Quantifying the antioxidant potential of various plants

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A research project submitted for the degree of Master of Science

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Master of Science is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Aaron Farrelly

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To my supervisors Dr. Vlachou and Dr. Gkrintzalis, it has been quite the journey- thank you very much for all your guidance and teaching over the years. To our collaborator, Dr. Aliferis, thank you very much for conducting metabolomics analyses on the included plant samples. The analyses themselves enriched the project very much.

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Plants	Polyphenols	Flavonoids	Tannins
Green Sichuan Pepper (Sp)	210±10	92±7	34±3
Urtete Herbal Tea (Ut)	223±8	73±8	20±3
Sumac (S)	250±50	23±3	2±0.2
Camellia Sinensis (Cs)	343±10	70±5	50±6
Red Sichuan Pepper (Rsp)	220±10	87±10	30±5
Ingefaerte Ginger Tea (Gt)	80±8	28±4	5±1

Table 1.2: Antioxidant potential/ radical concentration expressed as nmole standard per milligram (mg) of extracted plant (nmoles standard/ mg of plant extracted). In the case of CuRP and FuRP the standards used were gallic acid. CuRP and FuRP values expressed as nmoles gallic acid/ mg extracted plant. In the case of ABTS the standard used was gallic acid. ABTS values expressed as nmoles gallic acid/ mg extracted plant. In the case of DPPH and ABTS+ the standard used was catechin. DPPH and ABTS+ values expressed as nmoles catechin/ mg extracted plant. ± refers to standard deviation.

Plants	CuRP	FeRP	ABTS	DPPH	ABTS+
Green Sichuan Pepper (Sp)	92±5	42±4	95±2	19±3	207±28
Urtete Herbal Tea (Ut)	120±6	80±3	155±10	28±8	252±21
Sumac (S)	220±35	174±27	238±27	71±12	599±32
Camellia Sinensis (Cs)	223±20	87±1	282±4	073±2	361±33
Red Sichuan Pepper (Rsp)	102±6	46±2	101±13	15±1	225±51
Ingefaerte Ginger Tea (Gt)	50±4	31±6	36±3	11±2	169±22

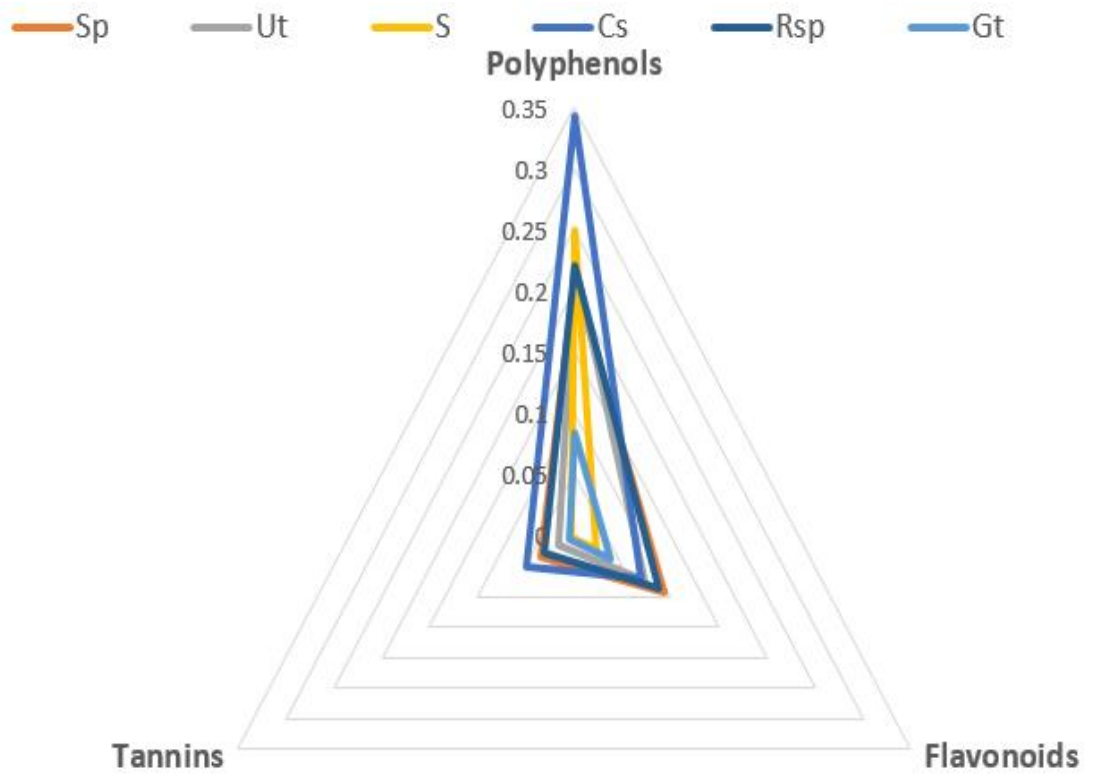


Figure 1.1: Radar chart demonstrating total polyphenols, flavonoids and tannins per plant extract.

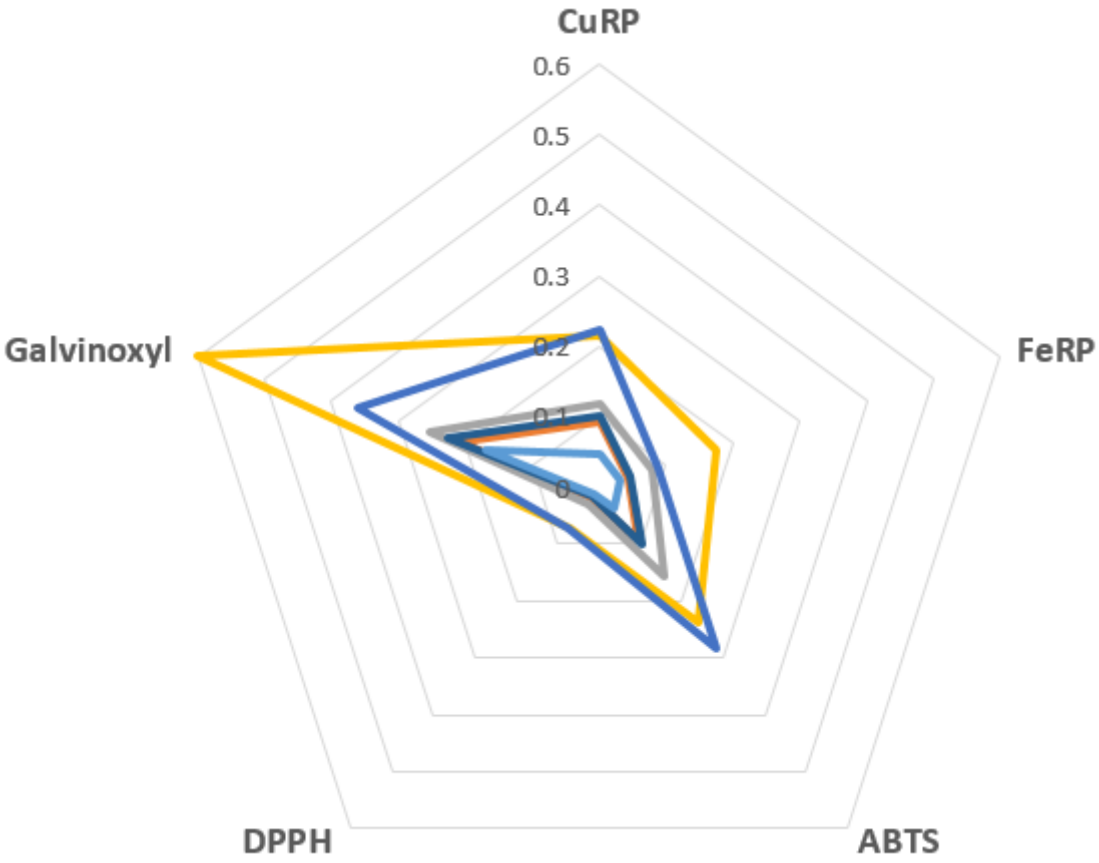


Figure 1.2: Radar chart demonstrating metal reduction and radical scavenging capacity per plant extract

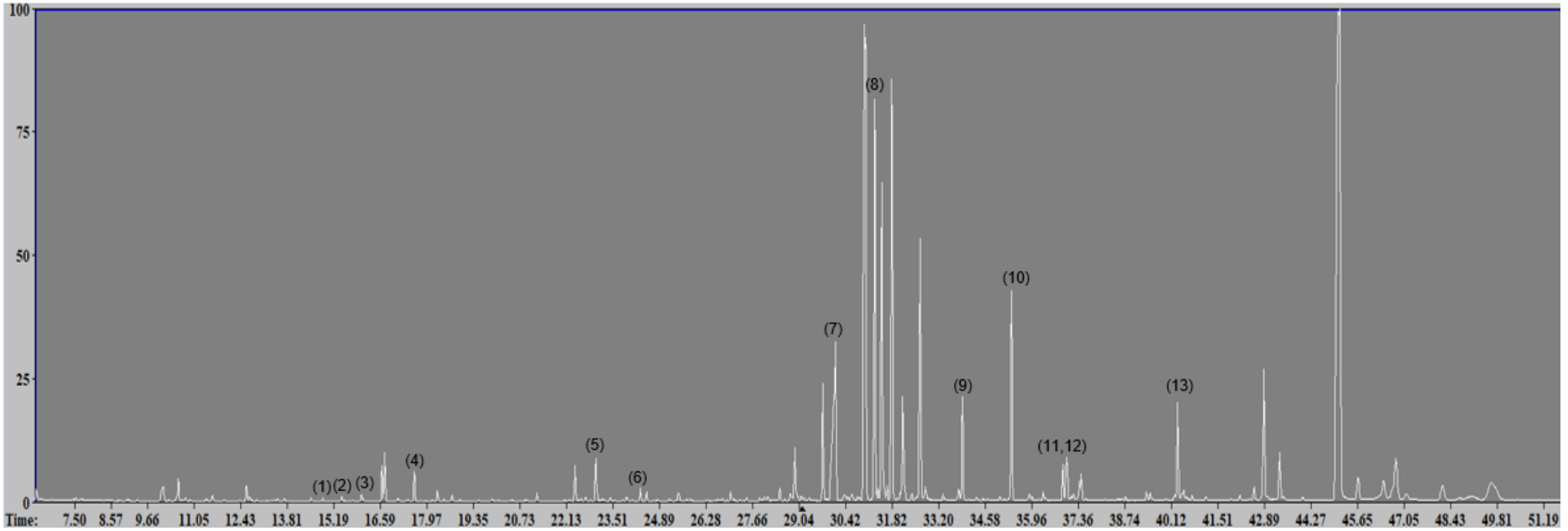
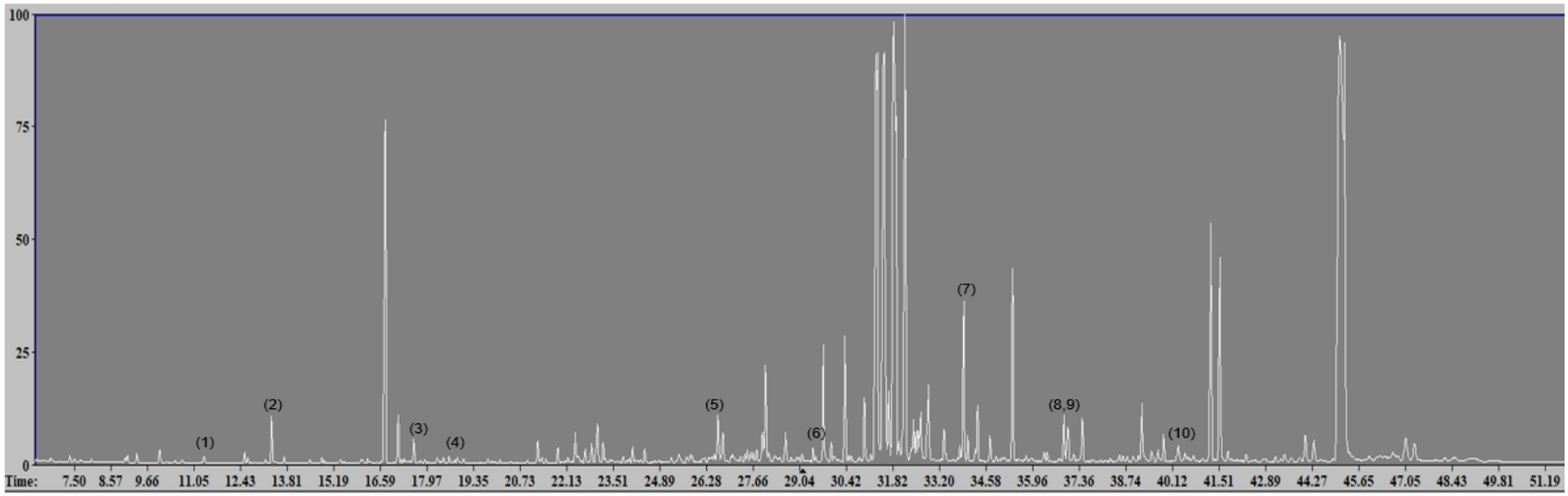


