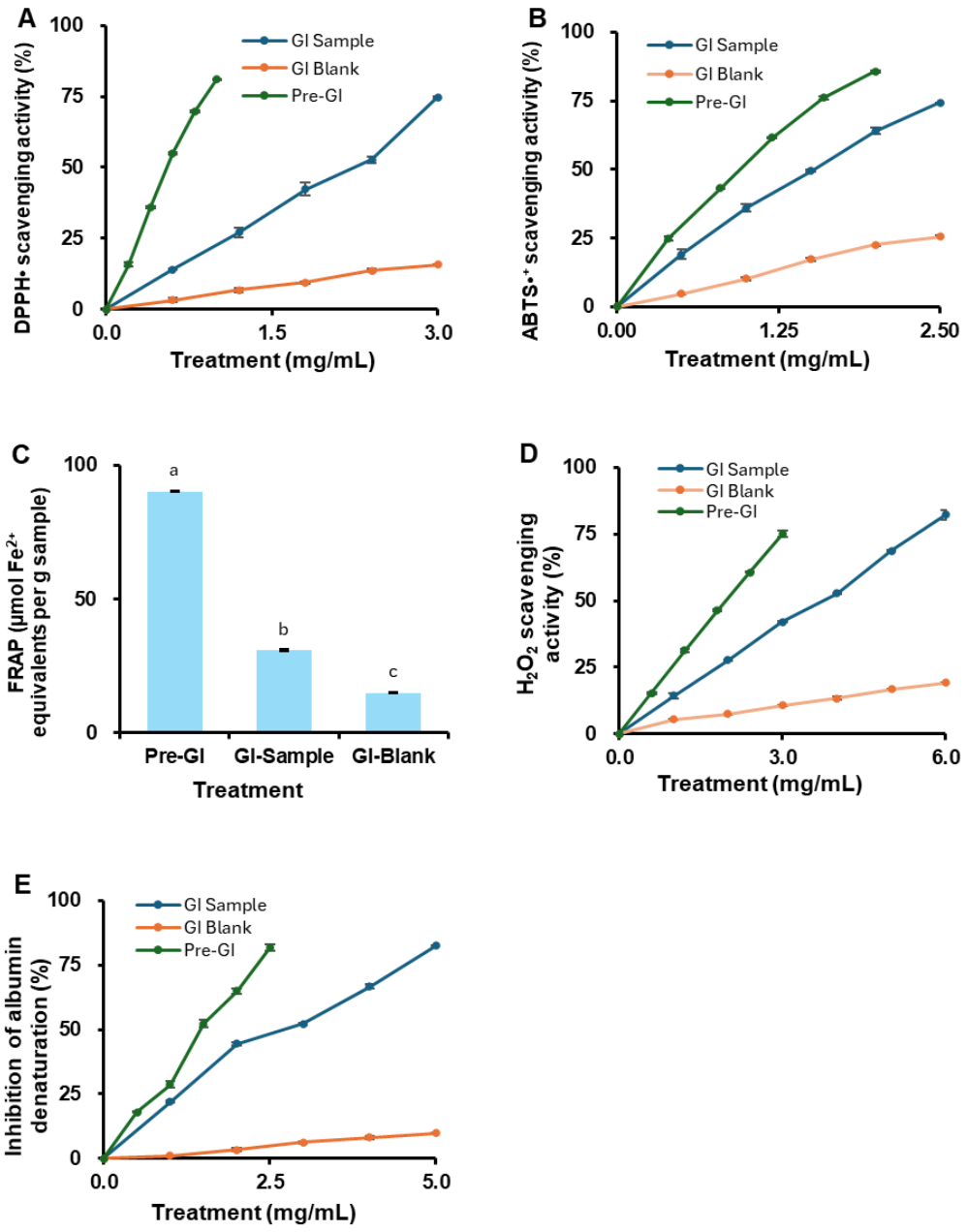

Figure 4.6: Effects of simulated gastrointestinal digestion on DPPH• scavenging activity (**A**), ABTS•$^{+}$ scavenging activity (**B**), FRAP (**C**), H_2O_2 scavenging activity (**D**), and inhibition of albumin denaturation (**E**) of the selected extract. For (**A**-**E**), GI: gastrointestinal digestion. Data are presented as mean ± standard error (n = 3). For (**C**), different superscript letters indicate significant differences ($p < 0.05$), as determined by Tukey's HSD multiple comparison test.

