



Synergistic, antagonistic, and additive effects on the resultant antioxidant activity in infusions of green tea with bee honey and *Citrus limonum* extract as additives

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ABSTRACT

Foods consumed by living organisms contain antioxidants of different antioxidant capacities. In a mixture of antioxidants, the resultant antioxidant potential can be different from the mathematical sum of antioxidant activities exerted by individual components. The possible interactions include synergism, antagonism, and additivity between the bioactive compounds. The study was focused on the synergistic, antagonistic, and additive interactions of bioactive components on antioxidant activity in a ternary mixture of green tea (*Camellia sinensis*) with the added bee honey and *Citrus limonum* extract. The resultant antioxidant activity was assessed in terms of total phenolic content (TPC), total flavonoid content (TFC), and Trolox equivalent antioxidant capacity (TEAC). Green tea brew held at each temperature with bee honey added and bee honey with *Citrus limonum* extract added were analyzed for the TPC, TFC, and TEAC. The observed values of TPC, TFC, and TEAC of the binary and ternary mixtures were compared with the expected values or the mathematical sum of each TPC, TFC, and TEAC of individual ingredients. The TPC and TEAC of each sample demonstrated synergism where the effect was antagonistic on TFC. Each sample demonstrated a significant difference ($p < 0.05$) in all TPC, TFC, and TEAC. The impact of the temperature of green tea brew at which the additives were added, particularly room (29 °C) and chilling temperatures (7 °C), was addressed considering the effect of additive and temperature collectively on the resultant antioxidant activity.

1. Introduction

Green tea (*Camellia sinensis*) has gained massive popularity over the last few years for its beneficial effects on human health. The health-promoting properties and the compounds responsible for those properties in green tea are intensively researched, which are potentially relevant to the prevention of diseases. Consumption of green tea is proven to be anticarcinogenic [1], antidiabetic [2], cardioprotective [3], and effective against obesity [4], and neurodegenerative diseases [5].

Bioactive constituents are the predominant compounds of interest that result in regulating and modulating metabolic processes leading to beneficial health effects. Green tea contains high contents of antioxidants, which helps reduce oxidative stress within the body. Most of the green tea antioxidants are flavonols commonly known as catechins namely, (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG), and (–)-epigallocatechin-3-gallate (EGCG).

Theobromine, theophylline, caffeine, and phenolic acids are also present in green tea as minor components [6,7].

The addition of certain ingredients like bee honey, ginger, cinnamon, and garlic is known to increase the effectiveness of ayurvedic medicines. Making this point the base for this research, the addition of bee honey and lemon juice extract (*Citrus limonum*) to green tea brew is addressed in this study.

The combination of green tea, bee honey, and *Citrus limonum* is not a novel thing, which has been a healthy practice among locals for ages. Even bee honey, along with *Citrus limonum* itself, is a home remedy for preventing infective respiratory diseases. Sometimes green tea may be incorporated either with bee honey or *Citrus limonum*.

Bee honey is a functional food due to its naturally high antioxidant potential, contributing to preventive action against certain illnesses [8]. Nevertheless, the application of bee honey in modern medicine is declined due to the advancement of new drugs.

Citrus limon L./Citrus limonum or generally known as lemon, is one of

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Abbreviations

TPC	Total phenolic content
TFC	Total flavonoid content
TEAC	Trolox equivalent antioxidant capacity
EC	(–)-Epicatechin
EGC	(–)-Epigallocatechin
ECG	(–)-Epicatechin-3-gallate
EGCG	(–)-Epigallocatechin-3-gallate
Trolox	6-Hydroxy-2,5,7,8 tetramethylchroma n-2-carboxylic acid
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
GAE	Gallic acid equivalent
QE	Quercetin equivalent
TE	Trolox equivalent
HMF	5-Hydroxymethylfurfural
DNA	Deoxyribonucleic acid

the well-known and most used species of the genus *Citrus*. The *Citrus limonum* fruit is a remarking source of bioactive compounds and is usually underestimated in modern physiotherapy [9]. The juice extract of *Citrus limonum* had been used as an ancient remedy for treating vitamin C deficiency (scurvy) before the discovery of vitamin C. *Citrus limonum* extract is also known as a traditional medicine for treating irregular menstruation, high blood pressure, and the common cold [10–12].

The action of food, when present in a matrix, on human biological systems is not the same as the corresponding actions of the individual food components [13]. The bioactive compounds of green tea are considered to exert a considerable amount of functional properties. Nonetheless, the extent to which the functional properties exerted by green tea gets changed when green tea is accompanied by certain add-ons. The correlation of functional properties of the mixture can either be equal to the mathematical cumulative of each component before mixing or possibly be greater or less than that of each component before mixing. If the magnitude to which the functional properties exerted by the resulting mixture is equal to the mathematical sum of that of the individual, prior to mixing, the interaction is termed additive. In the same way, when the extent of the functional properties of the mixture is greater than the cumulative individual contribution, the interaction is said to be synergistic. If the resultant mixture possesses reduced amounts of the selected functional properties than that of the cumulative of the individual's input, the interaction is termed antagonistic.