Figure 1.3. Chromatographic analysis of *Camelia sinensis*. X = Time (minutes) Y = Relative Abundance (total ion count). Characteristic highly abundant signals identified as 1) valine 2) urea 3) serine 4) succinic acid 5) pyroglutamic acid 6) threonic acid 7) caffeine 8) fructose 9) palmitic acid 10) inositol 11) linoleic acid 12) α-linonelic acid 13) oleamide



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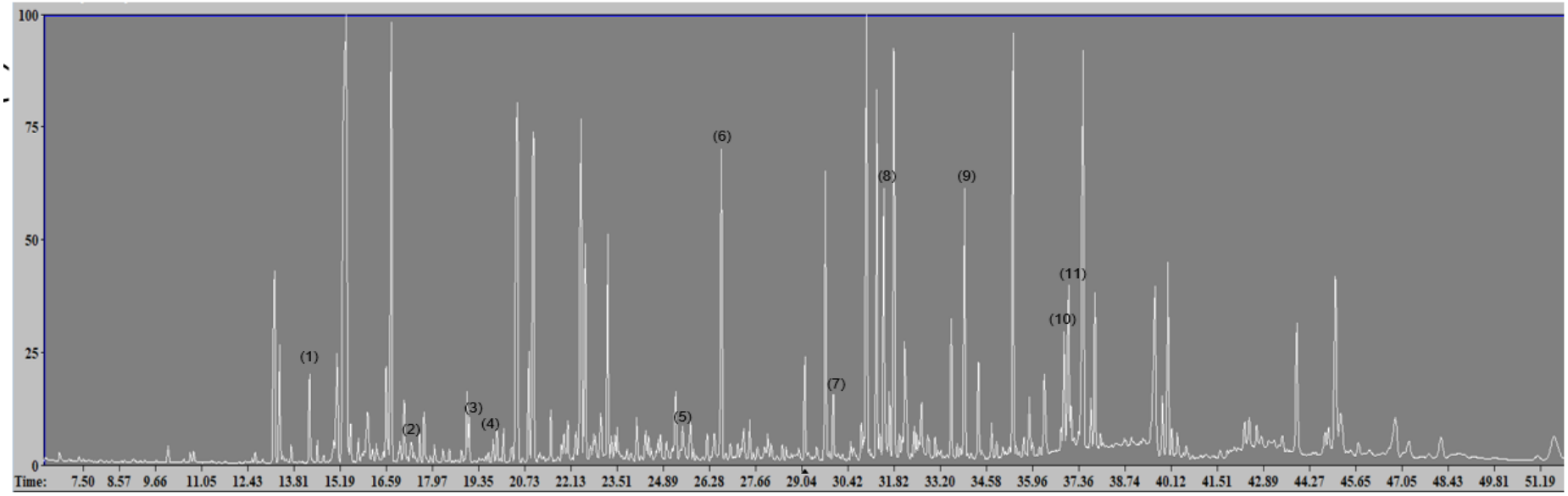

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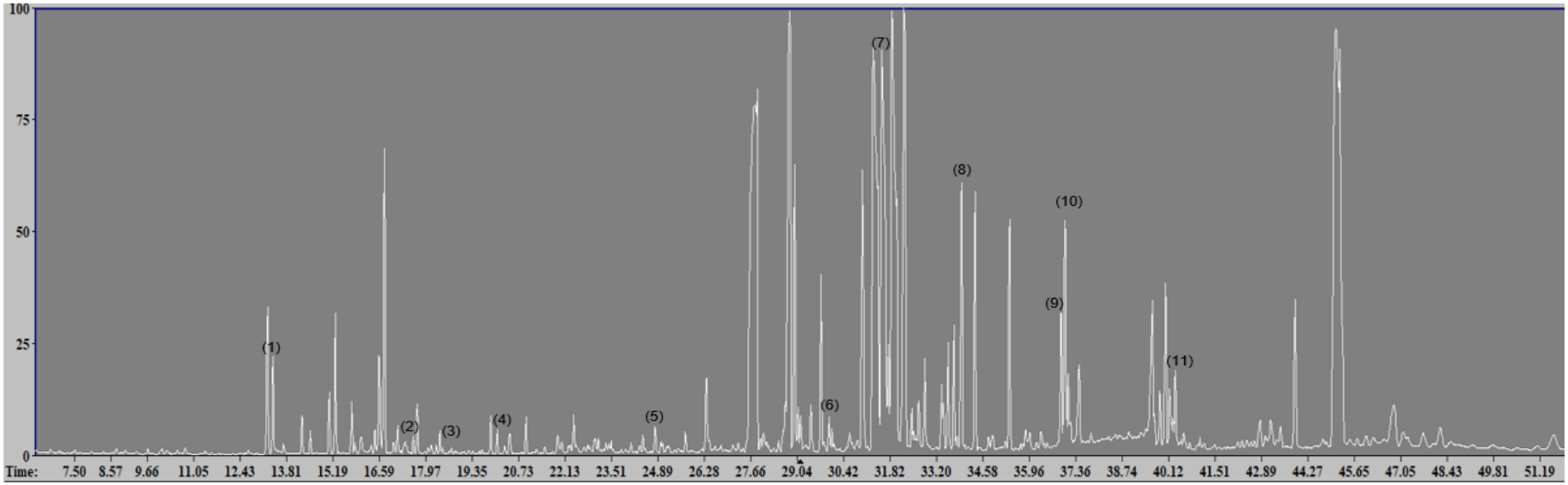

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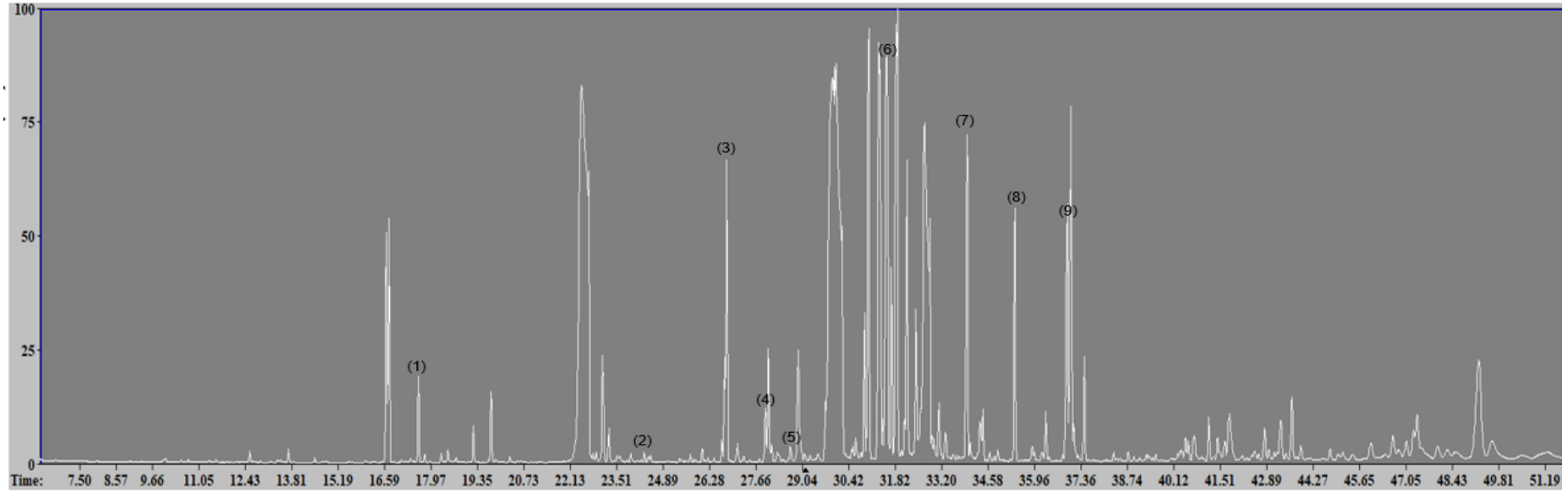

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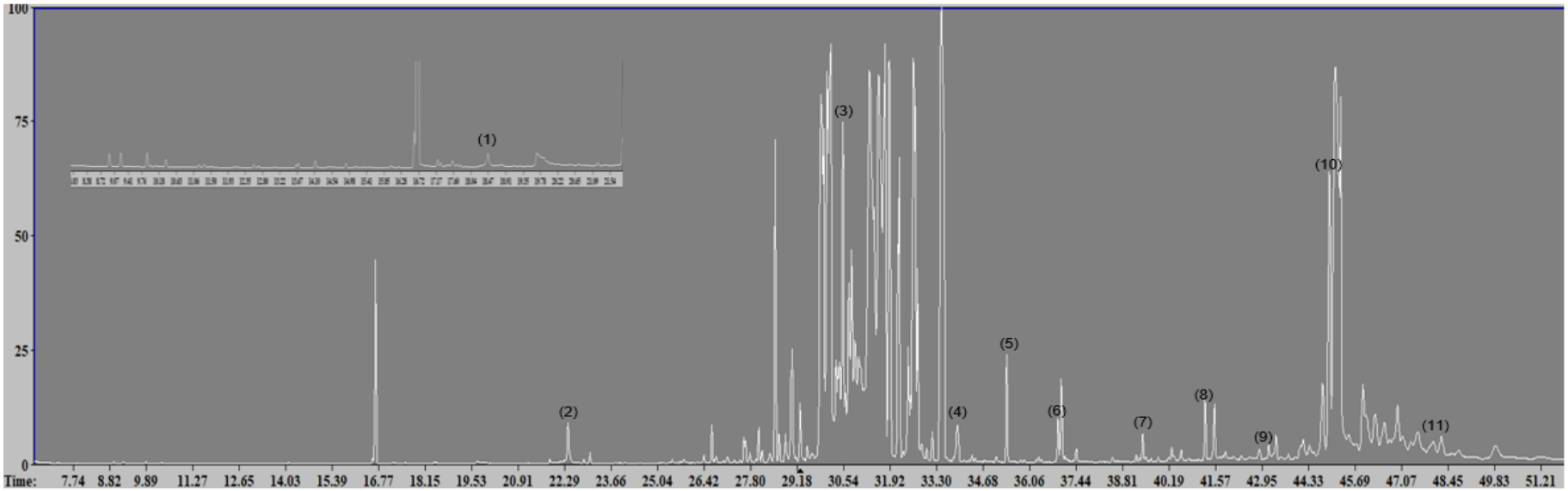

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Project title: Quantifying the antioxidant potential of various plants
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Abstract

Plants are an excellent natural source of phytochemical molecules which makes them good candidates for promoting healthy living in humans. This project focused on the assessment of the antioxidant potential and the metabolic characteristics of six common plants used as beverages or food supplements; sumac, green tea, herbal tea, ginger tea, red Sichuan pepper and green Sichuan pepper. The total polyphenol, flavonoid and tannin content was quantified with biochemical methods, while the metal reducing potential for iron and cupper and the radical scavenging capacity were also assessed. For a more detailed analysis, a metabolomic assessment with gas chromatography coupled with mass spectrometry (GC-MS) was performed to identify key metabolites in the plant extracts. Results revealed a varied antioxidant profile, with different plants being characterised by unique performance in each assay. It was determined that green tea performed consistently well across all assays. Ginger tea in contrast performed sub-optimally relative to other included plant extracts. Metabolomics revealed a varied metabolite profile across samples, however there was consistency among samples with metabolites such as palmitic acid present in relative abundance across all plant extracts. In conclusion, the plants analysed in this project presented a varied antioxidant profile *in vitro*. Future research should aim to confirm this antioxidant profile *in vivo* while also broadening our understanding of the metabolic processes underpinning antioxidant molecule action through further metabolic analyses.

Keywords: Plants, Oxidation, Antioxidants, Phytochemicals, Polyphenols, Radical Scavenging, Metal Reduction, Metabolomics, GC-MS

1. Introduction

The phytochemicals found in plants can be utilised to treat various diseases and have been in use as traditional remedies for millennia (Dias et al, 2012). For example, Sumerians; as a society with a history of five thousand years, utilised plant extracts to treat disease (Swerdlow et al, 2000; Zare et al, 2022). While imbuing health benefits phenotypically, the exact molecular mechanisms underpinning these beneficial effects were not common knowledge to ancient societies (Nutton, 2012). This lack of molecular insight, however, did not hinder the practice of utilising plants to treat disease and promote healthy living. The knowledge pertaining to plants deemed efficacious was passed on from generation to generation. In modern times, our ability to understand the phytochemical make up of plants has increased with the advent of sophisticated biochemical methods (Barros et al, 2011). As a result, we now possess the ability to catalogue detailed understandings that demonstrate why plants provide health and mental health benefits. The scope of this project will be to further our understanding of specific plant extracts in relation to their antioxidant potential.

Plants are an excellent natural source of phytochemical molecules, thus they provide an excellent means to treat diseases and promote health in humans (Loizzo et al, 2016; Engwa, 2018). It is known, for example, that phytochemicals within various plants such as aid humans in their response to biological stress (Kasote et al, 2015). For example, St John's wort was recently demonstrated to bring about positive changes in depressive symptoms (Benitez et al, 2022). This is one of many examples highlighting the efficacy of phytochemicals in promoting health through mitigating stress. Essentially, stress in a biological system manifests as excessive oxidative challenges, which cause cellular damage (Sies et al, 2017). These oxidative challenges result from a disturbance in the prooxidant-antioxidant balance in favour of the former (Sies & Cadenas, 1985). Such a prooxidant environment is detrimental to cellular health (Sotler et al, 2019). Interestingly, one way in which this prooxidant shift can be combated is through the consumption of foods rich in antioxidants. Plants contain varied profiles of phytochemicals, with many of these phytochemicals understood to be antioxidant. Molecules such as polyphenols for example, are found in many plant species and are known to be antioxidant (Farhan and Rizvi, 2022). These antioxidants can essentially balance the cellular environment, shifting it from prooxidant, thus reducing stress and subsequently cellular damage (Black, 2004).

