PRODUCTION OF BLACK INSTANT TEA POWDER AND EVALUATION OF ITS PHYSICOCHEMICAL AND ANTICANCER PROPERTIES

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ABSTRACT

PRODUCTION OF BLACK INSTANT TEA POWDER AND EVALUATION OF ITS PHYSICOCHEMICAL AND ANTICANCER PROPERTIES

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Black tea is the most consumed beverage in the world behind water. During black tea production, a substantial amount of waste, known as black tea fiber, was generated. In this study, black tea waste fibers were valorized to produce various instant teas, a growing and increasingly popular product category of black tea. The water-to-tea ratio of 30:1 ml/g, the superfine particle size range (<100 μm), the infusion conditions at 100 ℃ for 6 min and at 25 ℃ for 2 h for the *hot* and *cold* infusion methods, respectively, were set as operational parameters to maximize the extraction yield of black tea waste fibers. Instant tea powders produced by freeze drying were also mixed with ginger and sage using superfine grinding to enhance their functional properties. Instant black teas enriched with ginger and sage were characterized based on the analyses of moisture content, hygroscopicity, color, morphological, solubility and turbidity. In addition, instant teas were investigated for their antioxidant capacities, total phenolic content and the composition of catechins (epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate), theaflavin, thearubigins, gallic acid, caffeine and theanine. Instant teas produced by

the hot infusion method had a higher antioxidant capacity and total phenolic content than those obtained by the cold infusion method for the same formulation ($p<0.05$). Instant black tea powders also demonstrated anticancer potential in a concentration-dependent manner against prostate cancer cells (PC3, 22Rv1 and LNCaP), brain tumor cells (U87MG and A172) and breast cancer cell (MCF7) in the *in vitro* studies.

Keywords: instant tea, black tea waste fiber, superfine grinding, phenolics, anticancer

ÖZ

SİYAH HAZIR TOZ ÇAY ÜRETİMİ VE FİZİKOKİMYASAL VE ANTİKANSER ÖZELLİKLERİNİN DEĞERLENDİRİLMESİ

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Siyah çay, sudan sonra dünyada en çok tüketilen içecektir. Siyah çay üretimi sırasında, siyah çay lifi olarak bilinen, önemli miktarda atık üretilmektedir. Bu çalışmada, atık siyah çay lifleri, büyüyen ve giderek daha popüler bir ürün kategorisi olan çeşitli hazır çayları üretmek için değerlendirilmiştir. 30:1 ml/g su-çay oranı, çok ince parçacık boyutu aralığı (<100 μm), sıcak ve soğuk infüzyon yöntemleri için sırasıyla 100°C'de 6 dakika ve 25°C'de 2 saat infüzyon koşulları, siyah çay atık liflerinin ekstraksiyon verimini en üst düzeye çıkarmak için belirlenmiştir. Dondurarak kurutma ile üretilen hazır çay tozları işlevsel özelliklerini geliştirmek için süper öğütme işlemi kullanılarak zencefil ve adaçayı ile karıştırılmıştır. Zencefil ve adaçayı ile zenginleştirilmiş hazır siyah çaylar nem içeriği, higroskopisite, renk, morfolojik, çözünürlük ve bulanıklık analizleri ile fiziksel olarak karakterize edilmiştir. Ek olarak, hazır çayların antioksidan kapasiteleri, toplam fenolik içerikleri ve kateşin (epikateşin, epikateşin gallat, epigallokateşin, epigallokateşin galat), theaflavin, thearubigins, gallik asit, kafein ve teanin miktarları belirlenmiştir. Aynı formülasyona sahip çaylar için, sıcak infüzyon yöntemiyle üretilelerin, soğuk

infüzyon yöntemiyle elde edilenlere göre daha yüksek antioksidan kapasiteye ve toplam fenolik içeriğe sahip olduğu anlaşılmıştır ($p<0.05$). Sıcak ve soğuk demleme yöntemleri ile üretilen hazır siyah çaylar, ayrıca, prostat kanseri (PC3, 22Rv1 ve LNCaP), meme kanseri (MCF7) ve beyin tümörü (U87MG ve A172) hücre hatlarında, in vitro çalışmalarda, konsantrasyona bağlı antikanser potansiyel göstermiştir.

Anahtar Kelimeler: hazır toz çay, siyah atık çay lifi, super öğütme, fenolikler, antikanser

To my beloved family

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LIST OF ABBREVIATIONS

ABBREVIATIONS

BTL	Black tea leaves
CI	Instant teas produced by cold infusion
CIG	Instant teas produced by cold infusion and mixing with ginger
CIS	Instant teas produced by cold infusion and mixing with ginger
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
GAE	Gallic acid equivalent
GBM	Glioblastoma multiforme
HI	Instant teas produced by hot infusion
HIG	Instant teas produced by hot infusion and mixing with ginger
HIF	Instant teas produced by hot infusion and mixing with sage
IC_{50}	Inhibitory concentration
PG	Pre-ground black tea waste fiber
PGBM	Pre-ground and ball milled black tea waste fiber
TF:TR	Theaflavin: Thearubigins

CHAPTER 1

INTRODUCTION

Black tea is the most consumed beverage in the world following water, and its global consumption has risen markedly in recent years (Raj et al., 2023). In addition, according to the report of Food and Agriculture Organization of the United Nations in 2022, black tea consumption, which accounts for 75% of global tea consumption, is expected to increase annually by 2% over the next decade (Zhang et al., 2019). Therefore, to meet this rising demand, black tea production has increased substantially around the world (FAO, 2022). Figure 1.1 demonstrates the amounts of black tea production in 2020 and also projected black tea production in 2030.

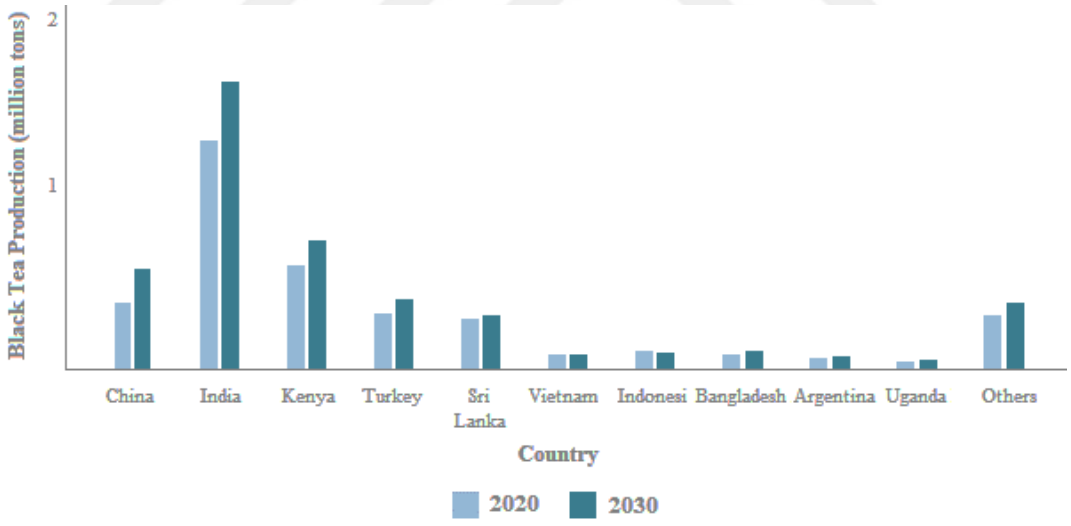


Figure 1.1 Black tea production in 2020 and 2030 (FAO, 2022)

1.1 Black Tea Production

The journey of tea commences with the meticulous processing of the leaves derived from *Camellia sinensis*, a member of the family *Theaceae* (Teshome, 2019). Then, a wide range of tea products are manufactured based on the post-harvesting processes. They are generally classified based on the fermentation process as unfermented white and green tea, semi-fermented oolong tea and completely fermented black tea (Carloni et al., 2013).

Black tea production starts with the crucial initial step of withering, where the moisture content of tea leaves is reduced to make them soft and leathery (Rahman et al., 2020). During withering, aroma compounds are formed and modified, which are started upon plucking through enzyme-assisted extractions, in the tea leaves (Bortolini et al., 2021; Feng et al., 2019). Moreover, withering creates volatile aroma compounds from the nonvolatile precursors in the leaves (Teshome, 2019). Furthermore, the phenolic profile is changed, as well as the caffeine and the free amino acid concentrations of the tea leaves increased during the withering process (Bortolini et al., 2021).

The rolling step is performed following the withering process to decrease the size of the tea leaves. The reduction in the size provides leaves to increase the exposing surface area to oxygen, which is essential in the subsequent fermentation step because of enhancing the formation of desired complex oxidation products in the tea leaves (Bortolini et al., 2021). Furthermore, during the rolling process, the polyphenol oxidase and peroxidase, which play a crucial role in black tea production due to the catalyzing oxidation reactions of the phenolic compounds, release from the chloroplast and the plant cell wall to initiate oxidation (Rahman et al., 2020; Salman et al., 2021; Serpen et al., 2012).

The fermentation process, which is intended to be oxidation in the tea literature, represents the serial enzyme-catalyzed oxidation of polyphenols, especially catechins, to the distinctive polymerized compounds of black tea, theaflavins and

thearubigins (Jia et al., 2023). Theaflavins are the first stable oxidation products, and they are further exposed to oxidation and generate more polymerized compounds, thearubigins (Samanta et al., 2015). Theaflavins and thearubigins give black tea its characteristic sensory properties such as astringent flavor and reddish color (Serpen et al., 2012).

Once the leaves have achieved the desired oxidation level, which directly affects the quality of the end product, the fermentation process is stopped to lock in the developed flavor profile of the black tea in the drying step by deactivating the responsible enzymes, oxidases (Bortolini et al., 2021). Moreover, the reduction of the moisture content in the drying process is also important for the storage stability of black tea products (Teshome, 2019).

As a result, after the serial processes of withering, rolling, fermentation, and drying, black tea is manufactured.

1.2 Black Tea Waste Fiber

Black tea is the most demanded beverage in the world, following water; therefore, the production of black tea is expected to increase extensively worldwide, as mentioned before (Figure 1.1) (Raj et al., 2023). During the production of black tea, it was inevitable that enormous amounts of black tea waste were also generated (Debnath et al., 2021). It was indicated that the waste in the range of 20-200 million kilograms was obtained per year during harvesting and production steps of black tea (Sayar et al., 2019). In 2021, the estimated amount of tea waste was 25 million kilograms in India, the highest black tea production country in the world (Debnath et al., 2023).

Black tea waste comprises of the discarded leaves, stems and stalks of tea, also known as black tea waste fiber (Sayar et al., 2019). Black tea waste fiber which is

supplied from the black tea production line of Doğadan Tea Company (Rize, Turkey) was demonstrated in Figure 1.2. It was separated from the end product after exposing all the production steps of black tea.

Black tea waste fiber primarily constitutes lignocellulosic biomass composed of cellulose, hemicellulose, lignin, polyphenols and tannins (Sayar et al., 2019). In addition, it also has a reservoir of biologically active compounds such as catechins, flavonoids, caffeine, theobromine, vitamins, minerals, terpenoids and amino acids with wide-ranging potential (Debnath et al., 2021).

Although tea waste is purchased and used by some industries for caffeine extraction and as animal feed, it is only a small portion of massive waste, and the rest is discarded (Debnath et al., 2021). Despite the inherent richness of tea waste, the disposal of it remains a concerning reality because it actually means the disposal of the bioactive and nutritionally valuable constituents. In addition, the factories are facing many challenges in dealing with tea waste since there is no proper waste management system available in the countries (Debnath et al., 2023). Tea waste is generally composted, burned or stocked in landfills, resulting in several environmental issues such as soil, water and air pollution (Debnath et al., 2021). Therefore, the potential and sustainable utilization of tea waste has been a major research priority nowadays.



Figure 1.2 Black tea waste fiber

Several studies have recently reported the valorization of tea waste for the extraction of antioxidant products (Abdeltaif et al., 2018; Sui et al., 2019), the reinforcement in polymer composites (Prabhu et al., 2021), the synthesis of crystalline cellulose derivatives (Guo et al., 2020), the production of adsorbents for waste water treatment (Nigam et al., 2019), the generation of bioenergy (Basumatary et al., 2018) and the fabrication of energy storage devices (Khan et al., 2020).

1.3 Black Tea Composition

The composition of black tea, which makes even the waste of it valuable, consists of 30-40% of polyphenols, 10-20% of catechins, 6-8% of flavonols, 2-5% of caffeine, 37.2% of cellulose, 14.7% of lignin, 12.1% of hot water insoluble proteins and 9.6% of hot water-soluble polysaccharides and proteins (Debnath et al., 2021). The variations in the composition of black teas are attributed to the differences in diversity, growing environment, cultivation method and process conditions (Boros et al., 2016; Lin et al., 2003; Ozdemir et al., 2018).

The four main catechins (epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG)) and condensed phenolics (theaflavin and thearubigins) have drawn attention because they give black tea its characteristic sensory profile and the health benefit potentials, especially antioxidant and anti-cancer features (Serpen et al., 2012; Yang & Liu, 2013; Yang & Wang, 2016). Moreover, the other essential bioactive compounds of caffeine (alkaloid), gallic acid (phenolic acid) and theanine (free amino acid) also deserve attention separately regarding their unique and functional properties (Cadoná et al., 2022; Endesfelder et al., 2017; Shojaei-Zarghani et al., 2021; Yang et al., 2023).

1.3.1 Catechins

Catechins, the subgroup of flavanols, are polyphenolic compounds that belong to the family of flavonoids, and they are naturally produced in plants (Kopustinskiene et al., 2021). The monomeric catechins are (+)-catechin and (-)-epicatechin, as well as their gallic acid conjugates, (+)-gallocatechin, (+)-catechin gallate, (+)-gallocatechin gallate, (-)-epicatechin gallate, (-)-epigallocatechin and (-)-epigallocatechin gallate (Abudureheman et al., 2022). The most abundant catechins in black tea are (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epigallocatechin gallate (EGCG), and their chemical structures are presented in Figure 1.3 (Yang & Liu, 2013).

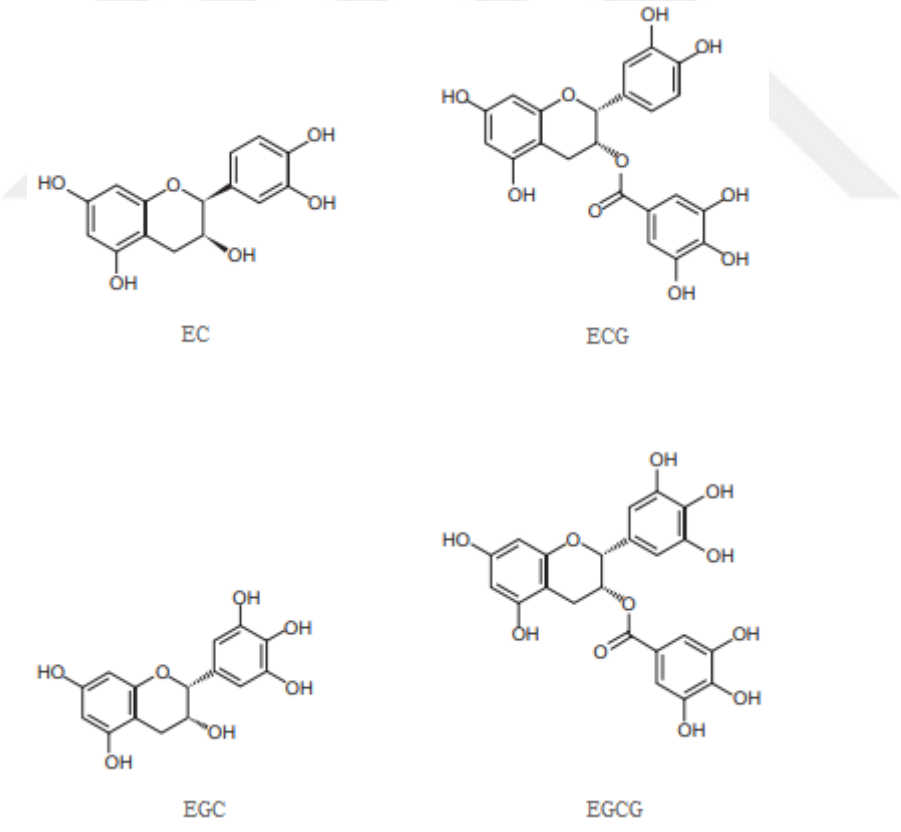


Figure 1.3 Structures of main catechins in black tea (Kopustinskiene et al., 2021)

Catechins are colorless compounds, and their esterified group with gallate are responsible for the distinctive flavor of tea products, bitter and astringent (Kopustinskiene et al., 2021). Therefore, the variation in the concentration of catechins directly affects the sensory attributes of different teas (Bortolini et al., 2021).

Several studies have confirmed the valuable health benefits of catechin compounds in black tea. Catechins are known for their high antioxidant properties (Cai et al., 2018). It enables them to protect cells from oxidative stress-induced damage by scavenging reactive oxygen species and free radicals (Bernatoniene & Kopustinskiene, 2018). The antioxidative activity of catechins not only preserves cellular integrity but also lowers the risk of chronic diseases such as cardiovascular and neurological disorders (Pedro et al., 2019). It was also revealed that tea catechins can inhibit the activity of the main protease in SARS-CoV-2, a novel severe acute respiratory syndrome-related coronavirus strain (Zhu & Xie, 2020). Furthermore, catechins have demonstrated the ability to modulate cellular signaling pathways involved in anticancer activities such as apoptosis (Bakhshandeh et al., 2023; Chen et al., 2020; Lin et al., 2008; Nam et al., 2001; Oeckinghaus & Ghosh, 2009; Yang et al., 2009). EGCG especially deserves special attention since it is considered the most effective compound in tea against the survival of cancer cells (Miyata et al., 2019).

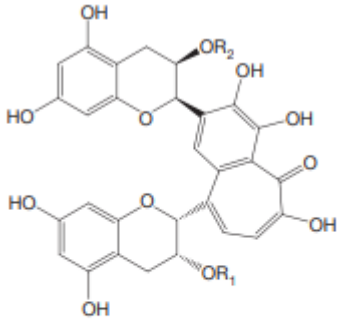
1.4 Theaflavins and Thearubigins

Catechins in the tea leaves are exposed to enzymatic transformation, including oxidation and polymerization, to generate complex secondary polyphenolic compounds, theaflavins and thearubigins, during the fermentation step of black tea production, a critical stage that sets black tea apart from other tea varieties (Abudureheman et al., 2022). The structures of the main theaflavins in black tea,

theaflavin, theaflavin 3-gallate, theaflavin 3'-gallate and theaflavin 3,3'-digallate, were demonstrated in Figure 1.4 (Sang, 2015). On the other hand, the chemistry of thearubigins is still unclear (Abudureheman et al., 2022; Long et al., 2023). However, it is considered that thearubigins are the products of further oxidation and polymerization of theaflavins (Ozdemir et al., 2018; Samanta et al., 2015).

Theaflavin and thearubigins are responsible for the characteristic sensory features of black tea. Theaflavins, having yellow-orange color, and thearubigins, having red-brown color, give black tea its unique reddish-brown appearance (Kelebek, 2016; Mao et al., 2021; Polat et al., 2022). Moreover, theaflavins play a significant role in enhancing the astringency, brightness and briskness of black tea, while thearubigins determine the strength and mouthfeel of the tea (Senthil Kumar et al, 2013).

The contribution of theaflavins and thearubigins to the health benefits of black tea also extensively studied for antioxidant effects (Leung et al., 2001; Long et al., 2023; Wu et al., 2017), cardioprotective effects (Stangl et al., 2007), anti-inflammatory effects (Gosslau et al., 2011), neuroprotective effects (Anandhan et al., 2012) and anticancer effects (Lin et al., 2008; Shan et al., 2021).



Theaflavin: R_1=R_2=H
Theaflavin 3-gallate: R_1=Galloyl R_2=H
Theaflavin 3'-gallate: R_1=H R_2=Galloyl
Theaflavin 3,3'-digallate: R_1=R_2= Galloyl

Figure 1.4 Structures of main theaflavins in black tea (Sang, 2015)

1.4.1 Gallic Acid

Gallic acid is a simple phenolic acid compound in black tea, and the chemical structure of it was demonstrated in Figure 1.5. It is the most abundant phenolic acid in black tea; even so, it was the only detectable phenolic acid in seven-grades of black tea originating in Turkey (Serpen et al., 2012).

Gallic acid is a crucial nutraceutical component of black tea due to its strong antioxidative nature, such that it has a promising effect on the oxidative damage is resulted from reactive species in pathologic conditions (Badhani et al., 2015; Chia et al., 2010; Shabani et al., 2020; Veluri et al., 2006). In addition, there were several studies that observed the effective defense of this valuable phenolic acid against the diseases of cardiovascular (Patel et al., 2011; Priscilla et al., 2009), liver (Ohno et al., 2001; Rasool et al., 2010) and neurodegenerative (Sun et al., 2014; Yu et al., 2019), as well as a variety of cancers (Kaur et al., 2009; Liu et al., 2013; Yang et al., 2022).

1.4.2 Caffeine

Caffeine is a stimulator bioactive compound and the predominant purine alkaloid, also known as methylxanthines, in black tea (Naveed et al., 2018). The chemical structure of caffeine was presented in Figure 1.5.

Caffeine is a versatile functional compound enhancing the health benefits of black tea products in addition to supporting their astringency (Pou, 2016; Zuo et al., 2023). Antioxidative (Endesfelder et al., 2017; Prasanthi et al., 2010; Ullah et al., 2015), neuroprotective (Endesfelder et al., 2014; Rivkees et al., 2011), immunomodulatory (Endesfelder et al., 2014; Tunc et al., 2013), anti-inflammatory (Valdez et al., 2011; Weichelt et al., 2013) and anticancer (Cadoná et al., 2022; Machado et al., 2020;

Niknafs, 1996; Yang et al., 2023) activities of caffeine were investigated in the several studies.

1.4.3 Theanine

Theanine, also known as L-theanine because it occurs in nature as L-enantiomeric form, is a unique non-protein amino acid in tea (Yang et al., 2023). The chemical structure of theanine is shown in Figure 1.5.

Theanine is the most abundant free amino acid in tea at more than 50% (Mu et al., 2015), and it has a specific flavor contribution to black tea as an umami taste (Shojaei-Zarghani et al., 2021). Apart from the contribution of theanine to the taste of tea, the relaxation and stress-alleviating effects of black tea are considered to result from theanine (Wang et al., 2022). Furthermore, the valuable health benefits of theanine were demonstrated in vascular diseases (Rogers et al., 2007), the immune system (Miyagawa et al., 2008), neuroprotection (Egashira et al., 2007), cognition (Wang et al., 2022) and cancer cells (Shojaei-Zarghani et al., 2021).

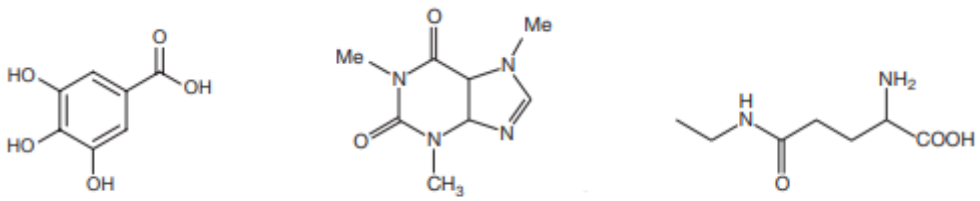


Figure 1.5 Structures of gallic acid, caffeine and theanine (Sang, 2015; Omar, 2017)

1.5 Black Tea Cytotoxicity

The anticancer activity has drawn special attention between the wide variety of health benefits of black tea components, as mentioned before, due to the extensiveness and severity of the disease (Bray et al., 2018). According to the World Health Organization, there will be approximately 27 million cases by 2050, with a yearly mortality rate of 17.5 million (Briguglio et al., 2020).

Several conventional cancer therapies, commonly chemotherapy, surgery, radiation, immunotherapy and hormone therapy, have been used to reduce the mortality of the diseases such as prostate cancer, brain tumor and breast cancer (Sidiqqui et al., 2006). However, it was found that these treatments have severe side effects on patients (Andleeb et al., 2021). Therefore, researchers have tried to find novel approaches and new potential anticancer agents with minimal side effects, such as plant-based cytotoxic components (Alserihi et al., 2023).

1.5.1 Prostate Cancer

Prostate cancer is the second most common cancer diagnosed in men, following lung cancer, and one of the most leading causes of death around the world (Rawla, 2019). The risk factors contribute to the development of prostate cancer include genetic predisposition, age and hormonal aspects (Rawla, 2019). The common symptoms of prostate cancer are urinary problems and erectile dysfunction, which are crucial to early detection of the disease (Bestmann et al., 2007).

As mentioned before, the cytotoxic effects of natural agents, rather than chemical agents, against prostate cancer cells are the main consideration of researches *in vivo*, *in vitro* and epidemiological studies to promote the maintenance of healthy conditions at the same time (Miyata et al., 2019). In this regard, the anticancer activities of biocompounds in black tea have been studied against prostate cancer

cells. The cytotoxicity of EGCG were widely investigated on prostate cancer cell lines of PC3 (Sun et al., 2008; Yang et al., 2009; Sun et al., 2013), 22Rv1 (Siddiqui et al, 2011; Perez et al., 2021) and LNCaP (Bakhshandeh et al., 2023; Eom et al., 2015) since it was considered as the most effective compound in tea against the survival of cancer cells (Miyata et al., 2019). Moreover, the anticancer activities of theaflavins and gallic acid were also revealed in the several studies against PC3, LNCaP and 22Rv1 (Kaur et al., 2009; Lin et al., 2008; Liu et al., 2011; Liu et al., 2013; Russel Jr et al., 2012).

PC3, LNCaP and 22Rv1 are mainly varied in terms of their androgen sensitivity, and it directly affects their responses to the cytotoxic activities (Alserihi et al., 2023). PC3, LNCaP and 22Rv1 are classified as androgen-independent, androgen-dependent and androgen-sensitive cancer cells, respectively (Lafront et al., 2020). Androgen sensitivity refers how susceptible prostate cancer cells are to androgens, which are male sex hormones, especially testosterone (Fujita & Nonomura, 2018). Androgens are required to develop and proliferate for androgen-dependent cancer cells, where androgens bind to specific androgen receptors resulting in the activation of signaling pathways that promote cell growth and survival (Grossman et al., 2001). On the other hand, while androgen-sensitive cells do not depend on androgen receptor for proliferation, they are positively stimulated by androgens (Lafront et al., 2020).