Synergistic, antagonistic, and additive effects of bioactive components when existing in a mixture, have been presented in literature and some studies have been discussed in this study. As per the study conducted by Pereira et al. [14] on 'the ability of honey to potentiate the antioxidant and cytotoxic properties of medicinal plants consumed as infusions for hepatoprotective effects,' it was proved that the addition of honey led to elevated antioxidant and cytoprotective properties in plant-based infusions. Cardamom tea was added with organic apple juice, and it was observed that the final mixture demonstrated a synergistic effect in total phenolic content and antioxidant activity. The antioxidant activity in the final beverage was twice the amount compared to the apple juice and tea individually [15]. A synergistic antioxidant activity has been observed for a mixture of naringenin and hesperetin [16]. An evaluation of the use of honey, along with various add-ons, demonstrated to be more beneficial in treating ailments in mice in terms of microbial infections and improving white blood cells to enhance immunity [17]. Furthermore, the study evidenced that Omani honey with turmeric showed to be more antimicrobial, and that was

greater than the sum of individuals, revealing their synergistic effect. Synergistic antioxidant activity in mixtures of *Cymbopogon citratus* and *Pterostemum tridentatum* or *Gomphrena globosa* infusions has been observed by Roriz et al. [18]. These are medicinal plants with antioxidant properties of their own nevertheless the antioxidant activity of the mixture tends to be enhanced than the individual cumulative.

Formation of hydrogen bonding between carbonyl groups in the ellagic acid and o-dihydroxyl groups in catechin, which hinders the hydrogen donating ability of catechin, was observed to be the causative of antagonistic effect on antioxidant activity in mixtures of catechin and ellagic acid [19].

The additive effect always denotes the absence of synergistic or antagonistic interactions between the targeted bioactive components [20]. This occurs when a single bioactive component acts independently on each other. One's action is not disrupted by any other bioactive component in the complex mixture.

Adding bee honey and *Citrus limonum* in green tea brew is more likely intended to increase the health-promoting attributes. This can alter the grassy, vegetal, and astringent notes of flavour in green tea brew positively up to some extent, yet the predominant aim is to enhance the functional properties collectively. The study was focused on assessing the interactions of bioactive compounds, namely, synergism, antagonism, and additivity, on the resultant antioxidant activity in infusions of green tea with bee honey and *Citrus limonum* extract as additives.

Besides, the impact of infusion temperature was evaluated at which bee honey and *Citrus limonum* extract were incorporated. The addition of additives was carried under reduced temperatures, particularly at room temperature (29 °C) and chilling temperature conditions (7 °C), since both the temperature and the duration of heat treatment affect the formation of 5-Hydroxymethylfurfural (HMF) in honey [21]. HMF and its derivatives may contribute to the toxicity of honey [22], which is reported to have a negative impact on human health. HMF easily gets absorbed from food through the gastrointestinal tract and metabolizes into different derivatives. HMF and its derivatives exert to be genotoxic, mutagenic, DNA-damaging, organotoxic, carcinogenic, and enzyme inhibitory [23].

2. Materials and methods

2.1. Materials

Gallic acid, quercetin, and Trolox were purchased from MP Bio-medicals (France). Sodium nitrite AR, potassium persulfate, and HCl were procured from Research-Lab Fine Chem (India), and Folin-Ciocalteu reagent was from Loba Chemie (India). Sodium carbonate anhydrous 99.5% was purchased from Techno Pharmchem (India), and aluminium chloride hexahydrate was obtained from Hopkin & Williams (Essex, England). Sodium hydroxide was obtained from HiMedia, India. Moreover, ABTS was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Methods

2.2.1. Sample preparation

Green tea was brewed at 80 °C using 220 ml of boiled/hot water per serving (one tea bag), dipped for 2 min considering the guidelines given in the packaging (70–80 °C for 2–3 min in 220 ml of water), and allowed to cool down to the room temperature (29 °C). Another set of samples was brought down to chilling temperature conditions (7 °C). Plain green tea, which was cooled down to room temperature, and the chilling temperature were analyzed for total phenolic content, total flavonoid content, and Trolox equivalent antioxidant capacity (TEAC) using ABTS^{•+} radical scavenging activity.

Bee honey and *Citrus limonum* extract were tested separately for total phenolic content, total flavonoid content, and Trolox equivalent antioxidant capacity (TEAC) to assess the antioxidant activity exerted by

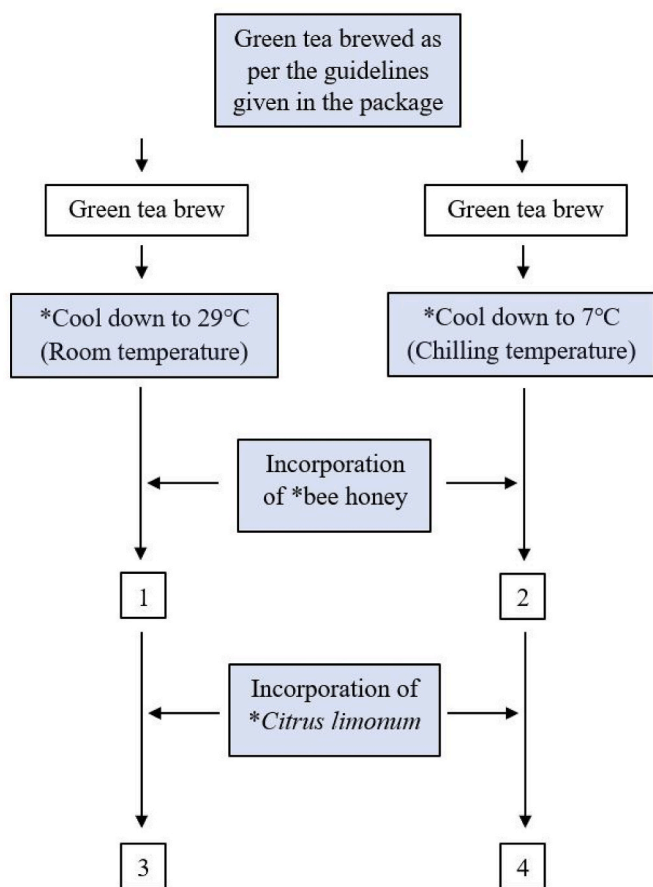
green tea, bee honey, and *Citrus limonum* extract individually. Bee honey was collected from a beekeeper located in Meegoda (Latitude: 6.82162, Longitude: 80.04744, Homagama, Colombo) Sri Lanka. Fruits of *Citrus limonum* were obtained from a randomly selected healthy tree cultivated in Kiriwattuduwa (Latitude: 6.92496, Longitude: 79.87228, Homagama, Colombo) Sri Lanka. Fruits were collected from the same branch, the same bunch, to minimize variations.

