JOURNAL OF
EMERGING INVESTIGATORS

Article

Antioxidative properties of Taiwanese high mountain tea infusions

Shang-Chieh Lien[1], Fabian Graßl[2]
[1] Kang Chiao International School Linkou Campus, New Taipei City 244, Taiwan
[2] School of Pharmacy, College of Medicine, National Taiwan University, Taipei 100, Taiwan

SUMMARY
Tea is an integral part of Taiwanese culture, and Taiwanese high mountain teas, in particular, have gained significant popularity in recent years, especially cherished for their unique mild and fresh taste. Beyond its taste, tea is appreciated for its health benefits, including anti-inflammation, improved heart health, and cancer prevention. These benefits are primarily attributed to the high antioxidant content found in certain types of tea. We aimed to study the factors influencing the antioxidant concentration in Taiwanese high mountain tea infusions and hypothesized that growing conditions, the climate, and processing of tea leaves play a substantial role in that regard. To assess how the above-mentioned factors affect antioxidative properties, we studied samples of Taiwanese high mountain green, oolong, and black teas and compared them to various green teas grown at lower altitudes and in different geographical regions. For quantification of antioxidant properties, we utilized the Folin-Ciocalteu assay, oxygen radical absorbance capacity assay, and thiobarbituric acid reactive substances assay, which each illuminated different aspects of the oxidation process. Our findings indicated that the antioxidative properties of Taiwanese high mountain teas are predominantly influenced by oxidation during tea processing, while no definitive conclusion could be drawn regarding the influence of cultivation altitude. The highly oxidized black teas tested in this study featured a low antioxidant content, while both high mountain green and oolong teas exhibited high levels, making them valuable sources of antioxidants. We also noticed that the spherical shape of high mountain tea leaves led to slower extraction compared to loose or needle-shaped tea leaves, suggesting that multiple infusions may be needed to fully harness their antioxidant potential. Findings of this study may assist consumers in selecting teas with potential health benefits and provide some guidelines to maximize the extraction of antioxidants from tea.

INTRODUCTION
Since tea (*Camellia sinensis*) was first introduced to Taiwan by Chinese traders in the 18th century, its cultivation and consumption have become an integral part of Taiwanese culture (1). Taiwan produces approximately 14,000 tons of tea every year, with oolong tea accounting for approximately 90% of total production and the residual 10% shared among green and black tea (2,3).

Tea contains a variety of secondary plant metabolites such as catechins, flavonoids, and caffeine. A large proportion of these secondary plant metabolites contain phenolic structures and polyphenolic compounds, in particular, and possess antioxidative properties, which contribute to a range of health-promoting effects (4). The mechanisms of action of antioxidants in the human body are diverse, including the scavenging of reactive oxygen species, the activation of antioxidant enzymes, the inhibition of proinflammatory mediators, the inhibition of lipid peroxidation, and the chelation of metal ions (5). A study conducted by Suzuki-Sugihara *et al.* found that green tea catechins could prevent the oxidation of low-density lipoprotein, thereby reducing the risk of atherosclerosis, which is further supported by Chung *et al.,* who found some correlation between the consumption of tea and a reduced risk of cardiovascular disease-related mortality (6,7). The anti-inflammatory effect, both *in vitro* and *in vivo,* of catechins, a subgroup of polyphenols, which are particularly abundant in green tea, has been extensively studied and can in part be associated with the regulation of interleukins like IL-6 and IL-8 as well as the transcription factor NF-κB (8-11). Further studies also indicate that tea consumption might decrease the risk of certain types of cancer, where the involvement of antioxidants is still under investigation (12). Together, these findings underscore the importance of antioxidants in the health-promoting effects of tea and emphasize the need for continued research on tea antioxidants.

Several factors influence the amount of secondary metabolites found in a tea infusion, starting with the climate and soil in which the tea plants are grown, followed by the processing of tea leaves after harvest, and lastly by the steeping method (13).

Taiwan is known for the tea cultivated in the central mountainous regions at heights above 1,000 meters. Due to the unique climate at this altitude, teas grown in such regions, also called high mountain teas, are exposed to less sunlight and lower air temperature (14). One study conducted on Chinese high mountain teas found that teas grown at higher altitudes possess a unique taste with less bitterness, which might stem from a lower concentration of bitter substances such as theanine, caffeine, and catechins and another study found that catechin content in oolong teas was inversely correlated to cultivation altitude (15,16).

The main processing steps of tea, after the harvest, include sun drying, withering, rolling, oxidation/fermentation, fixing, shaping, and drying (2,17). Among the three main types of tea produced in Taiwan, black teas undergo the highest levels of fermentation and oxidation, oolong teas are semi-fermented, and green teas are typically considered unfermented (17). Differences in fermentation affect the antioxidative properties of the teas, as previous research has shown that unfermented or only lightly fermented green tea,

https://doi.org/10.59720/24-318

in general, features a higher polyphenol content compared to heavily fermented black tea (18). Additionally, both the total amount of antioxidants present in the tea, as well as the amount that can realistically be extracted during tea steeping, strongly depend on the shape and particle size of the tea leaves (19). Oolong teas, in general, and Taiwanese high mountain teas, in particular, are shaped into spheres (gunpowder style), while Japanese sencha are formed into needles (17). While whole tea leaves are considered to be of higher quality, most of the tea consumed around the world is prepared from tea bags, which in many cases are produced from the fine tea dust from the tea-making process (20). Finally, the amount of plant ingredients extracted from the tea leaves during steeping is highly influenced by steeping time and steeping temperature (21). It remains to be studied how the processing of Taiwanese high mountain teas influences antioxidant concentrations, and how antioxidative properties of Taiwanese high mountain teas compare to teas grown and processed in other geographical locations.

In this study, we evaluated how tea processing and unique growing conditions affect the antioxidant properties of Taiwanese high mountain black, green, and oolong teas by comparing them to common green teas, such as low-altitude Taiwanese green tea, Japanese sencha, and Vietnamese jasmine tea. We hypothesized that both the growing conditions and processing of tea leaves influence the antioxidant concentration in tea infusions. We expected higher antioxidant concentration for green tea compared to black tea, with oolong tea being situated in the middle and an inverse correlation between antioxidant concentration and cultivation altitude. We were also interested to see how the particle size and shape of the tea samples would influence the extraction behavior. To quantify the antioxidative properties of tea infusion, we utilized three detection methods: the Oxygen Radical Absorbance Capacity (ORAC) assay, measuring the capability of a sample to prevent oxidative processes, the Folin-Ciocalteu method, measuring the overall antioxidative capacity of a sample, and the thiobarbituric reactive substances (TBARS) assay, measuring the capability of a sample to prevent lipid peroxidation.