1.5.2 Brain Tumor

Glioblastoma multiforme (GBM) is one of the most aggressive cancers, with an average overall survival of less than 15 months, and it is also the most frequent invasive brain and central nervous system tumor (Zheng et al., 2022). The studies suggest that GBM is markedly more common in men, and glioma risk increases with age while median survival remains low (Grochans et al., 2022). The symptoms of

GBM encompass seizures, cerebral edema, infections, depression, cognitive dysfunction and fatigue (Alifieris & Trafalis, 2015).

The anticancer activity of black tea components against glioblastoma multiforme cells was demonstrated in the several studies. The cytotoxicity of tea catechins such as ECG, EGC and EGCG was revealed on U87MG cells (Das et al., 2010; Grube et al. 2018; Shervington et al., 2009; Yokoyama et al., 2000) and A172 cells (Sachinidis et al., 2000; Siegelin & Gaiser, 2008). In addition, the reduced cell viability of U87MG cells against gallic acid (Yang et al., 2022) and caffeine (Li et al., 2018) was confirmed.

U87MG and A172 are aggressive glioma cells because of having genetic mutations at the gene of phosphatase and tensin homolog deleted on chromosome ten (PTEN) which is a tumor suppressor gene (Elhag et al., 2015). Therefore, the mutation of PTEN gene stimulates uncontrolled cell growth and tumor formation (Song et al., 2012). Moreover, A172 cells also have a mutation at another tumor suppressor gene, TP53 which encodes the p53 protein, and it provides additional resistance to the aggressive A172 cells against anticancer agents (Elhag et al., 2015).

1.5.3 Breast Cancer

Breast cancer is a significant global health concern since it is the most diagnosed cancer in the world (Wilkinson & Gathani, 2021). The disease is the main cause of cancer mortality in women worldwide (Wilkinson & Gathani, 2021). The symptoms of breast cancer include swelling in armpit, pain and tenderness in breast, change in breast color and unusual nipple bloody discharge (Sengar et al., 2021). The most important risk factor of breast cancer is age, and the oldest females have the highest age-specific incidence rates (Wilkinson & Gathani, 2021). Moreover, breast cancer risk increases with younger menarche age, older menopause age, late age at marriage and less breast-feeding exposure (Kashyap et al., 2022).

The anticancer potential of black tea against the most common used breast cancer cells of MCF7 was confirmed in the studies of Friedman et al. (2007), Koňariková et al. (2015) and Way et al. (2009). In addition, catechins such as EC, ECG, EGC and EGCG, theaflavins and theanine were found as the effective anticancer agents for MCF7 cells (Friedman et al., 2007). Furthermore, gallic acid (Aborehab et al., 2020; Rezaei-Seresht et al., 2019; Wang et al., 2014) and caffeine (Machado et al., 2020; Niknafs, 1996) could reduce the cell viability of MCF7 cells.

1.6 Instant Black Tea Production

The demand for black tea, caused by its unique characteristic sensory properties and extensive health promoting effects, is changing its direction to adapt to dynamic global standards. For instance, while people still desire black tea, they are also looking for more convenience. Therefore, various black tea products, such as instant tea, have been developed to meet this demand. Instant tea is a growing and increasingly popular product category of black tea thanks to the simplicity in the preparation (Kraujalytė et al., 2016).

Instant black tea is a fully soluble solid tea powder (Alasalvar et al., 2013). Broken mixed fanning, which is the blend of green leaves, fermented particles obtained from the rolling of the leaves, black tea and rejected fractions of the end product, are mostly utilized as a raw material for instant tea manufacture because of the cheapness (Perera et al., 2015; Liu et al., 2021). The principle of instant black tea production is based on the drying infusion of black tea (Alasalvar et al., 2013). Therefore, the extraction of black tea and the drying method play a critical role in the quality of produced instant tea powder.

Infusion is a crucial step of the production of instant tea because of the effects of extraction efficiency on the soluble solid content, sensory profile, nutritional value and antioxidant capacity of the end product (Chandini et al., 2011; Liu et al., 2021).

The cold infusion method is becoming an increasingly attractive alternative to the hot infusion method due to the potential of cold infusion to improve the extraction of valuable bioactive components (Hajiaghaalipour et al., 2016; Rodrigues et al., 2015; Venditti at al., 2010; Yang et al., 2007;). Moreover, the detailed infusion conditions, which are the water-to-tea ratio, the particle size range of the raw material, the infusion temperature and the infusion time, were found to significantly impact on the extraction efficiency (Bindes et al., 2019; Ma & Hung, 2020; Vuong et al., 2011b).

Superfine grinding has also drawn attention in the black tea infusion research area because of the ability to increase extraction efficiency of infusions (Hu et al., 2012; Park et al., 2001; Zhao et al., 2009). Superfine grinding decreases the particle size of food components to the superfine range, which is typically spanning from 1 nm to 100 μm, by the mechanical pulverization methods such as ball milling (Zhao et al., 2009; Zhao et al., 2023). The mechanism behind the promising effects of superfine grinding was attributed to the destruction of the cell wall, which decreases the mass transfer resistance and accelerates the diffusion of chemical compounds (Xiao et al., 2017; Zhang et al., 2022). Moreover, it was also suggested that superfine grinding improved the solubility, dispersibility and flowability of food powders (Xiao et al., 2017; Zhong et al., 2016).

The capacity of the superfine grinding process, where the particle size of pre-ground black tea waste fiber was reduced to the superfine range by ball milling, can be better understood on black tea waste fiber (Figure 1.2) in Figure 1.6.



a b

Figure 1.6 Pre-ground black tea waste fiber (a), Pre-ground and ball milled black tea waste fiber (b)

Spray-drying and freeze-drying are commonly used methods to obtain instant tea powder from the infusion, although vacuum-drying is also used for instant tea production (Alasalvar et al., 2013). While spray-drying is considered as a more economical alternative, the freeze-drying method provides the production of instant tea, which has more aroma compounds than that produced by spray-drying (Liu et al., 2021). This is due to the working principles of the spray-drying and freeze-drying methods, which are based on exposing liquid droplets to hot air and sublimation under a vacuum (Kraujalytė et al., 2016). Heat sensitive volatile compounds are retarded in the low temperature conditions of freeze-drying (Kraujalytė et al., 2016; Polat et al., 2018).

After the production of instantly soluble tea powders, as a further step, instant tea powders can be mixed with herbs such as ginger and sage to promote their functional properties, which are the concern of consumers nowadays.

1.7 Ginger

Ginger, also known as *Zingiber officinale*, belongs to the family of *Zingiberaceae* (Mao et al., 2019). Ginger has been widely consumed both as a spice and a medicinal herb for a long time (Bieżanowska-Kopeć & Piątkowska, 2022).

Ginger is known for its high antioxidant capacity, especially in the dried form (Figure 1.7), and it was attributed to the phenolic compounds of ginger (Abolaji et al., 2017; Ji et al., 2017). The main phenolic compounds of ginger are gingerols, shogaols and paradols (Mao et al., 2019). In fresh ginger root, the primary polyphenols are gingerols, and when subjected to heat or stored for extended periods, they can undergo a transformation into their corresponding shogaol forms and further into paradols (Stoner, 2013). On the other hand, several studies have found that there are also various non-phenolic antioxidative compounds in ginger, especially terpenes which are the main constituents of ginger essential oils such as borneol, camphene, cineole, zingiberene and α-terpineol (El-Ghorab et al., 2010; Wei & Shibamoto, 2007; Yeh et al., 2014; Zancan et al., 2002).



Figure 1.7 Ginger powder

Ginger has been widely investigated for its preventive effects against several diseases such as neurodegenerative diseases (Ho et al., 2013; Huh et al., 2018; Park et al., 2013), cardiovascular diseases (Akinyemi et al., 2016; De Las Heras et al., 2017), respiratory disorders (Townsend et al., 2013) and various cancer types (Nile et al., 2015; Saha et al., 2014; Zhang et al., 2016).

1.8 Sage

Sage, also known as *Salvia officinalis*, belongs to the family of *Lamiaceae*, and it has long been utilized as a spice and a medicinal herb (Hamidpour et al., 2014; Zaccardelli et al., 2020).

Sage has been renowned for its diverse biological activities, which have led to their application in the prevention and treatment of various diseases. This is associated with its secondary metabolites isolated from several parts of the plant, flowers, leaves and stem (Zaccardelli et al., 2020). The powder form of sage obtained from these parts of the plant can be seen in Figure 1.8.



Figure 1.8 Sage powder

The essential secondary metabolites of sage are considered as phenolic acids such as rosmarinic acid, caffeic acid and salvianolic acids, terpenoids such as α- and β-thujone, camphor and 1,8-cineole as well as terpenes such as borneol, carnosic acid, ursolic acid and carnosol (Wu et al.,2012).

The health benefits of sage were demonstrated in the several studies such as for its antidiabetic (Eidi & Eidi, 2009), neuroprotective (Ayoub et al., 2022), anti-inflammatory (Kolac et al., 2017; Vieira et al., 2020) and cytotoxic (Jiang et al., 2016; Russo et al., 2013) properties.

1.9 Objective of the study

Black tea is the most demanded beverage in the world behind water, and its global consumption and production have risen significantly in recent years. In addition, the generation of black tea waste fiber, including discarded leaves, stems and stalks, has also increased with the progress of production. The potential valorization of black tea waste fiber has been a major research priority due to its bioactive and nutritionally valuable components.

The main objective of this research is to valorize the black tea waste fiber in the production of instant tea. Within this framework, the specific objectives were listed as follows.

- Evaluate the hot and cold infusion methods to extract black tea waste fiber.
- Determine the best infusion conditions based on the water-to-tea ratio, the particle size range of the raw material, the infusion temperature and the infusion time to get the maximum efficiency from the black tea waste fiber.
- Mix the tea powders with ginger and sage via superfine grinding to promote their functional properties.
- Investigate the instant teas for their chemical properties (*antioxidant capacity, total phenolic content, phenolic profile including epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate, theaflavin, thearubigins, gallic acid, caffeine and theanine*) and physical properties (*moisture content, hygroscopicity, color, morphological, solubility and turbidity*) to give meaning to the effects of different infusion methods and superfine grinding with ginger and sage on the end products.
- Demonstrate the anticancer potentials of the instant black teas, produced by freeze-drying hot and cold infusions of back tea waste fiber, against the cell lines of prostate cancer (PC3, 22Rv1 and LNCaP), brain tumor (U87MG and A172) and breast cancer (MCF7) in *in vitro* studies

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Black tea fiber was obtained from a tea producing company, Doğadan, as a waste of black tea production (Ankara, Turkey). In addition, black tea leaves, the end product of the same tea company, as well as ginger and sage, in the form of fragmented leaves and roots, were also supplied from Doğadan Tea Company.

In order to characterize the instant teas, several chemicals were used. ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) reagent, DPPH (2,2-diphenyl-1-picrylhyrazyl) reagent, methanol (CH_3OH), potassium persulfate ($K_2S_2O_8$), Folin-Ciocalteu's phenol reagent, sodium carbonate (Na_2CO_3), gallic acid ($C_7H_6O_5$), HPLC standards of epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate, gallic acid, caffeine and theanine, acetonitrile (CH_3CN), acetic acid (CH_3COOH), isobutyl methyl ketone ($C_6H_{12}O$), diphenyl boric acid-2-aminoethyl ester ($(C_6H_5)_2BOCH_2CH_2NH_2$), ethanol (C_2H_6O), sodium bicarbonate ($NaHCO_3$), oxalic acid ($H_2C_2O_4$), fetal bovine serum, MTT (3-(4,5-dimethythiazol-2yl)-2,5-diphenyltetrazolium bromide) and nonessential amino acids were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). RPMI-1640 medium, DMEM medium, HEPES ($C_8H_{18}N_2O_4S$), penicillin-streptomycin ($C_{37}H_{57}N_9O_{16}S$) and sodium pyruvate ($C_3H_3NaO_3$) were purchased from Thermo Fisher Scientific (Grand Island, NY, USA).

2.2 Production of Instant Tea Powders

2.2.1 Preparation of Black Tea Waste Fibers for Infusion

Black tea waste fibers with irregular structures were exposed to high shear grinding (TM5, Vorwerk Thermomix, Germany) at 10,700 rpm for 3 min, and the pre-ground black tea waste fibers (PG) ready to be an infusion were obtained.

2.2.2 Ball Milling of Pre-ground Black Tea Waste Fibers

Coarsely ground black tea waste fibers were also subjected to a subsequent pulverization treatment, *planetary ball milling* (LT-M0610, Liya Test, Turkey), at 600 rpm for 5, 15 and 25 min to obtain *pre-ground and ball milled black tea waste* fibers (PGBM) which were in the range of 'superfine particle' size.

2.2.3 Determination of Particle Size Distribution

A laser diffraction particle size analyzer with a dry dispersion unit (Mastersizer 2000, Malvern Instruments, UK) was used in the evaluation of the particle size distributions of black tea leaves, the end product of Doğadan Tea Company, PG and PGBM at METU Central Laboratory. The refractive index for black teas was taken as 1.59 (Quyang et al., 2020). D_{10}, D_{50} and D_{90}, representing the diameters at which 10%, 50% and 90% of the particles, respectively, are smaller, as well as the span of the samples were calculated by the software of the particle size analyzer.

2.2.4 Determination of Cellular Disruption Ratio

The cellular disruption ratio measures how effectively cell walls are broken down to release cellular components and was calculated for black tea leaves, PG and PGBM based on the relation established by Xiao et al. (2017).

$$\text{Cellular disruption ratio } (\%) = 1 - \left(1 - \frac{10}{D_{50}}\right)^3 \quad \text{when } D_{50}>10\ \mu m$$

In addition, cellular disruption ratio was accepted as 100% when $D_{50}<10$ µm since the diameter of the plant cells was considered as 10-20 µm (Xiao et al., 2017).

2.2.5 Determination of Infusion Parameters

Infusion parameters of black tea waste fibers were specified in terms of water-to-tea ratio, particle size range, infusion temperature and infusion time to maximize the extraction yield.

Infusions of black tea were performed using an oil bath. In addition, infusions were mixed for 15 s every 3 min in the hot infusion method and for 15 s every 30 min in the cold infusion method to prevent sedimentation during extraction. After the infusions, the supernatants were collected using polypropylene filters at 50 µm for PG and 10 µm for PGBM.

2.2.5.1 Water-to-Tea Ratio

The effect of the water-to-tea ratios of 10:1, 20:1, 30:1 and 40:1 ml/g on extraction efficiency was investigated for PG and PGBM separately. When evaluating the

impacts of water-to-tea ratios, infusion temperature and time were kept constant at the highest temperature and time of the infusion methods, 100 ℃ and 15 min, respectively.

2.2.5.2 Particle Size Range, Infusion Temperature and Infusion Time

The hot infusion method was performed for different extraction temperatures (80 ℃ and 100 ℃) and times (6, 10 and 15 min) considering the previous studies (Ramolho et al. 2013; Rodrigues et al., 2015; Kelebek et al., 2016; Vinci et al., 2022; Yu et al., 2021). Moreover, the cold infusion method was conducted at room temperature (25 ℃) for 2 h and refrigerator temperature (4 ℃) for 6 and 12 h (Hajiaghaalipour et al., 2015; Lantano et al., 2015; Rodrigues et al., 2015; Venditti et al., 2010; Yang et al., 2007).

2.2.6 Determination of Extraction Yield

The extraction yield values of PG and PGBM in the infusions were calculated using the method described by Ozdemir et al. (2018) with some modifications. After the infusion, filtrate was dried in an oven at 105 ℃ until a constant weight of extract (W_e) was obtained. Then, the extraction yield was determined using the equation given subsequently.

$$\text{Extraction Yield } (\%) = \frac{W_e}{W_s} \times 100$$

where W_s is the weight of the dry black tea waste fiber, determined drying in an oven at 105 ℃ until a constant weight.

2.2.7 Freeze Drying

Infusions of PGBM produced at the optimum extraction parameters were frozen in the freezer at -18 ℃ for 24 h and then put in a freeze dryer (TF-10D, Beijing Songyuan Huaxing Technology Development Co., Ltd., China) for 48 h to have a final moisture content of instant teas in the range of 4.0-4.5% (wet basis).

2.3 Production of Instant Tea Powders Enriched with Ginger and Sage

2.3.1 Preparation of Herbs for Mixing

Ginger and sage, supplied as fragmented leaves and roots, were exposed to high shear grinding (TM5, Vorwerk Thermomix, Turkey) at 10,700 rpm for 3 min, and the pre-ground herbs were sieved by vibratory sieve shaker (Fritsch, Germany).

2.3.2 Mixing Instant Tea Powders with Ginger and Sage

Instant teas, produced by freeze-drying hot and cold infusions of black tea waste fibers, were mixed with pre-ground and sieved ginger and sage at 600 rpm for 1 min to reduce the particle size of herbs into the range of superfine. The instant tea-to-herb ratios were determined as 90:10 g/g based on the preliminary sensory studies conducted by Doğadan Tea Company.

D_{10}, D_{50}, D_{90} and span values of ginger and sage were measured as mentioned in Sections 2.2.3 where the refractive index values of herbs were accepted as 1.52. In addition, the cellular disruption ratios of herbs were also calculated as described in Section 2.2.4.

2.3.3 Chemical Analysis of Instant Teas Enriched with Ginger and Sage

2.3.3.1 Extraction of Phenolic Compounds

The phenolics of instant teas were extracted according to the procedure described by International Organization for Standardization 14502-1 (2005). The solution at a concentration of 40% (w/v) was prepared with samples and methanol:water mixture (70:30 v/v) that had previously been heated to 70 ℃ in a water bath. The solution was immediately vortexed and kept in a water bath at 70 ℃ for 5 min. The vortex and heating steps were repeated once for a further 5 min. The extraction solutions were rested at room temperature to cool. Then, the solutions were centrifuged (MF80, Hanil Scientific Inc., South Korea) at 2422 g for 10 min, and the supernatant was collected carefully.

2.3.3.2 Determination of Antioxidant Capacity

The antioxidant capacities of instant black tea samples were determined using two free radical scavenging assays, ABTS and DPPH.

2.3.3.2.1 ABTS Free Radical Scavenging Assay

The antioxidant capacities of samples were measured using modified version of ABTS assay performed by Kelebek (2016). According to the method, firstly, the ABTS solution was prepared mixing 7 mM ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) and 2.54 mM potassium persulfate, and the solution was kept at room temperature for 12-16 h. Before starting the assay, the ABTS solution was diluted to obtain the absorbance of 0.70 ± 0.05 using a UV-VIS

spectrometer (Optizen Pop, Nano Bio, South Korea) at 734 nm with methanol:water mixture (70:30 v/v), also taken as control. Then, 3 ml of diluted ABTS solution was added to test tubes including instant tea extracts at different concentrations (0.097-100 µg/ml), prepared with methanol:water mixture (70:30 v/v). After vortex, the test tubes were allowed to stand at room temperature for 30 min, and the absorbance values were read at 734 nm according to the blank solution of methanol:water mixture (70:30 v/v).

Antioxidant capacities of instant tea samples were expressed as IC_{50} (inhibitory concentration) values which is the concentration of the instant tea extract that is required to suppress 50% of the initial free radicals (Rahimi et al., 2021). IC_{50} values were determined from the curves of the extract concentration (µg/ml) versus the inhibition capacity (%). The equation to calculate the percent inhibition capacity of instant tea, also known as the scavenging capacity (%), was given below.

$$\text{Inhibition (\%)} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}}$$

An example of the related regression curves was given in Appendix A.

2.3.3.2.2 DPPH Free Radical Scavenging Assay

The antioxidant capacities of samples based on DPPH were determined using the procedure described by Brand-Williams et al. (1995). According to the method, 4 ml of 50 µM DPPH (2,2-diphenyl-1-picrylhyrazyl) solution, prepared with methanol:water mixture (70:30 v/v), was mixed with 2 ml of instant tea extracts at different concentrations (0.097-100 µg/ml), prepared with methanol:water mixture (70:30 v/v). After vortex, the test tubes were kept at room temperature for 30 min,

and the absorbance values were read at 515 nm according to the blank solution of methanol:water mixture (70:30 v/v). The absorbance of 50 μM DPPH solution was also measured as control.

IC_{50} values were determined from the curves of the extract concentration (μg/ml) versus the inhibition capacity (%), as mentioned in ABTS assay. An example of the related regression curves for DPPH assay could be seen in Appendix A.

2.3.3.3 Determination of Total Phenolic Content

The content of phenolic compounds was analyzed using Folin-Ciocalteu's phenol reagent based on the method described by International Organization for Standardization 14502-1 (2005). 0.5 ml of sample extract was diluted with distilled water to the concentration of 1% (v/v). Then, 1 ml of dilutes sample extract was mixed with 5 ml of Folin-Ciocalteu's reagent (0.2 N) in a test tube. Within 3-8 min, 4 ml of sodium carbonate solution (7.5% (w/v)) was added to the test tube, and the mixture was vortexed. Samples were allowed to stand at room temperature in the dark for an hour, and the absorbance values of the blue colored solutions were read at 765 nm according to the blank solution of distilled water.

The total phenolic contents of the instant teas were expressed regarding the gallic acid calibration curve, i.e., gallic acid equivalent (GAE), which had been prepared using the same procedure of total phenolic content analysis at different gallic acid concentrations (10-90 μg/ml). The calibration curve could be seen in Appendix A.

2.3.3.4 Determination of Catechins (EC, ECG, EGC, EGCG), Gallic Acid, Caffeine and Theanine Contents by High Pressure Liquid Chromatography (HPLC)

Catechins (EC, ECG, EGC, EGCG), gallic acid, caffeine and theanine compounds were extracted by the method of Serpen et al. (2012) with some modifications. 500 mg of instant tea samples were extracted with 5 ml of distilled water at 80 °C. The infusion was mixed well for 5 min and then centrifuged (CF-10, Wisd Laboratory Instruments, Germany) at 9,055 g for 5 min. The supernatant was collected, and the residue was further extracted with 5 ml of distilled water at 80 °C. Combined supernatants were filtrated through 0.45 µm nylon filter and transferred to vials. Catechins, gallic acid, caffeine and theanine compounds in instant black teas were quantified by an HPLC system (Shimadzu Scientific Instruments, Japan) consisting of a degasser (DG-20A5), a pump (LC-20AD), an autosampler (SIL-20AHT), a column oven (CTO-20A), and a PDA detector (SPD-M20A). The chromatographic analyses of bioactive compounds were performed with a Hypersil GOLD Phenyl HPLC column (250 x 4.6, 5µm, Termo Fisher Scientific, USA) and the sample injection volume and column temperature were 10 µL and 25°C, respectively.

For catechins, gallic acid and caffeine compounds, a linear gradient method was used with a mobile phase of acetonitrile (Solvent A) and acetic acid (Solvent B) (0.1 v/v) at a flow rate of 1 ml/min (Serpen at al., 2012). The gradient elution program was adopted as: 0-15 min, linear gradient from 10 to 20% A; 15-25 min, linear gradient elution from 20 to 40% A; 25-30 min, linear gradient elution from 40 to 10% A. The peaks were identified at 275 nm by comparing the retention time of tea sample with the standard compounds and calibration curves were prepared with standard solutions.

The chromatographic analysis of theanine was performed according to the method described by Murugesh et al. (2018). Analysis was conducted by a linear gradient elution with mobile phase of acetonitrile (Solvent A) and water (Solvent B) at a flow

rate of 1 ml/min. The gradient elution program was adopted as: 0-5 min, 2% A; 5-12 min, linear gradient elution from 2 to 50% A; 12-30 min, 50% A; 30-40 min, linear gradient elution from 50 to 2% A; 40-45 min, 2% A. The peaks were identified at 200 nm and calibration curve was prepared by using theanine standard.

2.3.3.5 Determination of Theaflavin and Thearubigins Contents

The quantification of theaflavin and thearubigins contents in instant black teas were performed using the modified version of Flavognost method (Obanda et al., 2004; Ozdemir et al., 2018). According to the method, firstly, the tea infusion at a concentration of 24 (w/v) was prepared with fresh-boiled distilled water and allowed to cool at room temperature. Then, 10 ml of the infusion was mixed with 10 ml of isobutyl methyl ketone (4-methylpentan-2-one, IBMK) using shaker (Daihan Scientific Co., Ltd., South Korea) at 300 rpm for 10 min. After mixing, the solution was rested to observe phase separation, and 2 ml of upper layer was vortexed with 4 ml of ethanol and 2 ml of Flavognost reagent which had been prepared previously dissolving 2 g of diphenyl boric acid-2-aminoethyl ester in 100 ml of ethanol. Mixtures were kept at room temperature for 15 min to see color development, and then the absorbance values of the solutions were read at 625 nm against IBMK: ethanol (1:1 v/v) as a blank. Theaflavin contents of instant teas were calculated using the equation given below.

$$\text{Theaflavin } \left(\frac{\text{mg}}{\text{100 g dry matter}}\right) = \frac{\text{Absorbance } \times 47.9 \times 10 \times 564.49}{\text{Dry matter (\%)}}$$

where 47.9 was the correction factor regarding the dilution. 564.49 g/mol was the molecular weight of theaflavin. The results of theaflavin contents were converted to the unit of mg/100 g and reported in that way.

The contents of thearubigins, on the other hand, were determined using the same infusion, but 10 ml of the infusion was mixed with 50 ml of IBMK at this time. After mixing the solution with shaker at 300 rpm for 10 min, the solution was rested to observe phase separation, and 4 ml of upper layer was diluted with methanol to 25 ml (solution A). In addition, 25 ml of upper layer was mixed with 25 ml of 2.5% aqueous sodium bicarbonate solution., and 4 ml of the mixture was diluted to 25 ml with methanol (solution B). Moreover, 2 ml of upper layer was mixed with 2 ml of saturated oxalic acid solution and 6 ml of distilled water, and the mixture was diluted to 25 ml with methanol (solution C). Then, the absorbance values of solutions A, B and C were read at 380 nm against distilled water as a blank. Thearubigins contents of instant tea samples were calculated using the equation given below.

$$\text{Thearubigins } \left(\frac{\text{mg}}{\text{100 g dry matter}}\right) = \frac{100000 \times 0.02 \times 6.25 \times (2 \times \text{Abs}_{\text{C}} + \text{Abs}_{\text{A}} - \text{Ab}_{\text{B}})}{0.733 \times 0.024 \times \text{Dry matter (\%)}}$$

The results of thearubigins were also converted to the unit of mg/100 g and reported in that way.