Two sets of green tea samples were added with bee honey to determine the impact of the addition of bee honey on the cumulative TPC, TFC, and TEAC exerted by green tea and bee honey at room temperature (29 °C) and chilling temperature (7 °C), respectively. 7.5g bee honey/cup of green tea brew was added with reference to the study done by Toydemir et al. [24] with modifications.

The resulting samples were added with *Citrus limonum* extract to determine the impact of incorporating *Citrus limonum* as an additive on the cumulative TPC, TFC, and TEAC exerted when green tea is mixed with bee honey and *Citrus limonum* at 29 °C and 7 °C, respectively. A quantity of 3 ml *Citrus limonum* per cup was considered, following the optimization to determine the quantity of *Citrus limonum* to be added.

The addition of additives was carried out in an order as given in Fig. 1 where bee honey was added as the initial step, followed by adding *Citrus limonum*.

Green tea brew cooled down to 29 °C (room temperature) and 7 °C (chilling temperature), bee honey, and *Citrus limonum* marked with an asterisk are controls. In the process of adding bee honey and green tea, samples 1 and 2 are binary mixtures and samples 3 and 4 are ternary mixtures which are of interest. Samples were analyzed for TPC, TFC, and TEAC at each 1,2,3, and 4 points.



*Controls

Fig. 1. The steps involved in introducing additives.

2.2.2. Determination of total phenolic content, total flavonoid content and Trolox equivalent antioxidant activity

2.2.2.1. Determination of total phenolic content

2.2.2.1.1. *TPC of green tea, binary and ternary mixtures.* Total polyphenolic content was determined using the Folin-Ciocalteu reagent as described by Anesini et al. [25] with few modifications. A stock solution of gallic acid 1 mg/ml was prepared by dissolving 50 mg of gallic acid in distilled water, making the volume up to 50 ml. Then 250 µl of the gallic acid stock solution was dissolved in 9750 µl of distilled water to make the standard solution of 25 µg/ml. A dilution series of 50, 100, 200, and 400 µl was prepared using distilled water.

The working solutions were prepared by dissolving 3 ml of the prepared green tea brews cooled down to ambient temperature and chilling temperature, respectively, in 100 ml of distilled water. 1 ml of working solution of green tea brew cooled down to ambient temperature, and chilling temperature/1 ml of standard solutions was reacted with 5 ml of 10% Folin-Ciocalteu reagent and 4 ml of 7.5% sodium carbonate solution which were then allowed to stand for 2 h in the dark at room temperature, and the absorbance was measured at 765 nm against distilled water. A standard curve was plotted between the concentrations and absorbance of gallic acid standards. The total phenolic content was expressed as gallic acid equivalent (GAE) in µg/ml of green tea.

2.2.2.1.2. *TPC of bee honey.* The method used for the determination of the total phenolic content of bee honey was conducted as proposed by Biesaga & Pyrzyńska [26], with minor modifications. 5 ml of 40% methanol/acidified water (v/v), (acidified with HCl to adjust the pH value to 2) was used to dissolve 1g of bee honey and was stirred for 15 min using a magnetic stirrer. 200 µl of bee honey extract was added with 2 ml of 10% Folin-Ciocalteu reagent and 1.8 ml of 7.5% (w/v) sodium carbonate. The samples were left to stand for 20 min in the dark, and the absorbance was measured at 765 nm. Referring to the standard curve, which was plotted between the concentrations and absorbance of gallic acid standard, the total phenolic content was expressed as gallic acid equivalent (GAE) in mg/g material.

2.2.2.1.3. *TPC of Citrus limonum.* The total phenolic content in *Citrus limonum* extract was determined by the previously reported Folin-Ciocalteu method by Abdullakassim et al. [27] with few modifications. 5 ml of freshly extracted *Citrus limonum* was mixed with 5 ml of 80% methanol and centrifuged at 4000 rpm for 20 min. 100 µl of the supernatant was mixed thoroughly with 100 µl (10%) Folin-Ciocalteu reagent and 3 ml of distilled water. After 10 min of incubation time at ambient temperature, 100 µl of 20% (w/v) sodium carbonate solution was added with immediate mixing and further incubated for 2 h at ambient temperature in the absence of light. The absorbance was then measured at 765 nm. Referring to the standard curve plotted, the total phenolic content of the sample was expressed in milligrams per ml as gallic acid equivalent (mg GAE/ml).