Table 4.6 shows the EC_{50} values for various bioactivities of "Pre-GI" and "GI Sample" treatments. In the investigation of antioxidant and anti-inflammatory activities, except for NO scavenging activity, the "Pre-GI" treatment has a lower EC_{50} when compared to the "GI Sample". EC_{50} of "GI Blank" for all bioactivities (except FRAP) were not determined as 50% of activities were not reached. The EC_{50} for the "GI Sample" were 3.61-, 1.48-, and 1.85-fold higher than that of "Pre-GI" treatment, respectively, in DPPH•, $ABTS^{\bullet+}$, and H_2O_2 scavenging activity. For inhibition of albumin denaturation, the EC_{50} for the "GI Sample" was 1.91-fold higher than that of the "Pre-GI" treatment. For NO scavenging activity, when the "GI Sample" was used at 15-times higher concentration than the EC_{50} of the "Pre-GI" treatment, the scavenging activity was still lower than 50%, which was 5.13 ± 0.47%. While 6.655 ± 0.001 mg/mL was the EC_{50} of the "Pre-GI" treatment, the EC_{50} of the "GI Sample" would likely exceed 100 mg/mL. Thus, the EC_{50} of the "GI Sample" in NO scavenging activity was not determined due to limited sample availability.

Table 4.6: Effects of simulated gastrointestinal digestion on EC_{50} of various bioactivities.

Bioactivities	**EC_{50} (mg/mL)**	
	Pre-GI	**GI Sample**
DPPH• scavenging activity	0.587 ± 0.000	2.118 ± 0.042
$ABTS^{\bullet+}$ scavenging activity	1.069 ± 0.003	1.585 ± 0.001
H_2O_2 scavenging activity	1.976 ± 0.006	3.658 ± 0.023
NO scavenging activity	6.655 ± 0.001	ND
Inhibition of albumin denaturation	1.527 ± 0.005	2.918 ± 0.023

GI: gastrointestinal digestion, ND: not determined. Data are presented as mean ± standard error (n = 3).

CHAPTER 5

DISCUSSIONS

5.1 Extraction Yield

Within either hot water extraction (HWE) or ultrasonic-assisted extraction (UAE), a linear relationship between yield and duration of extraction was not observed. However, the yield data were presented based on one replicate, which was not sufficient for statistical analysis. When compared to the "No heating or sonication" extract, the "HWE for 15 min" and "HWE for 30 min" extracts had higher yield while the "HWE for 60 min" extract had lower yield, as shown in **Table 4.1**. This may indicate that prolonged heating reduces yield (Zhang *et al.*, 2023), while heating up to 30 minutes may still be beneficial to increase yield.

Among three variations in UAE, only the "UAE for 20 min" extract had a higher yield when compared to the "No heating or sonication" extract, as demonstrated in **Table 4.1**. This is likely to indicate that plant extract remains stable at the temperature used in UAE (Nguyen *et al.*, 2021). When exposed to short-term duration, i.e. 5 minutes and 10 minutes, some plant materials (readily extracted even without any treatment) may be degraded by the high frequency used in UAE. This then caused a lower yield than the "No heating or sonication" extract. Both sequential hybrid extractions showed higher yield than their individual compartments. This may indicate the effectiveness of combining more than one extraction method in improving extraction yield.

5.2 Effect of Extraction Methods on Phytochemical Contents

The total phenolic content (TPC) and total flavonoid content (TFC) for the "No heating or sonication" extract were significantly lower than all other types of extracts. This indicates that some phenolics and flavonoids of *Tradescantia zebrina* were readily extracted even without the assistance of heating or sonication. Additional phenolics and flavonoids present in other extracts could be proofs of the effectiveness of heating and sonication.

A decreasing trend can be established in TPC and TFC of various HWE samples with increasing duration of extraction. This finding was expected because of the thermolabile characteristics of phenolic compounds, where extended duration of extraction leads to degradation of polyphenols (Muala, Desobgo and Jong, 2021; Pattnaik *et al.*, 2022). In contrast, TPC and TFC of various UAE treatments possessed an increasing trend with increasing duration of extraction. The findings in this study showed similarities with UAE on other plant extracts. Ji *et al.* (2022) discovered that the phenolic content (sum of few listed compounds) in *Coffea arabica* increases from 5 to 20 minutes of extraction, and the difference between the "UAE for 5 min" and the "UAE for 10 min" extracts was not significant. Another study on flavonoids content in *Daphne genkwa* also showed a similar trend (Xiang *et al.*, 2024). Kumar, Srivastav and Sharanagat (2021) mentioned that upon an initial increment in time, the cavitation effect of ultrasound would enhance different processes which elevate the exposure of solute to the extraction solvent. Different trends between HWE and UAE could be due to the heat-sensitivity of some phytochemicals present in *T. zebrina* at