2.4 Physical Analysis of Instant Teas Enriched with Ginger and Sage

2.4.1 Moisture Content

The moisture content of black teas was calculated based on the weight difference during drying in an oven at 105 °C until the samples reached a constant weight (Chen & Weng, 2010; Wu et al., 2021)

2.4.2 Hygroscopicity

Hygroscopicity values of instant black tea powders were measured using the method described by Zokti et al (2016) with some modifications. Petri dish, on which 0.2 g of the sample was weighed (W_i) and spread, was placed in a cabinet that was set to 25 ℃ and 75% RH (Relative Humidity). When a constant weight of the sample was obtained (W_f), after 120 min, the weight difference was immediately determined and the hygroscopicity was calculated using the equation given subsequently.

$$\text{Hygroscopicity}\ (\%) = \frac{W_f - W_i}{W_i} \times 100$$

2.4.3 Morphology

Scanning electron microscopy (SEM) (Thermo Fisher Scientific, Quanta 400F, US), having a resolution of 1.2 nm, was used to investigate the morphological properties of instant tea powders at METU Central Laboratory. Samples were coated with gold and palladium in 3 nm, and the measurements were conducted with SEM at 30 kV.

2.4.4 Solubility

The solubility of instant tea powders in water was characterized by following the method of Nadeem et al. (2011) with some modifications. Instant teas (W_t) mixed with the distilled water, at ambient temperature, at the concentration of 10% (w/v) using a magnetic stirrer (Wisd Laboratory Instruments, Germany) at 500 rpm for 5 min. Then, the solution was centrifuged at 3136 g for 5 min, and the supernatant was dried at 105 ℃ until the constant weight (W_c) was obtained. Finally, the solubility was calculated using the equation given below.

$$\text{Solubility } (\%) = \frac{W_c}{W_t} \times 100$$

2.4.5 Turbidity

Turbidity values of the instant tea samples, prepared at a concentration of 2.5 (w/v) based on preliminary studies, were measured using a turbidimeter (Milwaukee, Mi415, USA) in the unit of FNU (Formazin Nephelometric Unit).

2.4.6 Color

Color measurements of instant tea powders and instant tea infusions, prepared at a concentration of 2.5 (w/v) based on preliminary studies, were conducted according to the CIE (International Comission on Illumination) L*, a*, b* system using a spectrocolorimeter (Serlab, Sl400, Turkey).

2.5 Anticancer Analysis of Instant Teas

2.5.1 Cell Culture

The cytotoxicity of instant black tea powders was investigated against prostate cancer cells (PC3, 22Rv1 and LNCaP), glioblastoma multiforme cells (U87MG and A172) and breast cancer cell (MCF7).

PC3, 22Rv1 and LNCaP cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, nonessential amino acids, sodium pyruvate and HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid).

U87MG, A172 and MCF7 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, nonessential amino acids, sodium pyruvate.

All cells were grown in humidified 5% CO_2 and 95% air atmosphere at 37 ℃.

2.5.1.1 Dissolving of the Cells

5 ml of growth medium, left at room temperature for a while, was added to a falcon tube in the biological safety cabinet. The cryogenic tube, retrieved from the nitrogen tank, was alcohol-wiped and placed inside the biological safety cabinet. Cells in the cryogenic tube were gently taken with a portion of the growth medium using a pipette from the falcon tube until the cells detached from the tube base. Once the cells were separated from the tube base, they were transferred to the falcon tube and centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded, and the cells were transferred with a portion of growth medium using a pipette to a petri dish, including 10 ml of growth medium. Then, the cells were placed in an incubator with 5% CO_2 .

2.5.1.2 Feeding of the Cells

The cells were taken from the incubator, and the growth medium was removed in a sterile cabinet. The cells were washed with phosphate buffered saline solution (PBS), and they were placed in an incubator by adding 10 ml of growth medium.

2.5.1.3 Passaging of the Cells

When the observed cell density reached approximately 75-80% under a microscope, the process was performed. The growth medium was discarded, and the cells were washed with PBS. 500 µl of trypsin was added to the cells, and they were left for 3-4 minutes in the incubator. Then, the cells that started to detach from the petri dish were gently pipetted using trypsin. After a fresh growth medium was added to a new petri dish, 200 µl of the cells were transferred.

2.5.1.4 Freezing of the Cells

The cells removed from the growth medium in a sterile cabinet were washed with 5 ml of PBS. The trypsin added cells were left for 3-4 minutes in the incubator. 10 ml of 10% dimethyl sulfoxide and fetal bovine serum mixture was prepared in a sterile falcon tube. The cells from the incubator were transferred with trypsin to a sterile falcon tube, including 5 ml of growth medium. Then, the falcon tube containing the cells was centrifuged at 2000 rpm for 5 minutes. After removing the supernatant, the pellet of cells was homogenized with 10% dimethyl sulfoxide solution. The homogenized cells were divided into cryogenic tubes. After keeping at -80 °C for 24 h, the cells were placed in a nitrogen tank.

2.5.1.5 Counting of the Cells

Cell counting was performed using the hemocytometry method. After removing the growth medium from the cells and washing them with PBS, the cells were detached from the petri dish with trypsin. The cells were diluted (1:10) and 100 µl was added to a haemocytometer, counting chamber. Then, the cells were counted under a microscope.

The total cell count was calculated from the number of cells in the middle square using the formula below.

$$\text{Total cell count (ml)} = \text{A} \times \text{DF} \times 10^4$$

where A is the number of cells in the square, DF is the dilution factor and 10^4 is the constant.

Then, the cells were seeded with growth medium into the plates including 96 wells such that each well included 4000 cells and 100 µl of growth medium.

2.5.2 Cell Viability

The cell viability of the cancer cells was determined with MTT (3-(4,5-dimethythiazol-2yl)-2,5-diphenyltetrazolium bromide) assay.

Instant teas were dissolved at 95 ℃ for 15 min in the isotonic solution at the concentrations of 0.001, 0.01, 0.1, 1, 1.25, 2.5, 5,8 and 10 mg/ml, and the solutions were filtered using the filter of 0.22 micron. Then, the sample solutions were added

to the wells, 4000 cells/well in 96-well plates, as 4 replicates. After treatment with the samples at various concentrations, the cells were incubated for 48 h. 20 µl of 5 mg/ml MTT solution in PBS was then added to each well from a stock solution and cultured for 4 h. After removing the growth medium, 100 µl of dimethyl sulfoxide solution was added to dissolve formazan. Finally, the absorbance values in each well were read at 560-620 nm using spectrophotometer (Optizen Pop, Nano Bio, South Korea).

2.6 Statistical Analysis

Each experiment was conducted in three replicates, except the cell viability assays (4 replicates), and the coefficient of variation less than 10% was determined as a criterion for analyses. Statistical analyses were conducted using MINITAB (Version 20, Minitab Inc., UK). Analysis of variance (ANOVA) was performed to observe the effect of parameters on the samples based on the general liner model regression approach, and the normality test was applied to check the assumption of ANOVA. In addition, when there was a significant difference, the samples were compared using Tukey's Comparison Test at the confidence interval of 95%. Results were expressed as mean ± standard deviation.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 Infusion of Black Tea Waste Fibers

3.1.1 Preparation of Black Tea Waste Fibers for Infusion

Black tea waste fibers with irregular structures that ranged in size from a few millimeters to several centimeters were exposed to high shear grinding to achieve a homogenous extraction process by reducing the variation in particle sizes. In addition to the uniformity of the particle size distribution ensuring a consistent extraction procedure, the particle size range also plays a crucial role in the efficiency of the process. Diminishing the large particle size effect boosts the extraction yield since it shortens the diffusion path and increases the contact surface area between the tea leaves and the water (Vuong et al., 2010).

The milling procedure had been performed so that the pre-ground black tea waste fibers (PG) had a similar particle size distribution to the black tea leaves (BTL), the end product of the same tea company. D_{10}, D_{50}, D_{90}, span and cellular disruption ratio values of BTL and PG were summarized in Table 3.1. The particle size of PG and BTL were in the range of 64.32-1026.16 μm and 173.53-861.23 μm, respectively. PG had a significantly lower average particle size than BTL ($p<0.05$). Furthermore, the cellular disruption ratio of BTL was less than PG indicating that the cell wall integrity of PG was disrupted more ($p<0.05$). It means that the cellular components in PG encounter less resistance during the extraction process (Xiao et al., 2017; Zhang et al., 2022). Although the particle size distribution of PG was wider than BTL ($p<0.05$), the high shear grinding process brought the particle size profile of black tea waste fibers closer to that of the reference black tea leaves (2.43 and 1.66, respectively).

Table 3.1 D_{10}, D_{50}, D_{90}, span and cellular disruption ratio values of pre-ground black tea waste fibers and black tea leaves

Sample	D_{10} (μm)	D_{50} (μm)	D_{90} (μm)	Span	Cellular Disruption Ratio (%)
PG	64.32 ± 0.447^{b}	395.96 ± 2.306^{b}	1026.16 ± 4.382^{a}	2.43 ± 0.017^{a}	7.39 ± 0.042^{a}
BTL	173.53 ± 1.685^{a}	413.64 ± 2.331^{a}	861.23 ± 4.344^{b}	1.66 ± 0.016^{b}	7.08 ± 0.039^{b}

*Different letters in the same column represents a significant difference ($p<0.05$)

3.1.2 Ball Milling of Pre-ground Black Tea Waste Fibers

Coarsely ground black tea waste fibers (PG) were incorporated into the subsequent pulverization treatment to investigate the effect of superfine particle size on the extraction efficiency. A wealth of scientific literature has consistently emphasized that superfine grinding increases the bioaccessibility of tea leaves (Xiao et al., 2017; Zhao et al., 2009). The mechanism behind it can be attributed to the destruction of the cell wall, which decreases the mass transfer resistance and accelerates the diffusion of chemical compounds (Xiao et al., 2017; Zhang et al., 2022). Moreover, the higher surface area created by lowering particle size improves solubility, dispersibility and flowability in addition to extraction efficiency (Hu et al., 2012; Park et al., 2001; Zhong et al., 2016).

Ball milling can reduce the particle size of food components to the superfine range, which is typically spanning from 1 nm to 100 μm (Zhao et al., 2009; Zhao et al., 2023). Table 3.2 showed the particle size distributions of the pre-ground and ball milled black tea waste fibers (PGBM) for different superfine grinding times. The particle sizes of PGBM were in the range of 4.12-955.18 μm, and their mean particle size values were 56.31, 19.39 and 17.94 for 5, 15 and 25 min, respectively. As the ball milling time was prolonged, the particle sizes and span values of the samples

decreased ($p<0.05$). On the other hand, the cellular disruption ratio rose from 44.3% to 91.32% with increasing milling duration ($p<0.05$).

The extreme particle size distribution and cellular disruption ratio values were obtained after 25 minutes of superfine grinding. However, the outcomes of the 15 minutes of grinding, which were very comparable to them, were also in the desired extent. At this point, it was considered that the temperature rose to 80 ℃ after 25 minutes of ball milling. The increasing of temperature in dry grinding results in a loss of bioactive ingredients (Hu et al., 2012; Islam et al., 2020). Therefore, it was decided to expose the pre-ground waste black tea fibers to the ball milling process for 15 minutes before the infusion.

Table 3.2 D_{10}, D_{50}, D_{90}, span and cellular disruption ratio values of pre-ground and ball milled black tea waste fibers for 5, 15 and 25 min

Sample	Ball Milling Time (min)	D_{10} (μm)	D_{50} (μm)	D_{90} (μm)	Span	Cellular Disruption Ratio (%)
PGBM	5	5.35 ± 0.008^{a}	56.31 ± 0.075^{a}	955.18 ± 1.318^{a}	16.87 ± 0.025^{a}	44.38 ± 0.048^{c}
	15	4.12 ± 0.016^{c}	19.39 ± 0.061^{b}	76.02 ± 0.226^{b}	3.71 ± 0.015^{b}	88.64 ± 0.114^{b}
	25	4.36 ± 0.019^{b}	17.94 ± 0.062^{c}	64.83 ± 0.213^{c}	3.37 ± 0.015^{c}	91.32 ± 0.113^{a}

*Different letters in the same column represents a significant difference ($p<0.05$)

3.1.3 Determination of Infusion Conditions of Black Tea Waste Fibers

The cold infusion method is becoming an increasingly popular way of extraction as a replacement for hot infusion (Yang et al., 2007). The reason for considering cold infusion as an alternative to hot infusion lies in its potential to enhance the extraction of valuable bioactive compounds (Hajiaghaalipour et al., 2016; Rodrigues et al., 2015; Venditti at al., 2010). Therefore, the conditions of the cold infusion method

were optimized in addition to the conventional hot infusion method to examine the effects of cold-water extraction on the end product of the study.

The extraction efficiency is a primary concern in the production of instant tea because it directly affects the nutritional value and sensory profile (Chandini et al., 2011). The higher the extraction efficiency is, the more desirable beneficial soluble compounds are obtained (Vuong et al., 2011a). Therefore, the infusion conditions of black tea waste fibers for hot and cold infusion methods were specified to maximize the extraction yield. In other words, the water-to-tea ratio, the particle size range of the samples, the infusion temperature and the infusion time, which have an immediate impact on the extraction yield, were optimized for both infusion techniques (Bindes et al., 2019; Ma & Hung, 2020; Vuong et al., 2011b).

3.1.3.1 Determination of Water-to-Tea Ratios for PG and PGBM

The recovery of the soluble solids is improved by increasing the ratio of the water-to-tea since the gradient concentration, which is the driving force for extraction, is maximized (Spigno & Faveri, 2009). Therefore, the effect of the water-to-tea ratio on the extraction efficiency was investigated for PG and PGBM at the highest infusion temperature and time of the hot infusion method, 100 ℃ and 15 min, respectively, at various water-to-tea ratios ranging from 10:1 to 40:1 ml/g.

As shown in Table 3.3, the highest extraction yield was obtained at 30:1 ml/g when the water-to-tea ratio was increased from 10:1 to 30:1 ml/g ($p<0.05$) for both samples. However, there was no significant increase in efficiency when the ratio reached 40:1 from 30:1 ml/g ($p>0.05$). Similar behaviour was also observed in the studies of Chandini et al. (2011) and Vuong et al. (2011b), in which the yield of the extraction process increased and subsequently reached the plateau after the water-to-tea ratio of 50:1 ml/g for catechins of black tea and polyphenolic compounds of green tea, respectively.

Table 3.3 Extraction yield values of pre-ground and pre-ground and ball milled black tea waste fibers for different water-to-tea ratios

Water-to-Tea Ratio (ml/g)	Extraction Yield (%)	
	PG	PGBM
10:1	23.48 ± 0.366^{b}	27.37 ± 0.491^{c}
20:1	23.49 ± 0.358^{b}	29.69 ± 0.476^{b}
30:1	25.05 ± 0.271^{a}	31.08 ± 0.507^{a}
40:1	24.22 ± 0.431^{ab}	30.92 ± 0.448^{ab}

*Different letters in the same column represents a significant difference ($p<0.05$)

Though the greatest extraction efficiency was attained at ratios of 30:1 and 40:1 ml/g ($p>0.05$) for PG and PGBM, the optimum water-to-tea ratio was actually 30:1 ml/g for both. It was attributed to the cost-effectiveness in terms of raw material and energy (Vuong et al., 2011a; Vuong et al., 2011b). The same efficiency was achieved with less volume of water and lower energy consumption required to heat. In the light of such information, the ideal water-to-tea ratio of PG and PGBM was determined as 30:1 ml/g, and it was used to optimize the particle size range, the infusion temperature and the infusion time of hot and cold infusions separately.

3.1.3.2 Determination of Particle Size Range, Infusion Temperature and Infusion Time of Black Tea Waste Fibers

After determining the water-to-tea ratios for PG and PGBM which represented the particle size ranges of black tea waste fibers, the hot infusion method was performed.

3.1.3.2.1 Hot Infusion Method

A detailed comparison of the extraction yield values for the hot brewed PG and PGBM was presented in Table 3.4.

Table 3.4 Extraction yield values of black tea waste fibers for different particle size ranges, infusion temperatures and infusion times in the hot infusion method

Particle Size Range	Infusion		Extraction Yield (%)
	Temperature (℃)	Time (min)	
PG[1]	80	6	24.78 ± 0.500^{cd}
		10	23.15 ± 0.102^{d}
		15	24.41 ± 0.066^{cd}
	100	6	23.16 ± 0.827^{d}
		10	23.23 ± 1.011^{d}
		15	25.05 ± 0.271^{c}
PGBM[2]	80	6	26.86 ± 0.837^{b}
		10	27.37 ± 0.634^{b}
		15	27.15 ± 0.216^{b}
	100	6	31.97 ± 0.259^{a}
		10	30.71 ± 0.582^{a}
		15	32.17 ± 0.839^{a}

[1] in the range of 64.32-1026.16 µm with the average particle size of 395.96 µm
[2] in the range of 4.12-76.02 µm with the average particle size of 19.39 µm
*Different letters in the same column represents a significant difference ($p<0.05$)

The particle size range, the infusion temperature and the infusion time were all parameters that had a significant effect on the extraction yield of black tea waste fibers ($p<0.05$). There was also an interaction between the parameters except the infusion time and the particle size range ($p<0.05$).

The extraction process was markedly improved by decreasing the particle size of black tea waste fibers to the superfine range ($p<0.05$). In other words, PGBM, with a mean particle size of 19.39 μm, have significantly higher extraction efficiency than PG, with a mean particle size of 395.96 μm ($p<0.05$). Parallel findings were also found in the previous study of Xiao et al. (2017), in which black tea powder in the range of superfine particle size showed a higher extraction yield of caffeine and total polyphenols over the coarsely ground samples. Moreover, it was also observed that superfine grinding enhanced the infusion of the total water-soluble solids due to the large cell wall disruption ratio and the conversion of macromolecules into soluble compounds. In addition, Zhang et al. (2017) indicated that the milling method which could increase the contents of extractable biopolymers in black tea powder was only superfine grinding such as ball milling. The consistent result was also observed in the recently published study of Zhang et al. (2022), in which the pulverization of black tea powder at a superfine scale improved the extraction process by decreasing insoluble dietary fiber contents because of the depolymerization of polysaccharides.

The extraction yield of black tea waste fibers significantly increased at 100 °C regarding the effect of infusion temperature on the extraction efficiency of PGBM ($p<0.05$). The same pattern was shown in other research conducted on the extraction of polyphenolic compounds from black tea (Vinci et al., 2022). It was demonstrated that higher amount of total phenolic content was extracted at 100 °C than those extracted at 80 °C. Moreover, the extraction of phenolic compounds at 100 °C and 80 °C from black tea was also investigated in detail by Kelebek (2016). According to the results of the study, the extraction of gallic acid derivatives, catechins, flavanols, theaflavin and purine alkaloids was markedly higher at the temperature of 100 °C. In addition, Yang et al., (2007) stated that the highest amounts of gallic acid and

catechins were extracted from black tea at 100 ℃. Similar behavior was also observed in the researches of Balcı & Ozdemir (2016), Liang & Xu (2003), Sánchez-López et al. (2020) and Wright et al. (2007), in which the extractability of tea components improved by increasing temperature. The reason for this impact of temperature was explained by the cell walls of PGBM becoming more permeable to water and compounds (Vuong et al., 2010). In addition, the diffusion coefficient and the solubility of the compounds increase with the rising temperature (Ramalho et al., 2013; Vuong et al., 2011b; Yang et al., 2007).

When it came to the effect of infusion time on the extraction efficiency of PGBM at 100 ℃, it can be seen in Table 3.4 that there was no significant difference between 6, 10 and 15 min ($p>0.05$) although the extraction yield of PG at 100 ℃ increased after 10 min ($p<0.05$). It indicates that the superfine particle size range enabled the extraction efficiency of black tea waste fibers to be the highest after a short time. Previous research on the extraction of black tea components also identified a similar pattern, in which the infusion of water-soluble solids increased at the initial stage and gradually reached a steady (Xiao et al., 2017). Moreover, it was also observed that superfine black tea powders reached the plateau earlier than coarsely ground samples. Farakte et al. (2016) explained this case with their study of the black tea infusion kinetics, in which decreasing the particle size range result in increasing the first-order rate constant, in other words, the faster infusion.

After all these aspects, it was concluded that black tea waste fibers had the highest extraction yield in the particle size range of 4.12-76.02 μm with a mean particle size of 19.39 μm (PGBM) at 100 ℃ for 6-15 min. The ideal extraction efficiency of black tea waste fibers was in the range of 30.71-32.17% (Table 3.4). The extraction yield of black tea is an international quality control standard (Ozdemir et al., 2018). The minimum extraction efficiency of black tea must be 32% according to the International Organization for Standardization (ISO) (Turkish Standards, 2003). The outcome of the current research was consistent with the ISO standard. In addition, it was also similar with the study, in which black tea leaves obtained from different

shooting periods and altitudes were investigated by Ozdemir et al. (2018). The extraction efficiency of twelve different black tea leaves were found in the range of 27.12-31.45%.

As a result, the optimum infusion time of PGBM at 100 ℃ was determined as 6 min since the same extraction efficiency was achieved with less time and so less energy consumption thanks to the high infusion rate of the superfine particle size range.

3.1.3.2.2 Cold Infusion Method

The extraction yield values of the cold brewed PG and PGBM were demonstrated in Table 3.5. The particle size range and the combination of infusion temperature and time have a significant effect on the extraction yield of black tea waste fibers ($p<0.05$). There was also an interaction between the particle size ranges and the infusion combinations ($p<0.05$).

The same combinations of infusion temperature and time at 4 ℃ had similar extraction yield values in terms of the particle size range ($p>0.05$) although the extraction yield of PGBM was markedly different from the extraction yield of PG at the infusion combinations of 25 ℃ ($p<0.05$). It can be attributed to the unpreventable tendency of powders in the range of superfine particle size to agglomerate, even under agitation, for a long infusion time at 4 ℃ (Bindes et al., 2019). Agglomeration of superfine powders hindered the diffusion of tea components and water and so reduced the extraction efficiency (Sari & Velioglu, 2011).

The extraction efficiency of black tea waste fibers increased at 4 ℃ for both PG and PGBM as the infusion time was prolonged ($p<0.05$). In addition, the highest extraction yield of black tea waste fibers for the cold infusion method was 17.46% obtained in the particle size range of 4.12-76.02 μm with a mean particle size of 19.39 μm (PGBM) at 25 ℃ for 2 h ($p<0.05$). Yang et al. (2007) also observed similar

findings in their study, in which the extraction of gallic acid, caffeine and catechins in black tea was investigated at 4 and 25 ℃. It was found that the extraction of compounds was higher at 25 ℃ and enhanced with time for both temperatures.

In conclusion, the extraction of PGBM at 25 ℃ for 2 h was set as operational for the cold infusion method due to the highest extraction efficiency.

Table 3.5 Extraction yield values of black tea waste fibers for different particle size ranges, infusion temperatures and infusion times in the cold infusion method

Particle Size Range	Infusion		Extraction Yield (%)
	Temperature (℃)	Time (h)	
PG[1]	4	6	7.54 ± 0.204^{c}
		12	10.99 ± 0.130^{b}
	25	2	11.16 ± 0.963^{b}
PGBM[2]	4	6	8.74 ± 0.580^{c}
		12	12.05 ± 0.377^{b}
	25	2	17.46 ± 0.496^{a}

[1] in the range of 64.32-1026.16 μm with the average particle size of 395.96 μm
[2] in the range of 4.12-76.02 μm with the average particle size of 19.39 μm
*Different letters in the same column represents a significant difference ($p<0.05$)

3.2 Production of Instant Teas Enriched with Ginger and Sage

Instant black teas, produced by freeze-drying hot and cold infusions of PGBM, were mixed with pre-ground and sieved herbs to enhance their functional properties. The

instant tea-to-herb ratios of the end products in the research were determined as 90:10 g/g based on preliminary studies.

Superfine grinding was used to blend instant black teas and herbs because it was known to enable powders to better interact with other ingredients and form a desired uniform mixture (Gao et al., 2020). Furthermore, superfine grinding also adds outstanding properties to powders in terms of bioavailability and antioxidant activity in addition to solubility, dispersibility and flowability as mentioned before (Gao et al., 2020; Zhong et al., 2016).

The conditions of superfine grinding for the production of instant teas enriched with herbs were determined for 1 min at 600 rpm to reduce the particle size of ginger and sage into the range of superfine. D_{10}, D_{50}, D_{90}, span and cellular disruption ratio values of ginger and sage were summarized in Table 3.6. The particle size of ginger and sage were in the range of 7.69-29.97 μm and 9.68-206.25 μm, respectively. Ginger had a significantly lower average particle size than sage ($p<0.05$). In addition, the cellular disruption ratio of ginger was higher than sage indicating that the cell wall integrity of ginger was more disrupted ($p<0.05$). The differences in particle size profiles can be explained by the distinction in the origin and the initial state of the herbs supplied as fragmented leaves and roots.

Table 3.6 D_{10}, D_{50}, D_{90}, span and cellular disruption ratio values of ginger and sage

Sample	D_{10} (μm)	D_{50} (μm)	D_{90} (μm)	Span	Cellular Disruption Ratio (%)
Ginger	7.69 ± 0.091^{b}	15.88 ± 0.097^{b}	29.97 ± 0.009^{b}	1.40 ± 0.017^{b}	94.93 ± 0.158^{a}
Sage	9.68 ± 0.049^{a}	66.26 ± 0.292^{a}	206.25 ± 0.073^{a}	2.97 ± 0.015^{a}	38.79 ± 0.144^{b}

*Different letters in the same column represents a significant difference ($p<0.05$)

As a result of all the discussions, the production parameters of instant teas enriched with herbs from PGBM were established and demonstrated in Table 3.7. The end products of the research can be categorized firstly as instant teas produced by the hot infusion (HI) and the cold infusion (CI) methods. Moreover, there were also instant teas produced by mixing with ginger and sage separately on the following steps of the hot infusion (HIG and HIS, respectively) and the cold infusion (CIG and CIS, respectively) methods.

Table 3.7 Production parameters of instant teas enriched with herbs from PGBM

Sample	Infusion		Herb Type	Herb Ratio (%)	Blending
	Temperature (℃)	Time (min)			
HI	100	6	-	-	-
HIG			Ginger	10	1 min at 600 rpm
HIS			Sage		
CI	25	120	-	-	-
CIG			Ginger	10	1 min at 600 rpm
CIS			Sage		

Instant tea powders were characterized by conducting chemical, physical and anti-cancer analysis, as mentioned in continuation.

3.3 Chemical Analysis of Instant Teas Enriched with Ginger and Sage

Antioxidant capacity, total phenolic content, phenolic profile including four main flavanols (epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate), two condensed phenolics (theaflavin and thearubigins) and one phenolic acid (gallic acid) as well as the contents of caffeine and theanine were investigated to identify instant teas enriched with ginger and sage.