2.2.2.2. Determination of total flavonoid content

2.2.2.2.1. *TFC of green tea, binary and ternary mixtures.* The total flavonoid content of sample extracts was determined by a method described by Makris et al. [28] with minor modifications. The calibration curve was plotted using quercetin as the standard. A stock solution of quercetin having a concentration of 0.5 mg/ml methanol was prepared. A dilution series was then prepared for 5, 10, 20, 30, 40, and 50 µg/ml methanol.

A 1 ml aliquot of the sample/standard was added to a volumetric flask containing 300 µl of 0.05% (w/v) sodium nitrite and allowed to stand for 5 min. After 5 min, 300 µl of 0.1% (w/v) aluminium chloride was added and left for 6 min. Then 2 ml of 1 M sodium hydroxide was put into the mixture and made the volume up to 10 ml with distilled water. The resulting solution was mixed properly, and the absorbance was measured at 510 nm. The concentrations of the samples were expressed as µg quercetin equivalent per ml material (µg QE/ml).

2.2.2.2. TFC of bee honey. From the same extract prepared for analyzing the TPC in bee honey, 5 ml was mixed with 300 μ l of 5% (w/v) sodium nitrite and allowed to stand for 5 min. Then 300 μ l of 10% (w/v) aluminium chloride was added, and the resulting solution was allowed to stand for 6 min in the dark. Then it was mixed with 2 ml of NaOH 1 M and topped the volume up to 10 ml with distilled water. The absorbance of each sample was read at 510 nm. The calibration curve was plotted using quercetin as the standard, and the concentrations of the samples were expressed as μ g quercetin equivalent per gram material (μ g QE/g).

2.2.2.3. TFC of *Citrus limonum*. The total flavonoid content of *Citrus limonum* extract was determined as reported by Wang et al. [29]. 300 μ l of 5% sodium nitrite was added to 1 ml of *Citrus limonum* extract and allowed to stand for 5 min at room temperature. 300 μ l of 10% aluminium chloride was mixed and incubated for 6 min again. Then 4 ml of 1 M sodium hydroxide was added to the resulting solution. After an incubation time of 15 min in the absence of light at room temperature, absorbance was measured at 510 nm. The total flavonoid content was expressed as μ g quercetin equivalent per ml *Citrus limonum* extract, referring to the calibration curve.

2.2.2.3. Determination of Trolox equivalent antioxidant activity

2.2.2.3.1. TEAC of green tea, binary and ternary mixtures. The Trolox equivalent antioxidant capacity (TEAC) of sample extracts was determined by a method described by Re et al. [30] and Rice Evans & Miller [31], with slight modifications. 360 mg of ABTS was dissolved in 100 ml of distilled water to obtain a 7.0 mM ABTS solution. 2.45 mM potassium persulfate solution was prepared by dissolving 66 mg of potassium persulfate in 100 ml of distilled water. ABTS^{•+} radical solution was prepared by mixing equal parts (v/v) of 7.0 mM ABTS and 2.45 mM potassium persulfate solutions. This solution was allowed to stand for 16 h at room temperature in the absence of light.

The Trolox stock solution was prepared by dissolving 0.375 mg of Trolox in methanol which was topped up to 50 ml in a volumetric flask. A standard dilution series was prepared within the range of 5–30 μ M/ml. The ABTS^{•+} radical solution was added with ethyl alcohol to adjust the absorbance value to 0.700 (± 0.02) at 734 nm.

200 μ l of green tea/standard was mixed with 1800 μ l ABTS^{•+} radical solution adjusted for its absorbance as mentioned above and allowed to react for 30 min at room temperature in the absence of light. The absorbance was measured at 734 nm. The calibration curve was plotted using Trolox as the standard, and the concentrations of the samples were expressed as μ M Trolox equivalent per ml material (μ M TE/ml).

2.2.2.3.2. TEAC of bee honey. 100 μ l of the same honey extract taken for the analysis of TPC and TFC was mixed with 900 μ l of ABTS^{•+} working solution and incubated at room temperature in the absence of light for 2.5 min. The absorbance was measured at 734 nm. The results were expressed as μ M Trolox equivalent per gram of bee honey (μ M TE/g), referring to the standard curve plotted.

2.2.2.3.3. TEAC of *Citrus limonum*. Freshly extracted *Citrus limonum* extract was centrifuged at 2800 rpm for 10 min. The supernatant was collected for analysis. 25 μ l of the supernatant of *Citrus limonum* extract was mixed with the working ABTS^{•+} radical solution to a final volume of 1 ml, and the absorbance of the samples was measured at 734 nm after 4 min of incubation in the dark. The concentrations of the samples were expressed as μ M Trolox equivalent per ml material (μ M TE/ml), referring to the calibration curve plotted.

All the tests were carried out in triplicates. The impact of additives on TPC, TFC, and TEAC of the resulting infusions was addressed considering the effect of additives as the sole variable on the resultant antioxidant activity of the mixture. The impact of temperature and additive effect together on TPC, TFC, and TEAC of the resulting infusions were then addressed, considering the effect of additive and temperature as two variables on the resultant antioxidant activity.

2.2.3. Interactions

The observed antioxidant activities through TPC, TFC, and TEAC were compared with the expected antioxidant activities or the mathematical sum of the antioxidant activities possessed by the individual component. The expected antioxidant activity of the ternary mixture was assessed per one cup of green tea with additives added and calculated per ml of the final infusion. An assumption was made as the infusion volume remained the same throughout the analysis.