With such knowledge established, it would be logical to assume that plants are commonly utilised as treatments in disease, however, the reliance on phytochemicals has waned due to the meteoric rise of the pharmaceutical industry (Tungmunnithum et al, 2018). Direct pharmaceutical intervention with phytochemicals is therefore less common, however, this has not stopped several industries developing based on utilising plants for their phytochemicals (Lubbe and Verpoorte, 2011). For example, plants are paramount to several industries including, but not limited to, functional foods, dietary supplements and recombinant protein manufacturing (Bigliardi and Galati, 2013; Egert and Rimbach, 2011; Xu et al, 2011). All of these industries screen, understand and utilise plants for an array of functions. While less common, there is clear merit still to understanding plants in our society. Thus, there is a necessity for researchers to progress our understanding of various phytochemicals. An example of this necessity is illustrated by the recent approval of a phytochemical (Cannabidiol) to treat paediatric forms of epilepsy such as Lennox-Gastaut syndrome and Dravet syndrome (Abu-Sawwa, Scutt and Park, 2020).

Western society has been experiencing major treatment resistance in diseases such as epilepsy (Kwan et al, 2011). Significant proportions of people with epilepsy worldwide receive no seizure remission from commonly prescribed antiepileptic drugs (Concepts et al, 2011). This segment of the epileptic population is categorised as treatment resistant. As a result, members of this cohort avail of several phytochemical-based drugs in order to receive remission from untreatable symptoms of epilepsy (Concepts et al, 2011). Recent advances in epilepsy research have demonstrated that specific phytochemicals such as cannabidiol are viable alternatives to conventional pharmaceutical methods. This research has culminated in the Federal Drug Administration (FDA) in the USA approving Epidoliex, a phytochemical-based therapy for the explicit treatment of epilepsy (Devinsky et al, 2017; 2018; Patel et al, 2017). This approval has provided a much-needed alternative in epilepsy treatment, which can now work in tandem with more commonly prescribed, and pharmaceutically conventional antiepileptic drugs (Devinsky et al, 2017;2018). The usage of phytochemicals in various diseases is not however limited to epilepsy (Bachtel & Israni-Winger, 2020), nor should it be.

Leveraging the inherent power within plants is only possible if we understand them. Therefore, it is salient to keep progressing our understanding of the molecular make up of various plants. For more accurate insights, researchers must begin categorising the myriad phytochemicals naturally available for use. Through an understanding of the molecular constituents of

previously under researched plants, we can begin to add to the natural catalogue of useful plants, thus strengthening the place they have in everyday life. With this in mind, the overarching question guiding this research project will be focused on which plants can provide antioxidant benefits. By subjecting specific plant species to various biochemical assays, we can determine their utility in terms of human health. The plants included in this project were; *Rhus tripartite* (known commonly as the sumac), *Camellia sinensis*, herbal tea, the ginger tea, and two types of pepper; the red and the green pepper.

The common name sumac extends for numerous species of the genus *Rhus*, which accounts for over 250 individual species of flowering plants (Rayne & Mazza, 2007). Particularly, *Rhus tripartite* belongs to the family *Anacandiaceae* (USDA, 2007). Sumacs are primarily found in temperate and tropical regions worldwide, with growth also possible in non-agriculturally viable regions (Rayne & Mazza, 2007). This has led to sumac being utilised for medicinal purposes by indigenous cultures (Van Wyk & Wink, 2004). Sumac's robust ability to grow in harsh environments makes it a good candidate for further analysis as its antioxidant capability is useful to an array of populations. Interestingly, sumac has already been associated with several health benefits including reducing cholesterol, balancing blood sugar, reducing muscle pain, reducing bone depletion and easing cancer (Abdallah et al, 2019; Shahrajabian and Wenli, 2022; Ehsani et al, 2022).

Camellia sinensis (green tea) is one of the most popular beverages consumed globally and was also included in this project (Nanita, Mukesh & Vijay, 2012). Green tea belongs to the *Theaceae* family and is native to mainland China, South Asia and South-east Asia (Nanita, Mukesh & Vijay, 2012). The plant itself contains an estimated 4000 bioactive compounds, with polyphenols making up the majority (Tariq, Naveed & Barkat, 2010). With demonstrable health benefits, green tea has also been shown to aid in treating diseases such as Parkinson's and Alzheimer's (Malar et al, 2020; Okello & Mathar, 2020).

Herbal tea and ginger tea were also analysed and compared within this project. These non-*Camellia* variants have formed a unique classification within Chinese tea culture (Xiao et al, 2011). While unique, these teas are reported to exhibit properties similar to *Camellia sinensis*. Research to date has particularly elucidated that these teas contain high levels of polyphenols, particularly flavonoids (Ming, 2015; Wang et al, 2016). It is known that this high flavonoid concentration imbues these teas with their health promoting qualities (Yao et al, 2004; Xiaro

et al, 2011; Ballard & Junior, 2019). While broad health benefits are associated with teas, specific effects have also been established within disease models (Ye et al, 2013; Jiang et al, 2014; Liu et al, 2014; Zhou et al, 2014).

Finally, emphasis was given to peppers which are known for having antioxidant properties (Bogusz et al, 2018). Two peppers were used; the red and green Sichuan pepper. This strain of pepper stems from the genus *Zanthoxylum*, and is known globally for its presence in cultures as a pungent spice (Zhang et al, 2021). Sichuan pepper is native to China, with varying production methods implicating various *Zanthoxylum* species from the plant family *Rutaceae* (See Zhang et al, 2017a, for a detailed review on production processes). Similar to all included plants, Sichuan pepper is rich in biomolecules such as polyphenols, as well as carotenoids, alkaloids and coumarins (Zhang et al, 2021). The polyphenol content is largely constituted by flavonoids (Nagy et al, 2015). Interestingly, Sichuan pepper was also analysed for efficacy within cancer models, where it demonstrated an ability to stimulate p53-mediated apoptotic signalling in tumour cells (Zhang et al, 2017b).

The overarching aim of this project was the analysis of the phytochemical constitution of the previously detailed plants. Essentially, this research focused on quantifying various phytochemicals within the aforementioned plants. Simultaneously, through the use of modern methods we aim to generate novel data pertaining to antioxidant capability and metabolic profile. Specifically, we aim to determine the total polyphenol, flavonoid and tannin content of each plant. The main rationale for targeting these molecules is their correlation with antioxidant potential. The major polyphenols found in plants are epicatechin, epigallocatechin, epicatechin-3-gallate and epigallocatechin-3-gallate (Anesini, Ferraro, Filip, 2008). Similar to polyphenols, flavonoids scavenge free radicals and promote a balanced molecular environment (Andarwulan et al, 2010; Ren, Nian, Perusselles, 2020; Al-Sayyed et al, 2022), with tannins purported to do the same (Ogawa & Yazaki, 2018). This project accurately targeted all of the aforementioned molecules within included plant samples, thus building a comprehensive phytochemical profile of each.

In tandem, other quantifications were also conducted to strengthen our knowledge of the antioxidant profile of included plant extracts. There are many assays currently validated for assessing antioxidant potential *in vitro* and *in vivo* (Alam, Bristi and Rafiquzzaman, 2013).

We chose to focus on metal reduction capability and radical scavenging capacity as indexes of antioxidant capability. Specific reducing assays were conducted, namely Ferric Reducing Power (FeRP) and Cupric Reducing Power (CuRP) assays. These analyses provide *in vitro* estimations pointing to the total reducing power of included plants. Coupled with reduction assays, this project also included radical scavenging assays. The particular assays implemented were the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging assay, the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay and the Galvinoxyl (ABTS+) radical scavenging assay. These assays are unique and analyse antioxidant capability *in vitro*. However, it must be stated that a determination of antioxidant potential *in vitro* cannot be extrapolated to *in vivo* systems (Kasote et al, 2015). From an *in vitro* perspective these assays are unified by a common mechanism involving a redox reaction, this mechanism being potentially different *in vivo* (Pulido, Bravo, Saura-Calixto, 2000). Through subjecting included plant extracts to these assays a robust *in vitro* phytochemical profile of each was developed.

Finally, this project aimed to build on, and progress our understanding of antioxidant potential by conducting metabolomics analysis on the included plant extracts. By utilising high throughput Gas Chromatography Mass Spectrometry (GC-MS) in partnership with an expert collaborator (Aliferis, Faubert, and Jabaji, 2014; Karamanou and Aliferis, 2020) we intended to illustrate a detailed metabolic profile of each plant extract in a similar manner to previous research (Al-Rubaye et al, 2017). Such metabolic analyses on the included plant extracts provide novel data on the metabolite profile of each plant. Such information will then be combined with other collected data to provide a detailed profile of each plant in terms of its overarching antioxidant capability and metabolic make up. Such amalgamation of metabolomics data with antioxidant data provides the current project with its novel edge and will provide the field with important information pertaining to metabolite content and antioxidant potential. Further and more specific information on the methods follows.

2. **Materials and methods**

(i) Preparation of plant material

The dried plant tissue was powdered using a pestle and mortar and 50 mg was extracted in 1 ml 80% HPLC-MS methanol:20% HPLC-MS via overnight shaking. Samples were cleared by centrifugation (ThermoFisher Sorval LegendMicro21) at 12,000 rpm for 10 minutes and the clear supernatant was collected for analysis. Samples were appropriately diluted for subsequent biochemical analysis.

(ii) Preparation of standard curves

For all biochemical assays conducted on phytochemicals and antioxidant characterisation results were expressed in equivalents of gallic acid or catechin. Initial stocks of gallic acid or catechin were prepared as 10 mM in methanol and diluted with deionised water to ranges between 10-100 and 100-1000 µM used in the corresponding standard curves (see Appendix).

(iii) Phytochemical characterisation of plant extracts

Polyphenols were quantified using the Folin reagent (Agbor et.al, 2014). An initial 0.1 ml of appropriately diluted extract (or gallic acid standard) was mixed with 0.1 ml of Folin reagent (diluted 4x in ddH_2O) and 0.1 ml 1.89 M Na_2CO_3. Mixtures were agitated and following incubation for 40 minutes at room temperature, absorbance was measured at 765 nm. The net absorbances, after subtracting the reagent blank were converted to equivalents of gallic acid from the linear standard curve (10-1000 μM).

Flavonoids were quantified using aluminium trichloride (Valyova et al, 2012). An initial 0.1 ml of appropriately diluted extract was combined with 20 mg of $NaNO_2$ (diluted in 1 ml ddH_2O), 75 mg $AlCl_3$ (diluted in 1 ml ddH_2O) and 2.8 g NaOH (diluted in 20 ml ddH_2O). These mixtures were agitated and following incubation for 10 minutes at room temperature, their absorbances were measured at 500 nm. The net absorbances after subtracting the reagent blank were converted to equivalents of catechin from the linear standard curve (10-1000 μM).

Tannins were quantified using their reaction with vanillin under acidic conditions (Price et al, 1978). An initial 0.1 ml of appropriately diluted extract was combined with 40 mg vanillin (diluted in 1ml methanol) and .05 ml H_2SO_4. These mixtures were agitated, and following

incubation for 10 minutes at room temperature, their absorbances were measured at 500 nm. The net absorbances after subtracting the reagent blank were converted to equivalents of catechin from the linear standard curve (10-1000 μM).

(iv) Antioxidant characterisation of plant extracts

Ferric reducing power (FeRP) was assessed by the reduction of ferric to ferrous ions which react with 2,4,6-tri-pyridyl-s-triazine (TPTZ) (Benzie & Strain, 1996). The FeRP reagent was prepared by mixing in a 10:1:1 ratio respectively, the following; 300 mM acetic acid, TPTZ and 0.54% $FeCl_3.6H_2O$. 300 mM acetic acid was created by dissolving 0.093 g sodium acetate anhydrous and 0.8 ml glacial in 48.2 ml ddH_2O. TPTZ was prepared fresh by dissolving 3.12 mg TPTZ in 500 µl methanol with another 500 µl of 40 mM HCl added to this solution. The 40 mM HCl was itself prepared fresh by diluting 0.5 ml concentrated 12 M HCl with 150 ml ddH_2O under stirring. 0.54% $FeCl_3.6H_2O$ was prepared fresh by dissolving 54 mg ferric chloride hexahydrate in 10 ml ddH_2O. The final FeRP reagent was created by mixing the aforementioned solutions in a 10:1:1 ratio. 0.125 ml sample was subsequently mixed with 0.125 ml FeRP reagent. The mixture was incubated for 40 minutes at room temperature with its absorbance measured at 595 nm. The net absorbances after subtracting the reagent blank were converted to equivalents of ferrous ions from the linear standard curve (nmoles).