3.3.1 Antioxidant Capacity

Antioxidant capacities of instant tea samples were measured based on the free radical scavenging assays, ABTS and DPPH, and expressed as IC_{50} (inhibitory concentration) values. IC_{50} (μg/ml) is the concentration of the instant tea extract that is required to suppress 50% of the initial ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) and DPPH (2,2-diphenyl-1-picrylhyrazyl) radicals (Rahimi et al., 2021). Therefore, the lower the IC_{50} value, the higher the ability to restrain free radicals (Dutta et al., 2013).

IC_{50} values of instant tea powders for ABTS and DPPH radicals were presented in Table 3.8. Antioxidant capacities of instant teas, on the strength of IC_{50} values, ranged from 1.42 to 3.97 μg/ml in ABTS and from 1.48 to 5.21 μg/ml in DPPH. Parallel findings were also observed in the study of Odumosu et al. (2015), in which the antioxidant capacities of six different instant black tea brands, originated in London, Nigeria and Sri Lanka, were analyzed. According to the results of the study, instant teas had IC_{50} values of 1.26-3.98 μg/ml.

Table 3.8 Antioxidant capacities of instant teas enriched with ginger and sage

Sample	Infusion		IC_{50} (μg/ml)	
	Temperature (℃)	Time (min)	ABTS	DPPH
HI	100	6	2.57 ± 0.189^{c}	2.90 ± 0.196^{b}
HIG			1.72 ± 0.097^{de}	1.85 ± 0.266^{cd}
HIS			1.42 ± 0.128^{e}	1.48 ± 0.117^{d}
CI	25	120	3.97 ± 0.040^{a}	5.21 ± 0.244^{a}
CIG			3.25 ± 0.130^{b}	2.95 ± 0.017^{b}
CIS			2.03 ± 0.035^{d}	2.15 ± 0.229^{c}

Different letters in the same column represents a significant difference ($p<0.05$)

Karimi et al. (2016) also observed the IC_{50} value of the black tea extract, purchased in Iran, as 8.00 μg/ml. In addition, the IC_{50} value of the black tea extract, purchased in Jordan, was found as 9.70 μg/ml in the previous research (Khalaf et al., 2008). However, the results of Qu et al. (2020) demonstrated that the IC_{50} values of the extracts of black tea samples, originated in China, fermented at different temperatures (25-35 ℃) ranged from 17.72 to 24.78 μg/ml.

The bioactive compounds of black tea have also drawn attention separately regarding their antioxidant potency. Chen et al. (2009) measured the antioxidant capacity of the polysaccharides of black tea, originated in China, as the IC_{50} value of 20.30 μg/ml. Moreover, the free radical scavenging capacity of the flavonoids in the infusion waste of black tea, purchased in China, was also investigated, and IC_{50} value of flavonoids found as 14.72 μg/ml (Xue et al., 2018).

The antioxidant capacities of instant black tea powders were much higher than those measured in the conventional black tea samples. The reason for this expected result

was the highly concentrated form of instant tea (Alasalvar et al., 2013; Kraujalytė et al., 2016). On the other hand, the variations in antioxidant capacities of the black tea samples in the studies could be attributed to the profile of bioactive compounds, which have the ability to scavenge free radicals. The variety, the growing environment (geographical origin, climate, altitude, use of fertilizer and the composition of the soil), the cultivation method (shooting period and plucking technique) and the process conditions (fermentation temperature and time) ultimately affect the antioxidative phytochemicals of the black tea (Venditti et al., 2010; Serpen et al., 2012; Ozdemir et al., 2018). In addition, the extraction conditions (the type of solvent, the extraction temperature and time) also play a significant role in determining the composition of bioactive compounds and the antioxidant capacities of the black tea (Bieżanowska-Kopeć & Piątkowska, 2022; Serpen et al., 2012).

The infusion conditions for the instant powders and the mixing with herbs using superfine grinding markedly affect the antioxidant capacities of the instant teas ($p<0.05$). There was also an interaction between the infusion conditions and the mixing of instant black tea powders with ginger and sage ($p<0.05$).

Instant teas produced by freeze-drying hot infusion of black tea waste fibers had a higher antioxidant capacity than those obtained by the cold infusion method in ABTS and DPPH assays ($p<0.05$). This was due to the increased extraction of antioxidative compounds during the hot infusion method (Hajiaghaalipour et al., 2016). Siah et al. (2011) also indicated that the best infusion combination to achieve the optimum antioxidant capacity of herbal tea was the high temperature and the short time. Although the 20 times longer extraction time in the cold infusion method was an advantage, it could not compensate for the impacts of the high temperature in the hot infusion method. Hajiaghaalipour et al. (2016) also observed a similar pattern in their study, in which the infusion of black tea, originated in Malaysia, at 100 ℃ for 5 min had a higher antioxidant capacity than the infusion at 28 ℃ for 2 h. The reason for this unpreventable impact of high temperature in a short time was explained by

increasing the permeability of the cell wall, the diffusion coefficient and the solubility of the compounds (Ramalho et al., 2013; Vuong et al., 2010; Vuong et al., 2011b; Yang et al., 2007), as previously mentioned in the section on the hot infusion method.

The addition of herbs using superfine grinding to tea powders significantly increased the antioxidant capacities of the instant teas in both ABTS and DPPH assays ($p<0.05$). Makanjuola et al. (2015) also found a synergistic effect between black tea and ginger, originated in Nigeria, in terms of their ABTS and DPPH free radicals scavenging abilities. In addition, sage also had the potency to increase the antioxidant capacity of black tea since it was observed in the study of Bender et al. (2014) that sage had a higher antioxidant capacity than black tea, purchased in Germany.

Even though sage increased the antioxidant capacity of instant teas more than ginger, this was significant only in the case of the instant teas produced by the cold infusion when considering the interaction between the infusion conditions and the mixing with herbs ($p<0.05$). In other words, although there was no significant difference between HIG and HIS ($p>0.05$), CIS had a higher antioxidant capacity than CIG in both free radical scavenging methods ($p<0.05$). Souza et al. (2017) also compared the antioxidant capacities of ginger and sage, originated in Portugal, in ABTS and DPPH assays, and they found that sage had a higher antioxidant capacity than ginger. In addition, Bieżanowska-Kopeć & Piątkowska (2022) also observed similar behavior in their study, in which they observed that sage had a higher antioxidant capacity when comparing commercial ginger and sage purchased in Poland. Furthermore, parallel findings were also found in different food matrixes, such as meat (Shadidi et al., 1995) and pork (El-Alim et al., 1999), where sage showed a higher antioxidant impact than ginger (Yanishlieva et al., 2006).

3.3.2 Total Phenolic Content

Total phenolic contents of instant black tea powders were measured based on the Folin-Ciocalteu reducing capacity assay and expressed as gallic acid equivalent per 100 grams of the sample (g GAE/100 g).

The total phenolic contents of instant teas were demonstrated in Table 3.9 and ranged from 7.11 to 11.74 g GAE/100 g. The total phenolic contents of spray dried instant black tea powders, originated in Turkey, were also investigated in the study of Alasalvar et al. (2013). They found that instant black tea powders produced from high- and low-quality black teas had total phenolic contents of 17.35 and 17.82 g GAE/100 g, respectively. In addition, Odumosu et al. (2015) were measured the total phenolic contents of six different instant black tea brands, originated in London, Nigeria and Sri Lank, in their study. According to the results of the study, the total phenolic contents of the instant teas were in the range of 28.8-36.0 g GAE/100 g. The variations in the total phenolic contents could be attributed to the differences in variety, growing environment, cultivation method, and process conditions, as mentioned before in the section on the antioxidant capacity (Ozdemir et al., 2018; Serpen et al., 2012; Venditti et al., 2010). Moreover, the raw material used in the current research, black tea waste fiber instead of the end product of any company, was also considered the reason for the lower total phenolic content of instant tea powders.

Instant teas, on the other hand, had higher total phenolic contents than the conventional black tea samples reported in the study of Ozdemir et al. (2018) thanks to the highly concentrated form of instant tea (Alasalvar et al., 2013; Kraujalytė et al., 2016). The total phenolic contents of Turkish black tea samples in the study were in the range of 4.75-6.97 g GAE/100 g. In addition, Khokhar & Magnusdottir (2002) observed similar total phenolic contents in the black tea, purchased in the UK, as 8.05-13.50 g GAE/100 g dry matter. Moreover, Karadeniz & Koca (2009) and Serpen et al. (2012) also found that the conventional black tea samples, originated in

Turkey, had the total phenolics of 7.29-8.39 g GAE/100 g dry matter and 7.52-8.29 g GAE/100 g, respectively.

Table 3.9 Total phenolic contents of instant teas enriched with ginger and sage

Sample	Infusion		Total Phenolic Content (g GAE/100 g)
	Temperature (℃)	Time (min)	
HI	100	6	11.11 ± 0.104^{b}
HIG			10.99 ± 0.009^{b}
HIS			11.74 ± 0.020^{a}
CI	25	120	7.36 ± 0.168^{d}
CIG			7.11 ± 0.020^{d}
CIS			8.48 ± 0.112^{c}

Different letters in the same column represents a significant difference ($p<0.05$)

The infusion conditions for the instant teas and the mixing with herbs using superfine grinding significantly affect the total phenolic contents of the instant tea powders ($p<0.05$). There was also an interaction between the infusion conditions and the mixing instant black tea powders with ginger and sage ($p<0.05$).

Instant teas produced by freeze-drying hot infusion of black tea waste fibers had significantly higher total phenolic contents than those obtained by the cold infusion method ($p<0.05$). The reason for this effect of high temperature in a short time was the enhanced extraction of the bioactive compounds because of increasing the permeability of the cell wall, the diffusion coefficient and the solubility of the compounds (Ramalho et al., 2013; Siah et al., 2011; Venditti et al., 2010; Vuong et

al., 2010; Vuong et al., 2011b; Yang et al., 2007), as mentioned before in the sections on the hot infusion method and the antioxidant capacity.

Although sage increased the total phenolic content of instant teas ($p<0.05$), there was no significant difference between the total phenolic contents of HI and HIG ($p>0.05$) as well as CI and CIG ($p>0.05$). The abundant phenolic concentration of sage was also observed in the study of Bieżanowska-Kopeć & Piątkowska (2022), which examined the total phenolic contents of 13 different herbs, purchased in Poland, including ginger. It was indicated that sage deserved attention because of the highest total phenolic content among the commercial herbs. Zeyada et al. (2007) and Souza et al. (2017) found that sage had a higher total phenolic content than ginger, purchased in Egypt and Portugal, respectively. On the other hand, Makanjuola (2017) demonstrated that the total phenolic content of the black tea-ginger mixing (2:1), originated in Nigeria, was lower than the black tea. The consistent result was also observed in other research of Makanjuola et al. (2016) for the total phenolic contents of black tea and black tea-ginger mixing (2:1), originated in Nigeria. Therefore, it could be considered that ginger-containing instant teas did not have a statistically lower total phenolic content because of the substantially higher instant tea-to-herb ratio (9:1).

3.3.3 Pearson Correlation Coefficients for $IC_{50,\,ABTS}$, $IC_{50,\,DPPH}$ and Total Phenolic Content

Pearson correlation analysis was performed on the antioxidant capacity and the total phenolic content analyses, and Pearson correlation coefficients were presented in Table 3.10.

IC_{50} values of ABTS were positively, highly and significantly correlated with IC_{50} values of DPPH ($r=0.946$, $p<0.05$). Sharapov et al. (2014) also confirmed the relationship between the free radical scavenging capacity analyses. The researchers

suggested that ABTS and DPPH analyses of plant extracts, where phenolic compounds were plentiful, generally showed a high correlation.

Total phenolic contents, on the other hand, demonstrated non-significant correlation with IC_{50} values of ABTS ($r=-0.793$, $p>0.05$) and DPPH ($r=-0.661$, $p>0.05$). While there were several studies where ABTS and DPPH analyses of black tea showed high and significant correlation with total phenolic contents (He et al., 2020; Kelebek, 2016; Venditti et al., 2010), it was considered after the elaborative examination of the results in the antioxidant capacity and the total phenolic contents of instant teas that the non-significant correlation in the current research was due to the different reaction trends of the free radicals of ABTS and DPPH as well as the Folin-Ciocalteu's reagent with bioactive compounds in the ginger. In other words, the dominant non-phenolic antioxidative compounds in ginger, especially borneol, camphene, cineole, zingiberene and α-terpineol, lead to non-correlation of the antioxidant capacity and the total phenolic contents analyses (El-Ghorab et al., 2010; Wei & Shibamoto, 2007; Zancan et al., 2002).

Table 3.10 Pearson correlation coefficients regarding $IC_{50,\ ABTS}$, $IC_{50,\ DPPH}$ and TPC

	Total Phenolic Content	$IC_{50,\ ABTS}$
$IC_{50,\ ABTS}$	-0.793	
$IC_{50,\ DPPH}$	-0.661	0.946*

*represents a significant difference ($p<0.05$)

3.3.4 Catechins, Theaflavin, Thearubigins, Gallic Acid, Caffeine and Theanine Contents

Phenolic profile including four main flavanols (epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate), two condensed phenolics (theaflavin and thearubigins) and one phenolic acid (gallic acid) was investigated to characterize instant powders. In addition, the contents of caffeine and theanine were also measured to specify instant black tea samples.

3.3.4.1 Catechins

Catechins, which are the subgroup of flavanols in black tea, have drawn attention due to the functional features such as their potential health benefits, especially antioxidant and anti-cancer properties (Yang & Wang, 2016). Therefore, epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG) were quantified to investigate the effects of black tea waste fibers as raw materials and the process journey of instant powders on the composition of major catechins in the end products (Yang & Liu, 2013).

The contents of EC, ECG, EGC and EGCG in instant black tea powders were summarized in Table 3.11.

Table 3.11 Epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate contents of instant teas

Sample	Infusion		Flavanols			
	Temperature (℃)	Time (min)	EC (mg/100g)	ECG (mg/100g)	EGC (mg/100g)	EGCG (mg/100g)
HI	100	6	3313.50 ± 69.623^{a}	353.94 ± 13.436^{a}	1499.55 ± 6.506^{a}	241.52 ± 17.301^{a}
HIG			2231.99 ± 58.455^{c}	268.26 ± 6.481^{b}	1069.19 ± 65.818^{bc}	237.73 ± 21.733^{a}
HIS			2741.11 ± 58.455^{b}	275.20 ± 15.549^{b}	1134.00 ± 42.631^{b}	262.30 ± 7.099^{a}
CI	25	120	2856.78 ± 148.963^{b}	278.60 ± 10.200^{b}	967.23 ± 78.474^{cd}	154.12 ± 13.903^{b}
CIG			2389.55 ± 6.365^{c}	255.09 ± 5.870^{bc}	917.84 ± 10.388^{d}	152.68 ± 12.251^{b}
CIS			2391.34 ± 10.100^{c}	238.37 ± 3.727^{c}	934.95 ± 20.802^{d}	144.45 ± 11.050^{b}

Different letters in the same column represents a significant difference ($p<0.05$)

The predominant catechin of instant teas was EC in the range of 2231.99-3313.50 mg/100 g. It was followed by EGC (917.84-1499.55 mg/100 g), ECG (238.37-353.94 mg/100 g) and EGCG (144.45-262.30 mg/100 g). Alasalvar et al. (2013) also measured the catechin contents of instant teas processed from low- and high-quality black teas in Turkey. EC contents in the present research were higher than those reported in the study of Alasalvar et al. (2013), as 889.00 and 524.00 mg/100 g, respectively. However, EGC contents of low- and high-quality instant black teas (3460.00 and 2004.00 mg/100 g) were higher than in the current research. In addition, ECG contents (428.00 and 315.00 mg/100 g) were also found to be higher, except for HI. On the other hand, EGCG levels were reported between the hot and cold infused instant teas (176.00 and 173.00 mg/100 g). Instant black teas were also found to have similar catechin contents to conventional black tea samples from different locations in the literature, could be seen in Table 3.12. EC of instant powders deserved special attention because they were higher than those reported in the studies although a clear trend could not be mentioned for other catechins (Table 3.12).

The infusion conditions of PGBM significantly affect the catechin contents of the instant powders ($p<0.05$). In addition, the mixing instant teas with ginger and sage using superfine grinding and the interaction between the parameter had statistically significant meaning on the catechins ($p<0.05$), except for EGCG ($p>0.05$), the least abundant catechin in instant black teas.

Instant teas produced by freeze-drying hot infusion of PGBM had significantly higher catechin contents than those produced by the cold infusion method, as expected from the results of the antioxidant capacities and the total phenolic compounds of powders ($p<0.05$). This was due to the increasing solubility and extraction rate of catechins with temperature (Yang et al., 2007; Pastoriza et al., 2016). The outcomes of the related study conducted by Yang et al. (2007) further corroborated the impact of infusion at high temperatures in a short time. They found

that the extraction of catechins in black tea, purchased in Taiwan, was higher at 100 ℃ for 4 min than at 25 ℃ for 16 h.

Instant tea powders without ginger and sage had markedly higher EC, ECG and EGC because of the dominant catechin ingredient of tea over herbs ($p<0.05$). On the other hand, instant teas, regardless of herb addition, had similar EGCG, the least abundant catechin in the samples ($p>0.05$). Moreover, HIS had more EC than HIG ($p<0.05$), although there was no difference between the samples mixing with herbs for others ($p>0.05$). A considerable amount of EC in sage was also drawn to attention by Rababah et al. (2011).

Table 3.12 Epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate contents of black teas in the literature

Number of black tea samples	Origin	Epicatechin (EC)	Epicatechin gallate (ECG)	Epigallocatechin (EGC)	Epigallocatechin gallate (EGCG)	Reference
			mg/100 g			
1	Taiwan*	117.00	193.00	1083.00	242.00	(Lin et al., 2003)
32	USA*	30.00-630.00	170.00-2680.00	0-1030.00	50.00-2840.00	(Friedman et al., 2005)
15	China*	121.00	359.00	310.00	346.00	(Wang et al., 2011)
7	Turkey	61.90-83.90	89.50-115.00	1038.00-1214.00	102.00-155.00	(Serpen et al., 2012)
1	South Korea*	362.86	456.86	817.36	1436.31	(Tong et al., 2019)
			mg/100 g dry matter			
17	Assam, China, Ceylon, Kenya, India	201.00-1004.00	214.00-767.00	502.00-2203.00	304.00-1540	(Fernández et al., 2000)
12	UK*	140.00-560.00	50.00-860.00	20.00-630.00	270.00-2520.00	(Khokhar & Magnusdottir, 2002)
12	Turkey	263.00-340.00	418.00-541.00	627.00-791.00	719.00-1007.00	(Ozdemir et al., 2018)

*purchased from local markets

3.3.4.2 Theaflavin and Thearubigins

Catechins were oxidized to condensed phenolics by a series of biochemical reactions during the fermentation step of black tea production (Kelebek, 2016). Theaflavins, the first stable oxidation products, undergo further oxidization to generate more polymerized compounds, thearubigins (Samanta et al., 2015).

Theaflavin and thearubigins play a crucial role in the characteristic features of black tea because they are responsible for reddish color and astringent flavor (Serpen et al., 2012). Ozdemir et al. (2018) indicated that a higher concentration of thearubigins was to be expected with decreasing theaflavin content. Therefore, the balance between condensed phenolics, i.e., the ratio of theaflavin to thearubigins (TF:TR), is considered a quality parameter of black tea. The optimum value should be 0.08-0.11 for good quality tea (Hossain et al., 2022; Gill et al., 2011; Someswararao & Srivastav, 2012). On the other hand, the antioxidant and anticancer properties of condensed phenolics also make an appearance of their values (Imran et al., 2018; Luo and Jiang, 2021).

The contents of theaflavin and thearubigins of instant black tea powders were 79.77-243.35 mg/100 g and 892.92-2574.36 mg/100 g, respectively, reported in Table 3.13. In addition, TF:TR was determined in the range of 0.08-0.10. Alasalvar et al. (2013) found theaflavin levels of instant tea samples from low-quality black teas originating in Turkey as 56.00 mg/100 g, lower those in the current research, and from high-quality black teas as 124.00 mg/100 g, between the values of instant teas produced by the hot and cold infusion methods. Theaflavin contents of instant tea, originating in India, also was between those which the infusion methods had, as 165.00 and 175.00 mg/100 g (Someswararao & Srivastav, 2012). In addition, lower theaflavin content of conventional black tea purchased in South Korea was measured as 74.06 mg/100 g in the study of Tong et al. (2019). Moreover, similar theaflavin contents were also observed in the conventional seven black teas, originating in Turkey, as 121.00-399 mg/100 g (Serpen et al., 2012) and thirty-two black teas, purchased in

the USA, as 0-540 mg/100 g (Friedman et al., 2005). On the other hand, thearubigins levels were detected in instant black tea samples originating in Turkey were 10161 and 12701 mg/100 g (Alasalvar et al., 2013) and originating in India were 1973 and 4526 mg/100 g (Someswararao & Srivastav, 2012), as well as in conventional black teas originating in Turkey were 5920.00-6830 mg/100 g for seven samples (Serpen et al., 2012) and were 8580-11970 mg/100 g for twelve samples (Ozdemir et al., 2018). Although TF:TR of instant black tea samples were in the range of good quality black tea according to the literature, thearubigins levels were dramatically lower than those in other studies in general. The raw material used in the current research, black tea waste fiber instead of the end product of any company, was considered the reason for the lower thearubigins contents of instant tea powders.

The infusion conditions for the instant powders and the mixing with ginger and sage using superfine grinding markedly affect the contents of condensed phenolics in the instant teas ($p<0.05$). For instant teas produced by the same infusion method, the contents of theaflavin and thearubigins in teas without herbs were higher as expected ($p<0.05$). Moreover, for instant teas with the same formulation, those produced by freeze-drying hot infusion had higher condensed phenolics levels ($p<0.05$) due to the more permeability of cell walls and increasing solubility of compounds at higher temperatures (Vuong et al., 2010).

3.3.4.3 Gallic Acid

Gallic acid is a crucial phenolic of black tea due to its strong antioxidative nature (Ntezimana et al., 2021). In addition, there were several studies that observed the effective defense of this simple nutraceutical compound against cancer cells (Shojaei-Zarghani et al., 2021). The gallic acid composition of instant teas, in the range of 738.37-1115.80 mg/100 g, was shown in Table 3.13. Alasalvar et al. (2013) indicated that gallic acid was the only phenolic acid identified in instant black teas

originating in Turkey. The contents of gallic acid in the study, 697.00 and 751.00 mg/100 g for instant teas produced from high- and low-quality black teas, respectively, were also lower than those reported in the current research, except for HIS. Moreover, five different conventional black teas originating in China, India and Sri Lanka were found to have lower gallic acids (250.00-450.00 mg/100 g) than instant powders (Cabrera et al., 2003). Furthermore, even the gallic acid contents of black tea samples, 96.80-116.00 mg/100 g, originating in Turkey in the study of Serpen et al. (2012) were almost 10-fold lower than instant black teas produced from PGBM.

The infusion conditions for the instant teas and the mixing with herbs using superfine grinding significantly affect the gallic acid contents of the powders ($p<0.05$). There was also an interaction between the infusion conditions and the mixing instant teas with ginger and sage ($p<0.05$).

Instant teas without herbs, regardless of the hot and cold infusion methods ($p>0.05$), had higher gallic acids than teas mixed with ginger and sage ($p<0.05$) due to the dominant gallic acid ingredient of black tea over herbs. In addition, for instant black teas mixed with herbs, the contents of gallic acid in powders produced by freeze-drying cold infusion of PGBM were higher ($p<0.05$). Instant teas containing ginger had also more gallic acids than those mixed with sage although this was significant only for instant black teas produced by the hot infusion method ($p<0.05$). A corroborating conclusion could be drawn from the other studies, where Ghasemzadeh & Ghasemzadeh (2011) quantified gallic acid, the most abundant phenolic acid in ginger, for different varieties of ginger as 176.29 and 186.65 mg/100 g dry matter, while Rababah et al. (2011) found a lower gallic acid content for sage, 9.10 mg/100 g.

3.3.4.4 Caffeine

Caffeine, the predominant purine alkaloid in black tea, is a potential functional compound to enhance the health benefits of tea products, especially their antioxidative and anticancer activities (Cadoná et al., 2022; Endesfelder et al., 2017; Yang et al., 2023). Caffeine levels of instant tea powders, varied from 2791.42 to 3415.36 mg/100 g, were demonstrated in Table 3.13. Instant teas produced from high- and low-quality black teas originating in Turkey were measured to have higher caffeine contents, 3964 and 4398 mg/100 g, respectively, in the study of Alasalvar et al. (2013) than those found in the present research. On the other hand, there were several studies published in the literature where the contents of caffeine in conventional black tea samples were found to be 2206.99 mg/100 g for a black tea purchased in South Korea (Tong et al., 2019), 1525–1809 mg/100 g for seven black teas originating in Turkey (Serpen et al., 2012), 1348–2344 mg/100 g for eleven black teas purchased in Hungary (Boros et al., 2016) and 350–2670 mg/100 g for thirty-two black teas purchased in the USA (Friedman et al., 2005).

The infusion conditions for the instant teas and the mixing with herbs using superfine grinding markedly affect the contents of caffeine in powders ($p<0.05$). For instant teas with the same formulation, those produced by freeze-drying cold infusion had higher caffeine levels, even though this was significant only for instant black teas containing ginger ($p<0.05$). It could be drawn conclusion that the long extraction time could compensate for the rate limitation of mass transfer in the cold extraction of caffeine (Fuller & Rao, 2017). Damiani et al. (2014) also observed similar behavior in eight white tea samples from Africa and China in their study, where the extraction of teas at 25 ℃ for 2 hours resulted in higher caffeine levels than hot infusion in a short time extraction (at 70 ℃ for 7 min). Moreover, for instant teas produced by the same infusion method, as expected, the contents of caffeine in teas without herbs were higher ($p<0.05$), except statistically for CIG ($p>0.05$). On the other hand, instant black teas, produced by the same infusion method, mixed with herbs had similar caffeine contents ($p>0.05$).