We were able to quantify the antioxidative properties of various tea infusions and showed, on a very basic level, how tea might prevent the harmful influence of reactive oxygen species (ROS). We found that among the measured samples, high mountain black tea contained the lowest concentrations of antioxidants overall. However, the antioxidative capacity of high mountain oolong teas was very similar to that of Vietnamese jasmine green tea and higher than that of Japanese sencha. We also found that tea leaf shape and particle size heavily influence the extent and rate of antioxidant extraction. Our results from this study emphasize the potential of Taiwanese high-mountain teas as valuable dietary sources of antioxidants, with significant implications for health and wellness. This study might also help to better understand how antioxidants are extracted from tea and might aid in the optimization of extraction processes for tea.

RESULTS

For this study, we evaluated 10 different types of dried tea as well as fresh tea leaves. Dried Taiwanese high mountain Oolong tea (TO3, TO4, TO5, TO7); green tea (TG9) and black tea (TR1); as well as regular Taiwanese green tea (TG6) and black tea (TR2); Vietnamese green tea (VG8); and Japanese green sencha tea (JG10) were purchased from commercial sources **(Table 1).** TR1 and TO3, in particular, were chosen because they have been stored for 10 years and therefore might also give some insight into the influence of prolonged storage on antioxidant capacity. Since Taiwanese high mountain oolong teas are typically only lightly fermented, the transition from oolong to green tea is somewhat fluent and generally not indicated on the packaging. The Japanese sencha, as well as the jasmine green tea, was therefore included in the experiments as examples of clearly defined common types of green tea.

Upon inspection of the dried tea samples, we noticed clear differences in the particle size and shape of the tea leaves. While Taiwanese high mountain teas were rolled into spheres (gunpowder style tea), the Japanese sencha

Entry	Product label (Chinese)	Product label (English)	Type	Country of origin (region)	Leaf shape
TR1	臺灣茶	Taiwan Tea	black tea (aged for 10 years)	Taiwan (Alishan)	spherical
TR2	紅玉紅茶	Ruby black tea	Black tea	Taiwan (Sun Moon Lake)	loose
TO3	阿里山金	Alishan tea	oolong tea (aged for 10 years)	Taiwan (Alishan)	spherical
TO4	杉林溪臺灣特色茶	Shan Lin xi specialty tea	oolong tea*	Taiwan (Shan Lin xi)	spherical
TO5	阿里山茶	Alishan tea	oolong tea*	Taiwan (Alishan)	spherical (partly crushed)
TG6	高海拔茗茶	Gao hai tea	green tea*	Taiwan (Luye)	spherical
TO7	阿里山茶	Alishan tea	oolong tea*	Taiwan (Alishan)	spherical
VG8	茉莉綠茶	Jasmine green tea	Jasmine green tea	Vietnam	loose
TG9	高山茶	high mountain tea	green tea*	Taiwan	spherical
JG10	玉煎茶	Jade sencha	green tea (sencha)	Japan (Kyoto)	needle

Table 1. Dried tea samples analyzed in this study. *No clear indication of tea type on the packaging. The type of tea was determined based on information found online or as indicated by the vendor. The first letter denotes country of origin: T (Taiwanese), V (Vietnamese), J (Japanese). The second letter denotes the type of tea: R (black tea), O (Oolong tea), G (green tea).

https://doi.org/10.59720/24-318

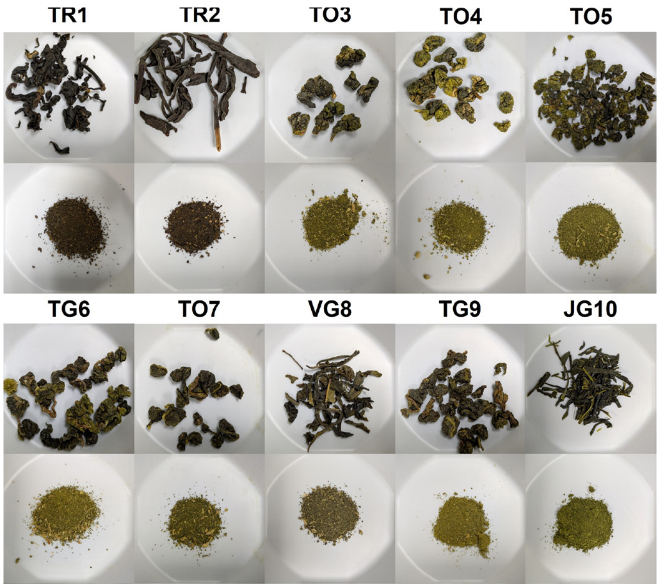

Figure 1: Tea samples evaluated in this study. Whole tea leaves (top) and ground tea samples (bottom). The first letter denotes country of origin: T (Taiwanese), V (Vietnamese), J (Japanese). The second letter denotes the type of tea: R (black tea), O (Oolong tea), G (green tea).

featured a needle-like shape, with other samples having an irregular loose shape (**Figure 1**). To ensure a more accurate comparison between the different tea types, we analyzed both whole leaves and finely ground samples of each type of tea.

Influence of particle size and steeping time on extraction yield

First, we investigated the influence of particle size and steeping time on the extraction yield of tea samples by steeping both ground and whole leaf samples of black tea TR1, as well as fresh tea leaves in boiling water, taking samples at different time points over a period of 25 minutes. We determined the total phenol content, a main indicator for antioxidant properties, by reacting the samples with Folin-Ciocalteu reagent. For better comparison with results from the literature, we used Gallic acid, a well-established antioxidative reference substance, as a standard and calculated the amount of antioxidants extracted from the tea samples as gallic acid equivalents (GAE), where one GAE corresponds to an amount of antioxidants extracted from 1 gram of dry substance equivalent to 1 mg gallic acid (22). Measurements of the gallic acid serial dilution indicated good linearity (R2 = 0.9983) and excellent reproducibility (RSD ≤ 3% between 3 replicates at defined concentrations) for the measurement method. Results from this experiment indicated that phenols are extracted at a high rate and with a high yield from ground tea leaves, with about 80% of the final phenol concentration at 25 minutes reached after only 1 minute of extraction. In total, 20 GAE were extracted from ground tea leaves. Steeping dried whole tea leaves of TR1, on the other hand, only yielded about 7 GAE after 25 minutes, with 80% of the final concentration reached after about 10 minutes. In both cases, phenol concentration stagnated after about 15 minutes. The lowest yield was extracted from whole fresh tea leaves with 1.3 GAE extracted after 25 minutes, even after correction for water content. Based on results from this experiment, an extraction time of 10 minutes was determined for further experiments, to still reflect actual steeping conditions suggested by tea manufacturers while also making sure that a high proportion of water-soluble antioxidants was extracted (**Figure 2**).

For further experiments, we extracted whole and ground tea leaf samples of each type of tea as described above with a 10-minute steeping time (aqueous infusions). Additionally, selected samples (TR1, TO3, VG8, JG10, and fresh tea leaves) were extracted with an ultrasound-assisted method using a mixture of water, acetone, and methanol as the extraction solvent (organic extracts). Organic extracts were evaluated to estimate the total amount of antioxidants present in the tea samples.