Cupric reducing power was determined by the complex of copper ions with neocuprine reagent (Apak et al, 2008). A specific Cu-neocuprine-ammonium acetate (CuRP) reagent was created by mixing in a 1:1:1 ratio respectively the following; 10 mM Cu^{+2}, 6 mM neocuprine and 1 M ammonium acetate. 10 mM Cu^{+2} was prepared fresh by dissolving 24.9 mg copper sulfate pentahydrate in 10 ml ddH_2O. 6 mM neocuprine was prepared fresh by dissolving 8 mg neocuprine in 5 ml ddH_2O. 1 M ammonium acetate was prepared fresh by dissolving 1.93 g ammonium acetate in 15 ml ddH_2O. The volume of this solution was then adjusted to 25 ml due to the solid ammonium acetate occupying some of the final solution. The final CuRP reagent was created by mixing the aforementioned solutions in a 1:1:1 ratio. 0.125 ml sample was subsequently mixed with 0.125 ml CuRP reagent. The mixture was incubated for 40 minutes at room temperature with its absorbance subsequently measured at 450 nm. The net absorbances after subtracting the reagent blank were converted to equivalents of gallic acid from the linear standard curve (nmoles).

Radical scavenging capacity was determined by utilising DPPH, ABTS and $ABTS^{\cdot+}$ radical scavenging assays. The DPPH radical was prepared fresh in this instance by dissolving 2 mg DPPH in methanol. This stock was then diluted by mixing 400 μl of the stock with 5.6 ml and 4 ml 0.1 M acetate buffer (pH 5.5). Prior to initiating experimentation, the DPPH radical was diluted with methanol acetate. 125 μl of this diluted stock was then mixed with 125 μl ddH_2O to act as the reagent blank. With the reagent blank created, 125 μl of sample was then mixed with 125 μl of the DPPH radical. This mixture was incubated for 10 minutes at room temperature in complete darkness with absorbance subsequently measured at 515 nm. The percentage of DPPH radical scavenging was calculated as follows: $100x[(A_{RB}-A_{ddH2O})-(A_S-AS_{ddH2O})]/(A_{RB}-A_{ddH2O})$. This scavenging capacity was then expressed as an equivalent of gallic acid antioxidant capacity (GEAC) values from the corresponding standard curve.

The ABTS radical was prepared fresh in this instance by mixing 14 mM ABTS radical cation with 5 mM potassium persulfate in a 1:1 ratio. 14 mM ABTS radical cation was prepared by dissolving 7.7 mg ABTS in 1 ml ddH_2O. 5 mM potassium persulfate was prepared by dissolving 1.35 mg potassium persulfate in 1 ml ddH_2O. These two reagents were then mixed together in a 1:1 ratio and incubated in the dark at room temperature for 12 hours before the experiment. Prior to initiating the experiment, the incubated ABTS solution was appropriately diluted with 125 μl ddH_2O to create a reagent blank. 125 μl of sample was then mixed with 125 μl of ABTS radical. This mixture was then incubated for 40 minutes at room temperature with absorbance subsequently measured at 734 nm. The percentage of ABTS radical scavenging was calculated as follows: $100x[(A_{RB}-A_{ddH2O})-(A_S-AS_{ddH2O})]/(A_{RB}-A_{ddH2O})$. This scavenging capacity was then expressed as an equivalent of GEAC values from the corresponding standard curve.

A fresh galvinoxyl radical was prepared prior to initiating the experiment which had an absorbance value of ~1.3 A at 435 nm. These values were necessary because they reside within the linear range of the spectrophotometer. To create this specific galvinoxyl radical 50 mg DPPH was initially mixed in 10 ml methanol. Subsequent dilution of this stock (200 µl) with methanol and citric acid (pH 6) in a 9:1 ratio (100 µl) then occurred with filtering of the resultant solution to 0.22 µM. From these serial dilutions the resulting galvinoxyl radical was stable and capable of absorbing at 435 nm. The radical also decolourised when scavenged. Upon completion of radical preparation, the plant samples (100 µl) were appropriately mixed with galvinoxyl radical solution (200 µl). Also, 200 µl of the galvinoxyl radical stock was mixed with 100 µl of 9:1 methanol:citric acid to form a reagent blank. Samples were

subsequently assayed for galvinoxyl scavenging capacity. After the assay, the samples were appropriately incubated for 10 minutes at room temperature in dark conditions. Absorbance was subsequently measured at 435 nm. The percentage of galvinoxyl radical scavenging was calculated as follows: $100x[(A_{RB}-A_{MethCitr})-(A_S-A_{MethCitr})]/(A_{RB}-A_{MethCitr})$. This percentage of galvinoxyl radical scavenging was then converted to equivalents of GEAC values.

(v) Metabolic characterisation of plant extracts

To further extend our analysis of included plant material, project collaborators conducted liquid/gas chromatography with mass spectrometry. To prepare samples for extraction they were initially pulverized. 60 mg of each was then put in plastic Eppendorf tubes (2 mL). For the metabolite extraction, 800 mL of a methanol – ethyl acetate mixture (50:50 v/v) (GC-MS grade purity 99.9% were purchased from Carlo Erba Reagents, Val de Reuil, France) was used. The resulting extracts were immediately sonicated for 20 min in an ultrasonic bath (Branson 1210, Danbury, USA), followed by stirring in horizontal rotary shaker (GFL 3006, Gesellschaft für Labortechnik mbH, Burfwedel, Germany) for 1 hour at 150 rpm, at 24ºC. For the removal of debris, extracts were filtered (0.2 mm pore diameter, Macherey-Nagel, Duren, Germany). The extracts were then evaporated using a vacuum concentration (Labconco, Kansas City, MO, USA). The derivatisation of the dry extracts was performed in a two-way step process. In a first step, the methoxymation was performed by adding 80 μL of methoxylamine hydrochloride (98.0%, w/w) [20 mg mL^{-1} in pyridine (98.0%, w/w)] (Sigma-Aldrich Ltd) and the solution was incubated for 2 hours at 30ºC in a water bath (Daihan Labtech, Gyeonggi-do, Korea). Then, for silylation 80 μL of MSTFA were added, followed by incubation for 1.30 hours at 37ºC. Finally, the derivatised samples were transferred to 180 μL microinserters (Macherey-Nagel, Duren, Germany) in 2 mL autosampler vials for analysis.

Metabolomics analysis was performed using an Agilent 6890 analytical platform (Agilent Technologies Inc.) equipped with 5973 inert mass selective detector (MSD), and a 7683 autosampler. Derivatised extracts (1 μL) were injected on column (HP-5MS, 30m long, 0.25 mm, 0.25 mm membrane thickness, Agilent Technologies Inc.) applying a 5:1 split ratio. The injector temperature was set at 230ºC and helium was the carrier gas (1 mL min^{-1}). The temperature of the oven was set initially at 70ºC, stable for 5 min, followed by an increase to 295ºC with a 5ºC min-1 rate, and kept finally stable for 5 min. The temperatures for the MS source and the quadrupole were set to 230ºC and 150ºC, respectively. Positive electron

ionization at 70eV was applied and full scan mass spectra were acquired over the range 50e800 Da, at a rate of 4 scans s^{-1}. A 10 min solvent delay was applied.

(vi) Statistical analysis

Data generated from all previously elucidated experiments was plotted and analysed using Excel software. The results were expressed as average±SD (see table 1.1 and 1.2) after construction of individual standard curves. Radar charts (see results) were produced using standardised values and the formula $z_i = \frac{x_i - \bar{x}}{s}$, whereby the mean is subtracted from the net value obtained from each assay and divided by the standard deviation of the same assay. This has allowed for the data gathered to be transformed and denoted 'z' whereby the mean is transformed to a standardised '0' and the standard deviation presents as '1' on the normalized standard curve.

3. **Results**

The initial assessment of the main phytochemical categories and antioxidant properties was used as first point to estimate the differences between plant extracts. Following this, a detailed metabolomics analysis was conducted to identify specific compounds.

(i) Total polyphenols, flavonoids and tannins

In relation to their phytochemical composition, there were marked differences among all plants when compared as a group (see Figure 1.1).

(See table 1.1: Total polyphenol, flavonoid and tannin concentrations per plant extract)

What is evident from this comparison is the close relationship the included plants share in terms of polyphenol content. *Camellia sinensis* contained the most polyphenols out of all the included plants in this project ($0.343\ \mu M$). While containing the highest polyphenol content relative to the other plants, it is clear that the content of polyphenols in green tea does not differ greatly from other plants, with all plants demonstrating polyphenol content within a narrow range (see Table 1.1). The exception to this range, and the plant which represents the minimum value in terms of total polyphenol content was the ginger tea ($0.08\ \mu M$).

(See figure 1.1: Radar chart demonstrating total polyphenols, flavonoids and tannins per plant extract)

Interestingly, ginger tea was the second lowest in flavonoids ($0.028\ \mu M$). This is in direct contrast to green Sichuan pepper which had the highest flavonoid content ($0.092\ \mu M$) out of all included plant samples. In terms of tannin content *Camellia sinensis* contained the highest concentrations ($0.05\ \mu M$) of any plant extract assayed in this study. This contrasts sumac, which presents with a very low concentration ($0.002\ \mu M$) of tannins. Sumac contained the second highest amount of total polyphenols, but contained simultaneously the lowest flavonoid ($0.023\ \mu M$) and tannin content of any included plant extract.

Further insight in terms of phenolic content was gained when consulting results pertaining to pepper extracts. Both green Sichuan pepper and red Sichuan pepper demonstrate a consistent profile both individually and collectively. The green Sichuan variant demonstrated a total

polyphenol concentration of 0.21 μM, a flavonoid concentration of 0.09 μM and a mean tannin concentration of 0.03 μM. Whilst the red Sichuan variant presented with a total polyphenol concentration of 0.22 μM, a mean flavonoid concentration of 0.08 μM and a mean tannin concentration of 0.04 μM. It is clear from results that both peppers share a phenolic profile.

(ii) Metal reduction and radical scavenging

(See table 1.2: The antioxidant potential of plant extracts)

Camellia sinensis (or green tea) contained the highest copper-ion/neocuprine complex concentration out of all included plants (0.223 μM), thus representing the maximum. This result aligns with previous results pertaining to total polyphenols, flavonoids and tannins where green tea also demonstrated the highest concentrations. Interestingly, and repeating trends from results pertaining to total polyphenols, *ginger tea* (0.05 μM) had the lowest concentrations of copper-ion/neocuprine complex. Therefore, this plant again represented the minimum. Of note, both teas included in this analysis represent both the maximum and minimum concentrations. In terms of ferrous ion concentrations, results are similar. Here again it is clear that *ginger tea* (0.031 μM) had the lowest concentrations of ferrous ions representing the group minimum, and thus the plant most inefficient in its reductive ability. *Camellia sinensis* in this instance is not the maximum concentration, but the second highest concentration (0.087 μM). In results pertaining to ferric reducing power, the plant which performed the best out of included plants was Sumac (0.17 μM). It must also be noted that within the cupric reducing power assay, sumac obtained the second highest concentration (0.22 μM). Therefore, in terms of both of these assays, sumac performed consistently well.

(See Figure 1.2: Radar chart demonstrating metal reduction and radical scavenging capacity per plant extract)

Immediately, it is clear from these results that ginger tea was once again the lowest performer in terms of radical scavenging capacity, maintaining previous discussion surrounding the correlation of concentrations on one assay to concentrations on another. Furthermore, this result was echoed across included radical scavenging assays. For example, ginger tea also

demonstrated the lowest concentrations of DPPH+ and galvinoxyl radicals, 0.01 μM and 0.17 μM, respectively. What is evident from these results is the fact that ginger tea was demonstrably the weakest plant included in this project in terms of antioxidant capability as measured through various assays. While ginger tea was the lowest performer, green tea performed best in radical scavenging assays out of all included plants.

In terms of green tea, and beginning with ABTS radical scavenging, it achieved the highest ABTS+ radical cation concentrations (0.3 μM). This was echoed in the DPPH assay with green tea obtaining the highest DPPH+ radical cation concentrations (0.07 μM). These results further demonstrate green tea as the plant providing optimal antioxidant capability out of all included plants. This result is not novel information however, with previous research pointing to the efficiency of *Camellia sinensis* in terms of overall antioxidant potential (Khalaf et al, 2008). The results obtained here do further confirm green tea as an efficient antioxidant phytochemical. In tandem with green tea, it is also worth noting the radical scavenging capacity of Sumac. Across ABTS, DPPH and galvinoxyl assays it achieved concentrations of 0.24 μM, 0.07 μM and 0.6 μM, respectively. The final concentration mentioned previously of 0.6 μM demarcates sumac as the top performer in terms of galvinoxyl radical scavenging (see Figure 1.2).

(iii) Metabolomic analysis of plant extracts

Acquired chromatograms (Figure 1.3 – 1.8) reveal a varied metabolite profile. Identification was carried out whereby metabolite mass spectra were matched (>95% match) to entries of the National Institute of Standards and Technology Library (NIST; Gaithersburg, MD, USA). Absolute annotation for selected metabolites was performed using analytical standards. Each plant extract revealed a varied molecular profile. >100 unique molecules were detected from chemical groups, such as amino acids, carbohydrates, carboxylic acids, fatty acids and phenylpropanoids. Specifically, the following molecules were identified as relatively abundant within the experimental group of plant samples. Metabolites such as valine, urea, serine, succinic acid, pyroglutamic acid, threonic acid, caffeine, fructose, palmitic acid, inositol, linoleic acid, α-linonelic acid, oleamide, alanine, proline, fumaric acid, asparagine, citric acid, serine, threonine, phenylanine, lactic acid, ribose, tricarballylic acid, malic acid, 2'-deoxyinosine, α-lucopyranoside, turanose and 2H-1-benzopyran. These metabolites were either consistently present across plant samples or present only in one sample. For example,

ribose was only relatively abundant in sumac, whilst palmitic acid was present across all samples in relatively abundant amounts.