2.2.4. Statistical analysis

The data of the TPC, TFC, and TEAC of green tea, bee honey, *Citrus limonum* extract samples and the above-mentioned combinations considering the additive effect as the sole factor were statistically evaluated by one-way analysis of variance (ANOVA) using Minitab-21 software. The significant differences between means were determined by Tukey's multiple comparison. All the test procedures were made at a 5% significance level.

The impact of holding temperature together with the additive effect of bee honey and *Citrus limonum* extract (as two variables) on the antioxidant activity (TPC, TFC, and TEAC) of the final infusion was statistically evaluated ($p < 0.05$) by two-way analysis of variance (ANOVA) using Minitab-21 software and Tukey pairwise comparison, accompanied by Tukey simultaneous tests for differences of means.

A paired *t*-test was conducted between the green tea brews held at room and chilling temperature at 0.05 significance level for TPC, TFC, and TEAC, respectively.

3. Results

3.1. Determination of total phenolic content, total flavonoid content and Trolox equivalent antioxidant activity

3.1.1. TPC, TFC and TEAC exerted by individual components

The antioxidant activities of individual ingredients namely, green tea held at room temperature and chilling temperature, bee honey and *Citrus limonum* extract are presented in terms of TPC, TFC and TEAC in Table 1.

There was no significant difference ($p > 0.05$) existed between the TPC of GT_R and GT_C, which indicates that the holding temperature of green tea brew had no significant effect on the TPC. Nevertheless, the holding temperature of the green tea brew on the TFC and TEAC of GT_R and GT_C demonstrated a significant difference ($p < 0.05$), where the TFC of GT_R appeared to be significantly higher than that of GT_C, and the TEAC of GT_C was significantly higher than that of GT_R.

3.1.2. The impact of additive on TPC, TFC and TEAC of the resulting infusions

3.1.2.1. The impact of additives on TPC. The TPC was significantly higher ($p < 0.05$) when both bee honey and *Citrus limonum* were added

Table 1
Antioxidant activities in terms of TPC, TFC and TEAC of individual components.

Control	TPC	TFC	TEAC
Green tea	GT _R 2276.0 $\pm$ 71.4 μ g GAE/ml	6.6 $\pm$ 0.2 μ g QE/ml	6.2 $\pm$ 0.2 mM TE/ml
	GT _C 2306.2 $\pm$ 86.9 μ g GAE/ml	5.4 $\pm$ 0.3 μ g QE/ml	6.9 $\pm$ 0.1 mM TE/ml
Bee honey	3.9 $\pm$ 0.2 μ g GAE/g	2.2 $\pm$ 0.1 μ g QE/g	0.3 $\pm$ 0.1 mM TE/g
<i>Citrus limonum</i> extract	475.6 $\pm$ 16.0 μ g GAE/ml	3.8 $\pm$ 0.1 μ g QE/ml	0.8 $\pm$ 0.1 mM TE/ml

Note: Data presented as mean $\pm$ SD ($n = 3$). TPC: μ g Gallic Acid Equivalent per ml infusion; TFC: μ g Quercetin Equivalent per ml infusion; TEAC: mM Trolox Equivalent per ml infusion; GT_R: green tea held at room temperature; GT_C: green tea held at chilling temperature.

to plain green tea brew which was 3160.2 ± 117.1 $\mu\text{g GAE/ml}$ infusion, followed by green tea with bee honey marked as 2566.3 ± 63.4 $\mu\text{g GAE/ml}$. As illustrated in Table 2, a significantly lower TPC was marked by the control, or the plain green tea held at room temperature as 2276.0 ± 71.4 $\mu\text{g GAE/ml}$.

As far as chilling temperature conditions are considered, the TPC of green tea added with both bee honey and *Citrus limonum* extract was significantly higher ($p < 0.05$). Green tea added with bee honey alone was significantly lower than the above-mentioned combination of bee honey and *Citrus limonum* extract but was significantly higher than the TPC of the control.

3.1.2.2. The impact of additives on TFC. The total flavonoid content exhibited to be decreased unlike total phenolic content with the addition of bee honey and *Citrus limonum* extract at room temperature. The total flavonoid content of the plain green tea held at room temperature, green tea added with bee honey alone and green tea added with bee honey and *Citrus limonum* extract at room temperature were significantly different ($p < 0.05$). As shown in Table 2, the highest TFC was expressed as 6.6 ± 0.2 $\mu\text{g QE/ml}$ infusion by the control GT_R . The addition of bee honey to green tea brew at room temperature has led to a reduction of TFC in the binary mixture to a level of 2.4 ± 0.1 $\mu\text{g QE/ml}$ infusion. The addition of *Citrus limonum* extract has increased the TFC to 4.9 ± 0.4 $\mu\text{g QE/ml}$ infusion in the $\text{GT}_R + \text{H} + \text{L}$, yet the control stands first in total flavonoid content out of all three.

Under chilling temperature conditions, control exhibited the highest TFC of 5.4 ± 0.3 $\mu\text{g QE/ml}$ infusion. The lowest TFC was marked by $\text{GT}_C + \text{H}$ which was 2.1 ± 0.2 $\mu\text{g QE/ml}$ infusion. With *Citrus limonum* extract added, the $\text{GT}_C + \text{H} + \text{L}$ set of samples demonstrated a TFC of 4.1 ± 0.2 $\mu\text{g QE/ml}$ infusion, as given in Table 2. Again, adding *Citrus limonum* extract has enhanced the TFC of the $\text{GT}_C + \text{H} + \text{L}$ yet is less than the TFC of GT_C .