Determination of total phenol content

To evaluate the overall antioxidant capacity of the tea samples, we then determined the total phenol content for the remaining aqueous infusions of whole and ground

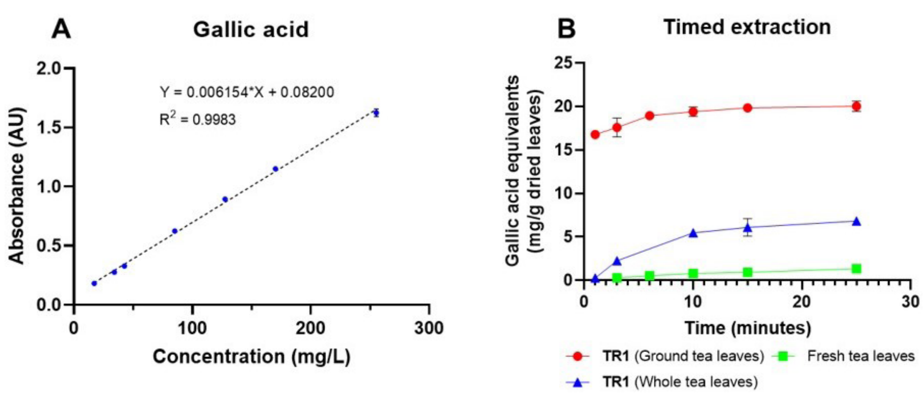

Figure 2: Optimization of the extraction method. (A) Gallic acid calibration curve for total phenol determination. Datapoints depict the concentration of gallic acid plotted against mean absorbance ± SD (n=3). Various concentrations of gallic acid were reacted with Folin-Ciocalteu reagent and absorbance was measured at 760 nm. The Calibration curve was calculated with simple regression analysis in GraphPad Prism. **(B)** Timed extraction of ground and whole tea leaves. Datapoints depict the extracted amount ± SD of phenols calculated as gallic acid equivalents at different timepoints (n=3). Ground (red) and whole (blue) tea leaves of sample TR1, as well as fresh tea leaves (green), were steeped in 100°C water without additional heating. Total phenol content at various time points was determined using the Folin-Ciocalteu reagent.

https://doi.org/10.59720/24-318

tea leaves as well as for the organic extracts. Comparing aqueous infusions of whole leaf samples, we could detect high variability between the single types of tea, with the least amount of phenols measured for black tea TR1 (4.5 GAE) and the highest amount measured for green tea VG8 (70 GAE). While total phenol content was generally on the lower end for highly oxidized black teas compared to less oxidized green and oolong teas, the more defining factor for high phenol concentration appeared to be the smaller particle size of tea samples TR2, VG8, and JG10. Grinding up the tea led to higher extraction yield across all samples, with the most relative increase (700%) measured for sphere-shaped oolong tea (TG6) and the least increase seen for needle-shaped sencha (JG10). Grinding also normalized phenol concentrations among all oolong and green teas used in the study, now ranging from 89 GAE for oolong tea (TO7) to 115 GAE for oolong tea (TO4) (difference of 30%), with JG10 being an outlier in that regard. After grinding up the leaves, aqueous infusions of black teas (TR1 and TR2) contained considerably less phenols, especially compared to the oolong teas. While the total phenol content of organic extracts was even higher than that of the ground leaves, the increase was low to moderate, indicating that aqueous extraction of ground tea leaves already yields a large proportion of the phenols. The total phenol content measured for the fresh tea leaves, even when corrected for water content, was lower than that of samples TO3 and VG8 (**Figure 3**).

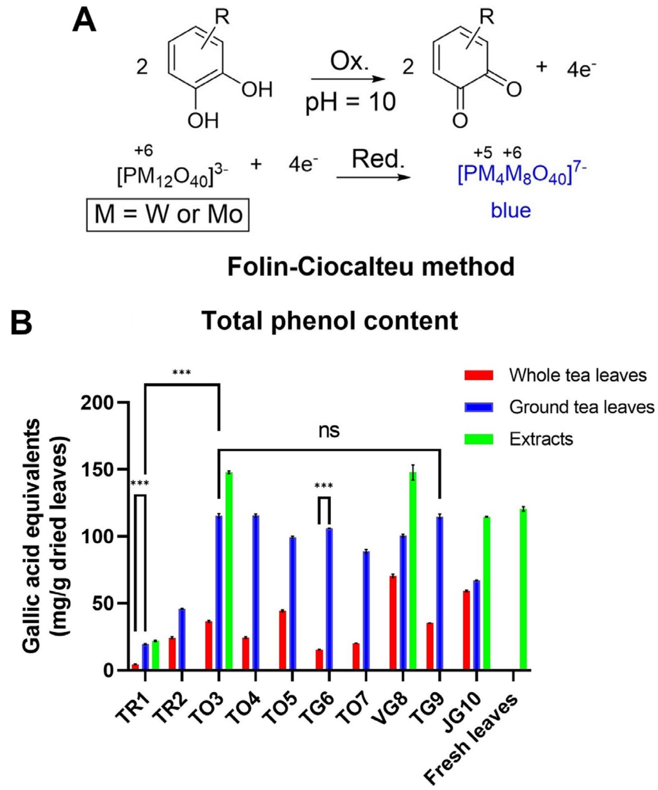

Figure 3: Determination of total phenol concentration. (A) Folin-Ciocalteu method. Redox reaction leading to the formation of blue molybdate and tungstate salts in the Folin-Ciocalteu method. **(B)** Total phenol contents of aqueous tea infusion and organic extracts. Bar graphs depict the mean ± SD extracted amount of phenols calculated as gallic acid equivalents (n=3). Whole (red) and ground (blue) tea leaf infusions of samples TR1, TR2, TO3, TO4, TO5, TG6, TO7, VG8, TG9, and JG10 were diluted to suitable concentrations and total phenol content was determined using Folin-Ciocalteu reagent. Additionally, organic solvent extracts of TR1, TO3, VG8, JG10 and fresh tea leaves were dissolved at suitable concentrations and total phenol content was determined using Folin-Ciocalteu reagent. Statistical significance was determined using 2-way ANOVA and post-hoc Tukey HSD. ns: no significance ($P>0.05$), ***: $P<0.001$.

Determination of radical scavenging capabilities

To see how well phenols detected with the Folin-Ciocalteu method could scavenge free radicals or ROS, the ORAC assay was conducted for all aqueous infusions. We used Trolox as a standard and calculated the radical scavenging capacity as Trolox equivalents, where one Trolox equivalent corresponds to a radical scavenging capacity per 1 gram of dry substance equivalent to 1 μmol of Trolox. Trolox calibration curves with a concentration range between 25 and 200 μmol/L were repeatedly measured with each experiment and displayed good linearity ($R2 = > 0.99$) and decent reproducibility between replications of the same experiment ($RSD \leq 7.5\%$ between 2 replicates at defined concentrations). Results gathered from the ORAC assay confirmed the findings from total phenol determination, with results, qualitatively speaking, closely correlating with each other. The lowest radical absorbance capacity was detected for the infusion of whole leaves of TR1, for which we measured 107 Trolox equivalents. The highest antioxidative capacity was determined for ground leaves of TG9 (1,674 Trolox equivalents). Some minor differences were observed between the ORAC assay and total phenol content. For example, no difference in antioxidant capacity was observed between TO3 and TG9 during the determination of total phenols, while a significant difference was detected in the ORAC assay (**Figure 4**).