3.3.4.5 Theanine

Theanine, also known as L-theanine since it occurs in nature as L-enantiomeric form, is a unique non-protein amino acid in tea (Yang et al., 2023). It was stated that theanine is the most abundant free amino acid in tea, even more than 50% (Mu et al., 2015). The reputation of theanine comes from the health benefits, especially anticancer potentials, as well as the specific flavor contribution as umami taste (Shojaei-Zarghani et al., 2021). The theanine content of instant black teas, in the range of 891.94-1367.73 mg/100 g, could be seen in Table 3.13. Instant teas produced from high- and low-quality black teas, originating in Turkey, in the study of Alasalvar et al. (2013) had lower theanine contents (512.80 and 572.20 mg/100 g, respectively) than reported in the current research. In addition, a conventional black tea purchased in Hungary was found to have lower theanine content as 513.00 mg/ 100 g (Boros et al., 2016). Two black tea samples from Cameroon and Sri Lanka had also lower theanine contents (480 and 520 mg/100 g dry matter, respectively), while the content of theanine in the four black tea samples from Kenyan, Rwandan and Ugandan (900-1900 mg/100 g dry matter) were similar with studied instant teas (Too et al., 2016).

The infusion conditions for the instant teas and the mixing with herbs using superfine grinding significantly affect the contents of theanine in instant tea powders ($p<0.05$). There was also an interaction between the infusion conditions and the mixing instant black tea powders with ginger and sage ($p<0.05$).

Instant tea without herbs had higher theanine than those mixed with herbs ($p<0.05$). Moreover, for instant teas with the same formulation, those produced by freeze-drying hot infusion had higher theanine levels ($p<0.05$). On the other hand, instant black teas, produced by the same infusion method, mixed with herbs had similar theanine contents ($p>0.05$).

Before concluding the discussions regarding the contents of catechins, theaflavin, thearubigins, gallic acid, caffeine and theanine in instant black teas produced from

PGBM, it was crucial to state that the variations in all these bioactive compounds were attributed to the differences in variety, growing environment, cultivation method, process conditions as well as extraction parameters as mentioned before in the sections of antioxidant capacity and total phenolic contents (Boros et al., 2016; Jian et al., 2020; Lin et al., 2003; Ozdemir et al., 2018).

Table 3.13 Theaflavin, thearubigins, gallic acid, caffeine and theanine contents of instant teas

Sample	Infusion		Condensed phenolics		Phenolic acid	Alkaloid	Free amino acid
	Temperature (°C)	Time (min)	Theaflavin (mg/100g)	Thearubigins (mg/100g)	Gallic Acid (mg/100g)	Caffeine (mg/100g)	Theanine (mg/100g)
HI	100	6	243.35 ± 2.704^{a}	2574.36 ± 33.342^{a}	1115.80 ± 13.638^{a}	3294.70 ± 201.462^{ab}	1367.73 ± 0.753^{a}
HIG			218.93 ± 5.408^{b}	2037.80 ± 62.804^{b}	888.33 ± 20.153^{c}	2791.42 ± 120.325^{d}	1149.92 ± 19.482^{b}
HIS			213.61 ± 8.879^{b}	2101.98 ± 42.899^{b}	738.37 ± 19.015^{d}	2954.68 ± 107.095^{cd}	1183.53 ± 2.372^{b}
CI	25	120	119.87 ± 1.561^{c}	1587.14 ± 75.551^{c}	1088.63 ± 24.982^{a}	3415.36 ± 69.779^{a}	1186.37 ± 30.657^{b}
CIG			82.92 ± 6.244^{d}	908.32 ± 90.879^{d}	974.81 ± 0.339^{b}	3134.19 ± 60.594^{abc}	891.94 ± 7.896^{c}
CIS			79.77 ± 1.352^{d}	892.92 ± 59.165^{d}	947.21 ± 5.531^{b}	3035.36 ± 69.779^{bcd}	900.12 ± 10.301^{c}

Different letters in the same column represents a significant difference ($p<0.05$)

3.4 Physical Analysis of Instant Teas Enriched with Ginger and Sage

Moisture content, hygroscopicity, color and morphological profiles of instant tea powders, as well as solubility, turbidity and color profiles of their infusions were investigated to evaluate the end products in terms of tea quality parameters.

3.4.1 Moisture Content

Moisture content is the crucial quality parameter of food powders because it has a voice in susceptibility to microbial damage and sensory properties of the end product (Li, 2022). The upper limit of the moisture content in black tea products produced in Turkey was determined as 7% (Turkish Food Codex, 2015). In addition, Othieno and Owuor (1984) indicated the black tea products had a moisture content of higher than 6.5% sensitive to chemical and microbiological deterioration. On the other hand, they also stated that the moisture content of lower than 2.5% result in black tea having a burnt flavor because of excessive drying.

The moisture contents of instant tea powders were in the range of 4.37-6.32%, shown in Table 3.14. Ozdemir et al. (2018) also found that conventional black tea samples, originating in Turkey, from different altitudes, shooting periods and grades had a similar moisture content of 4.78-5.45%.

While there was no effect of infusion methods ($p>0.05$), the addition of herbs using superfine grinding significantly increased the moisture contents of instant powders ($p<0.05$). The reason for this expected result was that although instant tea powders were put in a freeze dryer to have a final moisture content of 4.0-4.5%, ginger and sage were exposed to only pulverization and sieving methods during all production journey of enriched teas. In other words, the increasing effects of herbs on the moisture content of instant teas were due to their high inherent moisture contents.

Table 3.14 Moisture content values of instant teas enriched with ginger and sage

Sample	Infusion		Moisture Content (%)
	Temperature (°C)	Time (min)	
HI	100	6	4.45 ± 0.131^{b}
HIG			6.32 ± 0.235^{a}
HIS			5.86 ± 0.193^{a}
CI	25	120	4.37 ± 0.195^{b}
CIG			6.18 ± 0.976^{a}
CIS			5.91 ± 0.116^{a}

Different letters in the same column represents a significant difference ($p<0.05$)

3.4.2 Hygroscopicity

Hygroscopicity, the ability to absorb water vapor from an environment with a higher relative humidity, is the critical subject of tea powder because of its physical, chemical and microbiological effects on the stability (Ocieczek et al., 2023). Dried herbal materials are considered a highly hygroscopic food matrixes due to the low moisture content and the presence of hydrophilic compounds including carbohydrates, organic acids, phenolics and amino acids, which form hydrogen bonds with water molecules (Chen et al., 2009; Phanındrakumar et al., 2005; Vladić et al., 2016; Zokti et al., 2016). In addition, freeze dried instant teas also have further potential to exhibit greatly hygroscopic behaviors due to their amorphous, i.e., disordered, structures (Moghaddam et al., 2017).

The hygroscopicity values of instant powders, in the range of 17.33-22.58%, were demonstrated in Table 3.15. The mixing with herbs using superfine grinding, unlike

the infusion conditions ($p>0.05$), markedly affect the hygroscopicity values of instant black teas ($p<0.05$). The hygroscopicity values of instant powders without herbs were higher than those containing ginger and sage ($p<0.05$). In addition, there was no significant difference between the addition of ginger and sage in the hygroscopicity values ($p>0.05$). The reason for the reducing effect of herbs on the hygroscopicity of fully water-soluble instant teas was their structure, including cross-linking of insoluble dietary fibers such as cellulose, hemicellulose, and lignin (Chang et al., 2014; Lu et al., 2022; Wang et al., 2020).

Table 3.15 Hygroscopicity values of instant teas enriched with ginger and sage

Sample	Infusion		Hygroscopicity (%)
	Temperature (℃)	Time (min)	
HI	100	6	22.14 ± 0.425^{a}
HIG			17.33 ± 0.582^{b}
HIS			17.75 ± 0.337^{b}
CI	25	120	22.58 ± 0.552^{a}
CIG			18.12 ± 1.490^{b}
CIS			18.44 ± 0.103^{b}

Different letters in the same column represents a significant difference ($p<0.05$)

3.4.3 Pearson Correlation Coefficient for Moisture Content and Hygroscopicity

Pearson correlation analysis was conducted on the moisture content and hygroscopicity of instant teas, and Pearson correlation coefficients were presented in Table 3.16.

Moisture contents of instant powders were negatively, highly and significantly correlated with hygroscopicity values ($r=-0.924$, $p<0.05$). This shows that the herbs acted as moisture binders thereby reducing the tendency of the instant teas to absorb water vapor from the environment (Zokti et al., 2016). In other words, higher initial moisture content of instant teas containing herbs results in reducing the ability to absorb water because of lower driving force for the mass transfer (Mohammadi & Nokken, 2013).

Table 3.16 Pearson correlation coefficients regarding moisture content and hygroscopicity

	Moisture Content
Hygroscopicity	-0.924*

*represents a significant difference ($p<0.05$)

3.4.4 Color Measurements of Instant Tea Powders

Color is the important quality determinant for tea products since it directly affects consumer acceptance (Kelebek, 2016). The CIE (International Commission on Illumination) L*, a*, b* system was commonly used to evaluate color characteristics of tea leaves and infusions. L*, a* and b* represent the intervals of lightness as dark

to white as well as color as green to red and as blue to yellow, respectively (Mao et al., 2021).

L*, a* and b* values of instant black tea powders were presented in Table 3.17. The infusion conditions for the instant teas significantly affect the color parameters of tea powders ($p<0.05$). In addition, the mixing with herbs using superfine grinding had a marked impact on L*, a* and b* values ($p<0.05$) despite herbs had similar effects ($p>0.05$).

Table 3.17 L*, a* and b* values of instant tea powders enriched with ginger and sage

Sample	Infusion		L*	a*	b*
	Temperature (℃)	Time (min)			
HI	100	6	16.60 ± 0.361^{c}	11.37 ± 0.058^{b}	$17.80\ 1.000^{d}$
HIG			17.17 ± 0.924^{c}	13.43 ± 0.346^{a}	22.63 ± 1.332^{a}
HIS			17.80 ± 0.265^{bc}	13.10 ± 0.808^{a}	21.33 ± 0.289^{ab}
CI	25	120	18.57 ± 0.058^{ab}	10.00 ± 0.346^{c}	15.27 ± 0.351^{e}
CIG			19.43 ± 0.208^{a}	11.23 ± 0.058^{b}	20.07 ± 0.379^{bc}
CIS			19.73 ± 0.252^{a}	11.07 ± 0.153^{b}	19.17 ± 0.379^{cd}

Different letters in the same column represents a significant difference ($p<0.05$)

Lower L* values, i.e., darker color, of instant tea powders produced by freeze-drying hot infusion of PGBM ($p<0.05$) could be attributed to the higher extraction of complex phenolic compounds, especially colored oxidation products, theaflavin and thearubigins (Dong et al., 2018; Kelebek, 2016). It was also confirmed in Section 3.4.5 with the negative, high and significant Pearson correlation coefficients ($p<0.05$). Moreover, Lantano et al. (2014) observed a similar pattern in their study,

in which the solution of black tea, purchased in Italy, produced by the cold infusion in a long time had lighter color than those produced by the hot infusion in a short time.

Higher a* and b* values, i.e., redder and yellower colors, respectively, of instant powders obtained by the hot infusion method ($p<0.05$) were also considered the response of higher concentrations of theaflavin having yellow-orange color and thearubigins having red-brown color (Kelebek, 2016; Mao et al., 2021; Polat et al., 2022). However, it could not be observed in the results of Pearson correlation coefficients ($p>0.05$). The reason for this was suggested to be that the different effects of ginger and sage on the parameters complicated the situation and suppressed the correlation coefficients on a* and b* values caused by the infusion methods. Reddish and yellowish color pigments in herbs, curcuminoids of curcumin, desmethoxycurcumin and bisdemethoxycurcumin in ginger and carotenoids of lutein, β-cryptoxanthin and β-carotene in sage, enhanced the density of red and yellow colors of powders (Iijimah & Joh, 2014; Maiani et al., 2008).

3.4.5 Pearson Correlation Coefficients for L*, Theaflavin and Thearubigins

Pearson correlation analysis was conducted on the color L*, theaflavin and thearubigins values of instant teas, and Pearson correlation coefficients were presented in Table 3.18.

Table 3.18 Pearson correlation coefficients regarding L*, theaflavin and thearubigins

	Theaflavin	Thearubigins
L*	-0.973*	-0,977*

*represents a significant difference ($p<0.05$)

3.4.6 Morphology

Scanning electron microscopy (SEM) is one the most effective method for structural morphology analyses to examine size, shape and porosity of biocompounds (Patel et al, 2018). Therefore, it was used to investigate the surface morphology characteristics of instant tea powders, which revealed differences in their microscopic structure based on the infusion methods, as shown in Figure 3.1-2.

Instant black tea powders produced by freeze drying hot infusion of PGBM had irregular shaped smooth lamellar and flat-like structures (Figure 1) (Parvez et al., 2022). On the other hand, cold-infused instant tea powders had the sight of irregular, coarse and cloudlike (Figure 2). In addition, instant teas produced by the cold infusion method were agglomerated, although isolated microparticles were observed in the hot infused tea powders. The reason for these distinctions in the structures of instant tea powders produced by different infusion methods was attributed to higher extraction temperature of the hot infusion method, i.e., more aggressive extraction condition. High extraction temperature resulted in the modification in the structures of tea compounds such as epimerization of catechins (Vuong et al., 2011). Moreover, the composition of produced instant tea powders was different due to the variation in the extraction capabilities of the infusion methods, as mentioned before (Lantano et al., 2015).

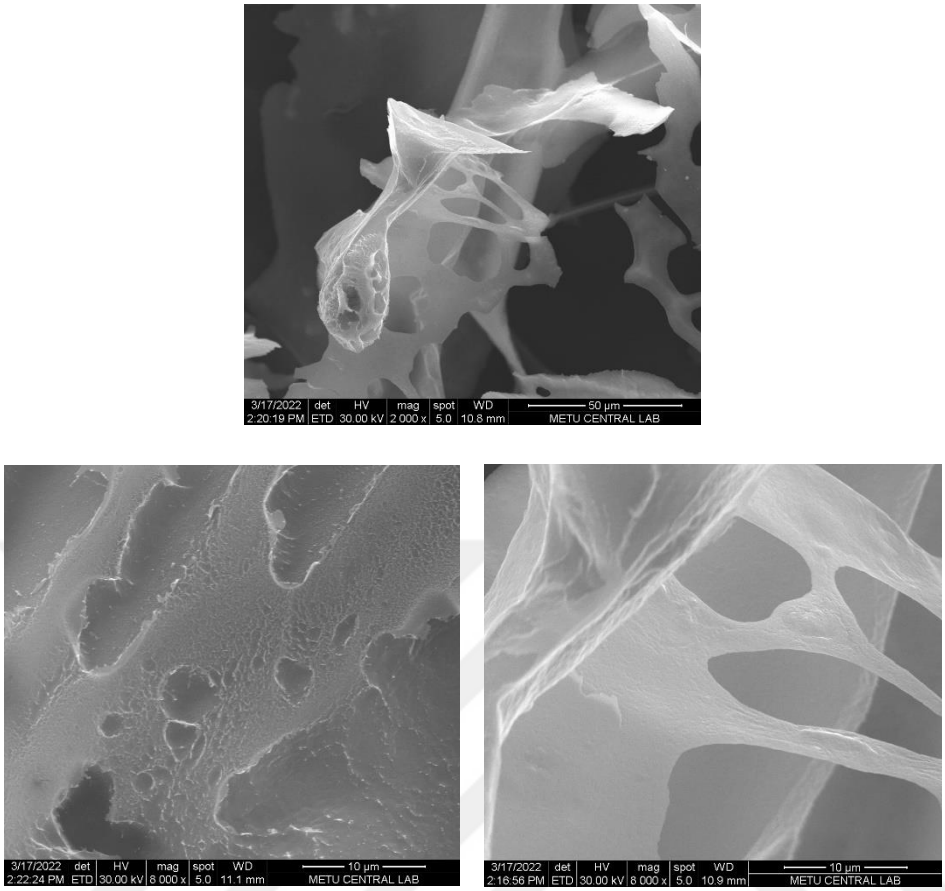


Figure 3.1 Scanning electron microscopy images of hot infused instant black tea powders.

Holes and channel-like structures in the instant tea powders, the common morphological properties of freeze-dried powders, also represented the location of ice-crystals which were formed during freezing of tea infusions (Millqvist-Fureby et al., 1999).

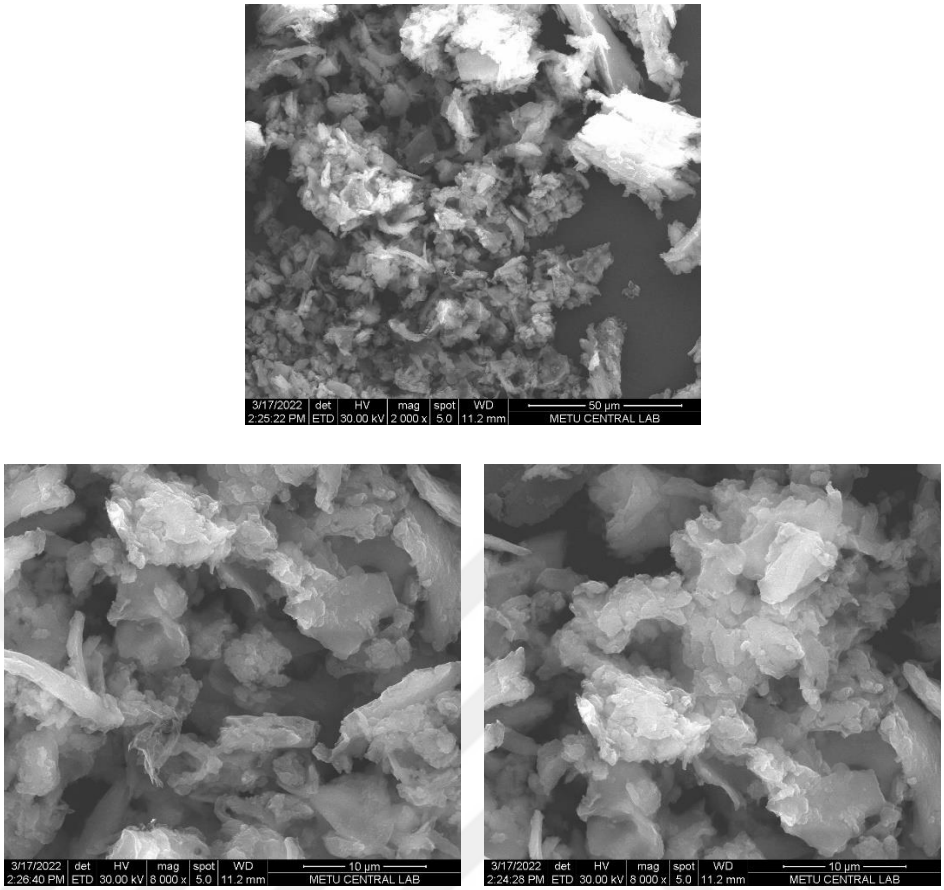


Figure 3.2 Scanning electron microscopy images of cold infused instant black tea powders.

3.4.7 Solubility

The investigation of water solubility in powders has yielded valuable insights into the qualification and convenience of instant tea products. Solubility values of instant black teas, in the range of 77.31-95.93%, could be seen in Table 3.19.

Tea cream formation, resulting from the complexation of phenolic polymers with other tea compounds such as polysaccharides, proteins, lipids and metal cations, is the main concern regarding the solubility of tea products because these spontaneous

chemical interactions upon the cooling of tea infusion cause cloudy and haze appearance (Bindes et al., 2019; Kim & Talkott, 2012; Quin et al., 2022). Although polyphenols, theaflavins and organic acids are also considered major components responsible for tea cream formation, caffeine is drawn special attention due to the ability to form strong self- and hetero- interactions with other compounds to generate cocrystals and complexes having distinct molecular weight, solubility and stability potentials (Bindes et al., 2019; Syed et al., 2019). In addition, tea cream formation in the infusions depends on the temperature, concentration and chemical composition of the tea products (Jöbstl et al., 2005; Syed et al., 2019). Similarly, in the current research, the infusion conditions for the instant teas and the mixing with ginger and sage using superfine grinding significantly affect the solubility of instant powders ($p<0.05$). Moreover, instant teas produced by freeze-drying hot infusion of black tea waste fibers had outstanding solubility properties even though this was significant only in the case of the instant teas produced without herbs ($p<0.05$). It could be attributed to the higher caffeine levels of those produced by freeze-drying cold infusion, for instant teas with the same formulation, as mentioned in Section 3.3.4.4. Although it was not significantly higher in statistic ($p>0.05$), it still could be meaningful within the context of solubility. Furthermore, the higher content of epicatechin in the hot infused instant teas could also enhance the solubility of powders because Syed et al. (2019) found that catechins without gallate structure increases the solubility of caffeine in the tea infusions. Furthermore, morphological structures of instant teas also affect the solubility of powders. Agglomerated clusters, observed in cold infused teas in Section 3.4.6, could decrease the solubility due to reduced surface area and porosity, limiting the penetration of solvent (Chen et al., 2017).

For instant teas produced by the same infusion method, on the other hand, the solubility of teas without herbs were higher ($p<0.05$). The reason for this expected result was the content of insoluble dietary fiber, i.e., non-starch polysaccharides, including cellulose, hemicellulose and lignin in the plants (Wang et al., 2020). In addition, ginger and sage containing teas had similar solubility values ($p>0.05$).

Table 3.19 Solubility values of instant teas enriched with ginger and sage

Sample	Infusion		Solubility (%)
	Temperature (℃)	Time (min)	
HI	100	6	95.93 ± 1.744^{a}
HIG			83.08 ± 0.973^{c}
HIS			79.38 ± 2.905^{cd}
CI	25	120	89.01 ± 0.817^{b}
CIG			80.59 ± 1.621^{cd}
CIS			77.31 ± 0.837^{d}

Different letters in the same column represents a significant difference ($p<0.05$)

3.4.8 Turbidity

Turbidity is the measure of cloudiness and haziness, i.e., relative clarity from another point of view, of tea infusions caused by the presence of suspended particles (Yu and He., 2022). Therefore, the precipitated complex substances, known as tea cream, during the cooling of infusions, mentioned in Section 3.4.7, are considered the main concern regarding the turbidity of black tea solutions (Bindes et al., 2019; Kim & Talkott, 2012).

The turbidity values of instant teas, in the range of 140.67-313.00 FNU, were shown in Table 3.20. The infusion conditions for the instant powders and the mixing with herbs using superfine grinding markedly affect the turbidity values of the instant teas ($p<0.05$). There was also an interaction between the infusion conditions and the mixing of instant black tea powders with ginger and sage ($p<0.05$).

Table 3.20 Turbidity values of instant teas enriched with ginger and sage

Sample	Infusion		Turbidity (FNU)
	Temperature (℃)	Time (min)	
HI	100	6	140.67 ± 2.307^{e}
HIG			237.67 ± 10.017^{c}
HIS			293.67 ± 7.095^{b}
CI	25	120	176.00 ± 7.000^{d}
CIG			280.67 ± 6.351^{b}
CIS			313.00 ± 2.646^{a}

Different letters in the same column represents a significant difference ($p<0.05$)

To reveal the high haziness issue of instant teas, the turbidity of infusion from black tea leaves, the end product of the same tea company as raw material of instant teas, was measured as 47.89 FNU. The reason behind the substantially higher turbidity of instant teas could be explained by the production journey of powders. After the extraction of black tea waste fibers, the infusions were frozen in the freezer at -18 ℃ prior to freeze-drying. At the drying stage, tea cream, originated from reactive agents in the tea infusions during the cooling period, was also dried. The formation of tea cream was induced by cold storage and increased at refrigerator temperature was hypothesized by Tim & Talcott (2012). It could also explain why those produced by freeze-drying cold infusion had higher turbidity levels for instant teas with the same formulation ($p<0.05$).

Turbidity values of instant teas were increasing in the order of without herbs, containing ginger and containing sage for the same infusion method ($p<0.05$). Denser turbidity of tea powders with herbs resulted from the insoluble constituents

of plants ($p<0.05$). In addition, the higher particle size range of sage, as mentioned in Section 3.2, the higher turbidity value ($p<0.05$).

The relationship between the solubility and turbidity values of instant teas was also confirmed in Section 3.4.9.

3.4.9 Pearson Correlation Coefficient for Solubility and Turbidity

Pearson correlation analysis was conducted on the solubility and turbidity values of instant teas, and Pearson correlation coefficients were presented in Table 3.21.

Solubility of instant powders were negatively, highly and significantly correlated with turbidity ($r=-0.983$, $p<0.05$) because the decreasing in the solubility of tea components gave rise to increase cloudiness and haziness of solutions. In other words, the more insoluble particles in the solution, the more scattering the light and turbid appearance (Chandini et al., 2013).

Table 3.21 Pearson correlation coefficients regarding solubility and turbidity

	Solubility
Turbidity	-0.983*

*represents a significant difference ($p<0.05$)

3.4.10 Color Measurements of Instant Tea Infusions

Color is one of the primary quality parameters for black tea products, as drawn attention in Section 3.4.4. Therefore, color measurements were also conducted on

the infusions of instant black tea powders, and the results were summarized in Table 3.22.

Unlike instant tea powders, L*, a* and b* values of instant tea solutions went against the theory of tea chemistry in terms of the infusion methods, where the higher the condensed phenolics, the darker and redder the infusions (Dong et al., 2018; Kelebek, 2016; Mao et al., 2021). Instant powders produced by freeze-drying of cold infusion gave rise to solutions having more dark, red and yellow colors, i.e., higher L*, a* and b* values ($p<0.05$). The reason for this unexpected outcome was that turbidity came to the forefront of the solutions and suppressed the impacts of complex compounds (Polat et al., 2022). On the other hand, for the same infusion method, the solutions including ginger and sage had similar L*, a* and b* values ($p>0.05$).

Table 3.22 L*, a* and b* values of instant teas enriched with ginger and sage

Sample	Infusion		L*	a*	b*
	Temperature (℃)	Time (min)			
HI	100	6	29.97 ± 0.058^{a}	0.90 ± 0.000^{c}	1.70 ± 0.100^{c}
HIG			29.27 ± 0.346^{ab}	1.33 ± 0.058^{ab}	2.23 ± 0.115^{ab}
HIS			29.10 ± 0.153^{b}	1.43 ± 0.153^{a}	2.30 ± 0.100^{ab}
CI	25	120	26.03 ± 0.379^{c}	1.17 ± 0.058^{b}	2.13 ± 0.058^{b}
CIG			25.40 ± 0.458^{cd}	1.40 ± 0.000^{a}	2.37 ± 0.252^{ab}
CIS			25.00 ± 0.100^{d}	1.53 ± 0.058^{a}	2.57 ± 0.115^{a}

Different letters in the same column represents a significant difference ($p<0.05$)

3.5 Anticancer Analysis of Instant Teas

There are several studies in which the anticancer activities of valuable tea extracts and biocompounds were demonstrated (Cadoná et al., 2022; Luo and Jiang, 2021; Shojaei-Zarghani et al., 2021; Yang et al., 2023). As a further step on this topic, the cytotoxic potentials of instant tea powders produced by black tea waste fibers were investigated in the current research.