90℃, while some other phenolics and flavonoids were stable at the conditions of UAE, namely 42 kHz and 60℃. However, the TPC and TFC of the "HWE for 15 min" and the "HWE for 60 min" extracts were significantly higher than those in all treatments of UAE, indicating higher efficiency in HWE. Cheng, Xue and Yang (2023) discovered that, by increasing the temperature of extraction, the quantity of polyphenols from various tea leaves would increase even up to 100℃. The authors explained that increasing water temperature would likely decrease its polarity, making it more compatible for extracting antioxidants. This effect is also supported by Leo *et al.* (2022).

TPC of sequential hybrid extractions were significantly higher than their individual methods, proving the effectiveness of combining two extraction methods. The difference in TPC of those extracts indicates that some phenolics were extracted only in UAE while some other phenolics were only present in HWE. Feihrmann *et al.* (2024) had previously identified malvidin-3,5-diglucoside and bixin in UAE of *T. zebrina* which were not found in a control experiment (without ultrasound). However, the temperature used in their control experiment was 60℃, which was lower than the temperature used in HWE in this study (90℃). Another study by Tran *et al.* (2023) also found that quinic acid was only present in UAE but not in Soxhlet extraction or maceration of *Rubus alceifolius* Poir leaves.

TFC, on the other hand, demonstrated a slightly different pattern. TFC of the "HWE for 15 min, followed by UAE for 20 min" extract was lower than "HWE for 15 min" but higher than "UAE for 20 min". Similar comparisons were

observed in the “UAE for 20 min, followed by HWE for 15 min” extract. However, this extract did not show any significant differences when compared to the “HWE for 15 min” extract. One possible explanation is that some flavonoids extracted using HWE were degraded during UAE. Huge mechanical energy generated from ultrasonic waves may destroy flavonoids, such as rutin, kaempferol, quercetin, etc., which were dissolved in solution (Fan *et al.*, 2020).

Total anthocyanin content (TAC) of “HWE for 30 min” and “HWE for 60 min” extracts were undetectable while the “HWE for 15 min” extract possessed quantifiable TAC, as shown in **Table 4.4**. Anthocyanins of *T. zebrina* were possibly sensitive to heat at 90°C. On the other hand, the “UAE for 5 min” extract had the highest TAC among all three variations in UAE. This finding was consistent with the one in a study conducted by Feihrmann *et al.* (2024), which suggests that the optimal duration of UAE was 6.25 minutes, rather than 2.5 or 10 minutes. This may be an indication that increasing duration of sonication may degrade anthocyanins present. According to Tiwari, O’donnell and Cullen (2009), sonochemical reactions during UAE such as generation of free radicals and enhancement of polymerisation or depolymerisation may cause degradation of anthocyanins. There was no significant difference between TAC of the “UAE for 10 min” and the “UAE for 20 min” extracts. After 10 minutes of sonication, the sensitive anthocyanins may have completely degraded, leaving the stable ones, hence similar TAC values. Some anthocyanins exhibit a high degree of stability to sonication (Tiwari, O’donnell and Cullen, 2009). In both cases (HWE and UAE), it seemed optimal to keep the duration short.

TACs of various UAE treatments were higher than the only detectable HWE treatment. Similar trends were recorded in water extraction of flowers of eight different species by Demasi, Caser and Scariot (2023). UAE has greater effectiveness than HWE in extracting anthocyanins. Anthocyanins are unstable glycosides of phenolic pigments that are susceptible to hydrolysis and isomerisation, whose structures may be degraded after thermal processing, especially at 90℃ heating (Liu *et al.*, 2022; Xu *et al.*, 2021). TAC of "No heating or sonication" extract was undetectable. This would likely indicate that minimal heating or sonication is necessary for the successful extraction of anthocyanins.