3.1.2.3. The impact of additives on TEAC. The TEAC of the control, green tea added with bee honey, and green tea added with bee honey and *Citrus limonum* extract at room temperature were significantly different ($p < 0.05$) and are given in Table 2. The highest TEAC was marked by $\text{GT}_R + \text{H} + \text{L}$ which was 10.8 ± 0.3 mM TE/ml infusion followed by $\text{GT}_R + \text{H}$ (7.3 ± 0.2 mM TE/ml infusion) and GT_R marks the

Table 2

Antioxidant activities in terms of TPC, TFC and TEAC of the resulting infusions added with bee honey and *Citrus limonum* extract as additives.

Temperature	Sample name	TPC ($\mu\text{g GAE/ml}$ infusion)	TFC ($\mu\text{g QE/ml}$ infusion)	TEAC (mM TE/ml infusion)
Room temperature	GT_R	2276.0 ± 71.4^c	6.6 ± 0.2^a	6.2 ± 0.2^c
	$\text{GT}_R + \text{H}$	2566.3 ± 63.4^b	2.4 ± 0.1^c	7.3 ± 0.2^b
	$\text{GT}_R + \text{H} + \text{L}$	3160.2 ± 117.1^a	4.9 ± 0.4^b	10.8 ± 0.3^a
Chilling temperature	GT_C	2306.2 ± 86.9^c	5.4 ± 0.3^a	6.9 ± 0.1^c
	$\text{GT}_C + \text{H}$	3366.6 ± 57.9^b	2.1 ± 0.2^c	8.1 ± 0.1^b
	$\text{GT}_C + \text{H} + \text{L}$	4032.0 ± 56.4^a	4.1 ± 0.2^b	11.6 ± 0.3^a

Note: Data presented as mean $\pm$ SD ($n = 3$). Mean values in a column superscripted by different letters are significantly different at $p < 0.05$. TPC: $\mu\text{g Gallic Acid Equivalent per ml}$ infusion; TFC: $\mu\text{g Quercetin Equivalent per ml}$ infusion; TEAC: $\text{mM Trolox Equivalent per ml}$ infusion; GT_R : green tea held at room temperature; $\text{GT}_R + \text{H}$: green tea held at room temperature + bee honey; $\text{GT}_R + \text{H} + \text{L}$: green tea held at room temperature + bee honey + *Citrus limonum*; GT_C : green tea held at chilling temperature; $\text{GT}_C + \text{H}$: green tea held at chilling temperature + bee honey; $\text{GT}_C + \text{H} + \text{L}$: green tea held at chilling temperature + bee honey + *Citrus limonum*.

lowest TEAC as 6.2 ± 0.2 mM TE/ml infusion. The addition of bee honey and *Citrus limonum* extract has a positive impact on increasing the TEAC of the ternary mixture considerably.

When chilling conditions are considered, the scenario remains the same, yet the Trolox equivalent antioxidant capacities are shown to be higher than that of room temperature conditions. As far as chilling conditions are concerned the lowest TEAC was reported by the green tea sample held at chilling temperature as 6.9 ± 0.1 mM TE/ml infusion as shown in Table 2. The addition of bee honey has led to an increase in the antioxidant activity to a level of 8.1 ± 0.1 mM TE/ml infusion, and furthermore, the incorporation of *Citrus limonum* extract has resulted in an elevation of TEAC in the ternary mixture. The highest antioxidant activity in terms of TEAC is marked by $\text{GT}_C + \text{H} + \text{L}$ as 11.6 ± 0.3 mM TE/ml infusion out of all.

3.1.3. The impact of temperature and additive effect on TPC, TFC and TEAC of the resulting infusions

3.1.3.1. The impact of temperature and additive effect on TPC. Both the factors of treatment and the temperature are statistically significant since the p-values for treatment and temperature are less than 0.05. The interaction of treatment temperature also shows a p-value less than 0.05, which indicates the treatment-temperature interaction is statistically significant. So that the treatment, temperature, and treatment-temperature interaction affect the total phenolic content of the infusion.

$\text{GT}_C + \text{H} + \text{L}$ and $\text{GT}_R + \text{H} + \text{L}$ exhibited a significant difference ($p < 0.05$) in TPC, and $\text{GT}_R + \text{H} + \text{L}$ demonstrated a significantly lower TPC compared to that of $\text{GT}_C + \text{H} + \text{L}$, indicating that the temperature, has an impact on total phenolic content of the ternary mixture with both bee honey and *Citrus limonum* extract added. Moreover, $\text{GT}_C + \text{H} + \text{L}$ marks the highest TPC amongst all the treatments being the most effective combination of treatments in terms of total phenolic content.

3.1.3.2. The impact of temperature and additive effect on TFC. Both the factors treatment and temperature are statistically significant since the p-values for treatment and temperature are less than 0.05. The interaction of treatment temperature also shows a p-value less than 0.05, which indicates the treatment-temperature interaction is statistically significant. So, the factors of treatment, temperature, and treatment-temperature interaction do impact the total flavonoid content of the infusion.

Again, the TFC of $\text{GT}_R + \text{H} + \text{L}$ and $\text{GT}_C + \text{H} + \text{L}$ are significantly different ($p < 0.05$), which means the addition of *Citrus limonum* extract has positively affected the final TFC of ternary solutions. Yet, the controls possess the greatest TFC of all. Unlike TPC, the TFC of green tea held at room temperature with respective additives is greater than that of green tea held at chilling temperature with additives.