3.5.1 Prostate Cancer Cells (PC3, 22Rv1 and LNCaP)

The cytotoxicity of instant black tea powders was confirmed on the prostate cancer cells, PC3, 22Rv1 and LNCaP. In addition, instant tea powders produced by the hot infusion method were analyzed for confirmation because they were considered to have more anticancer potential due to outstanding antioxidant capacity and extraction ability for beneficial tea compounds, as detailed in Section 3.3.

The cell viabilities of PC3, 22Rv1 and LNCaP cells against hot infused instant black tea powders were presented in Figure 3.3. According to the tetrazolium microculture assay (MTT), it was validated that instant teas resulted in suppression of the cell viability of all studied prostate cancer cells ($p<0.05$). It was also concluded the cell viability of PC3, LNCaP and 22Rv1 cells decreased in a concentration-dependent manner ($p<0.05$). In addition, the prostate cancer cells had different susceptibilities to the anticarcinogenic effects of instant teas over the concentration of 0.1-10 mg/ml ($p<0.05$). The distinct responses of cell lines against the cytotoxicity of instant black tea powders could be attributed to the variation in their inherent aptitudes such as androgen sensitivity (Alserihi et al., 2023).

The most sensitive prostate cancer cell line was determined as the androgen-independent cell of PC3 since its cell viability significantly decreased even at 0.1 mg/ml ($p<0.05$). The markedly decreased cell viabilities of PC3 were 87.85%, 79.13% and 34.27%, respectively. Moreover, the cell viability of 22Rv1, the androgen-independent but sensitive cell line, started to reduce significantly at 1.0 mg//ml, 71.56% and followed with the viability of 13.30% at 10 mg/ml ($p<0.05$). On the other hand, the androgen-dependent prostate cancer cell of LNCaP was considered the most resistant cell line in the current study due to the fact that the growth inhibition ability of instant teas was observed only at the concentration of 10.0 mg/ml as 11.54% ($p<0.05$).

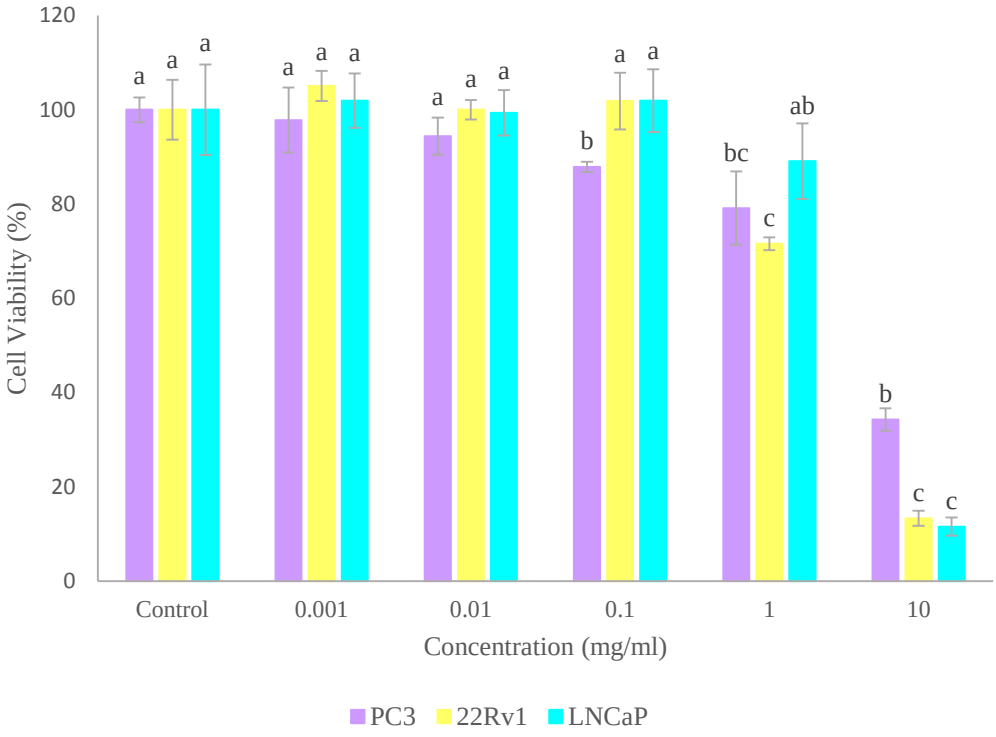


Figure 3.3 The cell viability of prostate cancer cells against instant teas at different concentrations. Different letters at the same concentration represents a significant difference when compared to control ($p<0.05$).

The anticancer potential of EGCG, was considered as the most effective compounds in tea against the survival of cancer cells, was investigated in the study of Sun et al. (2008), in which the concentration-dependent reduction in cell viability of PC3 due to EGCG were verified using the MTT analysis (Miyata et al., 2019). In addition, it was demonstrated in the research of Yang et al. (2009) that the reason for EGCG induced apoptosis and necrosis of PC3 cells was the mitochondrial dysfunction, i.e., mitochondria-mediated pathway. Apoptosis and necrosis are the two main cell death mechanisms, and they represent controlled active autonomous cellular dismantling and passive uncontrolled destruction caused by environmental disturbances, respectively (Fink & Cookson, 2005). In the mitochondria-mediated pathway, the intermembrane proteins such as cytochrome c were released into the cytosol because of the increased permeabilization of the mitochondrial membrane. In addition, the mitochondrial membrane lost its potential, and the synthesis of ATP was inhibited. Sun et al. (2013) also observed the mitochondria-mediated pathway for PC3 cells against two types of black tea extracts, obtained from China and India, and theaflavins. Moreover, in these studies, the activation of caspase 3 (Sun et al., 2013) and caspase 9 (Yang et al., 2009), which are members of the protease family and responsible for apoptosis, was attributed to the presence of EGCG, black tea extracts, and theaflavins. The concentration-dependent reduction in cell viability of PC3 due to gallic acid, which is the essential phenolic acid in instant black tea samples, emerged in the studies of Liu et al. (2011) and Liu et al. (2013). The mechanism under the anticancer activity of gallic acid was explained that it induced DNA damage and also prohibited the DNA repair by modifying DNA repair genes, which are the ataxia telangiectasia mutated, ataxia-telangiectasia and Rad3-related, O^6-methylguanine-DNA methyltransferase, DNA-dependent serine/threonine protein kinase and p53 mRNA expressions (Liu et al., 2013).

Reactive oxygen species (ROS) also cause cell death because of generating oxidative stress in cells, known as ROS-mediated pathway (Villalpando-Rodriguez & Gibson, 2021). ROS-mediated pathway in the cytotoxicity of EGCG was investigated in PC3 and 22Rv1 cell lines in the study of Alserihi et al. (2023). A significant increase of

reactive oxygen species (ROS) formation in PC3 cells was observed, unlike in 22Rv1 cells. In addition, the dramatic reduction in spheroid sizes of cancer cells was also demonstrated in the study. The attenuation of spheroid sizes was the important outcome of the study because it was known that tumors that had larger sizes exhibited higher resistance (Perez et al., 2021). The reduction of spheroid size due to EGCG was indicated as significantly higher in PC3 cells than 22Rv1 cells, similar with the current research, where the higher resistance of 22Rv1 cells than PC3 cells against instant black tea powder was determined. Moreover, the induction of apoptosis in cancer cells because of EGCG was supported in the study. However, unlike the earlier studies (Sun et al., 2013; Yang et al., 2009), a significant loss of mitochondrial membrane potential was not observed in prostate cancer cells because of EGCG in the recently published study. Therefore, they hypothesized that there also could be the mitochondrial independent mechanism of EGCG for cytotoxicity and ROS formation. Siddiqui et al. (2011) also revealed the reduction in cell viability of androgen-sensitive prostate cancer cells of 22Rv1 due to the ability of EGCG to decrease the expression of androgen receptor protein. Furthermore, Kaur et al. (2009) unveiled the anticancer potential of gallic acid on 22Rv1 cells, which was in the concentration-dependent trend, via the MTT assay. They also highlighted that the cytotoxic effect of gallic acid was caused by the induction of apoptosis in 22Rv1 cells.

The concentration-dependent inhibitory effect of EGCG on the cell viability of LNCaP, on the other hand, was found to be stronger than PC3 in the study of Eom et al. (2015), unlike instant black tea powders in current research. Bakhshandeh et al. (2023) also demonstrated that EGCG decreased the proliferation of LNCaP cells in a concentration-dependent manner. Nam et al. (2001) revealed that the ester bond in EGCG was responsible for the anticancer activity of the compound on LNCaP cells because it arrested the proteasome in cell and caused the accumulation of proteasome substrates, $p27^{Kip1}$ and IκB-α, which inhibited the transcription factor NF-κB, plays a crucial factor for cell proliferation and survival (Oeckinghaus & Ghosh, 2009). It was also suggested that EGCG stimulated protein kinase regarding

apoptosis, AMP-activated protein kinase (AMPK), in cells (Bakhshandeh et al., 2023; Chen et al., 2020). Moreover, Lin et al. (2008) investigated the chemoreceptive potentials of black tea theaflavins, which were theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3,3'-digallate, in addition to EGCG on androgen-dependent prostate cancer cell line of LNCaP via the MTT assay. They indicated that all biocompounds had concentration-dependent inhibitory on LNCaP by suppressing the activity of 5α-reductase, the responsible enzyme for the development and progression of prostate cancer cells, although theaflavin-3-gallate was the most effective component. It was also concluded in the study that EGCG and theaflavins prohibited the expression of androgen receptors in LNCaP cells. Furthermore, Russel Jr et al. (2012) confirmed the induction of gallic acid to decrease the cell viability of LNCaP, and they explained the mechanism under the cytotoxicity of gallic acid as a ROS-mediated pathway.

3.5.2 Brain Tumor Cells (U87MG and A172)

After confirming the anticancer potentials of instant black teas on prostate cancer cell lines (PC3, 22Rv1 and LNCaP), the effect of different infusion methods used in the production of instant powders on cytotoxicity was investigated on glioblastoma multiforme (GBM), aggressive brain tumor, cell lines (U87MG and A172).

The cell viability of U87MG and A172 cells against instant black teas produced by freeze-drying hot and cold infusions of black tea waste fibers were demonstrated in Figure 3.4. It was concluded from the results of the MTT assay that both treated instant black tea powders had the concentration-dependent chemoreceptive ability on the aggressive glioma cell lines ($p<0.05$). In addition, the infusion method significantly affected the anticancer activity of instant teas ($p<0.05$). Hot infused instant black teas exhibited a higher reduction in cell viability of U87MG and A172 cells than those produced by the cold infusion method ($p<0.05$), except for U87MG

cells at concentration groups of 5 and 8 mg/ml ($p>0.05$). The reason for this expected result was attributed to the outstanding contents of phenolics such as catechins and theaflavins whose noticeable cytotoxicity was already known, as mentioned in Section 3.3 (Lin et al., 2008; Sun et al., 2013). The higher content of EGCG in hot infused instant black tea powders ($p<0.05$) deserved to draw special attention because it was considered the most effective anticancer-promising agent in tea (Miyata et al., 2019; Wong et al., 2023).

Hot infused instant black tea powders achieved decreased the cell viability of U87MG cells, when compared with control, to 2.46%, 2.17% and 2.54% for the concentrations of 2.5, 5 and 8 mg/ml, respectively, where there was no significant difference ($p>0.05$). On the other hand, the cell viability of A172 cells reduced only to 97.67%, 83.16% and 81.18% ($p>0.05$) for the concentrations of 2.5, 5 and 8 mg/ml, respectively, against instant teas produced by the hot infusion method. In addition, cold infused instant teas could reduce the cell viability of U87MG cells to 2.54% and 2.57% ($p>0.05$) and A172 cells to 91.70% and 90.12% ($p>0.05$) for the concentrations of 5 and 8 mg/ml, respectively.

It should also be highlighted from the outcomes of the MTT assay on brain tumor cancer cells that instant black tea powders had a higher cytotoxic impact on U87MG cells over A172 cells ($p<0.05$). In other words, A172 cells were more resistant to instant tea cytotoxicity than U87MG cells. The difference between the responses of glioma cells to instant teas was based on their variation in genomic basis, such as carrying genetic alterations resulting in adding resistance to glioblastoma cells against treatments (Elhag et al., 2015). U87MG and A172 cells have genetic mutations at the gene of phosphatase and tensin homolog deleted on chromosome ten (PTEN) which is a tumor suppressor gene (Elhag et al., 2015). The principal catalytic function of PTEN, lipid phosphatase, is to dephosphorylate phosphatidylinositol- (3,4,5)-trisphosphate (PIP3), which is the activator of phosphatidylinositol-3 kinase (PI3K) (Hopkins et al., 2014). The PI3K pathway is a crucial signal transduction mechanism that regulates survival and cell growth

(Knowles et al., 2009). Therefore, the mutation of PTEN caused the increment in PIP3 and derepression of the PI3K pathway, which stimulates uncontrolled cell growth and tumor formation (Song et al., 2012). Moreover, A172 cells also have a mutation at another tumor suppressor gene, TP53, which encodes the p53 protein (Elhag et al., 2015). Although the p53 protein is known as the guardian of the genome, the mutation of p53 changes its reputation to that of the guardian of cancer cells (Mantoani et al., 2019). The p53 protein, a transcription factor, is responsible for the regulation of crucial cellular responses such as cell division and differentiation (Marei et al., 2021). When cells are exposed to a variety of stress signals, the p53 protein is activated to stimulate several transcriptional mechanisms including DNA repair, cell cycle arrest and apoptosis, p53-mediated pathway (Mirzayans et al., 2012). Therefore, the mutated p53, which has an oncogenic character, promotes the development of glioma cells through an unrestricted cell cycle and the escape of cancer cells from apoptosis (Marei et al., 2021). Thus, the p53 mutation provides additional resistance to the aggressive glioblastoma cells of A172 against anticancer agents (Elhag et al., 2015).

Das et al. (2010) found the apoptosis induction ability of EGCG in U87MG cells, and they also revealed the mechanism as increasing the level of intracellular ROS. In addition, they stated that EGC and EGCG increased the activation of caspase 8, which stimulated mitochondria-mediated pathway for apoptosis in U87MG cells. Grube et al. (2018) also demonstrated that EGCG led to the concentration-dependent decrease in the cell viability of U87MG cells, and they supported the ROS-mediated pathway that EGCG increased cellular ROS formation in U87MG cells, which caused apoptosis in the glioma cells due to creating oxidative stress. The concentration-dependent reduction in cell viability of U87MG because of EGCG was also observed via the MTT assay in the study of Yokoyama et al. (2000). In addition, Shervington et al. (2009) reported that EGCG inhibited cell proliferation in a concentration-dependent manner and telomerase protein, which is associated with the level of malignancy in glioma cells, of U87MG, ultimately caused apoptosis. On the other hand, gallic acid was also confirmed to decrease the cell viability of

U87MG in a concentration-dependent manner in the recent study by Yang et al. (2022). They also suggested that the anticancer effect of gallic acid was based on the ROS generation independent pathway, and p38 MAPK (mitogen-activated protein kinase) activation, playing a role in various cellular processes such as cell cycle regulation and apoptosis, stimulated by gallic acid could be the apoptosis induction mechanism of U87MG cells. Moreover, Li et al. (2018) showed that the cell viability of U87MG cells was suppressed because of caffeine, following the concentration-dependent trend, through the MTT assay.

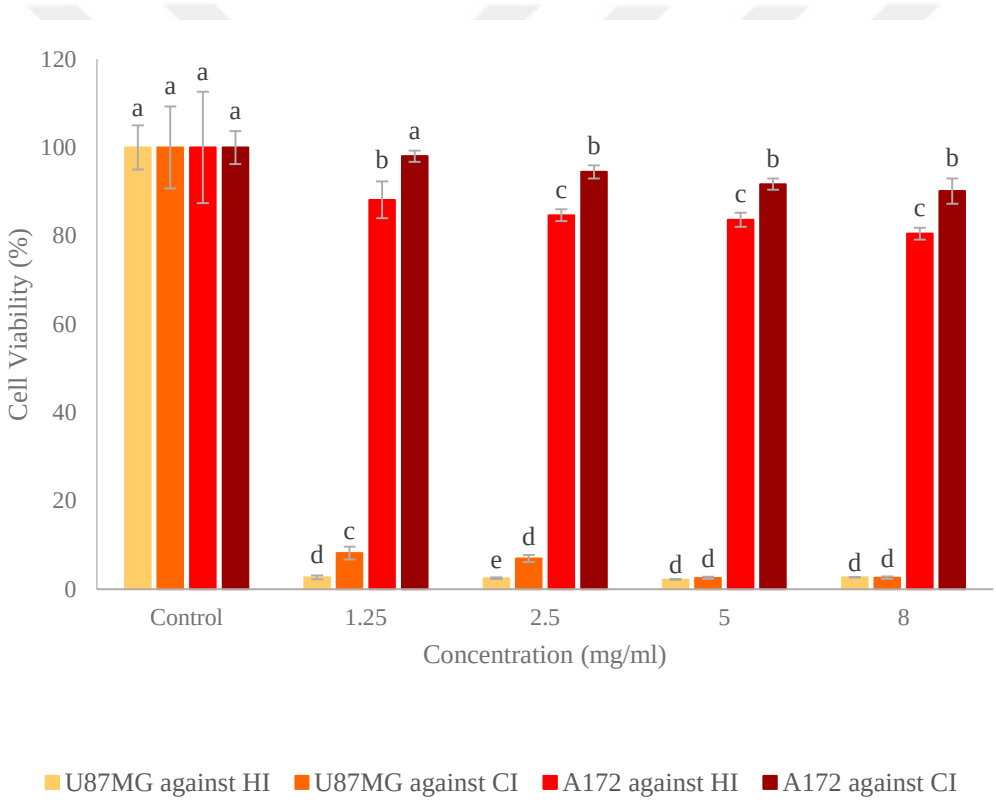


Figure 3.4 The cell viability of glioma cells against instant teas at different concentrations. Different letters at the same concentration represents a significant difference when compared to control ($p<0.05$).

The induction of apoptosis in the aggressive glioblastoma cells of A172 against EGCG, on the other hand, was demonstrated in the study of Siegelin and Gaiser (2008). They found that EGCG enhanced the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated cytotoxicity, also mentioned as TRAIL-induced pathway, in A172 cells by suppressing apoptosis inhibitory proteins of survivin and phosphoprotein enriched in astrocytes (PEA15). The TRAIL was known for its strong apoptosis activator character in cancer cells (Szliszka et al., 2009). It was also indicated that the mutation of p53 was not considered critical in the TRAIL-induced pathway because A172 was found to be susceptible to the TRAIL-induced pathway. In addition, Sachinidis et al. (2000) investigated the cytotoxic effect of tea catechins (GC, ECG and EGCG) on A172 cells. They revealed that the anticancer activity of gallate including catechins on A172 cells was caused by inhibiting the activity of the platelet-derived growth factor receptor β (PDFG-Rβ), which promotes cell proliferation (Heldin & Lennartsson, 2013; Hou et al., 2004; Li et al., 2022).

3.5.3 Breast Cancer Cell (MCF7)

The cytotoxic behaviors of hot and cold infused instant black tea powders, whose anticancer potentials have already been confirmed, were also investigated on the breast cancer cell line MCF7.

The cell viability of MCF7 cells against instant black teas produced by freeze-drying hot and cold infusions of black tea waste fibers were presented in Figure 3.4. According to the results of the MTT assay, both instant tea powders followed the concentration-dependent cytotoxic trend on the breast cancer cell line ($p<0.05$). In addition, the infusion method markedly affected the anticancer activity of instant tea powders ($p<0.05$). Cold infused instant tea powders had a higher reduction in cell viability of MCF7 than hot infused instant tea powders ($p<0.05$), except at the

concentration of 1.25 mg/ml ($p>0.05$). It was suggested for this unexpected outcome that some other bioactive molecules in cold infused instant tea powders other than catechins, theaflavin and thearubigins were responsible for its cytotoxic effect. Then, they came to the forefront of instant tea powders and suppressed the effects of the higher phenolic content in hot infused instant teas. Way et al. (2009) also observed in their study, where they investigated six different species of *Camellias*, including *Camellia sinensis*, that *Camellia tenuifolia* had the highest apoptosis induction ability following *Camellia sinensis* on MCF7 cells despite its lowest content of catechins. Therefore, they indicated that the anticancer activity of *Camellia tenuifolia* was based on other bioactive compounds in the plant instead of catechins. Moreover, the interactions between compounds during hot and cold infusions could affect the anticancer potential of powders on MCF7 cells. Friedmen et al. (2007) also supported the hypothesis that the competitive, additive and antagonistic interactions among the flavonoids could contribute to the cytotoxicity of teas because they found the levels of catechins and theaflavins did not correlate with the anticancer activity of commercial four black tea samples purchased in USA. On the other hand, the different morphological structures of instant tea powders, as mentioned in Section 3.4.6, could also affect their anticancer activity because it was revealed that the morphological properties of anticancer agents affected their cytotoxic abilities, such as cellular uptake (Wang et al., 2016; Esparza González et al., 2021; Jaragh-Alhadad et al., 2022).

Instant black tea powders, regardless of the hot and cold infusion methods ($p>0.05$), could start to decrease the cell viability of MCF7 cells at 2.5 mg//ml when compared with control. In addition, hot infused instant black tea powders decreased the cell viability of MCF7 cells to 29.93% and 21.65% for the concentrations of 5 and 8 mg/ml, respectively, where there was no significant difference ($p>0.05$). On the other hand, cold infused instant black teas reduce the cell viability of MCF7 cells to 18.33% and 11.90% ($p>0.05$) for the concentrations of 5 and 8 mg/ml, respectively.

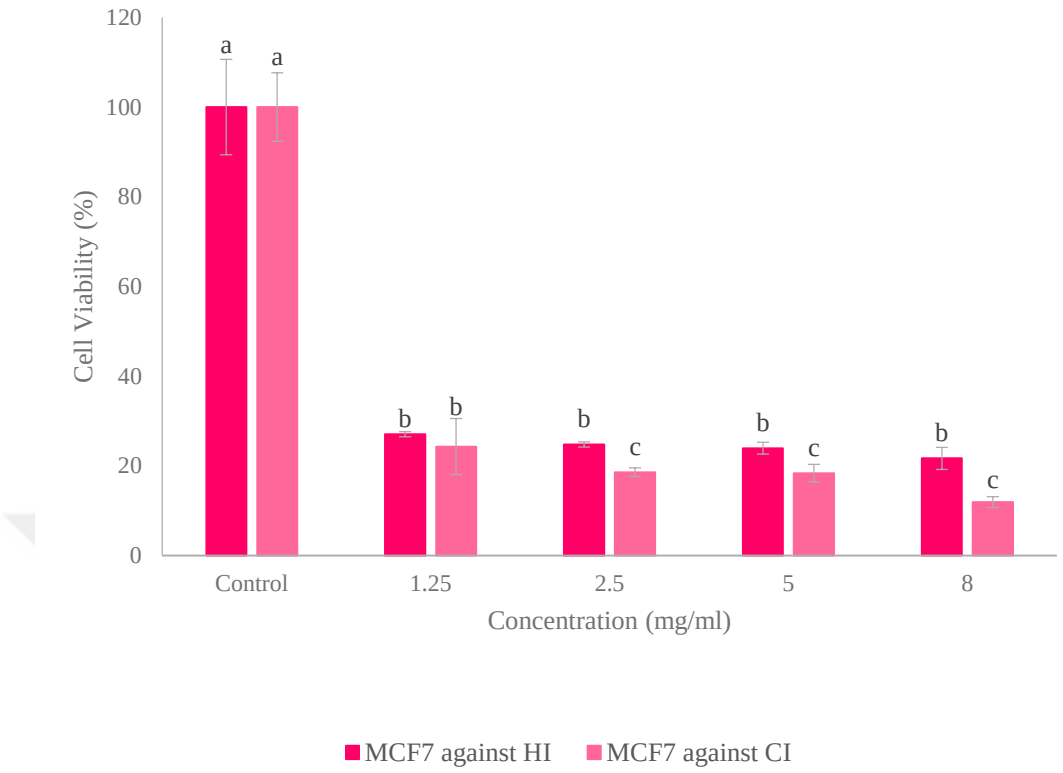


Figure 3.5 The cell viability of breast cancer cell against instant teas at different concentrations. Different letters at the same concentration represents a significant difference when compared to control ($p<0.05$).

The concentration-dependent cell viability reduction of MCF7 cells was also demonstrated in fresh tea leave extract originating in Taiwan using the MTT assay (Way et al., 2009). It was also stated from the observation of caspase 3 cleaving, i.e., activating, two apoptosis proteins, poly-ADP ribose polymerase (PARP) and BH3 interacting domain death agonist (BID), that fresh tea leaves induced apoptosis in MCF7 cells. In addition, the anticancer potential of black tea was confirmed in the study of Koňariková et al. (2015), where the concentration-dependent cytotoxic effect of the black tea extract was demonstrated on MCF7 cells using MTT assay. Moreover, it was also found that black tea extract resulted in DNA strand breaking and oxidative DNA damage. Friedman et al. (2007) supported the cytotoxicity of

black tea by revealing decreased cell viability of MCF7 cells in the presence of four commercial black tea samples purchased in the US. They also demonstrated the concentration-dependent anticancer activity of catechins (C, CG, EC, ECG, EGC, EGCG and GCG), theaflavins (theaflavin, theaflavin-3-gallate and theaflavin-3,3'-digallate) and theanine individually against MCF7 cells. The ability of EGCG to cause a reduction in the cell viability of MCF7 cells was also detected in the studies of Schroder et al. (2019), Tran et al. (2010), Wang et al. (2010) and Zhang et al. (2009) and using MTT assay. Moreover, Tran et al. (2010) determined that EGCG suppressed the expressions of heat shock proteins (HSPs), HSP70 and HSP90, which were stress response proteins and increased the resistance of the cells to apoptosis, by inhibiting the expressions of their heat shock transcription factors (HSFs), HSF1 and HSF2, of MCF7 cells in a concentration-dependent manner. Furthermore, the cell viability of MCF7 cells was decreased due to the cytotoxic effect of caffeine (Machado et al., 2020; Niknafs, 1996). The reason for the induction of MCF7 cells into apoptosis was oxidative stress generated by caffeine, which increased the lipid peroxides in the membrane (Machado et al., 2020). Aborehab et al. (2020), Rezaei-Seresht et al. (2019) and Wang et al. (2014) also revealed that gallic acid led to cell death in the breast cancer cells of MCF7.

CHAPTER 4

CONCLUSION AND RECOMMENDATIONS

Black tea waste fiber, whose generation significantly increased in recent years because of the risen production of black tea products, was valorized as instant tea, a growing product category of black tea, in the current study.