In the "UAE for 20 min, followed by HWE for 15 min" extract, both TPC and TFC were greater than the other type of sequential hybrid extraction. UAE relies on cavitation, which the formation and collapse of microbubbles generating microjets that disrupt tissues and enhance solvent penetration (Raspe *et al.*, 2021). Hence, more phytochemicals were better extracted by the high-efficiency HWE. Unlike TPC and TFC, TAC showed an opposite comparison between the two sequential hybrid extractions. Some anthocyanins extracted by UAE at a lower temperature cannot withstand the high temperature of HWE, even just for 15 minutes. This matches with the explanation by Xu *et al.* (2021) that anthocyanins begin to degrade when the temperature exceeds 80℃.

5.3 Effect of Extraction Methods on Antioxidant Activities

Among all extractions, the "No heating or sonication" extract showed the highest EC_{50} in DPPH• and $ABTS^{•+}$ scavenging activities with the lowest FRAP. These findings were consistent with the findings for TPC and TFC. According to Baghalpour *et al.* (2021), antioxidant activity can be originated from the redox potential of polyphenols, which scavenges free radical. The authors pointed out the presence of flavonoids and phenols in *T. zebrina* that can manifest its antioxidant activity. Among three variations in HWE, "HWE for 15 min" exhibited the highest activity. This is likely to indicate the presence of antioxidants that cannot withstand consistent, prolonged exposure to heat at 90℃. However, the presence of concentration-dependent activity of the "HWE for 60 min" extract served as a proof for the presence of certain antioxidants that were not affected by such prolong heating. The trends of decreasing activity (increasing EC_{50} and decreasing FRAP) as duration increases matched with trends in both TPC and TFC for HWE. It is thus observable that phenolics, or specifically flavonoids, could be responsible for various antioxidant activities in HWE. Hydroxyl groups may provide hydrogen atoms while the double bonds present may supply electrons required for the antioxidant mechanisms in DPPH• scavenging and FRAP, respectively (Pisoschi *et al.*, 2016; Rumpf, Burger and Schulze, 2023).

Among 3 variations of UAE, "UAE for 20 min" and "UAE for 10 min" extracts showed no significant differences in DPPH• and $ABTS^{•+}$ scavenging activity. However, both extracts were concurrently better than "UAE for 5 min". Further

increments in duration of extraction most likely do not increase radical scavenging antioxidants after 10 minutes. While increasing sonication time leads to increasing TPC and TFC, it also causes a linear increment in hydroxyl radical concentration (Wang *et al*., 2020), which may cause degradation of phytochemicals like cyanidin-3-glucoside, rutin, and caffeic acid. The overall compensation may explain the static trend of antioxidant activity. The optimal time could be between 5 and 10 minutes. Referring to **Figure 4.**4, in $ABTS^{\bullet+}$ scavenging activity, "UAE for 5 min" showed no significant difference when compared to "No heating or sonication". While 5 minutes may be sufficient for extracting certain phytochemicals, this duration may be insufficient to release functional antioxidants. The FRAP of "UAE for 20 min" was significantly better than the other two treatments in UAE, which did not show any significant differences. Unlike DPPH• and $ABTS^{\bullet+}$ scavenging activity, the trend in FRAP was in conjunction with TPC and TFC.

On a general basis, HWE outperformed UAE in DPPH• scavenging activity and FRAP. Possible reason is the presence of semi-polar compounds, which may be extracted better in HWE due to the decrease in water polarity at high temperatures as mentioned in **Section 5.2** by Cheng, Xue and Yang (2023) and Leo *et al.* (2022). These semi-polar compounds are likely rich in hydroxyl groups and double bonds, whose antioxidant activities were detected in the two assays.

Sequential hybrid methods are generally better than their individual methods. Across three evaluations of antioxidant activity, both hybrid methods had a lower EC_{50} in DPPH• and ABTS•$^{+}$ scavenging activity, along with higher FRAP values. This proved the effectiveness of combining two methods one after another. “UAE for 20 min, followed by HWE for 15 min” extract was better than the other sequential hybrid method in DPPH• and ABTS•$^{+}$ scavenging activity, as shown in **Figures 4.2 to 4.4**. It is notable that the sequence of the hybrid methods is important, which would cause differences in activity. Similar observations were found in a study conducted by Airouyuwa *et al.* (2024), when the sequence of hybrid methods was reversed, the FRAP activity of date seed extracts differed significantly. Antioxidants from UAE could be stable at 90℃ while some other antioxidants from HWE may not be suitable to be subjected to sonication. Another possible reason is that sonication creates cavitation bubbles, leading to fragmentation of cellular structure that allows better extraction of antioxidants using HWE. This mechanism is possibly identical to the one explained in **Section 5.2** by Raspe *et al*. (2021).