3.1.3.3. The impact of temperature and additive effect on TEAC. As of the statistical analysis, both temperature and the treatment are significant factors affecting the TEAC of the resulting infusion ($p < 0.05$). The interaction of temperature-treatment exhibits a p-value greater than 0.05, which indicates that the temperature-treatment interaction is not statistically significant. So that the interaction of temperature treatment has no significant impact on the Trolox Equivalent Antioxidant Capacity of the resulting infusion.

$\text{GT}_C + \text{H} + \text{L}$, and $\text{GT}_R + \text{H} + \text{L}$, also demonstrate a significant difference between the antioxidant contents with respect to TEAC. The highest TEAC was marked by green tea added with bee honey and *Citrus limonum* extract at chilling temperature.

3.2. Interactions

As illustrated in Table 3, the observed TPC of the binary and ternary mixtures are greater than the expected TPC or the mathematical

Table 3

Observed TPC, TFC and TEAC vs expected TPC, TFC and TEAC held at room temperature and chilling temperature.

TPC ($\mu\text{g GAE/ml}$)			
Sample	Observed value	Expected value	Type of interaction
GT _R + H	2566.3 $\pm$ 63.4	2217.7 $\pm$ 69.6	Synergism
GT _C + H	3366.6 $\pm$ 57.9	2247.2 $\pm$ 84.7	Synergism
GT _R + H + L	3160.2 $\pm$ 117.1	2194.9 $\pm$ 68.7	Synergism
GT _C + H + L	4032.0 $\pm$ 56.4	2223.9 $\pm$ 83.6	Synergism
TFC ($\mu\text{g QE/ml}$)			
GT _R + H	2.4 $\pm$ 0.1	6.5 $\pm$ 0.2	Antagonism
GT _C + H	2.1 $\pm$ 0.2	5.4 $\pm$ 0.3	Antagonism
GT _R + H + L	4.9 $\pm$ 0.4	6.5 $\pm$ 0.2	Antagonism
GT _C + H + L	4.1 $\pm$ 0.2	5.3 $\pm$ 0.3	Antagonism
TEAC (mM TE/ml)			
GT _R + H	7.3 $\pm$ 0.2	6.0 $\pm$ 0.2	Synergism
GT _C + H	8.1 $\pm$ 0.1	6.7 $\pm$ 0.1	Synergism
GT _R + H + L	10.8 $\pm$ 0.3	6.0 $\pm$ 0.2	Synergism
GT _C + H + L	11.6 $\pm$ 0.3	6.6 $\pm$ 0.1	Synergism

Note: Data presented as mean $\pm$ SD (n = 3). Mean values in a column superscripted by different letters are significantly different at $p < 0.05$. TPC: μg Gallic Acid Equivalent per ml infusion; TFC: μg Quercetin Equivalent per ml infusion; TEAC: mM Trolox Equivalent per ml infusion; GT_R: green tea held at room temperature; GT_R+ H: green tea held at room temperature + bee honey; GT_R+ H + L: green tea held at room temperature + bee honey + *Citrus limonum*; GT_C: green tea held at chilling temperature; GT_C+ H: green tea held at chilling temperature + bee honey; GT_C+ H + L: green tea held at chilling temperature + bee honey + *Citrus limonum*.

cumulative of individual ingredients. So that the type of interaction between green tea, bee honey and *Citrus limonum* extract on the TPC of the ternary mixture is synergism. The degree of synergism has increased with the addition of *Citrus limonum* extract compared to the addition of bee honey alone. Moreover, the synergistic total phenolic content of samples held at chilling temperature is greater than that of samples held at room temperature.

The addition of bee honey to green tea held at room temperature and the chilling temperature has resulted in a reduction of the TFC of the binary mixture, which indicates an antagonistic effect on the TFC. However, unlike TPC, the TFC of GT_C+ H is less than the TFC of GT_R+ H. The addition of *Citrus limonum* to GT_R+ H and GT_C+ H has increased the TFC, yet less than the TFC expected as of the mathematical sum of TFC possessed by the individual components. So that the combinations of GT_R+ H + L and GT_C+ H + L have also led to an antagonistic effect on the TFC of the ternary mixture. Again, the GT_C+ H + L combination exerts a reduced TFC compared to that of GT_R+ H + L.

As far as Trolox equivalent antioxidant activity is considered the type of interaction is synergism. The addition of *Citrus limonum* extract has a greater impact on the TEAC when compared with the TEAC exerted by the GT_R+ H, GT_C+ H combinations. The lower the temperature of holding green tea brew, the greater the TEAC of the resulting infusion.

4. Discussion

The mechanisms behind the interactions have hardly been explained due to the complex nature of the mixtures, particularly plant extracts. The explanations for synergism, antagonism and additivity are presented by Olszowy-Tomczyk [32] as discussed below.

Formulation of more robust antioxidants out of weaker antioxidants, formation of highly stable intermolecular complexes, dimers, adducts, or/and new phenolic compounds with elevated antioxidant activity compared to the parent component, differences in solubility and phase distribution of antioxidants near and at the interface and occurrence of uncertain interactions between the compounds are endorsed in justifying the synergistic antioxidant activity in complex mixtures of bioactive components by Olszowy-Tomczyk [32].