Correlation between total phenol content and radical scavenging capabilities

In order to determine how radical scavenging capabilities, determined by ORAC assay, compare to the total phenol content of the tea infusions, total phenol content, expressed as GA equivalents, measured for whole and ground tea leaf infusions, was plotted against respective Trolox equivalents determined with the ORAC assay. A simple linear regression analysis was performed, revealing high linearity ($R2 = 0.96$) between the total phenol content measured and radical scavenging capabilities of the tea infusions, with 1 mg GA equivalents approximately equaling 12 μmol Trolox equivalents (**Figure 5**).

In vitro protective effect against ROS

Lastly, to evaluate how antioxidants in the different tea infusions could prevent oxidative damage by ROS under physiological conditions found in the human body, the TBARS assay was performed with the tea infusions. For this assay, egg yolk was chosen as a cheap and readily available source of phospholipids, which are a main target of ROS in the human cell membrane (23,24). While all samples exhibited a dose-dependent increase in antioxidative capacity, the lowest inhibitory capacity was measured for both ground and whole tea leaves of TR1, which only started to inhibit peroxidation at a concentration of 5 or 10 mg/mL, respectively. Ground tea leaf infusions in general exhibited higher antioxidative capacity, reaching up to 70% inhibition at 20 mg/mL compared to the negative control, while whole tea leaf extracts only

https://doi.org/10.59720/24-318

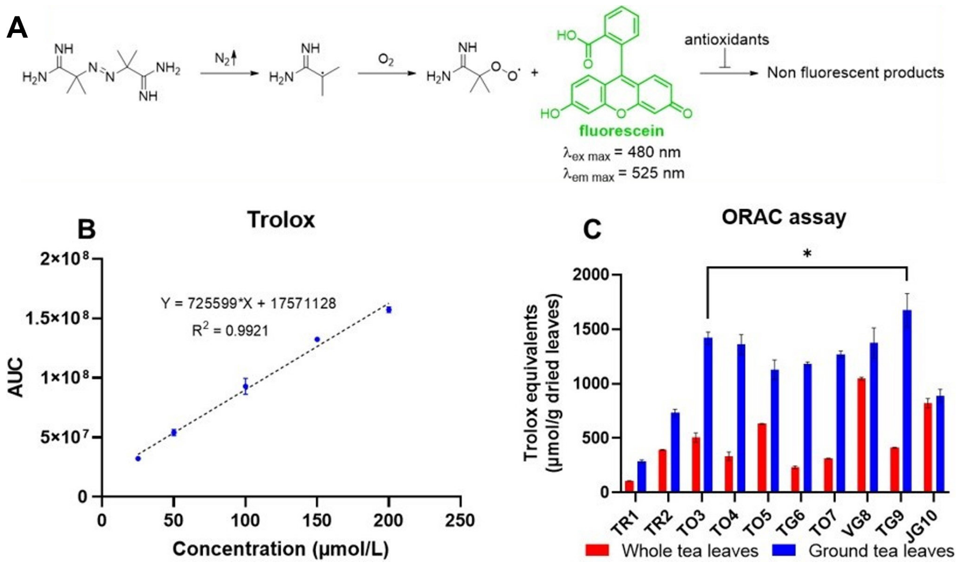

Figure 4: Determination of radical scavenging capabilities. (A) ORAC assay. Photobleaching reaction of fluorescein mediated by 2,2'-Azobis(2-amidinopropane) (AAPH) during the ORAC assay. **(B)** Trolox calibration curve for total phenol determination. Datapoints depict the concentration of Trolox plotted against mean AUC ± SD of fluorescence measured over time (n=2). ORAC was determined for various concentrations of Trolox and the Calibration curve was calculated with simple regression in GraphPad Prism. **(C)** Radical scavenging capabilities of aqueous tea infusion as well as organic extracts. Bar graphs depict mean ± SD ORAC for aqueous tea infusions calculated as Trolox equivalents (n=2). Whole (red) and ground (blue) tea leaf infusions were diluted to suitable concentrations and measured in the ORAC assay. Statistical significance was determined using 2-way ANOVA and post-hoc Tukey HSD. *: P<0.05.

reached at most 50% inhibition at the same concentration. Ground tea samples TO3 – VG8 also plateaued at 10 mg/mL with only a very slight increase observed afterward, while a gradual increase of inhibition was observed for whole tea leaf samples. While results from this experiment showed the same trends as observed for the ORAC and the Folin-Ciocalteu assay, results of this assay varied more strongly with RSD over 50% for some replicates. We also noticed a difference in results measured on different days when a new egg yolk emulsion was used. Inhibition determined for ground

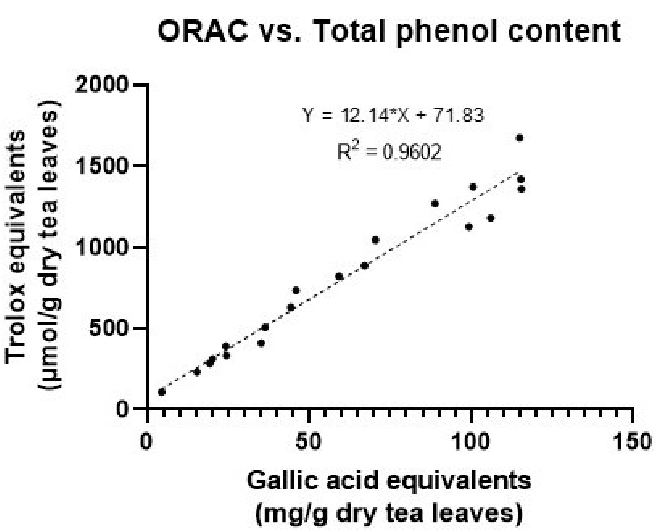

Figure 5: Correlation between ORAC and total phenol content. Mean values of total phenol content of aqueous tea infusions expressed as GAE (n=3) are plotted against the respective radical scavenging capacity determined with the ORAC assay expressed as Trolox equivalents (n=2). A simple linear regression curve of the correlation was calculated using GraphPad Prism.

tea leaves TO3 – VG8 measured on one day tended to be higher than that of ground tea leaves TR1, TR2, TG9, and JG10 on another day (**Figure 6**).