Different overall trends were observed across three assessments of antioxidant activities. It can be explained by differences in solvents used for radical generation, such as ethanol for DPPH• and water for ABTS•$^{+}$. HWE may help release bound semi-polar antioxidants, which are more effectively detected by DPPH• than by ABTS•$^{+}$. Hence, the activities of “HWE for 30 min” and “HWE for 60 min” were better than “UAE for 10 min” and “HWE for 20 min” only in DPPH• scavenging activity. This is in conjunction with the idea by Cheng, Xue and Yang (2023) that increasing water temperature would likely decrease its

polarity. The overall different trends could also be ascribed to different mechanisms of antioxidant activities such as the hydrogen atom transfer (HAT) mechanism for ABTS•$^{+}$ scavenging activity and the non-radical single electron transfer (SET)-based method for FRAP, whereas DPPH• scavenging activity employs both mechanisms (Rumpf, Burger and Schulze, 2023).

5.4 Effect of Simulated Gastrointestinal Digestion on Phytochemical Contents

Simulated GI digestion significantly reduced both TPC and TFC in the selected extract, with a more drastic effect on TFC. The significant reduction may be explained by biotransformation by GI enzymes such as pepsin and various enzymes present in pancreatin, which were implied in this research. Phytochemicals may also be affected by the effects of pH adjustment after each phase. Cavia *et al.* (2023) mentioned that polyphenols can undergo modifications during the digestive process, which increases the solubilisation. Free phenols may be transformed into complex derivatives which are hardly detected using spectrophotometric method. Hydrolytic enzymes like pepsin or pancreatin can cleave hydrogen bonds in onion quercetin and grape resveratrol. Along with the change in pH as a primary factor, this may cause irreversible breakdown of the two compounds mentioned (Lee *et al.*, 2020). At pH 6.5 to 7.0 in the intestinal phase, some phenolic compounds may experiment autooxidation, polymerisation, and complex formation with fibre, which may cause a drastic decrease in its amount, like flavon-3-ols (Fernández-Jalao *et al.*, 2020).

Detectable TPC and TFC in the "GI Blank" suggested the presence of residual GI components that were not efficiently removed by SPE. These GI components like enzymes and bile salts may interfere with the assay. However, TPC and TFC detected in this situation were significantly lower than the values in "GI Sample". This is likely to indicate that TPC and TFC from the "GI Sample" were not solely originated from GI components, indicating that not all TPC and TFC were degraded after simulated GI digestion. For instance, Jakobek *et al.* (2023) found out that after an initial decrease in the first 10 minutes of gastric phase, the amount of chlorogenic acid from apples' flesh and peel did not change significantly throughout gastric and intestinal digestion. While Feihrmann *et al.* (2024) proved the presence of chlorogenic acid in *T. zebrina* aqueous extract, it may be true that some phytochemicals like chlorogenic acid would still be present in the "GI Sample" even after GI digestion.

After simulated GI digestion, the absence of TAC in the "GI Blank" and a non-significant reduction of TAC was observed. An estimated recovery of 66.65% of anthocyanins in this research was close to other studies. Zhang *et al.* (2017) detected a recovery of 71.8 ± 0.3% after performing simulated GI digestion on purple potatoes freeze-dried powder. Malvidin-3-*O*-glucoside, pelargonidin-3-*O*-rutinoside, cyanidin-3-*O*-rutinoside, and cyanidin-3-*O*-glucoside showed 63.97 to 82.54% stability even after intestinal phase (Victoria-Campos *et al.*, 2022). Although anthocyanins are in the most stable structure at low pH (pH 3.0 in gastric phase), the 2-hour incubation with pepsin may greatly reduce the chemical stability. The conditions in the intestinal phase (pH 7.0) cause the transition of anthocyanins from flavylium cation form to quinoidal base form,

which is very unstable (Teixeira *et al.*, 2024). Continuous exposure to these effects should explain the reduction of TAC. However, food matrix can influence the release and stability of anthocyanins (Wanyo *et al.*, 2024).