Focusing now on each plant sample, *Camellia sinensis* contained an abundance of varying metabolites (See Figure 1.3). Of note, it contained high amounts of caffeine, fructose, palmitic acid, inositol and oleamide. The metabolite with the highest relative abundance in green tea was fructose, while the lowest in terms of relative abundance was valine. Ginger tea also contained various metabolites (see Figure 1.2). In contrast to green tea, it contained a relative abundance of proline, asparagine, linoleic acid and α-linolenic acid. The metabolite with the highest recorded and identified relative abundance in ginger tea was palmitic acid, while the lowest was alanine. Interestingly, both green tea and ginger tea presented with relatively abundant levels of palmitic acid. Herbal tea (Figure 1.6) had a similar metabolic profile to other tea samples included in this project. For example, it contained palmitic acid in a similar manner to other teas. Also, fructose was the metabolite with the highest relative abundance in herbal tea. This result is an exact echo of *camellia sinensis*. Inositol was also present in relatively high abundance in herbal tea, again this is similar to green tea. In contrast, herbal tea contained high amounts of turanose, this being the only plant extract demonstrating this content.

The pepper species also presented with varying metabolite profiles. Interestingly, the metabolic profile of green Sichuan pepper (see Figure 1.4) was similar to the included teas. It was particularly rich in asparginine, fructose, palmitic acid, linoleic acid and α-linolenic acid. From comparison, it is evident that green Sichuan pepper shared a very similar profile to ginger tea, with many of the same metabolites identified across these analyses. The red Sichuan variant (Figure 1.3) further echoed this similarity containing relatively abundant amounts of fructose, palmitic acid, linoleic acid, linolenic acid and proline. It is not surprising the red and green Sichuan peppers had a very similar metabolic profile, however, it is worth noting the relative abundance of each metabolite varied. For example, red pepper had a much higher relative abundance of fructose when compared to its green counterpart.

Finally, and of note, sumac (Figure 1.5) had a similar profile to other included plant samples. It contained fructose, palmitic acid, inositol and linoleic acid. All of these metabolites were present in varying amounts across other previously mentioned plant samples. In contrast, Sumac was unique in the fact that it contained relatively high amounts of ribose, which was not present in any of the other plant samples.

(iv) Discussion

The total polyphenol, flavonoid and tannin content of six different plants was determined in tandem with their metal reduction and radical scavenging capacity. Metabolomics analyses were also conducted on each plant extract to provide further insight into the plants antioxidant capability. What is evident from the results obtained is a varied profile in terms of total polyphenols, flavonoids and tannins. This varied profile of each plant extends also into the analyses on metal reduction and radical scavenging capacity. Finally, the metabolomics analysis provides further evidence of variation between samples. It is important to note that while each plant sample was varied in terms of its constitution and antioxidant capability, there were also clear similarities among the included plant extracts. The following is a discussion of the obtained data, particularly as it relates to interpreting the presently obtained results. Future directions for phytochemical quantification research are also provided throughout.

(i) Total polyphenol, tannin and flavonoid content

It is known that the antioxidant capability of phenolic structures comes from their inherent ability to scavenge free radicals, donate hydrogen atoms or electrons and carry out metal reduction (Amarowicz et al, 2004). Phenolic content was screened in all included plant extracts, with each demonstrating the presence of phenolic contents. With such content being correlated with antioxidant capability, it can be said that each included plant has inherent antioxidant capability based on the presence of phenolic content. This phenolic content was not trivial, except in the case of ginger tea, which had the lowest observed concentrations. In contrast, and based on phenolic contents, green tea was demonstrated to be optimal relative to the other plants included in this project. The results obtained here provide a useful comparison between all plant extracts in terms of phenolic contents, creating a hierarchy. This hierarchy can be consulted to determine potential antioxidant activity of each plant, as phenolic content and antioxidant capability are known to be correlated (Vasco, Ruales and Kamal-Eldin, 2008; Machu et al, 2015).

It is worth mentioning that while results obtained here provide guidance in terms of phenolic contents and potentially antioxidant capability, they are not unequivocal. For example, the solvent utilised to assay each plant extract, as well as its polarity, is known to affect final phenolic concentrations (Medini et al, 2014). Essentially, the recovery of phenolic contents is

influenced by the solubility of phenols in the utilised solvent (Galves et al, 2005; Naczk and Shahidi, 2006). As a result, it is very important to acknowledge the fact that no one single assays exists as a standard to determine phenolic contents. As such, results obtained in this instance should be interpreted as a reflection of potential phenolic content, as measured by assays used. If other assays to determine phenolic content were used in this project, we may observe differing results. An interesting extension of this research would be to assay the same included plant extracts but with different solvents and re-compare data.

Furthermore, physiological stage affects phenolic content (Murakami et al, 2013). In this project, the developmental stage of included plant extracts was unknown, therefore results may be comparing plants at different stages of physiological development. Again, the results obtained here are a useful guide, but in some instances, they may be comparing plants at differing developmental stages. With this in mind, future research determining phenolic contents should endeavour to determine the developmental stage of included plants as this has major effects on phenolic concentrations. Moreover, the environmental growing conditions of the plants should also be determined. In the case of this project, this was also an uncontrolled variable. The environmental conditions in which the plant is grown should be accounted for as this affects phenolic concentrations (Fratianni et al, 2007). Future research, while endeavouring to control for developmental stage, should also control for environmental growing conditions. If a plant that experiences drought is compared to a plant which does not, the phenolic contents will differ naturally (Kolahi et al, 2021). Sarker and Oba (2018) demonstrated that specific plants enhance their nutritional and bioactive compounds, particularly phenolic acids, in response to drought conditions. This is an extreme example meant to illustrate the importance of environmental parameters.

Data obtained here establishes green tea as the highest in terms of its total polyphenol content, but this result must be viewed in light of caveats. Results obtained here do line up with the commonly held cultural idea that green tea is inherently antioxidant. Moreover, this result is also further confirmation of previous analytical work conducted on green tea, specifically within the lens of total polyphenols (Lou et al, 2020). Therefore, it is safe to conclude that green tea has the highest polyphenol content within this project, but that may not necessarily hold up outside the remit of this project.

Another interesting observation within the polyphenol assay arm of this project was the positive correlations between total polyphenols, flavonoids and tannins. Catechins are a particular sub-group of polyphenols known as flavonoids (Mahindrakar et al, 2020), so we should therefore expect to see flavonoid concentrations somewhat correlated with polyphenol content. Consulting obtained results for green tea, it is clear that this correlation exists between flavonoid concentration and total polyphenols. While total polyphenol content is clearly a good measure of overarching antioxidant capability, it is important to further breakdown the obtained results in terms of their flavonoid and tannin content, which was measured for exactly this purpose. The main rationale for this idea stems from the fact that these specific polyphenolic sub-groups such as flavonoids and tannins also imbue the included plants with their antioxidant potential (Xiang et al, 2019; Peng et al, 2020). What is evident from consulting flavonoid and tannin values in tandem with total polyphenols is a recapitulation of antioxidant profile across assays (i.e. if polyphenols are high, flavonoids and tannins will also be relatively high based on phenolic content).

It is also interesting to note that plants of similar species obtained similar results on polyphenols, flavonoids and tannins assays. Similar observation of this cross species consistency was also demonstrated previously in different plants exhibiting similar antioxidant molecule fingerprints (Feduraev et al, 2019). This cross species recapitulation of antioxidant fingerprint was localised by the researchers. Interestingly, it was found to be most pronounced in the reproductive organs of their included plants. This insight was also demonstrated previously in *Crocus Sativas* (Baba et al, 2015). This idea that reproductive organs are richer in phenolic contents also ties in nicely with the previously mentioned caveat surrounding developmental stage.

Overall, what is evident from the results obtained pertaining to total polyphenol, flavonoid and tannin content per plant is a varied molecular fingerprint. While varied, there is apparent consistency across the concentrations with an apparent correlation between their total polyphenols, flavonoids and tannin content. As previously alluded to in this project, the global populations that can gain the most from using plants as medicine are in many cases environmentally harsh. In tandem, plants directly obtained from these environments are apparently richer in antioxidant molecules. Therefore, these environments may have a powerful treatment for certain diseases on their doorstep. Examining this idea also may provide good

directions for future research. This process is already being analysed and tested in Ghana and India (Sen & Chakraborty, 2017; Krah, de Kruijf & Ragno, 2018).

(ii) Antioxidant capacity of plants

Antioxidant capacity within this project was indexed as performance on metal reduction assays and radical scavenging assays. It is clear from obtained results that a varied profile exists across plant samples in relation to their antioxidant capability.

An interesting place to begin a discussion of the plant extracts' antioxidant capability is comparing this variable directly to phenolic content. Previous research has demonstrated that extracts with high antioxidant capability (as measured by radical scavenging and metal chelation assays) also have a high phenolic content (Ying et al, 2015). In the case of this project this fact is positively confirmed. Observing the metal reduction assay results, an almost complete recapitulation of the hierarchy set out in phenolic results is apparent. This occurs across both chelation assays. As such, results obtained here echo previous results where phenolic contents were successfully correlated to antioxidant activity (Mariod et al, 2009; Locatelli et al, 2010). Such correlations have led to hypotheses that antioxidant effect is therefore a synergistic dynamic between total phenolic content, metal chelation and radical scavenging.

In terms of metal reduction, it is clear that green tea and sumac were top performers with ginger tea a consistent low performer. As this result is consistent across both included metal chelation assays, it can be concluded that in terms of metal chelation, green tea is optimal, sumac is second best and ginger tea was the worst. What is further interesting when plant extract performance is analysed across each metal chelation assay is the fact that differing scores were obtained. This highlights the variability between assay results. Such differences can be observed for numerous reasons, either based on the samples themselves or the assay characteristics.

For example, both FeRP and CuRP rely on reaction time. Variation within the unique samples included in this project could have caused different temporal endpoints of the reduction reaction to be registered. As a result, sample characteristics may have affected final scores on both of the assays. In order for future research to avoid such an issue, a kinetic matching

approach can be implemented to express antioxidant capability in a more consistent manner (Magalhaes et al, 2012; Hao et al, 2021).

In terms of the assay's effects on obtained results, particularly focusing on FeRP, it must be highlighted that any electron donating molecule without antioxidant properties that possessed redox potential lower than that of the Fe(III)/Fe(II) pair would have skewed obtained results (Nilsson et al, 2005). While the FeRP assay itself is rapid and inexpensive (Prior et al, 2005), the fact that non-antioxidant molecules can potentially add to the final readout value is a definite limitation of this assay. In the case of results obtained here, this phenomenon does not seem to have occurred, with CuRP values being consistently higher. This may be explained by the fact that some limitations of FeRP are addressed in CuRP. The CuRP reagent itself has a lower redox potential for example, so it is more reactive to true antioxidants (Apak et al, 2008). Concomitantly, this lower redox potential makes CuRP a more sensitive assay (Prior et al, 2005). Therefore, the CuRP results may be more reflective of true antioxidant potential as compared with FeRP scores.

This reflection of true antioxidant potential by CuRP can be further delineated by analysing the pH level of each reaction. For example, FeRP assays require an acidic pH (3.6) which is far from physiological pH levels (Benzie & Strain, 1996). While the obtained FeRP results are useful, the lack of physiological pH level makes it difficult to extrapolate these results to *in vivo* scenarios. In contrast, the CuRP assay is performed at a pH of 7 (Apak et al, 2004). Arguably, this pH simulates a physiological environment more optimally, resulting in data which is more representative of potential reducing power *in vivo*. An interesting direction for future research would be to analyse the predictive utility of both reduction assays in terms of their ability to predict *in vivo* antioxidant activity.

As well as metal reduction, radical scavenging capacity was also utilised as an index of antioxidant capability within this project. The assays used to determine antioxidant capability include the ABTS, DPPH and galvinoxyl radical scavenging assays. These assays are still consistently utilised to determine radical scavenging capacity in varying environments (Couttolenc et al, 2022). At first glance, it is obvious that green tea was the optimal performer in terms of radical scavenging, while ginger tea was the weakest. The interesting thing about this result is the fact that radical scavenging capacity was not conserved across species. While interesting, such differences in the antioxidant potential of different tea species have been observed previously (Sirichaiwetchakoon, Lowe and Eumkeb, 2020). Also, while ginger tea was different to green tea, herbal tea was similar to green tea. Therefore, it could just as easily

be postulated from this result that ginger tea is just an outlier in terms of its overarching antioxidant capability. Further confirming this is ginger tea's poor performance across all assays. Worth mentioning here also is the performance of sumac (*Rhus coriaria L)* on radical scavenging assays. Sumacs results line up with recent research in the area and confirm it as a proficient antioxidant and phytochemical representing an interesting avenue for future research (Fereidoonfar et al, 2019; Alawsy & Al-Jumaili, 2020; Batiha et al, 2022).

Interestingly, and similar to the metal reduction results, all of the plant extracts' performance across all radical scavenging assays was predicted by their initial total polyphenols. Again, the idea that the variables total phenolic content and antioxidant capability are correlated gains confirmation from the results in this project. There is clear merit to the correlations observed consistently across time (Mariod et al, 2009; Locatelli et al, 2010; Ying et al, 2015).