DISCUSSION

Results from the series of experiments conducted in this study indicated that oxidation during tea processing reduces antioxidant concentration, as observed for black teas TR1 and TR2 compared to oolong and green teas. However, at least among the samples evaluated for this study, antioxidant concentrations between green and oolong teas were very comparable, with Taiwanese high mountain oolong tea (TO3) containing more antioxidants than green sencha (JG10) and being on par with the Vietnamese jasmine green tea (VG8). Total phenol contents measured for the organic extract of TO3 are comparable to phenol contents previously measured for Taiwanese oolong tea, thereby validating the extraction and measurement method (14). Compared to further examples from the literature, antioxidant concentrations determined for oolong and green tea samples in this study were well within the expected concentration range (13,15,25). These results could be explained by the fact that Taiwanese high mountain oolong tea is only very slightly fermented during processing, thereby conserving the antioxidant capacity of the tea (26). On the other hand, the lower concentration of antioxidants in the Japanese sencha could stem from differences in the tea cultivar as well as time of harvest (27). We could not observe a clear difference in antioxidative properties between green tea TG6, grown in the lower altitude Luye region of Taiwan, and the Taiwanese high mountain teas. This, however, does not disprove the influence of growing altitude on antioxidant concentration in tea, as various other factors like time of harvest, tea cultivar, and extent of processing play a large role in this matter (14,15). But it highlights that no generalized assumption regarding health benefits based solely on a single

https://doi.org/10.59720/24-318

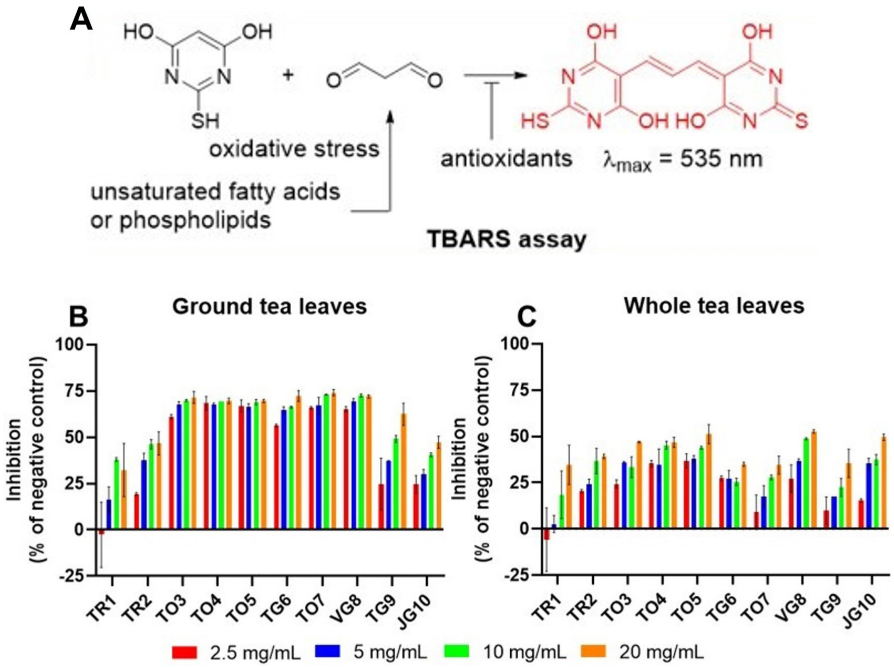

Figure 6: TBARS assay. (A) Formation of red thiobarbituric acid condensation product from thiobarbituric acid and malonaldehyde formed during lipid peroxidation. Inhibition of lipid peroxidation by aqueous **(B)** whole tea leaf infusions and **(C)** ground tea leaf infusions. Bar graphs depict mean inhibition ± SD of lipid peroxidation in percent of negative control (blank) at different concentrations of tea infusions (n=2). Aqueous tea infusions or blank (DI water) were co-incubated with egg yolk emulsion and FeSO4 for 45 minutes at 37°C and afterwards reacted with thiobarbituric acid for 10 minutes at 80°C. Colored reaction products were extracted with n butanol and absorbance was measured at 535 nm.

factor, such as the growing altitude of the tea, can be drawn. The total phenol content of the organic extract obtained from fresh tea was similar to that found in organic extracts of TO3 and JG10, further supporting the low oxidation levels in high mountain oolong teas. Interestingly, while in all experiments the antioxidative effect was lowest for the aged black tea (TR1), the antioxidative effect of aged oolong tea (TO3) was very similar to other oolong and green teas, indicating that only a little of the antioxidative effect was lost during storage. This could be explained by the fact that Taiwanese whole leaf teas are generally vacuum-sealed in non-transparent packaging, which prevents oxidation as long as the seal remains intact.

However, we also observed that while better at preserving the aroma of the tea, the sphere-shaped Taiwanese high mountain teas released antioxidants at a significantly slower rate and to a lesser extent than unshaped, needle-shaped, and more notably ground tea samples (28). The slower extraction rate of the sphere-shaped tea can be attributed to the gradual unfurling of the leaves during the initial steeping phase and the smaller surface area of the larger tea particles. The lower extraction yield may be partially due to the gradual decrease in water temperature, as no additional heat was applied during the extraction process. Based on these findings, unshaped loose teas or ground teas, as they are found in teabags, are more suitable for single extractions, while sphere-shaped whole leaf teas are more suited for multiple extractions. This aligns with manufacturer recommendations for the preparation of oolong tea, which suggest three or more steeping cycles for the same tea leaves (29). While the results were consistent across the different tea samples, one limitation of this study is that the tea samples were only extracted once. As a result, variations in antioxidant content, which might occur between multiple infusions of the same type of tea, were not captured.

Comparing the measurement methods for antioxidants, total phenol contents determined for the aqueous tea infusions with the Folin-Ciocalteu method correlate very well with the ORAC of the respective samples (R = 0.960) (**Figure 5**). This further validates the robustness of both methods for the determination of antioxidants in tea and reveals that secondary plant metabolites found in the tea samples are able to elicit an antioxidative effect via electron transfer (ET) as measured by the Folin-Ciocalteu method, as well as via the hydrogen atom transfer pathway (HAT) as indicated by the ability to scavenge radicals produced by AAPH (30).

Lastly, the TBARS assay was conducted to measure the effect of tea antioxidants in a very simplified, *in vitro* model for eukaryotic cells. While several substrates like meat, low-density lipoprotein (LDL), liver microsomes, brain homogenate, or lecithin are frequently used for the screening of antioxidative activity, we decided, both for ethical and cost reasons, to use egg yolk as a phospholipid-rich substrate for this experiment (31). We could indeed demonstrate that antioxidants from Taiwanese high mountain tea could inhibit lipid peroxidation caused by FeSO4 in a dose-dependent manner, which could have implications for the protective effect of tea antioxidants against ROS for phospholipids. While results were similar to the ORAC and Folin-Ciocalteu assay and showed the same trends, a more precise analysis of the results gathered from this experiment was not possible due to the high variation between measurements and especially the inter-day variability. As the egg yolk emulsion had to be prepared fresh, due to the splitting of the emulsion after longer storage and potential spoiling, the phospholipid content of the egg yolk emulsion might have varied between experiments conducted on different days. We could nonetheless qualitatively detect larger differences in the antioxidative capacity of tea samples

https://doi.org/10.59720/24-318

using the TBARS assay with egg yolk as a cheap and readily available phospholipid source.