5.5 Effect of Simulated Gastrointestinal Digestion on Antioxidant and Anti-Inflammatory Activities

Across all measured parameters (inclusive of antioxidant and anti-inflammatory activities), the bioactivities in the chosen *T. zebrina* extract greatly reduced after simulated GI digestion, as demonstrated in **Figure 4.6**. Similar findings reported that after simulated GI digestion, 77% loss in FRAP for ora-pro-nobis leaf extract, (Cruz *et al.*, 2024), 55.73% loss of $ABTS^{\bullet+}$ scavenging activity from gastric phase and only 17.86% recovery of $ABTS^{\bullet+}$ scavenging activity from intestinal phase for *Phoradendron brachystachyum* leaf infusion (Gutiérrez-Grijalva *et al.*, 2022). The significantly increased EC_{50}, indicative of reduced activities, may be explained by the extensive loss of phenolics and flavonoids, which was demonstrated in **Section 4.4**. As discussed extensively in **Section 5.3**, antioxidant activities of *T. zebrina* extracts seemed likely to originate from its phenolics and flavonoids. Antioxidants present in the extract may have used up their capacity during the GI procedure. Despite having different solvents of the radical generation system and mechanism of detecting antioxidants (Rumpf, Burger and Schulze, 2023), the trends (declining activity) were consistent over all the parameters measured. These trends altogether proved the negative impacts of human digestion.

However, the presence of activity in the "GI Blank" was demonstrated previously in **Section 4.5, Table 4.6**. Different bioactivities remained detected despite the absence of plant extract, which is not related to anthocyanins as the "GI Blank" did not exhibit any TAC. As shown in **Figure 4.6**, the detected antioxidant activities (DPPH•, ABTS•$^{+}$, H_2O_2, and NO scavenging activities) of the "GI Blank" could be an outcome of autolysis of enzymes during simulated GI digestion, as suggested by Zhang, Noisa and Yongsawatdigul (2020). Since all antioxidant and anti-inflammatory activities of the "GI Sample" were generally higher than the "GI Blank", it is justified that such activities in "GI Sample" were not solely coming from GI components.

Measuring the activities of H_2O_2 scavenging, NO scavenging and inhibition of albumin denaturation would give a better representation in the human system. H_2O_2 is a type of ROS which is commonly produced in physiological systems, whose build-up brings cellular damage by affecting different macromolecules (Aranda-Rivera *et al.*, 2022; Masenga *et al.*, 2023). Besides, albumin is the main protein in serum with many essential biological functions, such as maintaining colloidal osmotic pressure, binding varied compounds, and providing antioxidant activity (Levitt and Levitt, 2016). Hence, investigating whether plant extracts can protect albumin from denaturation could bring understandings about the benefits of that extract to the human body. Presence, although lower, of activity such as H_2O_2 scavenging activity and inhibition of albumin denaturation would indicate that, prior to consumption, the phytochemicals may still be beneficial to the human's health.

NO, on the other hand, is categorised as non-free radicals under the group of reactive nitrogen species (RNS) (Aranda-Rivera *et al.*, 2022). Similar to H_2O_2, understanding the effects of human digestion on retaining NO scavenging activity could carry biological and physiological meanings. Sadly however, this activity dropped drastically, as shown in the "GI Sample" after the simulated GI digestion. A 50% activity was achievable at less than 10 mg/mL of the "pre-GI" sample whereas the "GI Sample" could not generate a minimal effect of 10% at 100 mg/mL of "GI Sample". The dramatically low NO scavenging activity was most likely due to the dramatic loss of phytochemicals that were present before simulated GI digestion and capable of scavenging NO. Another possible explanation is that GI components can transform the phytochemicals into other metabolites that were increasingly light-sensitive, which degraded when incubating for 2.5 hours under a fluorescent light source.

5.6 Limitations and Future Research Directions

5.6.1 Optimisation of Extraction Parameters using Response Surface Methodology

In this research, the selection of extraction parameters that produced the overall best activities were performed manually via observations. However, efficiency of extraction greatly depends on multiple factors such as temperature, duration of extraction, and solid-to-liquid ratio (Ramos-Arcos *et al.*, 2023). Response surface methodology (RSM) is useful to model complex relationships between different factors and to optimise the extraction procedure (Papazidou *et al.*,

2025). For instance, Feihrmann *et al.* (2024) had applied RSM to the optimisation of UAE of *Tradescantia zebrina*, considering independent variables like amplitude, time and temperature while using TPC and TAC as response variables.