The water-to-tea ratio of 30:1 ml/g, the superfine particle size range (<100 μm), the infusion conditions at 100 ℃ for 6 min and at 25 ℃ for 2 h for the hot and cold infusion methods, respectively, were set as operational to maximize the extraction yield of black tea waste fibers, which is the primary concern in the production of instant tea because of its direct effects on the nutritional value and sensory profile of the end products. In addition, instant black teas produced by freeze drying hot and cold infusions of black tea waste fiber were also mixed with ginger and sage using superfine grinding to evaluate the effects of herbs on the functional properties of the powders.

Instant tea powders were characterized in terms of chemical (antioxidant capacity, total phenolic content, bioactive composition (epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate, theaflavin, thearubigins, gallic acid, caffeine and theanine) and physical (moisture content, hygroscopicity, color, morphological, solubility and turbidity) profiles.

Instant teas produced by the hot infusion method had a higher antioxidant capacity and total phenolic content than those obtained by the cold infusion method for the same formulation ($p<0.05$). Moreover, the addition of herbs increased the antioxidant capacity of the powders ($p<0.05$), while only sage could enhance the total phenolic content values ($p<0.05$). It was also suggested that the dominant non-

phenolic antioxidative compounds in ginger lead to non-correlation of the antioxidant capacity and the total phenolic contents analyses.

Some variations, albeit to different extents, were observed for the contents of bioactive compounds in instant teas ($p<0.05$). It should be highlighted that the hot infusion method could extract more epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate, theaflavin, thearubigins and theanine than the cold infusion method ($p<0.05$). However, it could not be mentioned for gallic acid and caffeine ($p>0.05$).

The addition of herbs increased the moisture content and decreased the hygroscopicity values of instant teas ($p<0.05$), for which there was a negative, high and significant correlation ($r = -0.924$, $p<0.05$). L* values of the powders were markedly correlated with the contents of theaflavin ($r = -0.973$, $p<0.05$) and thearubigins ($r = -0.977$, $p<0.05$). In addition, the addition of herbs enhanced a* and b* values of powders ($p<0.05$). It was also revealed that the infusion method differentiated the surface morphology characteristics of the powders.

Solubility of instant powders were negatively, highly and significantly correlated with turbidity ($r = -0.983$, $p<0.05$) because the decreasing in the solubility of tea components gave rise to increase cloudiness and haziness of solutions. Higher solubility and lower turbidity values of hot infused instant teas than those produced by the cold infusion method were attributed to the variation in composition and morphology ($p<0.05$). Moreover, herbs decreased solubility and increased turbidity of instant teas because of their insoluble dietary fiber content ($p<0.05$). It was also suggested for the color parameters of instant tea solutions that turbidity came to the forefront and suppressed the impacts of complex compounds for L*, a* and b* values.

Anticancer potentials of instant tea powders produced by black tea waste fibers were also investigated in the current research. The cytotoxicity of hot infused instant black teas was confirmed on the prostate cancer cells, PC3, 22Rv1 and LNCaP. Then, it

was revealed that hot and cold infused instant black teas could reduce the cell viability of glioblastoma multiforme cells (U87MG and A172) and breast cancer cell (MCF7). The cell viability of all studied cancer cells decreased in a concentration-dependent manner ($p<0.05$).

The resistances of prostate cancer cells and glioma cells were in the increasing order of PC3, 22Rv1, LNCaP and U87MG, A172, respectively ($p<0.05$). It was stated that the distinct responses of cell lines against the cytotoxicity of instant black tea powders were attributed to the variation in their genomic basis.

Hot infused instant black teas were more effective against glioma cells because of the outstanding contents of phenolics such as catechin, theaflavin and thearubigins whose noticeable cytotoxicity was already known in hot infused teas ($p<0.05$). On the other hand, cold infused instant black teas had higher cytotoxicity on breast cancer cell ($p<0.05$). It was hypothesized that some other bioactive molecules in cold infused instant tea powders other than catechins, theaflavin and thearubigins were responsible for its cytotoxic effect.

This comprehensive studying of instant teas produced by black tea was fiber can guide targeted applications in food waste industry.

The effects of ginger and sage on the properties of instant tea powders indicate the potential interactions between the bioactive compounds in herbs and instant black teas. Evaluating the impact mechanisms and expanding the range of herbs could yield new functional products with health benefits.

Further exploration is encouraged to investigate the extraction potential of cold infusion method. Isolating and identifying the exact biocompounds could help researchers better understand the unique properties of cold-infused tea infusions.

The cytotoxic effects of instant teas suggest more research into the underlying mechanisms. Identifying the pathways that instant teas exert their effects could open new ways for the development of new products with anticancer capabilities.

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APPENDICES

A. Calibration Curves

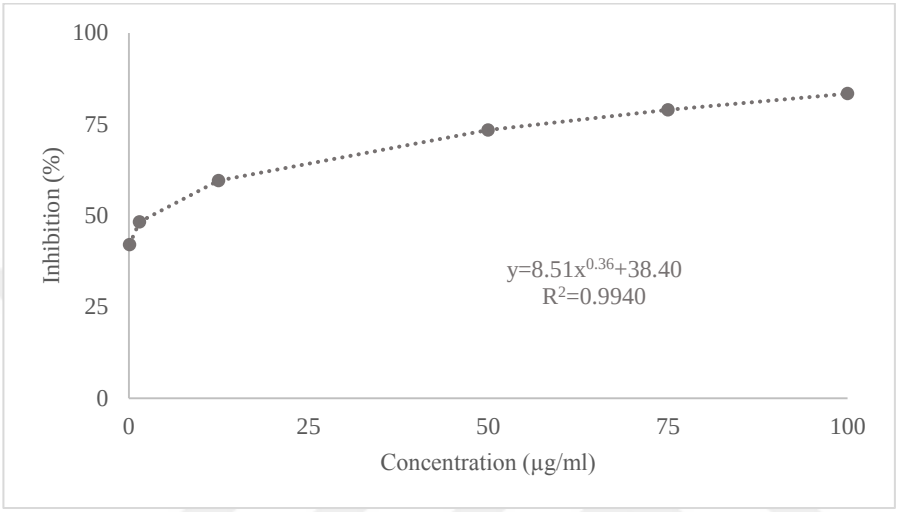


Figure A.1 An example of regression curve for ABTS assay to determine IC_{50} value of instant black tea powder (HI)

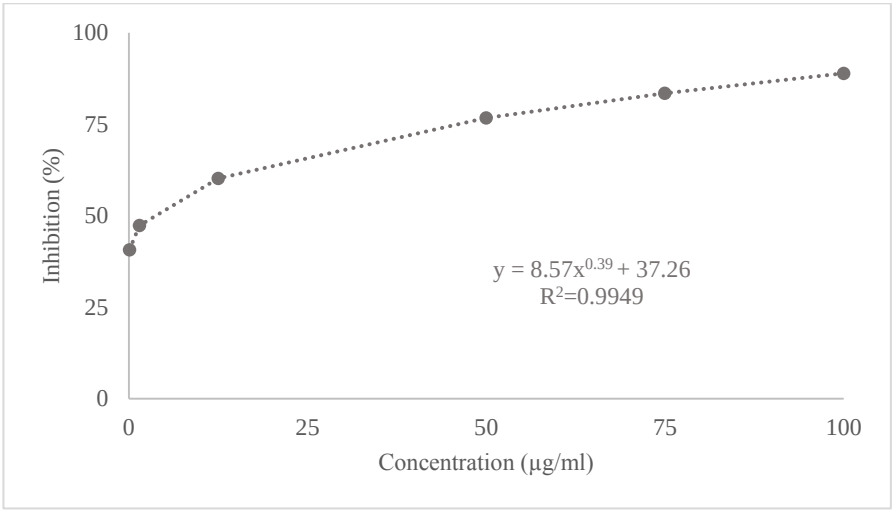


Figure A.2 An example of regression curve for DPPH assay to determine IC_{50} value of instant black tea powder (HI)

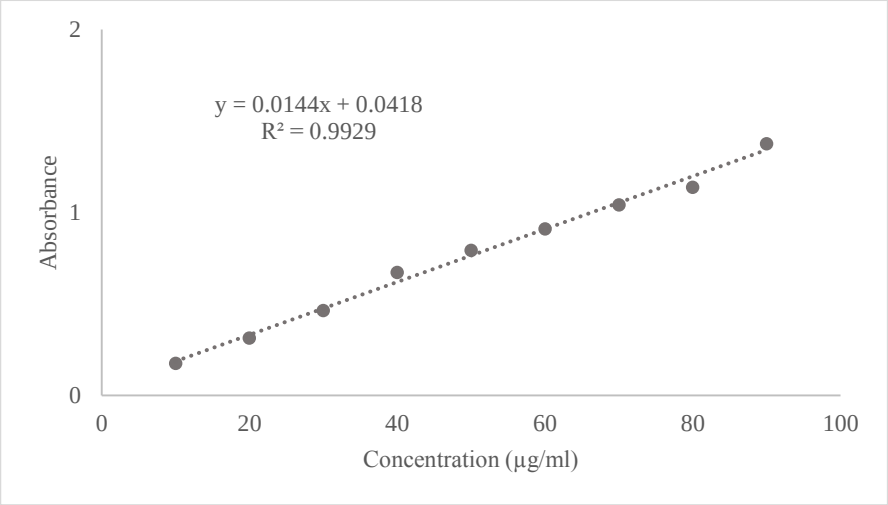


Figure A.3 Calibration curve for Folin-Ciocalteu reducing capacity assay prepared by gallic acid to determine total phenolic content of instant black tea powders

B. Statistical Analyses

Table A.1 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing D_{10} values of pre-ground black tea waste fibers and black tea leaves

Factor Information

Factor	Type	Levels	Values
Sample	Fixed	2	Pre-ground BTWF; Reference BTL

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	1	17893.2	17893.2	11777.36	0.000
Error	4	6.1	1.5		
Total	5	17899.3			

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
Reference BTL	3	173.534	A
Pre-ground BTWF	3	64.315	B

Means that do not share a letter are significantly different.

Table A.2 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing D_{50} values of pre-ground black tea waste fibers and black tea leaves

Factor Information

Factor	Type	Levels	Values
Sample	Fixed	2	Pre-ground BTWF; Reference BTL

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	1	469.03	469.033	87.24	0.001
Error	4	21.51	5.377		
Total	5	490.54			

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
Reference BTL	3	413.642	A
Pre-ground BTWF	3	395.959	B

Means that do not share a letter are significantly different.

Table A.3 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing D_{90} values of pre-ground black tea waste fibers and black tea leaves

Factor Information

Factor	Type	Levels	Values
Sample	Fixed	2	Pre-ground BTWF; Reference BTL

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	1	40859.8	40859.8	2145.97	0.000
Error	4	76.2	19.0		
Total	5	40935.9			

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
Pre-ground BTWF	3	1026.16	A
Reference BTL	3	861.12	B

Means that do not share a letter are significantly different.

Table A.4 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing span values of pre-ground black tea waste fibers and black tea leaves

Factor Information

Factor	Type	Levels	Values
Sample	Fixed	2	Pre-ground BTWF; Reference BTL

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	1	0.882180	0.882180	3232.53	0.000
Error	4	0.001092	0.000273		
Total	5	0.883272			

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
Pre-ground BTWF	3	2.42916	A
Reference BTL	3	1.66227	B

Means that do not share a letter are significantly different.

Table A.5 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing cellular disruption ratio values of pre-ground black tea waste fibers and black tea leaves

Factor Information

Factor	Type	Levels	Values
Sample	Fixed	2	Pre-ground BTWF; Reference BTL

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	1	0.142386	0.142386	87.00	0.001
Error	4	0.006546	0.001637		
Total	5	0.148932			

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
Pre-ground BTWF	3	7.38696	A
Reference BTL	3	7.07887	B

Means that do not share a letter are significantly different.

Table A.6 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing D_{10} values of pre-ground and ball milled black tea waste fibers for 5. 15 and 25 min

Factor Information

Factor	Type	Levels	Values
Sample	Fixed	3	5; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	2	2.56534	1.28267	5299.36	0.000
Error	6	0.00145	0.00024		
Total	8	2.56680			

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping		
5	3	5.352	A		
30	3	4.364		B	
15	3	4.116			C

Means that do not share a letter are significantly different.

Table A.7 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing D_{50} values of pre-ground and ball milled black tea waste fibers for 5. 15 and 25 min

Factor Information

Factor	Type	Levels	Values
Sample	Fixed	3	5; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	2	2836.92	1418.46	324164.91	0.000
Error	6	0.03	0.00		
Total	8	2836.95			

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping		
5	3	56.310	A		
15	3	19.394		B	
30	3	17.943			C

Means that do not share a letter are significantly different.

Table A.8 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing D_{90} values of pre-ground and ball milled black tea waste fibers for 5. 15 and 25 min

Factor Information

Factor	Type	Levels	Values
Sample	Fixed	3	5; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	2	1565776	782888	1280744.09	0.000
Error	6	4	1		
Total	8	1565780			

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
5	3	955.181	A
15	3	76.022	B
30	3	64.827	C

Means that do not share a letter are significantly different.

Table A.9 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing span values of pre-ground and ball milled black tea waste fibers for 5. 15 and 25 min

Factor Information

Factor	Type	Levels	Values
Sample	Fixed	3	5; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	2	355.574	177.787	500211.69	0.000
Error	6	0.002	0.000		
Total	8	355.577			

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping

5	3	16.8679	A	
15	3	3.7076	B	
30	3	3.3673		C

Means that do not share a letter are significantly different.

Table A.10 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing cellular disruption ratio values of pre-ground and ball milled black tea waste fibers for 5. 15 and 25 min

Factor Information

Factor	Type	Levels	Values
Sample	Fixed	3	5; 15; 30

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	2	4170.46	2085.23	222869.80	0.000
Error	6	0.06	0.01		
Total	8	4170.52			

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping	
30	3	91.3249	A	
15	3	88.6355	B	
5	3	44.3753		C

Means that do not share a letter are significantly different.

Table A.11 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing extraction yield values of pre-ground and pre-ground and ball milled black tea waste fibers for different water-to-tea ratios

Factor Information

Factor	Type	Levels	Values
Water-to-Tea Ratio	Fixed	4	10; 20; 30; 40

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Water-to-Tea Ratio	3	26.403	8.8009	38.00	0.000
Error	8	1.853	0.2316		

Total	11	28.256

Grouping Information Using the Tukey Method and 95% Confidence

Water-to-Tea Ratio	N	Mean	Grouping		
30	3	31.0848	A		
40	3	30.9217	A	B	
20	3	29.6946		B	
10	3	27.3742			C

Means that do not share a letter are significantly different.

Table A.12 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing extraction yield values of black tea waste fibers for different particle size ranges. infusion temperatures and infusion times in the hot infusion method

Factor Information

Factor	Type	Levels	Values
Temperature	Fixed	2	80; 100
Time	Fixed	3	6; 10; 15
Grinding	Fixed	2	PG; PGBM

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Temperature	1	39.436	39.436	109.98	0.000
Time	2	7.026	3.513	9.80	0.001
Grinding	1	263.347	263.347	734.45	0.000
Temperature*Time	2	2.444	1.222	3.41	0.050
Temperature*Grinding	1	51.709	51.709	144.21	0.000
Time*Grinding	2	1.266	0.633	1.77	0.193
Temperature*Time*Grinding	2	4.714	2.357	6.57	0.005
Error	24	8.606	0.359		
Total	35	378.546			

Grouping Information Using the Tukey Method and 95% Confidence

Temperature	N	Mean	Grouping	
100	18	27.7139	A	
80	18	25.6206		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Time	N	Mean	Grouping	
15	12	27.1958	A	
6	12	26.6915	A	B
10	12	26.1145		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Grinding	N	Mean	Grouping	
PGBM	18	29.3719	A	
PG	18	23.9626		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Temperature*Time	N	Mean	Grouping		
100 15	6	28.6107	A		
100 6	6	27.5644	A	B	
100 10	6	26.9665		B	
80 6	6	25.8185			C
80 15	6	25.7809			C
80 10	6	25.2624			C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Temperature*Grinding	N	Mean	Grouping		
100 PGBM	9	31.6170	A		
80 PGBM	9	27.1268		B	
80 PG	9	24.1144			C
100 PG	9	23.8107			C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Time*Grinding	N	Mean	Grouping		
15 PGBM	6	29.6618	A		
6 PGBM	6	29.4153	A		
10 PGBM	6	29.0386	A		
15 PG	6	24.7298		B	
6 PG	6	23.9676		B	C
10 PG	6	23.1903			C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Temperature*Time*Grinding	N	Mean	Grouping		
100 15 PGBM	3	32.1712	A		
100 6 PGBM	3	31.9727	A		
100 10 PGBM	3	30.7072	A		
80 10 PGBM	3	27.3700		B	
80 15 PGBM	3	27.1524		B	
80 6 PGBM	3	26.8580		B	
100 15 PG	3	25.0502			C
80 6 PG	3	24.7791			C D
80 15 PG	3	24.4094			C D
100 10 PG	3	23.2258			D
100 6 PG	3	23.1562			D
80 10 PG	3	23.1548			D

Means that do not share a letter are significantly different.

Table A.13 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing extraction yield values of black tea waste fibers for different particle size ranges. infusion temperatures and infusion times in the cold infusion method

Factor Information

Factor	Type	Levels	Values
Method	Fixed	3	25 °C.2 h; 4 °C.12 h; 4 °C.6 h
Grinding	Fixed	2	PG; PGBM

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Method	2	114.532	57.2658	201.01	0.000
Grinding	1	36.603	36.6035	128.48	0.000
Method*Grinding	2	26.719	13.3597	46.89	0.000
Error	12	3.419	0.2849		
Total	17	181.273			

Grouping Information Using the Tukey Method and 95% Confidence

Method	N	Mean	Grouping		
25 °C.2 h	6	14.3085	A		
4 °C.12 h	6	11.5169		B	
4 °C.6 h	6	8.1391			C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Grinding	N	Mean	Grouping
PGBM	9	12.7475	A
PG	9	9.8955	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Method*Grinding	N	Mean	Grouping	
25 °C.2 h PGBM	3	17.4571	A	
4 °C.12 h PGBM	3	12.0476	B	
25 °C.2 h PG	3	11.1599	B	
4 °C.12 h PG	3	10.9862	B	
4 °C.6 h PGBM	3	8.7378		C
4 °C.6 h PG	3	7.5403		C

Means that do not share a letter are significantly different.

Table A.14 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing D_{10} values of ginger and sage

Factor Information

Factor	Type	Levels	Values
Sample	Fixed	2	Ginger; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	1	5.92225	5.92225	1092.66	0.000
Error	4	0.02168	0.00542		
Total	5	5.94393			

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
Sage	3	9.681	A
Ginger	3	7.694	B

Means that do not share a letter are significantly different.

Table A.15 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing D_{50} values of ginger and sage

Factor Information

Factor	Type	Levels	Values
Sample	Fixed	2	Ginger; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	1	3807.82	3807.82	80585.06	0.000
Error	4	0.19	0.05		
Total	5	3808.01			

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
Sage	3	66.261	A
Ginger	3	15.877	B

Means that do not share a letter are significantly different.

Table A.16 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing D_{90} values of ginger and sage

Factor Information

Factor	Type	Levels	Values
Sample	Fixed	2	Ginger; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	1	46614.1	46614.1	17368158.41	0.000
Error	4	0.0	0.0		
Total	5	46614.1			

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
Sage	3	206.252	A
Ginger	3	29.968	B

Means that do not share a letter are significantly different.

Table A.17 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing span values of ginger and sage

Factor Information

Factor	Type	Levels	Values
Sample	Fixed	2	Ginger; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	1	3.66777	3.66777	14342.62	0.000
Error	4	0.00102	0.00026		
Total	5	3.66879			

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
Sage	3	2.96662	A
Ginger	3	1.40291	B

Means that do not share a letter are significantly different.

Table A.18 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing cellular disruption ratio values of ginger and sage

Factor Information

Factor	Type	Levels	Values
Sample	Fixed	2	Ginger; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	1	4727.73	4727.73	206662.13	0.000
Error	4	0.09	0.02		
Total	5	4727.82			

Grouping Information Using the Tukey Method and 95% Confidence

Sample	N	Mean	Grouping
Ginger	3	94.9278	A
Sage	3	38.7867	B

Means that do not share a letter are significantly different.

Table A.19 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing antioxidant capacities of instant teas enriched with ginger and sage in terms of ABTS

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	6.2955	6.29545	384.54	0.000
Herb	2	7.1271	3.56357	217.67	0.000
Infusion*Herb	2	0.7443	0.37216	22.73	0.000
Error	12	0.1965	0.01637		
Total	17	14.3634			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
CI	9	3.08551	A
HI	9	1.90272	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
None	6	3.26870	A
Ginger	6	2.48622	B
Sage	6	1.72742	C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping
CI None	3	3.97233	A
CI Ginger	3	3.25080	B
HI None	3	2.56507	C
CI Sage	3	2.03340	D
HI Ginger	3	1.72165	D E
H Sage	3	1.42145	E

Means that do not share a letter are significantly different.

Table A.20 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing antioxidant capacities of instant teas enriched with ginger and sage in terms of DPPH

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	8.3035	8.30349	211.62	0.000
Herb	2	16.0937	8.04684	205.08	0.000
Infusion*Herb	2	2.1688	1.08439	27.64	0.000
Error	12	0.4708	0.03924		
Total	17	27.0368			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
CI	9	3.43592	A
HI	9	2.07753	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
None	6	4.05108	A
Ginger	6	2.40045	B
Sage	6	1.81865	C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping
CI None	3	5.20583	A
CI Ginger	3	2.94730	B
HI None	3	2.89633	B
CI Sage	3	2.15463	C
HI Ginger	3	1.85360	C D
HI Sage	3	1.48267	D

Means that do not share a letter are significantly different.

Table A.21 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing total phenolic contents of instant teas enriched with ginger and sage

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	59.3577	59.3577	6762.48	0.000
Herb	2	3.8346	1.9173	218.44	0.000
Infusion*Herb	2	0.3205	0.1603	18.26	0.000
Error	12	0.1053	0.0088		
Total	17	63.6182			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping	
HI	9	11.2801	A	
CI	9	7.6482		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping		
Sage	6	10.1082	A		
None	6	9.2339		B	
Ginger	6	9.0503			C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping			
HI Sage	3	11.7392	A			
HI None	3	11.1100		B		
HI Ginger	3	10.9911		B		
CI Sage	3	8.4772			C	
CI None	3	7.3578				D
CI Ginger	3	7.1095				D

Means that do not share a letter are significantly different.

Table A.22 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing epicatechin contents of instant teas

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	210552	210552	15.05	0.002
Herb	2	1868381	934191	66.79	0.000
Infusion*Herb	2	323082	161541	11.55	0.002
Error	12	167852	13988		
Total	17	2569867			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
HI	9	2762.20	A
CI	9	2545.89	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
None	6	3085.14	A
Sage	6	2566.22	B
Ginger	6	2310.77	C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping
HI None	3	3313.50	A
CI None	3	2856.78	B
HI Sage	3	2741.11	B
CI Sage	3	2391.34	C
CI Ginger	3	2389.55	C
HI Ginger	3	2231.99	C

Means that do not share a letter are significantly different.

Table A.23 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing epicatechin gallate contents of instant teas

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	7855	7855.5	76.43	0.000
Herb	2	13086	6542.9	63.66	0.000
Infusion*Herb	2	2954	1477.2	14.37	0.001
Error	12	1233	102.8		
Total	17	25129			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping	
HI	9	299.133	A	
CI	9	257.352		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping	
None	6	316.269	A	
Ginger	6	261.675		B
Sage	6	256.784		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping		
HI None	3	353.941	A		
CI None	3	278.597		B	
HI Sage	3	275.198		B	
HI Ginger	3	268.260		B	
CI Ginger	3	255.089		B	C
CI Sage	3	238.370			C

Means that do not share a letter are significantly different.

Table A.24 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing epigallocatechin contents of instant teas

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	389590	389590	180.74	0.000
Herb	2	197566	98783	45.83	0.000
Infusion*Herb	2	129237	64619	29.98	0.000
Error	12	25867	2156		
Total	17	742261			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
HI	9	1234.25	A
CI	9	940.01	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
None	6	1233.39	A
Sage	6	1034.47	B
Ginger	6	993.52	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping		
HI None	3	1499.55	A		
HI Sage	3	1134.00		B	
HI Ginger	3	1069.19		B	C
CI None	3	967.23			C D
CI Sage	3	934.95			D
CI Ginger	3	917.84			D

Means that do not share a letter are significantly different.

Table A.25 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing epigallocatechin gallate contents of instant teas

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	42138.5	42138.5	196.37	0.000
Herb	2	209.0	104.5	0.49	0.626
Infusion*Herb	2	1004.1	502.1	2.34	0.139
Error	12	2575.0	214.6		
Total	17	45926.7			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
HI	9	247.182	A
CI	9	150.414	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
Sage	6	203.374	A
None	6	197.818	A
Ginger	6	195.201	A

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping
HI Sage	3	262.299	A
HI None	3	241.520	A
HI Ginger	3	237.727	A
CI None	3	154.116	B
CI Ginger	3	152.676	B
CI Sage	3	144.449	B

Means that do not share a letter are significantly different.

Table A.26 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing theaflavin contents of instant teas

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	77355.7	77355.7	2925.69	0.000
Herb	2	4358.9	2179.4	82.43	0.000
Infusion*Herb	2	134.6	67.3	2.55	0.120
Error	12	317.3	26.4		
Total	17	82166.4			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
HI	9	225.297	A
CI	9	94.186	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
None	6	181.612	A
Ginger	6	150.926	B
Sage	6	146.687	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping
HI	3	243.352	A
HIG	3	218.932	B
HIS	3	213.609	B
CI	3	119.873	C
CIG	3	82.920	D
CIS	3	79.765	D

Means that do not share a letter are significantly different.

Table A.27 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing thearubigins contents of instant teas

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CB; HB
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	5530290	5530290	1361.92	0.000
Herb	2	1420237	710118	174.88	0.000
Infusion*Herb	2	37892	18946	4.67	0.032
Error	12	48728	4061		
Total	17	7037147			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
HB	9	2238.04	A
CB	9	1129.46	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
None	6	2080.75	A
Ginger	6	1497.45	B
Sage	6	1473.06	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping
HB None	3	2574.36	A
HB Ginger	3	2101.98	B
HB Sage	3	2037.80	B
CB None	3	1587.14	C
CB Sage	3	908.32	D
CB Ginger	3	892.92	D

Means that do not share a letter are significantly different.