Upon further analysis of radical scavenging results, it is clear that the standardised scores for each plant extract are different depending on the assay consulted (see Table 1.2). While there is between group consistency in terms of where each assay ranks green tea and sumac in a hierarchy relative to all plant extracts, the scores each assay obtains are varied. This variability could exist for numerous reasons.

Similar to other assays, the solvent utilised (Apak et al, 2007) can greatly affect radical scavenging. Magalhaes et al (2008) demonstrated this, finding that above a specific water content of solvent, radical scavenging decreases because part of the DPPH radical coagulates and becomes somewhat inaccessible to antioxidant reactions. This provides an explanation for the consistently low scores seen across the DPPH assay when compared to other included assays.

For reasons such as the aforementioned, it may be logical to assume the other assays are more efficient determinants of radical scavenging ability (Floegel et al, 2011). While this seems logical, it is important to remember that antioxidant capability as measured by different assays will always vary somewhat (Wootton-Bearda et al, 2010). With that in mind, if one is attempting to determine the antioxidant potential of extracts *in vivo*, then maybe the optimal assay is the one which utilises a pH close to that of the *in vivo* system one wishes to screen. As well as pH, solvent type has also been shown to affect final radical scavenging values,

particularly within galvinoxyl/ABTS+ assays. Yong et al (2013) demonstrated that galvinoxyl radical scavenging was most efficient when chloroform was the solvent versus methanol (and aqueous). This efficiency of chloroform over methanol was consistent, and when chloroform was used as a solvent in both ABTS+ and DPPH assays, ABTS+ still demonstrated more sensitive results. Future research should endeavour to use the same plant extracts included here but alter the solvent used in radical scavenging assays. Obtained results could then be compared to those obtained here. Such comparison would provide important information regarding solvent used and observed radical scavenging capability.

Overall, and interestingly, between all plants we see a consistent profile across each assay pointing to an overall antioxidant capability. It would seem that results in one assay can reliably predict performance in other assays (Zhao et al, 2008; Rafi et al, 2018). It is thought that this correlation between total polyphenol content and subsequent antioxidant ability can be explained by the fact that the assays used to obtain the results all share a similar mechanism of action. Essentially, the total polyphenol assay shares similar electron transfer as the reactions occurring across assays. It is due to such a conserved mechanism of action at the molecular level that assays utilised in this project can obtain consistent results when compared to one another. With this in mind, another interesting avenue for future research would be the construction of a model that accurately predicts overall antioxidant capability, based on information obtained from assays deemed the cheapest, quickest and easiest to conduct. Such a model would be parsimonious and ultimately aid in determining the antioxidant potential of a broad range of plants efficiently. Preliminary research in this area is already ongoing, where researchers utilised Fourier transformations in infrared spectroscopy to predict the antioxidant capability of plant material (Johnson et al, 2020).

(iii) Metabolomics

Metabolomics analyses revealed the presence in relative abundance of various metabolites (>100). Some of these metabolites were present across all or most of the plant extracts, these included; palmitic acid, α-linonelic acid, linoleic acid and fructose. Specifically, the optimal performing plant, namely green tea, was relatively abundant in simple sugars, carbocyclic sugar and methylxanthine. Particularly, the simple sugar fructose was present in relatively high abundance across all plants with high antioxidant capability. Interestingly, fructose itself is implicated in chronic inflammation *in vivo* (Maguira-Alfaro et al, 2021), yet is present in

significant amounts within top performing plant extracts included in this project. It is important going forward to delineate the specific role of this molecule as it relates to antioxidant processes. Future research should specifically focus on this. Furthermore, sumac contained fructose, as well as ribose in relatively abundant amounts. In terms of sumacs antioxidant capacity, it could be hypothesised that it gains antioxidant capability from the presence of ribose. Such a line of thought stems from the fact that ribose was only present in sumac, which had a high antioxidant capability (second to green tea). Again, future research should delineate these nuanced metabolic fingerprints in terms of how they play into overarching antioxidant capability.

In contrast to top performing plant extracts, and consulting the metabolite profile of ginger tea (lowest antioxidant capability), a relative abundance of turanose can be observed. Turanose is a reducing disaccharide (Han, Lee and Yo, 2021) and its presence in ginger tea may partially explain the plants low antioxidant capability. While a potential explanation based on the fact that no other plant contained turanose, it is important to highlight that the mechanisms through which these metabolites influence antioxidant capacity is not fully understood. As a result, future work should be moving in this direction.

Palmitic acid is a common saturated fatty acid present in the human body. It carries out many fundamental biological functions. Its concentrations are guaranteed by its endogenous biosynthesis (Carta et al, 2015). However, particularly over long timeframes, this endogenous biosysnthesis may cease, manifesting as deficiencies in an organism. Contribution of palmitic acid by dietary means is relevant, with exogenous intake of palmitic acid deemed essential in some cases (Carta et al, 2017). All plant extracts included in this project contained palmitic acid, as a result they represent an exogenous source of this fatty acid. Future research should endeavor to determine how much *in vitro* palmitic acid content can predict *in vivo* levels of the fatty acid.

While beneficial in some contexts, palmitic acid has also been observed to be detrimental to human health by some research. Essentially, mutagenic metabolites can form from the peroxidation of palmitic acid (Beeharry et al, 2003). Interestingly, linoleic acid and α-linolenic acid were demonstrated to prevent damage from palmitic acid (Katsoulieris et al, 2009). What is interesting here is that metabolomics revealed the presence in relative abundance of both linoleic acid and α-linolenic acid across included plant extracts. What is evident from this is

that plant extracts contain a unique mixture of metabolites which inevitably interact to bring about potentially myriad effects. For example, it has been demonstrated that palmitic acid induced lipotoxicity within microglia was ameliorated by unsaturated fatty acids such as linoleic acid and α-linolenic acid (Urso and Zhou et al, 2011). This provides a good example of how two molecules, contained in all plants included in this project, can simultaneously cause and treat toxicity depending on context.

Metabolomics further revealed other molecules across plant extracts including; fructose, asparganine and inositol. While interesting to get a metabolic breakdown per-plant, it is important to highlight the fact that no differentiation was made between aspects of any one plant i.e. no stem-flower differentiation was made. As a result, data obtained here does not have a localized or region specific metabolic breakdown. Such a breakdown would be insightful and represents another fruitful avenue for future research. For example, recent research has already made distinct demarcations of metabolite profiles contained in specific regions of *Salvia hispanica L.* (de Falco et al, 2021). Such breakdowns provide more detailed pictures of antioxidant potential to researchers.

Furthermore, important considerations for future research in the realm of plant metabolomics relate to the effect environmental parameters can have on metabolic profile. Kolahi et al (2021) for example, demonstrated that differing levels of water stress/drought conditions can have significant impact on the secondary metabolite profile of plants. Unfortunately, the environmental growing parameters of included plant material in this project could not be known. As a result, while interpreting the metabolic profile of each plant, it must be kept in mind that they were arguably grown in unique conditions. In line with Kolahi et al (2021), another fruitful avenue for research would be to determine environmental parameters that produce optimal metabolite profiles. For example, Kolahi et al (2021) found that they could produce more optimal metabolic profiles with less water, thus industries concerned with producing plant-based supplements can save energy whilst producing more potent yields. Future research should compare data obtained here, to novel data, where growing parameters and plant region are considered as variables also. Such data would enrich our collective understanding of how metabolic processes play into antioxidant processes.

While segmenting the plants by region and understanding environmental growth parameters is important, it is equally important to fractionate and identify metabolites further in order to determine how each one is potentially contributing to overarching antioxidant capability.

Furthermore, understanding how these metabolites interact and act synergistically to potentially bring about antioxidant phenotypes is another avenue for future research. Fractionation methods such as the one developed by Salem et al (2017) can be utilised going forward to further illustrate and contextualize the metabolome of each included plant.

(v) Conclusions

Quantification of antioxidant potential was carried out on numerous plant species. Antioxidant potential was indexed as results on phenolic based assays, metal reduction assays, radical scavenging assays and metabolomics analyses. While all included plants demonstrated an antioxidant profile, it can be specifically concluded that *Camellia sinensis* is the best antioxidant as determined through this *in vitro* design. This is in direct contrast to ginger tea, which was determined to be the least efficient plant in terms of its antioxidant capability across all assays. Various points of discussion highlight why particular plant extracts may have been more efficient antioxidants. It is important to remember that if other methods were utilised to quantify antioxidant potential results may have varied. This is due to the fact that the antioxidant potential of the six plant extracts measured in the present study is an approximation based on the artificial radicals DPPH, ABTS, and galvinoxyl. Approximation via these methods is necessary due to the lack of available methodologies that simulate antioxidant potential on natural free radicals (superoxide radical and hydroxyl radical). Therefore, the obtained/not obtained parameter variations among included plants may not reflect actual natural levels due to the artificial radical-based antioxidant potential measuring methodologies used in this study.

Also, the current work is *in vitro* in nature. The antioxidant profiles obtained here cannot be applied to *in vivo* systems. It has long been known that *in vitro* results do not necessitate similar concentrations *in vivo* (Fraga, 2007). Future work, while endeavouring to take on the ideas throughout, should ultimately aim to translate results demonstrated here into *in vivo* systems, particularly models of disease. While methodological rigour and plant characteristics are important caveats, it is of paramount importance to determine if these plants have a role to play in treating certain diseases.