5.6.2 In-Depth Assessment of Effects of Simulated Gastrointestinal Digestion

For a more comprehensive understanding of the effects of simulated GI digestion, determining activities in pre- and post-digested samples were insufficient to provide a clearer picture. Phytochemical contents, along with antioxidant and anti-inflammatory activities can be recorded after each digestive phase by collecting samples at the intervals. The effects of individual phases were examined by many researchers (Feihrmann *et al.*, 2024; Gutiérrez-Grijalva *et al.*, 2022; Wanyo *et al.*, 2024).

Many studies (Fernández-Jalao *et al.*, 2020; Lee *et al.*, 2020; Teixeira *et al.*, 2024) found that phytochemicals, especially anthocyanins, are sensitive towards changes in pH. However, in this study, the causes of decreasing TPC, TFC, and TAC were not clearly investigated. Degradation of phytochemicals and loss of various bioactivities could be due to changes in pH, or interference of enzyme components, or both. Hence, GI digestion can be performed without enzymes and bile, to clarify the underlying cause of changes in parameters measured. This investigation was recommended by Brodkorb *et al.* (2019) and was applied by many researchers (Drawbridge, Apea-Bah and Beta, 2023; Ma *et al.*, 2024; Polmann *et al.*, 2025).

5.6.3 Exploitation of Methods to Enhance Bioaccessibility

Considering the convergent trends from different aspects, which pointed out the loss of bioactivities after simulated GI digestion, future work can be performed to discover methods that can enhance bioaccessibility. The effectiveness of spray drying microencapsulation was studied by Feihrmann *et al.* (2024) on the bioaccessibility of the optimised UAE for *T. zebrina*. They discovered that encapsulation was effective to protect anthocyanins and phenolic compounds from instability at high pH conditions. The same technique could be examined for its ability to elevate the antioxidant and anti-inflammatory activities in the promisingly beneficial sequential hybrid extract in this study.

Several studies have recently shown that complexes formation with proteins can likely raise anthocyanins' stability and bioaccessibility against GI conditions. Protein isolates can be used to capture, concentrate, and stabilise polyphenols, including anthocyanins, from berry sources. The capturing process is likely due to the natural affinity between polyphenols and proteins (Xiong *et al.*, 2020). However, the effectiveness of this technique was not studied specifically on *T. zebrina* extracts.

CHAPTER 6

CONCLUSION

To summarise, this study compared the extraction yield, phytochemical contents (TPC, TFC, and TAC), and antioxidant activities (measured as DPPH• and ABTS•$^{+}$ scavenging activity, as well as FRAP) of various extraction methods performed on *Tradescantia zebrina* leaves that include HWE and UAE, and the sequential hybrid extractions. Overall, the "UAE for 20 min, followed by HWE for 15 min" extract showed the highest TPC and TFC. This extract also showed the lowest EC_{50} in DPPH• and ABTS•$^{+}$ scavenging activity, and a highest FRAP value. This could possibly be explained by the mechanisms of UAE that increased penetration of solvent into the materials, boosting the efficiency of HWE. Hence, it is selected to proceed with simulated GI digestion based on the INFOGEST 2.0 protocol with minor modifications. Simulated GI digestion significantly declined TPC and TFC of the selected extract but only caused a non-significant decrease in TAC. In terms of various antioxidant and anti-inflammatory activities measured, simulated GI digestion drastically decreased the activities. The loss of activities may be explained by the extensive loss of phenolics and flavonoids during GI digestion due to the action of enzymes (pepsin and pancreatin) and the effect of pH.

Consistency across all measured bioactivities together provided an understanding about the negative consequences of human GI digestion on these activities present in *T. zebrina*. This could likely lay a foundation for future

investigations on the *in vivo* stability of phytochemicals and bioactivities during GI digestion. Both *in vitro* (like this study) and *in vivo* studies could be linked together to lead to a clearer image of the impact of human GI digestion on those parameters.

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APPENDICES

Appendix A

Composition of simulated digestive fluids used in INFOGEST 2.0

Salt Solution	Volume in 1.25 × SSF (mL)	Volume in 1.25 × SGF (mL)	Volume in 1.25 × SIF (mL)
0.5 M KCl	15.1	6.9	6.8
0.5 M KH_2PO_4	3.7	0.9	0.8
1 M $NaHCO_3$	6.8	12.5	42.5
2 M NaCl	-	11.8	9.6
0.15 M $MgCl_2•6H_2O$	0.5	0.4	1.1
0.5 M NH_4HCO_3	0.06	0.5	-
6 M HCl	0.092	1.3	0.725
Water	373.748	365.7	338.475