Table A.28 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing gallic acid contents of instant teas

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	35953	35953	134.11	0.000
Herb	2	208604	104302	389.07	0.000
Infusion*Herb	2	41794	20897	77.95	0.000
Error	12	3217	268		
Total	17	289568			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
CI	9	1003.55	A
HI	9	914.17	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
None	6	1102.22	A
Ginger	6	931.57	B
Sage	6	842.79	C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping
HI None	3	1115.80	A
CI None	3	1088.63	A
CI Ginger	3	974.81	B
CI Sage	3	947.21	B
HI Ginger	3	888.33	C
HI Sage	3	738.37	D

Means that do not share a letter are significantly different.

Table A.29 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing caffeine contents of instant teas

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	148341	148341	11.64	0.005
Herb	2	569834	284917	22.35	0.000
Infusion*Herb	2	59702	29851	2.34	0.138
Error	12	152966	12747		
Total	17	930844			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
CI	9	3195.16	A
HI	9	3013.60	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
None	6	3355.32	A
Sage	6	2995.02	B
Ginger	6	2962.81	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping		
CI None	3	3415.93	A		
HI None	3	3294.70	A	B	
CI Ginger	3	3134.19	A	B	C
CI Sage	3	3035.36		B	C	D
HI Sage	3	2954.68			C	D
HI Ginger	3	2791.42				D

Means that do not share a letter are significantly different.

Table A.30 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing theanine contents of instant teas

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	261179	261179	1048.89	0.000
Herb	2	242727	121363	487.39	0.000
Infusion*Herb	2	8466	4233	17.00	0.000
Error	12	2988	249		
Total	17	515361			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
HI	9	1233.73	A
CI	9	992.81	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
None	6	1277.05	A
Sage	6	1041.83	B
Ginger	6	1020.93	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping		
HI None	3	1367.73	A		
CI None	3	1186.37		B	
HI Sage	3	1183.53		B	
HI Ginger	3	1149.92		B	
CI Sage	3	900.12			C
CI Ginger	3	891.94			C

Means that do not share a letter are significantly different.

Table A.31 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing moisture content values of instant teas enriched with ginger and sage

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	0.0174	0.01742	0.07	0.799
Herb	2	11.4254	5.71270	22.19	0.000
Infusion*Herb	2	0.0274	0.01369	0.05	0.948
Error	12	3.0898	0.25749		
Total	17	14.5600			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
HI	9	5.54512	A
CI	9	5.48290	A

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping	
Ginger	6	6.24932	A	
Sage	6	5.88568	A	
None	6	4.40703		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping	
HI Ginger	3	6.32217	A	
CI Ginger	3	6.17647	A	
CI Sage	3	5.90667	A	
HI Sage	3	5.86469	A	
HI None	3	4.44848		B
CI None	3	4.36557		B

Means that do not share a letter are significantly different.

Table A.32 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing hygroscopicity values of instant teas enriched with ginger and sage

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	1.7848	1.7848	3.39	0.090
Herb	2	80.1694	40.0847	76.18	0.000
Infusion*Herb	2	0.1130	0.0565	0.11	0.899
Error	12	6.3146	0.5262		
Total	17	88.3818			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
CI	9	19.7147	A
HI	9	19.0849	A

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping	
None	6	22.3769	A	
Sage	6	18.0946		B
Ginger	6	17.7279		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping	
CI None	3	22.5843	A	
HI None	3	22.1694	A	
CI Sage	3	18.4358		B
CI Ginger	3	18.1240		B
HI Sage	3	17.7535		B
HI Ginger	3	17.3318		B

Means that do not share a letter are significantly different.

Table A.33 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing L* values of instant tea powders enriched with ginger and sage

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	19.0139	19.0139	98.07	0.000
Herb	2	4.2633	2.1317	10.99	0.002
Infusion*Herb	2	0.1011	0.0506	0.26	0.775
Error	12	2.3267	0.1939		
Total	17	25.7050			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
CI	9	19.2444	A
HI	9	17.1889	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
Sage	6	18.7667	A
Ginger	6	18.3000	A
None	6	17.5833	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping		
CI Sage	3	19.7333	A		
CI Ginger	3	19.4333	A		
CI None	3	18.5667	A	B	
HI Sage	3	17.8000		B	C
HI Ginger	3	17.1667			C
HI None	3	16.6000			C

Means that do not share a letter are significantly different.

Table A.34 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing a* values of instant tea powders enriched with ginger and sage

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	18.605	18.6050	87.67	0.000
Herb	2	7.960	3.9800	18.75	0.000
Infusion*Herb	2	1.053	0.5267	2.48	0.125
Error	12	2.547	0.2122		
Total	17	30.165			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
HI	9	12.6333	A
CI	9	10.6000	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
Ginger	6	12.1833	A
Sage	6	11.9833	A
None	6	10.6833	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping		
HI Ginger	3	13.4333	A		
HI Sage	3	13.1000	A		
HI None	3	11.3667		B	
CI Ginger	3	10.9333		B	C
CI Sage	3	10.8667		B	C
CI None	3	10.0000			C

Means that do not share a letter are significantly different.

Table A.35 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing b* values of instant tea powders enriched with ginger and sage

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	26.402	26.4022	48.49	0.000
Herb	2	76.448	38.2239	70.21	0.000
Infusion*Herb	2	0.148	0.0739	0.14	0.874
Error	12	6.533	0.5444		
Total	17	109.531			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
HI	9	20.5889	A
CI	9	18.1667	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
Ginger	6	21.3500	A
Sage	6	20.2500	A
None	6	16.5333	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping			
HI Ginger	3	22.6333	A			
HI Sage	3	21.3333	A	B		
CI Ginger	3	20.0667		B	C	
CI Sage	3	19.1667			C	D
HI None	3	17.8000				D
CI None	3	15.2667				E

Means that do not share a letter are significantly different.

Table A.36 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing solubility values of instant teas enriched with ginger and sage

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	65.85	65.851	24.06	0.000
Herb	2	649.63	324.817	118.67	0.000
Infusion*Herb	2	21.75	10.876	3.97	0.047
Error	12	32.85	2.737		
Total	17	770.08			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
HI	9	86.1291	A
CI	9	82.3037	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
None	6	92.4695	A
Ginger	6	81.8366	B
Sage	6	78.3432	C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping		
HI None	3	95.9321	A		
CI None	3	89.0069		B	
HI Ginger	3	83.0793			C
CI Ginger	3	80.5938			C D
HI Sage	3	79.3760			C D
CI Sage	3	77.3104			D

Means that do not share a letter are significantly different.

Table A.37 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing turbidity values of instant teas enriched with ginger and sage

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	4769.4	4769.4	113.41	0.000
Herb	2	66286.1	33143.1	788.08	0.000
Infusion*Herb	2	437.4	218.7	5.20	0.024
Error	12	504.7	42.1		
Total	17	71997.6			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
CI	9	256.556	A
HI	9	224.000	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
Sage	6	303.333	A
Ginger	6	259.167	B
None	6	158.333	C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping
CI Sage	3	313.000	A
HI Sage	3	293.667	B
CI Ginger	3	280.667	B
HI Ginger	3	237.667	C
CI None	3	176.000	D
HI None	3	140.667	E

Means that do not share a letter are significantly different.

Table A.38 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing L* values of instant teas enriched with ginger and sage

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	70.8050	70.8050	833.00	0.000
Herb	2	2.8544	1.4272	16.79	0.000
Infusion*Herb	2	0.0433	0.0217	0.25	0.779
Error	12	1.0200	0.0850		
Total	17	74.7228			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping	
HI	9	29.4444	A	
CI	9	25.4778		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping	
None	6	28.0000	A	
Ginger	6	27.3333		B
Sage	6	27.0500		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping		
HI None	3	29.9667	A		
HI Ginger	3	29.2667	A	B	
HI Sage	3	29.1000		B	
CI None	3	26.0333			C
CI Ginger	3	25.4000			C D
CI Sage	3	25.0000			D

Means that do not share a letter are significantly different.

Table A.39 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing a* values of instant teas enriched with ginger and sage

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	0.09389	0.093889	16.90	0.001
Herb	2	0.65444	0.327222	58.90	0.000
Infusion*Herb	2	0.03444	0.017222	3.10	0.082
Error	12	0.06667	0.005556		
Total	17	0.84944			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
CI	9	1.36667	A
HI	9	1.22222	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping
Sage	6	1.48333	A
Ginger	6	1.36667	B
None	6	1.03333	C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping		
CI Sage	3	1.53333	A		
HI Sage	3	1.43333	A		
CI Ginger	3	1.40000	A		
HI Ginger	3	1.33333	A	B	
CI None	3	1.16667		B	
HI None	3	0.90000			C

Means that do not share a letter are significantly different.

Table A.40 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing b* values of instant teas enriched with ginger and sage

Factor Information

Factor	Type	Levels	Values
Infusion	Fixed	2	CI; HI
Herb	Fixed	3	Ginger; None; Sage

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Infusion	1	0.34722	0.34722	18.38	0.001
Herb	2	0.86333	0.43167	22.85	0.000
Infusion*Herb	2	0.06778	0.03389	1.79	0.208
Error	12	0.22667	0.01889		
Total	17	1.50500			

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping	
CI	9	2.35556	A	
HI	9	2.07778		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Herb	N	Mean	Grouping	
Sage	6	2.43333	A	
Ginger	6	2.30000	A	
None	6	1.91667		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion*Herb	N	Mean	Grouping		
CI Sage	3	2.56667	A		
CI Ginger	3	2.36667	A	B	
HI Sage	3	2.30000	A	B	
HI Ginger	3	2.23333	A	B	
CI None	3	2.13333		B	
HI None	3	1.70000			C

Means that do not share a letter are significantly different.

Table A.41 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing the cell viability of the prostate cancer cells

Concentration: 0.001 mg/ml

Factor Information

Factor	Type	Levels	Values
Cells	Fixed	3	22rv1; LNCaP; pc3
Concentration	Fixed	2	0.001; Control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Cells	2	39.41	19.70	1.30	0.308
Concentration	1	11.46	11.46	0.76	0.401
Cells*Concentration	2	39.41	19.70	1.30	0.308
Error	12	181.68	15.14		
Total	17	271.96			

Grouping Information Using the Tukey Method and 95% Confidence

Cells	N	Mean	Grouping
22Rv1	6	102.523	A
LNCaP	6	100.962	A
PC3	6	98.910	A

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping
0.001	9	101.596	A
Control	9	100.000	A

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Cells*Concentration	N	Mean	Grouping
22Rv1 0.001	3	105.046	A
LNCaP 0.001	3	101.923	A
22Rv1 Control	3	100.000	A
LNCaP Control	3	100.000	A
PC3 Control	3	100.000	A
PC3 0.001	3	97.819	A

Means that do not share a letter are significantly different.

Concentration: 0.01 mg/ml

Factor Information

Factor	Type	Levels	Values
Cells	Fixed	3	22rv1; LNCaP; pc3
Concentration	Fixed	2	0.01; Control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Cell Line	2	60.82	30.412	3.36	0.069
Concentration	1	38.20	38.200	4.22	0.062
Cell Line*Concentration	2	60.82	30.412	3.36	0.069
Error	12	108.67	9.056		
Total	17	268.52			

Grouping Information Using the Tukey Method and 95% Confidence

Cell Line	N	Mean	Grouping
22Rv1	6	100.000	A
LNCaP	6	99.679	A
PC3	6	95.950	A

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping
Control	9	100.000	A
0.01	9	97.086	A

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Cells*Concentration	N	Mean	Grouping
PC3 Control	3	100.000	A
LNCaP Control	3	100.000	A
22Rv1 0.01	3	100.000	A
22Rv1 Control	3	100.000	A
LNCaP 0.01	3	99.359	A
PC3 0.01	3	91.900	A

Means that do not share a letter are significantly different.

Concentration: 0.1 mg/ml

Factor Information

Factor	Type	Levels	Values
Cells	Fixed	3	22Rv1; LNCaP; PC3
Concentration	Fixed	2	0.1; Control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Cells	2	168.69	84.35	5.71	0.020
Concentration	1	32.50	32.50	2.20	0.166
Cells*Concentration	2	168.69	84.35	5.71	0.020
Error	11	162.47	14.77		
Total	16	468.91			

Grouping Information Using the Tukey Method and 95% Confidence

Cells	N	Mean	Grouping
LNCaP	6	100.962	A
22Rv1	6	100.917	A
PC3	5	93.925	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping
Control	9	100.000	A
0.1	8	97.203	A

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Cells*Concentration	N	Mean	Grouping
LNCaP 0.1	3	101.923	A
22Rv1 0.1	3	101.835	A
22Rv1 Control	3	100.000	A
LNCaP Control	3	100.000	A
PC3 Control	3	100.000	A

PC3 0.1	2	87.850	B

Means that do not share a letter are significantly different.

Concentration: 1 mg/ml

Factor Information

Factor	Type	Levels	Values
Cells	Fixed	3	22Rv1; LNCaP; PC3
Concentration	Fixed	2	1; Control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Cells	2	232.3	116.13	5.51	0.020
Concentration	1	1812.6	1812.63	85.94	0.000
Cells*Concentration	2	232.3	116.13	5.51	0.020
Error	12	253.1	21.09		
Total	17	2530.3			

Grouping Information Using the Tukey Method and 95% Confidence

Cells	N	Mean	Grouping	
CnCaP	6	94.5513	A	
PC3	6	89.5639	A	B
22Rv1	6	85.7798		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping	
Control	9	100.000	A	
1	9	79.930		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Cell Line*Concentration	N	Mean	Grouping
22Rv1 Control	3	100.000	A
LNCaP Control	3	100.000	A

PC3 Control	3	100.000	A		
LNCaP 1	3	89.103	A	B	
PC3 1	3	79.128		B	C
22Rv1 1	3	71.560			C

Means that do not share a letter are significantly different.

Concentration: 10 mg/ml

Factor Information

Factor	Type	Levels	Values
Cells	Fixed	3	22rv1; LNCaP; pc3
Concentration	Fixed	2	10; Control

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Cells	2	479.6	239.8	122.41	0.000
Concentration	1	29014.2	29014.2	14809.41	0.000
Cells*Concentration	2	479.6	239.8	122.41	0.000
Error	12	23.5	2.0		
Total	17	29997.0			

Grouping Information Using the Tukey Method and 95% Confidence

Cell Line	N	Mean	Grouping
PC3	6	67.1340	A
22Rv1	6	56.6514	B
LNCaP	6	55.7692	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping
Control	9	100.000	A
10	9	19.703	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Cells*Concentration	N	Mean	Grouping

LNCaP Control	3	100.000	A	
PC3 Control	3	100.000	A	
22Rv1 Control	3	100.000	A	
PC3 10	3	34.268	B	
22Rv1 10	3	13.303		C
LNCaP 10	3	11.538		C

Means that do not share a letter are significantly different.

Table A.42 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing the cell viability of the brain tumor cells

Concentration: 1.25 mg/ml

Grouping Information Using the Tukey Method and 95% Confidence

Cells	N	Mean	Grouping	
A172	16	96.5443	A	
U87MG	16	52.7062		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping	
C	16	100.000	A	
1.25	16	49.250		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping	
CI	16	76.5466	A	
HI	16	72.7038		B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Cells*Concentration	N	Mean	Grouping		
A172 C	8	100.000	A		
U87MG C	8	100.000	A		
A172 1.25	8	93.089		B	
U87MG 1.25	8	5.412			C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Cell Lines*Infusion	N	Mean	Grouping
A172 CI	8	99.0119	A
A172 HI	8	94.0767	B
U87MG CI	8	54.0813	C
U87MG HI	8	51.3310	D

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration*Infusion	N	Mean	Grouping
C CI	8	100.000	A
C HI	8	100.000	A
1.25 CI	8	53.093	B
1.25 HI	8	45.408	C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Cells*Concentration*Infusion	N	Mean	Grouping
A172 C CI	4	100.000	A
U87MG C HI	4	100.000	A
A172 C HI	4	100.000	A
U87MG C CI	4	100.000	A
A172 1.25 CI	4	98.024	A
A172 1.25 HI	4	88.153	B
U87MG 1.25 CI	4	8.163	C
U87MG 1.25 HI	4	2.662	D

Means that do not share a letter are significantly different.

Factor Information

Factor	Type	Levels	Values
Cells	Fixed	2	A172; U87MG
Concentration	Fixed	2	1.25; C
Infusion	Fixed	2	CI; HI

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value

Cells	1	15374.2	15374.2	5777.06	0.000
Concentration	1	20604.2	20604.2	7742.27	0.000
Infusion	1	118.1	118.1	44.39	0.000
Cells*Concentration	1	15374.2	15374.2	5777.06	0.000
Cells*Infusion	1	9.5	9.5	3.59	0.070
Concentration*Infusion	1	118.1	118.1	44.39	0.000
Cells*Concentration*Infusion	1	9.5	9.5	3.59	0.070
Error	24	63.9	2.7		
Total	31	51671.9			

Concentration: 2.5 mg/ml

Grouping Information Using the Tukey Method and 95% Confidence

Cell Lines	N	Mean	Grouping
A172	16	94.7838	A
U87MG	16	52.3442	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping
C	16	100.000	A
2.5	16	47.128	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
CI	16	75.3446	A
HI	16	71.7835	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Cells*Concentration	N	Mean	Grouping
A172 C	8	100.000	A
U87MG C	8	100.000	A
A172 2.5	8	89.568	B
U87MG 2.5	8	4.688	C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Cells*Infusion	N	Mean	Grouping
A172 CI	8	97.2332	A
A172 HI	8	92.3345	B
U87MG CI	8	53.4561	C
U87MG HI	8	51.2324	D

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration*Infusion	N	Mean	Grouping
C CI	8	100.000	A
C HI	8	100.000	A
2.5 CI	8	50.689	B
2.5 HI	8	43.567	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Cell Lines*Concentration*Infusion

Grouping Information Using the Tukey Method and 95% Confidence

Cells*Concentration*Infusion	N	Mean	Grouping
A172 C CI	4	100.000	A
U87MG C CI	4	100.000	A
U87MG C HI	4	100.000	A
A172 C HI	4	100.000	A
A172 2.5 CI	4	94.466	B
A172 2.5 HI	4	84.669	C
U87MG 2.5 CI	4	6.912	D
U87MG 2.5 HI	4	2.465	E

Means that do not share a letter are significantly different.

Factor Information

Factor	Type	Levels	Values
Cells	Fixed	2	A172; U87MG
Concentration	Fixed	2	2.5; C
Infusion	Fixed	2	CI; HI

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Cells	1	14409.0	14409.0	24123.65	0.000

Concentration	1	22363.5	22363.5	37441.25	0.000
Infusion	1	101.5	101.5	169.86	0.000
Cells*Concentration	1	14409.0	14409.0	24123.65	0.000
Cells*Infusion	1	14.3	14.3	23.96	0.000
Concentration*Infusion	1	101.5	101.5	169.86	0.000
Cells*Concentration*Infusion	1	14.3	14.3	23.96	0.000
Error	24	14.3	0.6		
Total	31	51427.3			

Concentration: 5 mg/ml

Grouping Information Using the Tukey Method and 95% Confidence

Cell Lines	N	Mean	Grouping
A172	15	93.7147	A
U87MG	16	51.1762	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping
C	16	100.000	A
5	15	44.891	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
CI	16	73.5588	A
HI	15	71.3321	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Cell Lines*Concentration	N	Mean	Grouping
U87MG C	8	100.000	A
A172 C	8	100.000	A
A172 5	7	87.429	B
U87MG 5	8	2.352	C

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Cell Lines*Infusion

Grouping Information Using the Tukey Method and 95% Confidence

Cells*Infusion	N	Mean	Grouping
A172 CI	8	95.8498	A
A172 HI	7	91.5796	B
U87MG CI	8	51.2678	C
U87MG HI	8	51.0845	C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration*Infusion	N	Mean	Grouping
C HI	8	100.000	A
C CI	8	100.000	A
5 CI	8	47.118	B
5 HI	7	42.664	C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Cells*Concentration*Infusion	N	Mean	Grouping
U87MG C HI	4	100.000	A
A172 C HI	4	100.000	A
U87MG C CI	4	100.000	A
A172 C CI	4	100.000	A
A172 5 CI	4	91.700	B
A172 5 HI	3	83.159	C
U87MG 5 CI	4	2.536	D
U87MG 5 HI	4	2.169	D

Means that do not share a letter are significantly different.

Concentration: 8 mg/ml

Factor Information

Factor	Type	Levels	Values
Cells	Fixed	2	A172; U87MG
Concentration	Fixed	2	5; C
Infusion	Fixed	2	CI; HI

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Cells	1	13897.1	13897.1	30602.25	0.000
Concentration	1	23324.3	23324.3	51361.33	0.000
Infusion	1	38.1	38.1	83.86	0.000
Cells*Concentration	1	13897.1	13897.1	30602.25	0.000
Cells*Infusion	1	32.1	32.1	70.62	0.000
Concentration*Infusion	1	38.1	38.1	83.86	0.000
Cells*Concentration*Infusion	1	32.1	32.1	70.62	0.000
Error	23	10.4	0.5		
Total	30	53286.6			

Factor Information

Factor	Type	Levels	Values
Cells	Fixed	2	A172; U87MG
Concentration	Fixed	2	8; C
Infusion	Fixed	2	CI; HI

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Cells	1	13810.2	13810.2	7174.93	0.000
Concentration	1	24995.7	24995.7	12986.20	0.000
Infusion	1	40.2	40.2	20.88	0.000
Cells*Concentration	1	13810.2	13810.2	7174.93	0.000
Cells*Infusion	1	39.6	39.6	20.59	0.000
Concentration*Infusion	1	40.2	40.2	20.88	0.000
Cells*Concentration*Infusion	1	39.6	39.6	20.59	0.000
Error	24	46.2	1.9		
Total	31	52822.0			

Grouping Information Using the Tukey Method and 95% Confidence

Cells	N	Mean	Grouping
A172	16	92.8258	A
U87MG	16	51.2773	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping
C	16	100.000	A
8	16	44.103	B

Means that do not share a letter are significantly different.

Tukey Pairwise Comparisons: Infusion

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
CI	16	73.1722	A
HI	16	70.9309	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Cells*Concentration	N	Mean	Grouping
A172 C	8	100.000	A
U87MG C	8	100.000	A
A172 8	8	85.652	B
U87MG 8	8	2.555	C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Cells*Infusion	N	Mean	Grouping
A172 CI	8	95.0593	A
A172 HI	8	90.5923	B
U87MG CI	8	51.2852	C
U87MG HI	8	51.2694	C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration*Infusion	N	Mean	Grouping
C CI	8	100.000	A
C HI	8	100.000	A
8 CI	8	46.344	B
8 HI	8	41.862	C

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Cells*Concentration*Infusion	N	Mean	Grouping
A172 C CI	4	100.000	A
A172 C HI	4	100.000	A

U87MG C CI	4	100.000	A		
U87MG C HI	4	100.000	A		
A172 8 CI	4	90.119		B	
A172 8 HI	4	81.185			C
U87MG 8 CI	4	2.570			D
U87MG 8 HI	4	2.539			D

Means that do not share a letter are significantly different.

Table A.43 ANOVA and Tukey's Comparison Test with 95% confidence level for comparing the cell viability of the breast cancer cells

Concentration: 1.25 mg/ml

Factor Information

Factor	Type	Levels	Values
Concentration	Fixed	2	1.25; C
Infusion	Fixed	2	CI; HI

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration	1	22096.5	22096.5	2247.46	0.000
Infusion	1	7.7	7.7	0.79	0.393
Concentration*Infusion	1	7.7	7.7	0.79	0.393
Error	12	118.0	9.8		
Total	15	22229.9			

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping
C	8	100.000	A
1.25	8	25.676	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
HI	8	63.5328	A
CI	8	62.1429	A

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration*Infusion	N	Mean	Grouping
C CI	4	100.000	A
C HI	4	100.000	A
1.25 HI	4	27.066	B
1.25 CI	4	24.286	B

Means that do not share a letter are significantly different.

Concentration: 2.5 mg/ml

Factor Information

Factor	Type	Levels	Values
Concentration	Fixed	2	2.5; C
Infusion	Fixed	2	CI; HI

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration	1	24536.8	24536.8	79684.18	0.000
Infusion	1	38.6	38.6	125.44	0.000
Concentration*Infusion	1	38.6	38.6	125.44	0.000
Error	12	3.7	0.3		
Total	15	24617.7			

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping
C	8	100.000	A
2.5	8	21.679	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
HI	8	62.3932	A
CI	8	59.2857	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration*Infusion	N	Mean	Grouping
C CI	4	100.000	A

C HI	4	100.000	A	
2.5 HI	4	24.786	B	
2.5 CI	4	18.571		C

Means that do not share a letter are significantly different.

Concentration: 5 mg/ml

Factor Information

Factor	Type	Levels	Values
Concentration	Fixed	2	5; C
Infusion	Fixed	2	CI; HI

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration	1	24880.3	24880.3	17814.74	0.000
Infusion	1	31.3	31.3	22.44	0.000
Concentration*Infusion	1	31.3	31.3	22.44	0.000
Error	12	16.8	1.4		
Total	15	24959.8			

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping
C	8	100.000	A
5	8	21.132	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
HI	8	61.9658	A
CI	8	59.1667	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration*Infusion	N	Mean	Grouping	
C CI	4	100.000	A	
C HI	4	100.000	A	
5 HI	4	23.932	B	
5 CI	4	18.333		C

Means that do not share a letter are significantly different.

Concentration: 8 mg/ml

Factor Information

Factor	Type	Levels	Values
Concentration	Fixed	2	8; C
Infusion	Fixed	2	CI; HI

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Concentration	1	27703.2	27703.2	14634.03	0.000
Infusion	1	95.0	95.0	50.19	0.000
Concentration*Infusion	1	95.0	95.0	50.19	0.000
Error	12	22.7	1.9		
Total	15	27916.0			

Grouping Information Using the Tukey Method and 95% Confidence

Concentration	N	Mean	Grouping
C	8	100.000	A
8	8	16.779	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Infusion	N	Mean	Grouping
HI	8	60.8262	A
CI	8	55.9524	B

Means that do not share a letter are significantly different.

Grouping Information Using the Tukey Method and 95% Confidence

Concentration*Infusion	N	Mean	Grouping		
C CI	4	100.000	A		
C HI	4	100.000	A		
8 HI	4	21.652		B	
8 CI	4	11.905			C

Means that do not share a letter are significantly differe