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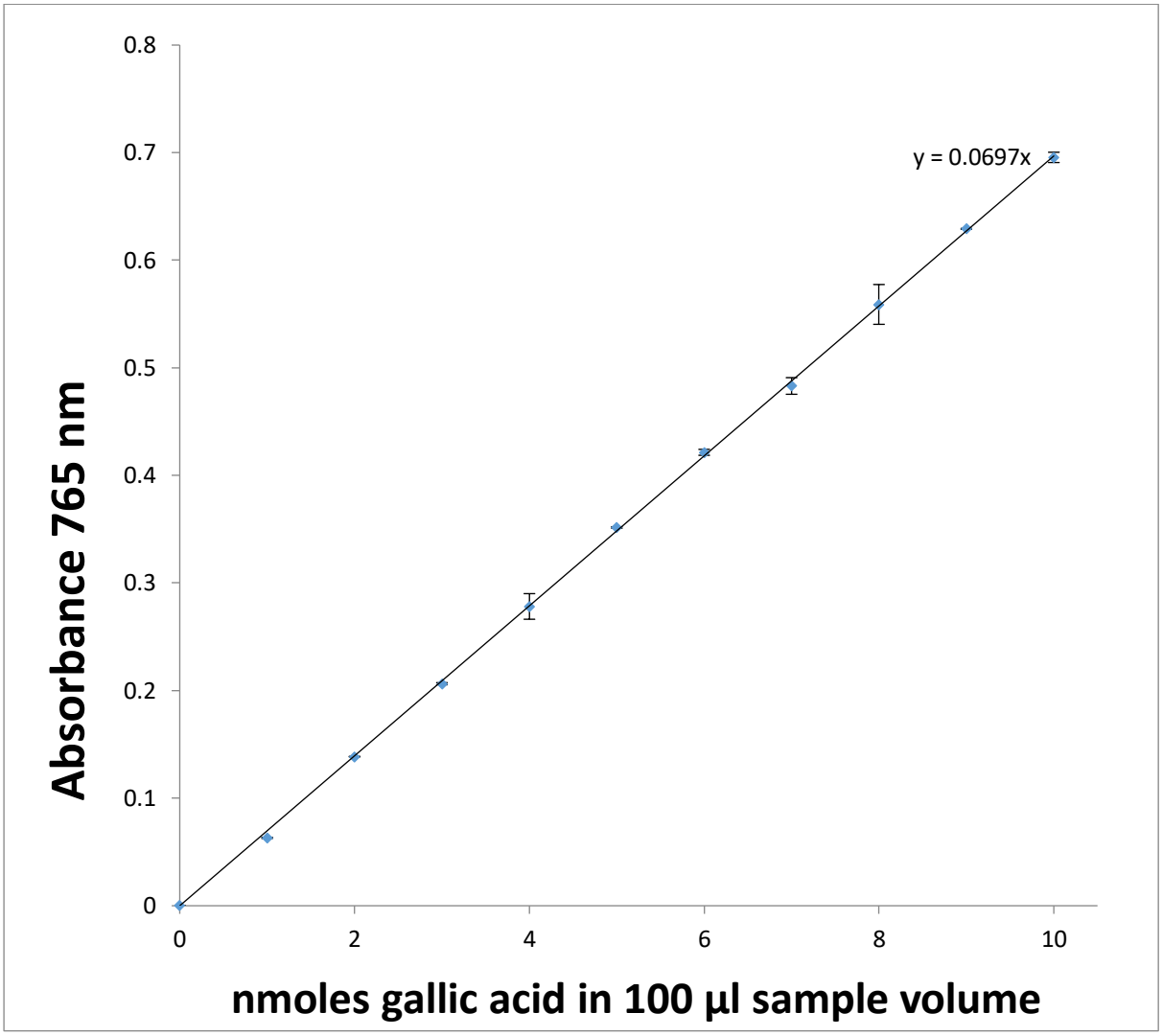
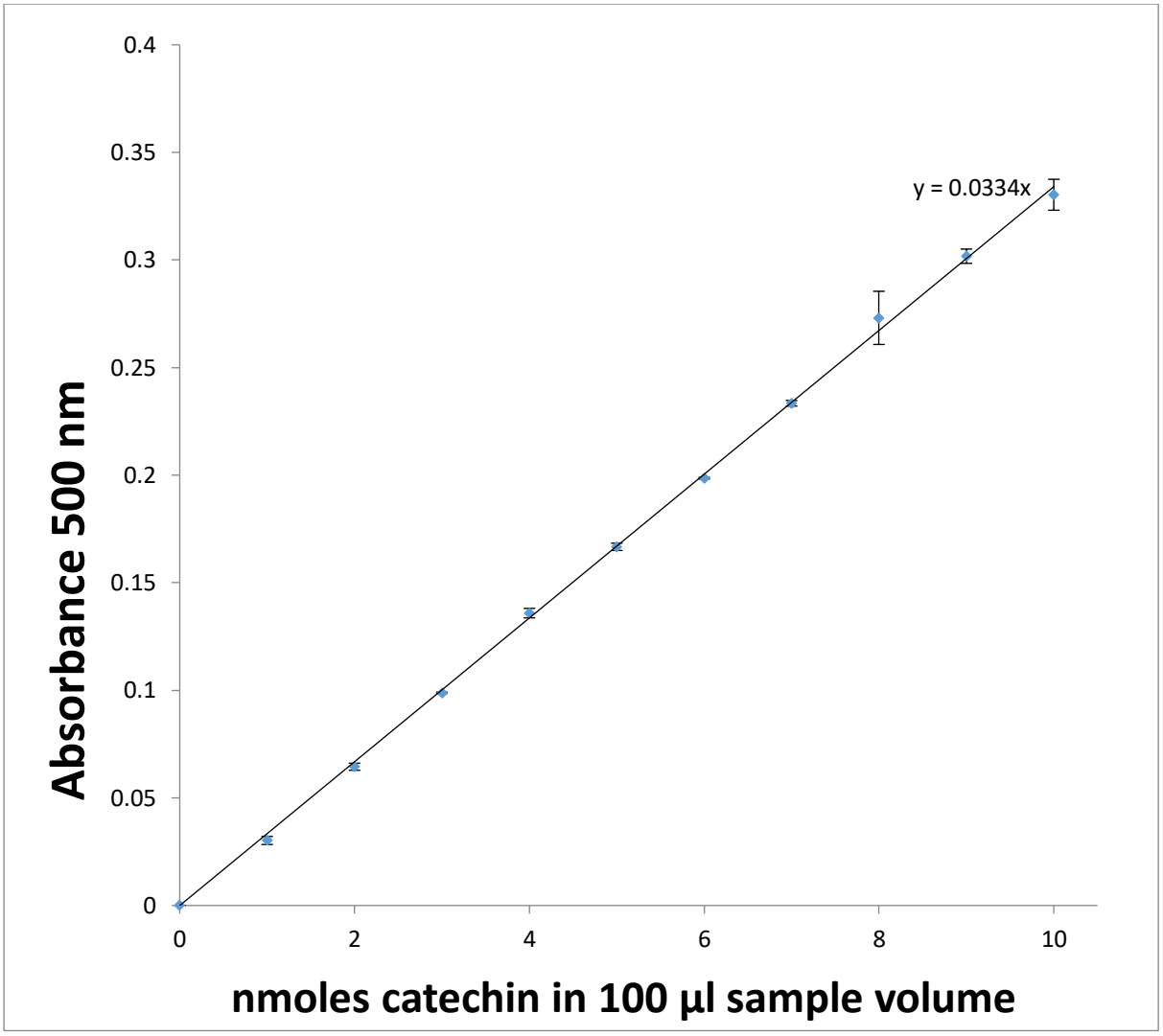
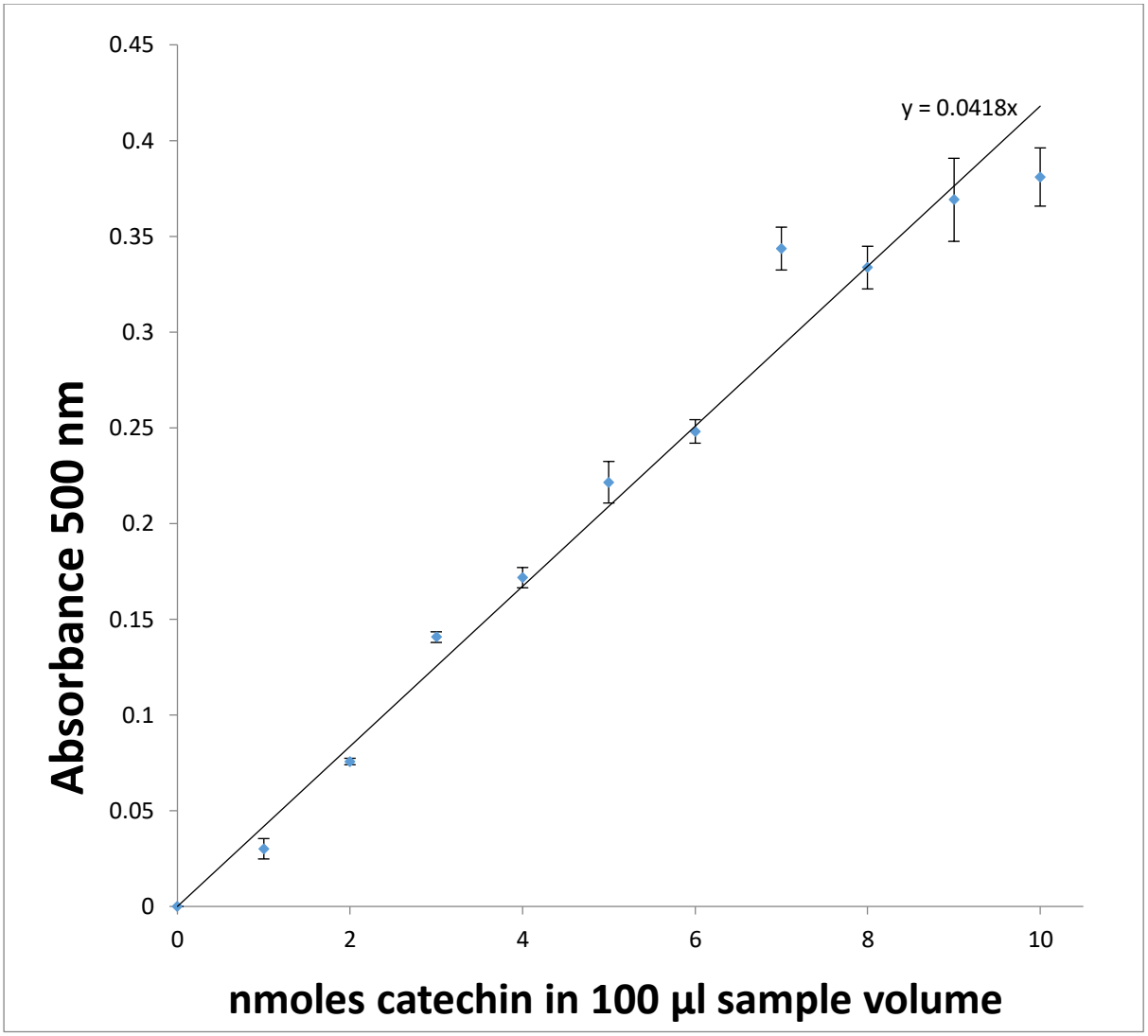
Appendix