Overall, our findings suggest that the antioxidative properties of Taiwanese high mountain teas are significantly affected by their fermentation and oxidation levels. While antioxidant content among black teas generally was low, both high mountain green and oolong teas showed similarly high levels, making them valuable sources of antioxidants. However, the spherical shape of high mountain tea leaves resulted in slower extraction of these antioxidants compared to regular loose or ground tea, indicating that multiple extractions could be necessary to harness the full antioxidative potential of such teas. Additionally, through various measuring methods for antioxidants, we shed light on how these substances may help mitigate damage caused by ROS. Findings from this study might make it easier for consumers to select teas with potential health benefits and provide a formula for how to more efficiently extract antioxidants from tea.

MATERIALS AND METHODS

Determination of loss on drying of fresh tea leaves

Fresh tea leaves (*Camellia sinensis*) were harvested from a local tea farm (Wen Sun Farm, New Taipei City, Taiwan). For better comparison to dried tea, the loss on drying of fresh tea leaves was determined by weighing a certain amount of fresh tea leaves and letting them dry at ambient temperature in a well-ventilated place for 14 days. The dried leaves were weighed again and the weight loss on drying was determined in percent. This factor was considered for the calculation of the total phenol content of fresh tea leaves.

Aqueous extracts

Various dried tea samples were obtained from local markets. Dried tea leaves were ground up in an electric spice mill for 30 seconds (ground tea leaves) or used whole (whole tea leaves) (see **Table 1**, **Figure 1**). Boiling water (50 mL) was added to 1.0 g of dried tea sample or 5.0 g of fresh whole leaves in a screw lid jar, resulting in concentrations of 20 mg/mL and 100 mg/mL tea leaves per volume extraction solvent, respectively. The vessel was closed and frequently agitated without further heating. For the timed extraction, samples were taken at fixed time points. For all other measurements, samples were taken after 10 minutes. Samples of ground teas were filtered through a 0.22 µm PTFE syringe filter directly after extraction.

Organic extracts

One gram of ground dry tea or 3.0 g of ground fresh tea, prepared by blending fresh tea leaves in an electric spice mill for 30 seconds, was added to 50 mL of a mixture consisting of acetone (uni-onward), methanol (duksan), and deionized (DI) water at a ratio of 7:7:6, respectively. The mixture was then placed in an ultrasonic bath for 15 minutes before the solvent was filtered off. The residue was recovered, and the extraction procedure was repeated two more times. Next, the organic extracts were combined and concentrated using a rotary evaporator at 40°C and further dried under high vacuum for 24 hours. Finally, the dried organic extracts were redissolved in DI water at a concentration of 4 mg/mL for further measurements. The total weight of dried organic extracts was divided by the initial weight of ground tea to obtain an extraction factor, which was taken into account for the determination of the total phenol content of organic extracts.

ORAC assay

The Oxygen Radical Absorbance Capacity (ORAC) assay measures the capability of an antioxidant to protect a fluorescent dye from degradation by free radicals formed from a radical starter (**Figure 4**). Antioxidant capacity is quantified by measuring the retardation of fluorescence decay in the sample (32).

The ORAC assay was adapted from a literature protocol (33). Tea samples were first diluted with phosphate buffer (70 mmol, pH 7.4) to appropriate concentrations (ranging from 10× to 200× times dilution based on results gathered from total phenol measurement). Trolox (Combi-blocks) standard was dissolved in phosphate buffer at concentrations ranging from 25-150 µmol/L fluorescein sodium salt (TCI) and AAPH (Thermo Scientific) were dissolved in phosphate buffer at a concentration of 11 µmol/L and 63.4 mmol/L, respectively. Fifteen microliters of sample, standard, or phosphate buffer (blank), and 150 µL of fluorescein solution were added to a black 96-well plate. To induce the formation of ROS, 56 µL of AAPH solution was added and the plate was immediately placed in a microplate reader (Spectramax iD3 Multi-Mode Microplate reader (Molecular Devices)) at 37°C, where fluorescence was measured every 3.5 minutes for 90 minutes. The excitation and emission wavelengths were set to 485 nm and 520 nm, respectively, along with 8 seconds of orbital shaking prior to measurement. All samples were prepared and measured in duplicate. AAPH solutions were prepared freshly and Trolox standards were remeasured for every well-plate. The area under the curve (AUC) of fluorescence intensity over time for each well was calculated using the SoftMax Pro 7 software (Molecular Devices LLC, San Jose, California, USA, www.moleculardevices.com). The average AUC of blanks was subtracted from the sample AUC, resulting in the blank-corrected values. Blank corrected values were averaged. The blank corrected average AUC of Trolox standard samples was plotted against the respective concentration to obtain a calibration curve and the linear equation was calculated using GraphPad Prism 9.5. Blankcorrected AUCs from the tea samples were inserted into the linear equation to calculate the Trolox equivalent concentration. Trolox equivalent concentrations were corrected for the dilution factor of the respective tea sample to obtain the amount of Trolox equivalents in 1.0 g of dried tea.

Total phenol content

Total phenol content was determined using the Folin-Ciocalteu reagent. This colorimetric assay is based on a redox reaction between phenols in the sample with phosphomolybdate and phosphotungstate in the reagent, leading to the formation of oxidized phenols, such as quinones and reduced blue metal complexes (**Figure 3**). This method gives a good indication of the overall concentration of antioxidants present in the measured sample (34).

The determination of the total phenol content was adapted from a literature protocol by Musci *et al.* (22). Tea samples or organic extract solutions were first diluted to appropriate concentrations with DI water (up to 20 times depending on antioxidant concentration), and gallic acid (AK scientific) was dissolved in DI water at concentrations ranging from 100 –

https://doi.org/10.59720/24-318

1500 µmol/L (17.0 – 255.2 mg/L). Two hundred microliters of sample solution or gallic acid solution was added to a 2 mL Eppendorf tube, along with 100 µL of Folin-Ciocalteu reagent (Sigma Aldrich), 700 µL water, and 1.2 mL 10.75% aqueous sodium carbonate solution. The mixtures were incubated for at least 30 minutes before 200 µL was transferred to a clear 96-well plate. The absorption of tea samples and gallic acid standard was measured at 760 nm using the Spectramax microplate reader. Both tea samples and the gallic acid standard solutions were prepared and measured in triplicate. The absorption of gallic acid standard solutions was plotted against the respective concentration and the linear equation was calculated using GraphPad Prism. The absorption values from the tea samples were inserted into the linear equation to calculate the gallic acid equivalent concentration. GAE concentrations were corrected for the dilution factor of the respective tea sample to obtain the amount of gallic acid equivalents in 1.0 g of dried tea.