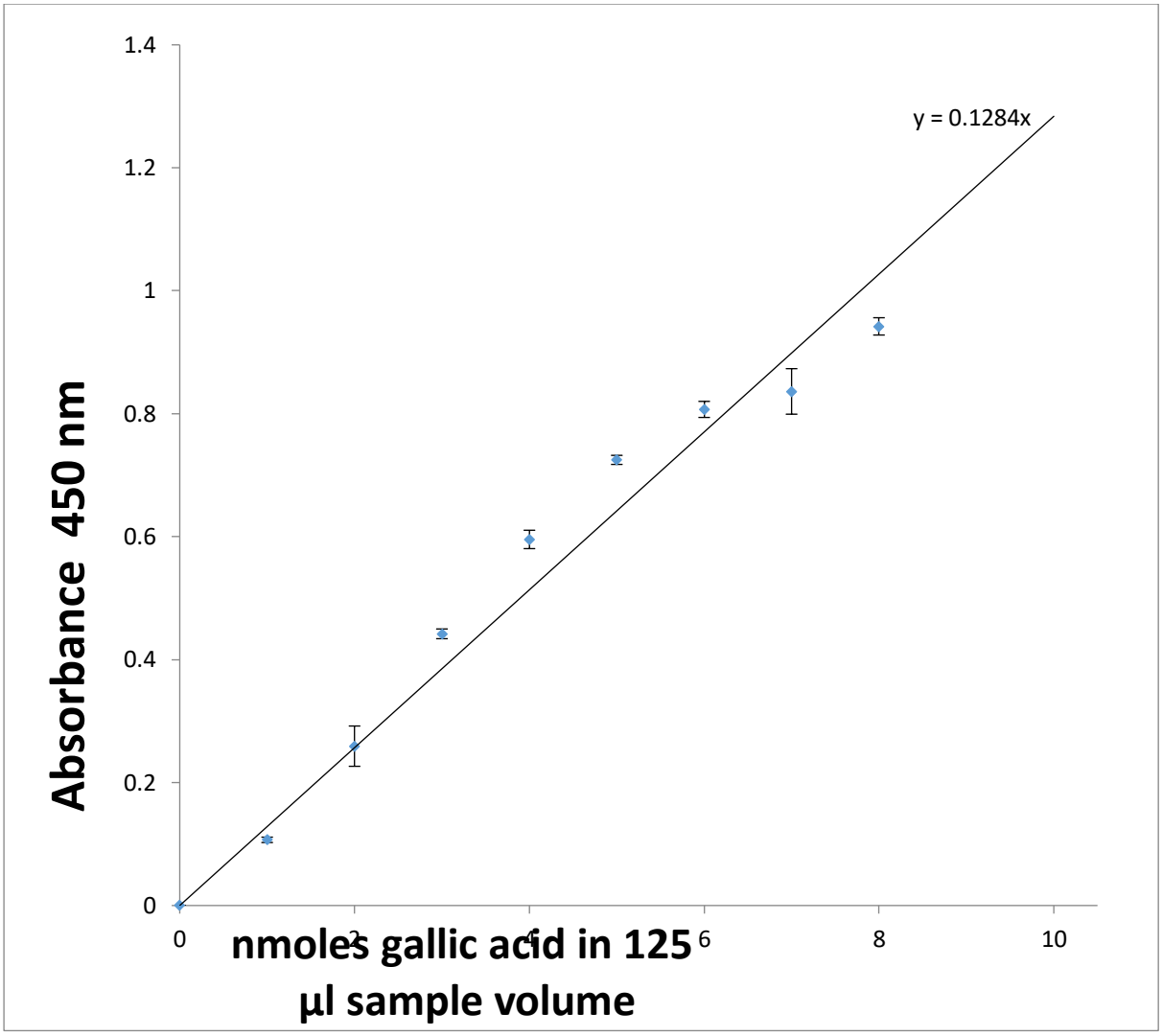
Polyphenols standard curve



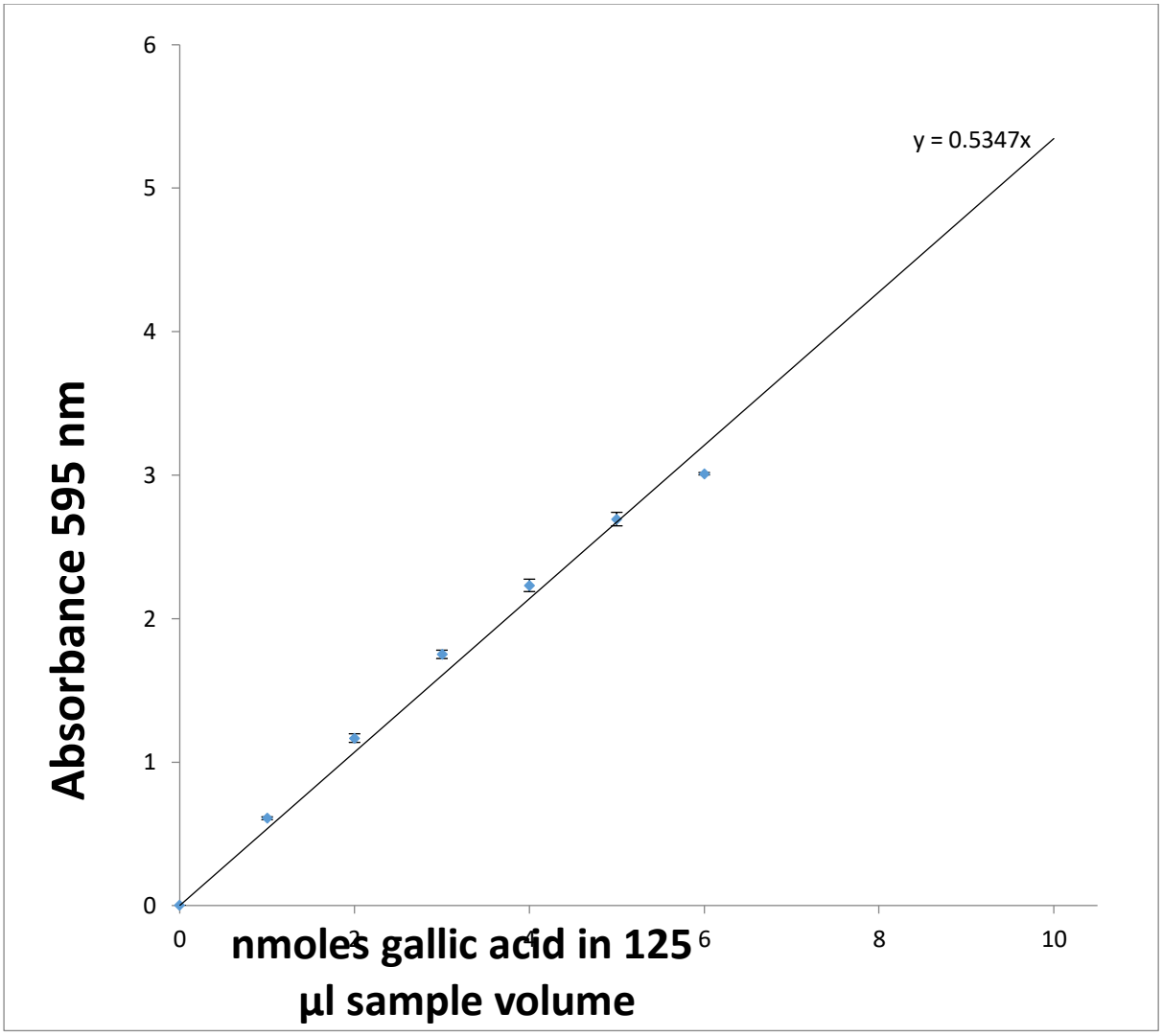
Flavonoids standard curve



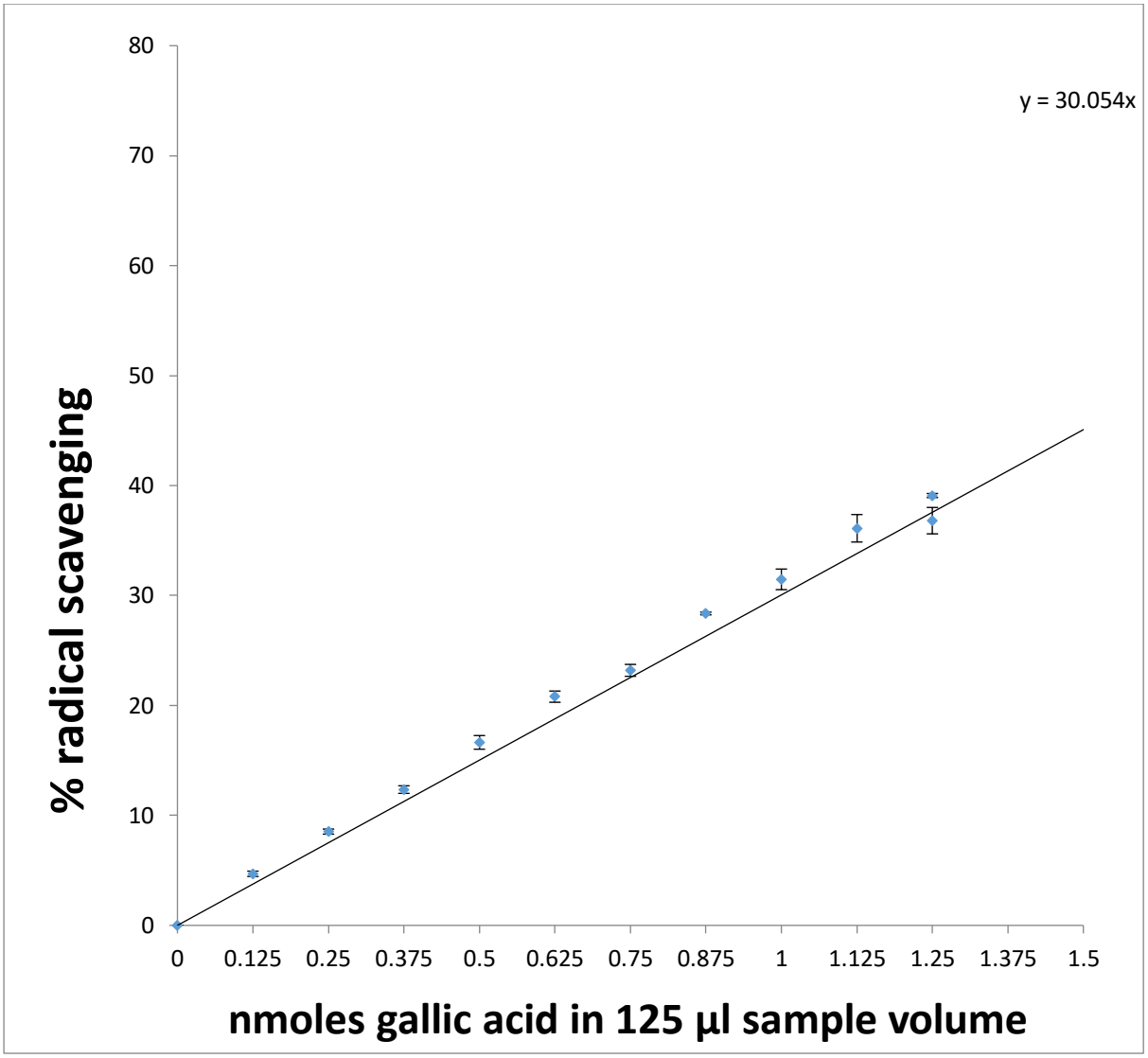
Tannins Standard Curve



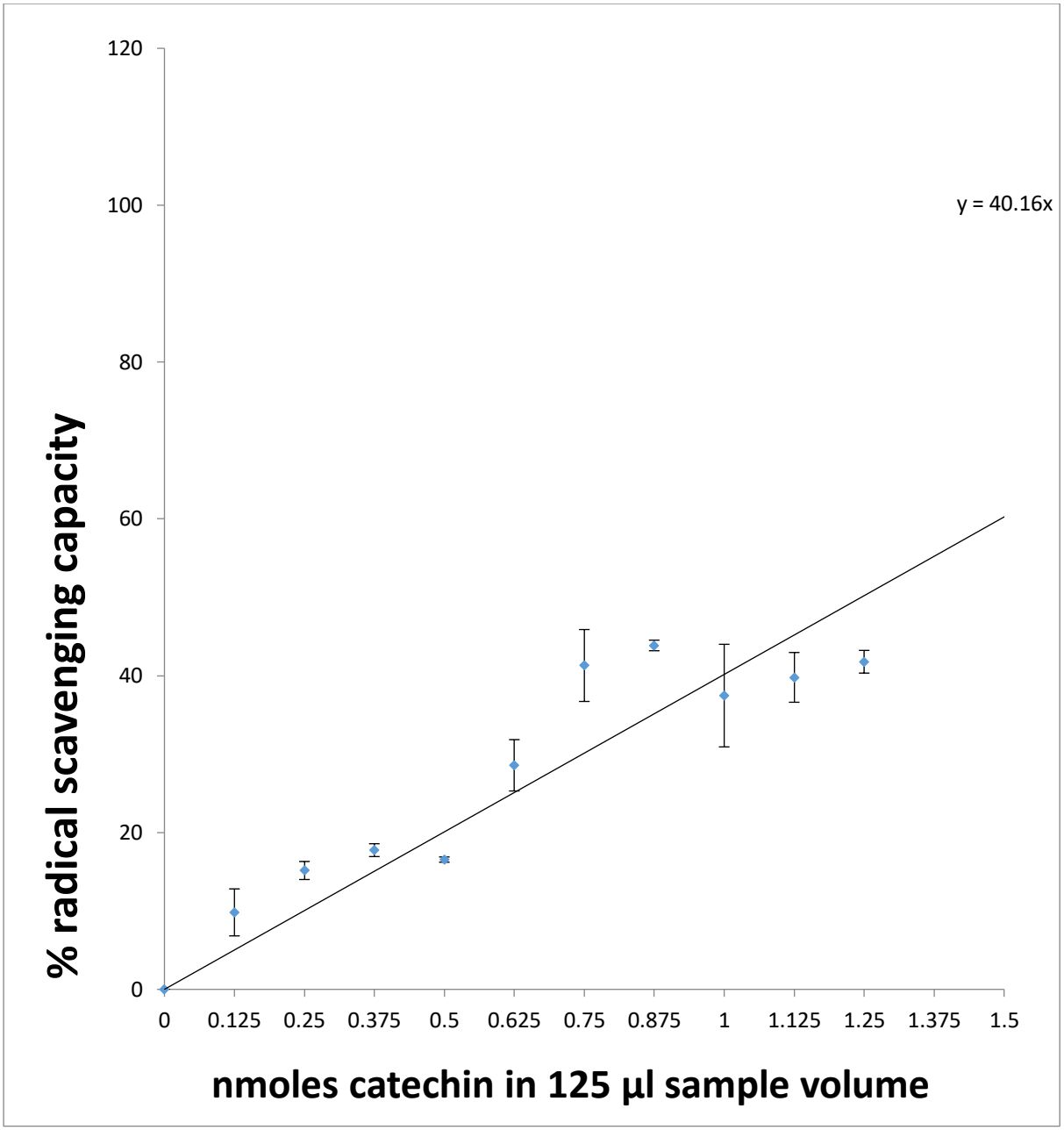
CuRP Standard Curve



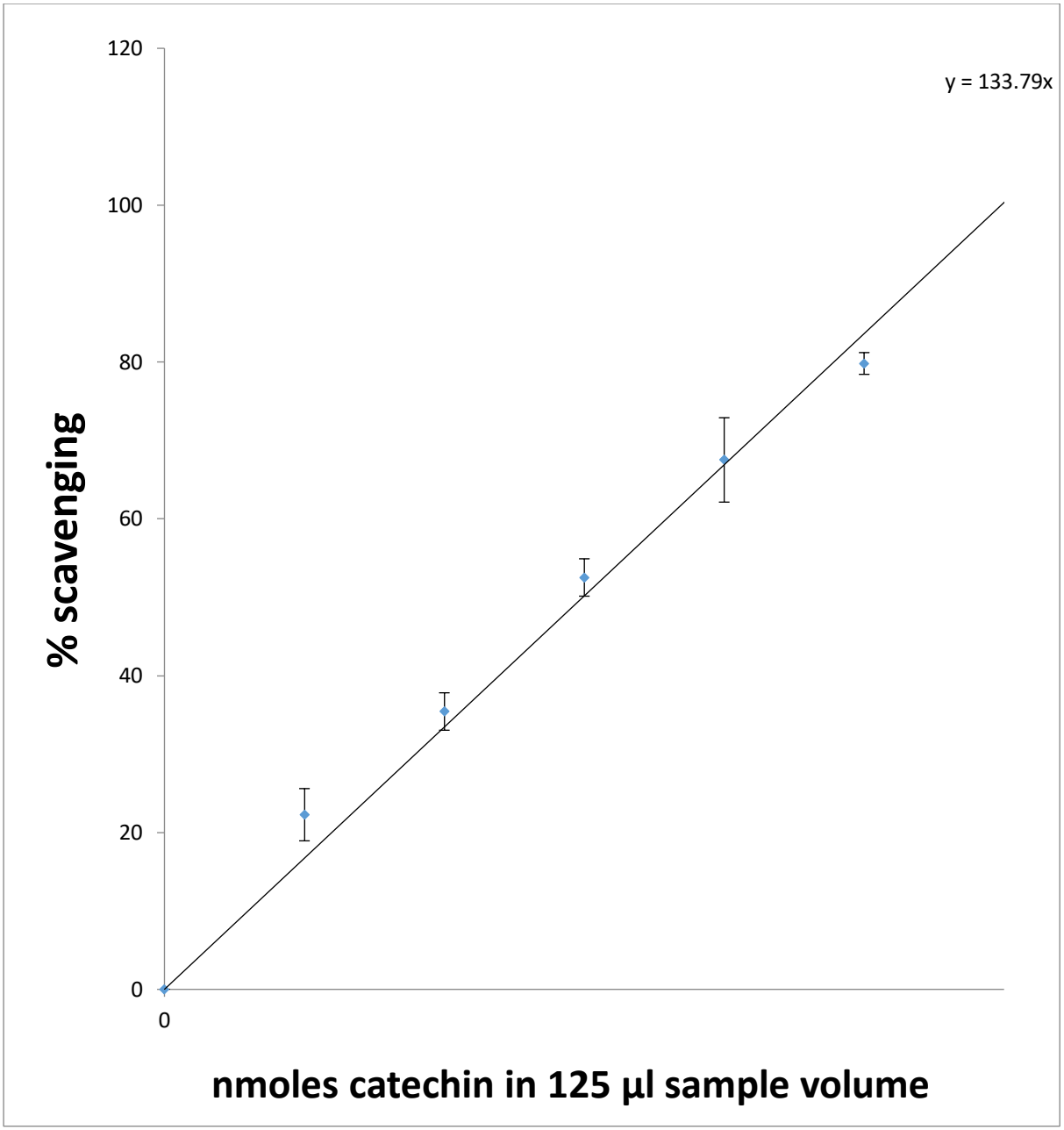
FeRP Standard Curve



ABTS Standard Curve



DPPH Standard Curve



Galvinoxyl/ABTS+ Standard Curve