TBARS assay

The thiobarbituric acid reactive substances (TBARS) assay was performed to evaluate how antioxidants present in the tea infusion might act under physiological conditions. The TBARS assay determines the amount of malondialdehyde (MDA) formed during peroxidation of unsaturated fats and phospholipids. MDA and other peroxidation products react with thiobarbituric acid to form pink colored products, which can be quantified by spectrophotometric methods. While the method is generally used for the quantification of MDA in foodstuffs, it can also be utilized to measure the ability of antioxidants to inhibit peroxidation (**Figure 6**) (35).

The TBARS assay was conducted according to a modified literature method (36). Tea samples were diluted to concentrations ranging from 2.5 to 20 mg/mL, calculated as the initial dry weight of tea leaves per extraction volume, in DI water. Egg yolk from whole eggs was separated from the egg white and diluted to a concentration of 100 g/L in DI water. A solution of 0.83% thiobarbituric acid (Sigma Aldrich) and 16.8% w/v trichloroacetic acid (Alfa Aesar) in 0.125 N HCl was prepared. 50 µL of the sample, or DI water (negative control) was added to 2 mL Eppendorf tubes containing 240 µL of the egg yolk mixture. Lipid peroxidation was initiated by the addition of 10 µL of a 10 mmol $FeSO_4$ solution in DI water. The mixtures were immediately placed in an incubator at 37°C and incubated under agitation for 45 minutes. After incubation, 500 µL of thiobarbituric acid solution was added to each tube, and the samples were placed in an 80°C water bath for 10 minutes. To stop the reaction, samples were quickly cooled on ice, and the reaction mixtures were partitioned with 500 µL of n-butanol (Acros). Samples were centrifuged at 5000 rpm for 3 minutes, 200 µL of the organic supernatant was transferred to a clear 96-well plate, and absorbance was measured using the Spectramax microplate reader. The absorption wavelength was set to 535 nm and corrected for turbidity at 620 nm. All samples were prepared and measured in duplicate. The corrected value was obtained by subtracting the absorption at 620 nm from the absorbance at 535 nm and the average value is reported. Inhibition (% negative control) was calculated according to the following formula:

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.5.0 (GraphPad Software, Boston, Massachusetts, USA, www.graphpad.com). Datapoints were depicted as mean ± standard deviation (SD) when applicable. Calibration curves were calculated by performing simple linear regression analysis. Statistical significance, comparing multiple groups with two variables, was determined using a 2-way analysis of variance (ANOVA) and post-hoc Tukey HSD test. Data from the determination of total phenol content and ORAC assay were correlated and the regression curve was calculated by simple linear regression analysis.

ACKNOWLEDGMENTS

We would like to thank Prof. Pi-Hui Liang for her support with this project, as well as for providing the laboratory and equipment. Helpful discussions with Mei-Ling Hsiao about the tea culture and manufacturing in Taiwan are gratefully acknowledged.

Received: October 23, 2024
Accepted: June 10, 2025
Published: March 23, 2026

REFERENCES

1. "The History of Taiwanese Tea Culture." *Path of Cha*. www.pathofcha.com/blogs/all-about-tea/the-history-of-taiwanese-tea-culture?srsltid=AfmBOop7Vut3K1Hn086W-cTgwMbdJMCrfz-jPgz7MSXfC1i0LPtm6vuq. Accessed September 20, 2024.
2. Chiu, Yi-Wen. "Environmental Implications of Taiwanese Oolong Tea and the Opportunities of Impact Reduction." *Sustainability*, vol. 11, no. 21, 2019, p. 6042, https://doi.org/10.3390/su11216042.
3. "Taiwan Tea show. Taiwan Tea Industry." *Tea and Beverage Research Station. Ministry of Agriculture, R.O.C.(Taiwan)* www.tbrs.gov.tw/en/ws.php?id=4860. Accessed September 20, 2024.
4. Shang, Ao et al. "Molecular Mechanisms Underlying Health Benefits of Tea Compounds." *Free Radical Biology and Medicine*, vol. 172, 2021, pp. 181-200, https://doi.org/10.1016/j.freeradbiomed.2021.06.006.
5. Yan, Zhaoming et al. "Antioxidant Mechanism of Tea Polyphenols and Its Impact on Health Benefits." *Animal Nutrition*, vol. 6, no. 2, 2020, pp. 115-23, https://doi.org/10.1016/j.aninu.2020.01.001.
6. Chung, Mei et al. "Dose–Response Relation between Tea Consumption and Risk of Cardiovascular Disease and All-Cause Mortality: A Systematic Review and Meta-Analysis of Population-Based Studies." *Advances in Nutrition*, vol. 11, no. 4, 2020, pp. 790-814, https://doi.org/10.1093/advances/nmaa010.
7. Suzuki-Sugihara, Norie et al. "Green Tea Catechins Prevent Low-Density Lipoprotein Oxidation Via Their Accumulation in Low-Density Lipoprotein Particles in Humans." *Nutrition Research*, vol. 36, no. 1, 2016, pp. 16-23, https://doi.org/10.1016/j.nutres.2015.10.012.
8. Fechtner, Sabrina et al. "Molecular Insights into the Differences in Anti-Inflammatory Activities of Green Tea Catechins on Il-1β Signaling in Rheumatoid Arthritis Synovial Fibroblasts." *Toxicology and Applied Pharmacology*, vol. 329, 2017, pp. 112-20, https://doi.org/10.1016/j.taap.2017.05.016.
9. Kim, Donghwa et al. "Photoprotective Effects of 2s,3r-

6-Methoxycarbonylgallocatechin Isolated from Anhua Dark Tea on Uvb-Induced Inflammatory Responses in Human Keratinocytes." *Journal of Photochemistry and Photobiology B: Biology*, vol. 202, 2020, p. 111704, https://doi.org/10.1016/j.jphotobiol.2019.111704.

10. Hisanaga, Ayami et al. "Anti-Inflammatory Activity and Molecular Mechanism of Oolong Tea Theasinensin." *Food & Function*, vol. 5, no. 8, 2014, pp. 1891-97, https://doi.org/10.1039/C4FO00152D.
11. Wang, Dongxu et al. "Green Tea Polyphenols and Epigallocatechin-3-Gallate Protect against Perfluorodecanoic Acid Induced Liver Damage and Inflammation in Mice by Inhibiting Nlrp3 Inflammasome Activation." *Food Research International*, vol. 127, 2020, p. 108628, https://doi.org/10.1016/j.foodres.2019.108628.
12. Yuan, Jian-Min et al. "Tea and Cancer Prevention: Epidemiological Studies." *Pharmacological Research*, vol. 64, no. 2, 2011, pp. 123-35, https://doi.org/10.1016/j.phrs.2011.03.002.
13. Wen, Bo et al. "Effects of Geographic Locations and Topographical Factors on Secondary Metabolites Distribution in Green Tea at a Regional Scale." *Food Control*, vol. 110, 2020, p. 106979, https://doi.org/10.1016/j.foodcont.2019.106979.
14. Chen, Yulong et al. "Variation in Catechin Contents in Relation to Quality of 'Huang Zhi Xiang' Oolong Tea (Camellia Sinensis) at Various Growing Altitudes and Seasons." *Food Chemistry*, vol. 119, no. 2, 2010, pp. 648-52, https://doi.org/10.1016/j.foodchem.2009.07.014.
15. Wang, Cong-ming et al. "Evaluation of Sensory and Safety Quality Characteristics of "High Mountain Tea"." *Food Science & Nutrition*, vol. 10, no. 10, 2022, pp. 3338-54, https://doi.org/10.1002/fsn3.2923.
16. Chen, Guan-Heng et al. "Catechin Content and the Degree of Its Galloylation in Oolong Tea Are Inversely Correlated with Cultivation Altitude." *Journal of Food and Drug Analysis*, vol. 22, no. 3, 2014, pp. 303-09, https://doi.org/10.1016/j.jfda.2013.12.001.
17. Wong, Melody et al. "Phytochemical Profile of Differently Processed Tea: A Review." *Journal of Food Science*, vol. 87, no. 5, 2022, pp. 1925-42, https://doi.org/10.1111/1750-3841.16137.
18. Carloni, Patricia et al. "Antioxidant Activity of White, Green and Black Tea Obtained from the Same Tea Cultivar." Food Research International, vol. 53, no. 2, 2013, pp. 900-08, https://doi.org/10.1016/j.foodres.2012.07.057.
19. Farakte, Raosaheb A. et al. "Role of Particle Size in Tea Infusion Process." *International Journal of Food Engineering*, vol. 12, no. 1, 2016, pp. 1-16, https://doi.org/10.1515/ijfe-2015-0213.
20. Bassi, Pratishtha et al. "Importance and Prior Considerations for Development and Utilization of Tea Bags: A Critical Review." *Journal of Food Process Engineering*, vol. 43, no. 1, 2020, p. e13069, https://doi.org/10.1111/jfpe.13069.
21. Castiglioni, Sara et al. "Influence of Steeping Conditions (Time, Temperature, and Particle Size) on Antioxidant Properties and Sensory Attributes of Some White and Green Teas." *International Journal of Food Sciences and Nutrition*, vol. 66, no. 5, 2015, pp. 491-97, https://doi.org/10.3109/09637486.2015.1042842.
22. Musci, Marilena and Shicong Yao. "Optimization and Validation of Folin–Ciocalteu Method for the Determination of Total Polyphenol Content of Pu-Erh Tea." *International Journal of Food Sciences and Nutrition*, vol. 68, no. 8, 2017, pp. 913-18, https://doi.org/10.1080/09637486.2017.1311844.
23. Freigang, Stefan. "The Regulation of Inflammation by Oxidized Phospholipids." *European Journal of Immunology*, vol. 46, no. 8, 2016, pp. 1818-25, https://doi.org/10.1002/eji.201545676.
24. Ali, Abdelmoneim H. et al. "Identification of Phospholipids Classes and Molecular Species in Different Types of Egg Yolk by Using Uplc-Q-Tof-Ms." *Food Chemistry*, vol. 221, 2017, pp. 58-66, https://doi.org/10.1016/j.foodchem.2016.10.043.
25. Kodama, Débora Harumi et al. "Flavonoids, Total Phenolics and Antioxidant Capacity: Comparison between Commercial Green Tea Preparations." *Food Science and Technology*, vol. 30, 2010, https://doi.org/10.1590/S0101-20612010000400037.
26. Jiang, Hong. "Commodity, Nature, and Taste: The Making of Taiwan's High-Mountain Tea." *Geographical Review*, pp. 1-16, https://doi.org/10.1080/00167428.2024.2319255.
27. Liu, Zhibin et al. "A Comparison of the Phenolic Composition of Old and Young Tea Leaves Reveals a Decrease in Flavanols and Phenolic Acids and an Increase in Flavonols Upon Tea Leaf Maturation." *Journal of Food Composition and Analysis*, vol. 86, 2020, p. 103385, https://doi.org/10.1016/j.jfca.2019.103385.
28. Ng, Kwan-Wai et al. "Oolong Tea: A Critical Review of Processing Methods, Chemical Composition, Health Effects, and Risk." *Critical Reviews in Food Science and Nutrition*, vol. 58, no. 17, 2018, pp. 2957-80, https://doi.org/10.1080/10408398.2017.1347556.
29. Hicks, Monique B. et al. "Tea Preparation and Its Influence on Methylxanthine Concentration." *Food Research International*, vol. 29, no. 3, 1996, pp. 325-30, https://doi.org/10.1016/0963-9969(96)00038-5.
30. Dudonné, Stéphanie et al. "Comparative Study of Antioxidant Properties and Total Phenolic Content of 30 Plant Extracts of Industrial Interest Using Dpph, Abts, Frap, Sod, and Orac Assays." *Journal of Agricultural and Food Chemistry*, vol. 57, no. 5, 2009, pp. 1768-74, https://doi.org/10.1021/jf803011r.
31. Ghani, Md Ahsan et al. "Measurement of Antioxidant Activity with the Thiobarbituric Acid Reactive Substances Assay." *Food Chemistry*, vol. 230, 2017, pp. 195-207, https://doi.org/10.1016/j.foodchem.2017.02.127.
32. Zulueta, Ana et al. "Orac and Teac Assays Comparison to Measure the Antioxidant Capacity of Food Products." *Food Chemistry*, vol. 114, no. 1, 2009, pp. 310-16, https://doi.org/10.1016/j.foodchem.2008.09.033.
33. Brown, Nicole et al. "Polyphenol Composition and Antioxidant Potential of Mint Leaves." *Food Production, Processing and Nutrition*, vol. 1, no. 1, 2019, p. 1, https://doi.org/10.1186/s43014-019-0001-8.
34. Pérez, Maria et al. "The Chemistry Behind the Folin-Ciocalteu Method for the Estimation of (Poly)Phenol Content in Food: Total Phenolic Intake in a Mediterranean Dietary Pattern." *J Agric Food Chem*, vol. 71, no. 46, 2023, pp. 17543-53, https://doi.org/10.1021/acs.jafc.3c04022.
35. van der Sluis, Addie A. et al. "An Improved, Rapid in Vi-

https://doi.org/10.59720/24-318

tro Method to Measure Antioxidant Activity. Application on Selected Flavonoids and Apple Juice." *Journal of Agricultural and Food Chemistry*, vol. 48, no. 9, 2000, pp. 4116-22, https://doi.org/10.1021/jf000156i.

36. Upadhyay, Richa et al. "Antioxidant Property of Aerial Parts and Root of Phyllanthus Fraternus Webster, an Important Medicinal Plant." *The Scientific World Journal*, vol. 2014, no. 1, 2014, p. 692392, https://doi.org/10.1155/2014/692392.