Besides, the antagonistic effect on antioxidant activity has been explained below. The regeneration of weaker antioxidants from the stronger ones, formation of complexes and adducts, polymerization reactions occurring between the antioxidants resulting in compounds owing to reduced antioxidant properties, reactions between free antioxidant radicals amidst the chain reactions causing neutralization, and unpredictable mutual interactions within antioxidants are stated to account for antagonism [32]. The study justifies the additive effect on the resultant antioxidant activity of a mixture of bioactive components by the lack of interactions between the antioxidants causing the bioactive components to behave the same way in the mixture as occurring individually and uncertain mutual interactions. The clarifications for all synergistic, antagonistic and additive interactions between the antioxidants are, at times, left undefined due to uncertain interactions between the compounds.

As per the results, incorporating bee honey and *Citrus limonum* into the green tea brew has led to elevated TPC and TEAC of the resulting mixtures, irrespective of the holding temperature of the brew. Compared to the individual effect of adding bee honey, incorporating both bee honey and *Citrus limonum* has intensified the observed TPC and TEAC. The synergistic effect of incorporating green tea with bee honey alone and both bee honey and *Citrus limonum* extract on TPC and TEAC can be disclosed with the above clarifications.

The facts mentioned above could account for the observed antagonistic effect on the TFC of the green tea brew added with bee honey (GT_R+ H, GT_C+ H). The interactions between the compounds at combined state, which lead to an antagonistic effect on the TFC of the resulting solutions, make the control the highest TFC. Adding *Citrus limonum* to the above combination has a synergistic effect on the resultant TFC. However, the addition of *Citrus limonum* has not exceeded the TFC exerted by the controls.

Unlike TPC and TEAC, the TFC of green tea brew itself, held at 7 °C, was significantly lower ($p < 0.05$) than green tea brew held at 29 °C, which might have a direct impact on TFC. Notably, the addition of bee honey has resulted in a significant decrease ($p < 0.05$) in the TFC of the resulting mixture.

The declination of the TFC has previously been reported by Toydemir et al. [24]. According to their study, adding flower honey to green tea has led to significant decreases ($p < 0.05$) in the TFC of resulting solutions. Whereas flower honey-added, green tea has given significantly higher ($p < 0.05$) values for TPC.

Considering the temperature of holding green tea brew, chilling temperature conditions demonstrated greater values for TPC and TEAC, unlike TFC of the resulting solutions. The study conducted by Toydemir et al. [24] revealed that the temperature of incorporating additives, particularly honey impacts the antioxidant activity exerted by the resulting infusions. Nevertheless, the study was focused on higher temperatures, and the literature sparingly discusses the infusions held at lower temperatures. As per the study, the TPC and TEAC of infusions held at chilling temperature are compelling compared to those held at room temperature, except for the TFC of infusions after incorporating bee honey alone. However, the retention of catechins can likely be improved by storing green tea in a refrigerator [33]. Nevertheless, this study is applicable to TPC and TEAC. Moreover, adjusting the pH to low by adding lemon juice also impacts the preserving tea catechins [33].

The afore-mentioned explanations for synergism, antagonism and additivity in exemplary antioxidant mixtures have been elaborated in Table 4.

Moreover, the concentration, combination of antioxidants [45], the structure of compounds, molecular ratio [42], type of solvent [46], and reaction time [47] have been considered to influence the synergistic, antagonistic or additive effect on the resultant antioxidant activity of the combined solution.

Table 4

Exemplary antioxidant mixtures with corresponding explanations for synergism, antagonism and additivity.

Interaction	Compounds of the mixture	Explanation	Reference
Synergism	Catechin/malvidin-3-o-glucoside	Generation of highly efficient antioxidant malvidin 3-glucoside	[34]
	Rosmarinic acid/quercetin, rosmarinic acid/caffeic acid	Formation of highly stable intermolecular complexes with elevated antioxidant activity compared to the parent component	[35]
	Bovine serum albumin/green tea catechins (ECG/EGCG/EC)	Formation of protein–catechin adducts with a higher antioxidant activity	[36]
	α -tocopherol/quercetin	Differences in solubility and phase distribution of antioxidants near and at the interface, regeneration of α -tocopherol by quercetin from its oxidized state	[37]
Antagonism	Gallic acid/resveratrol	Uncertain interactions between the compounds	[38]
	Catechin/ellagic acid	Formation of hydrogen bonding between carbonyl groups in the ellagic acid and o-dihydroxyl groups in catechin which hinders the hydrogen donating ability of catechin	[39]
	Glutathione/galangin	Galangin forms covalent adducts with glutathione which consumes glutathione	[40]
	Resveratrol/catechin/quercetin	Polymerization reaction between resveratrol, catechin, and quercetin which causes the decrease of hydroxyl group availability	[41]
	Glutathione/quercetin	Reactions between free antioxidant radicals amidst the chain reactions causing neutralization, formation of adducts between the antioxidants	[42]
	Rosmarinic acid/rutin	Undefined mutual interactions between the antioxidants	[43]
Additivity	Ascorbic acid/Trolox, ascorbic acid/gallic acid, ascorbic acid/uric acid, Trolox/gallic acid, Trolox/uric acid, gallic acid/uric acid	The absence of interactions between the antioxidants	[44]
	Catechin and resveratrol	Uncertain mutual interactions between the antioxidants.	[38]

Adopted by Olszowy-Tomczyk [32].

5. Conclusion

The observed TPC and TEAC values of the resulting infusions were greater than the mathematical sum of the TPC and TEAC exerted by the individual components. A synergistic effect on the TPC and TEAC of the resulting infusion was observed after adding bee honey and *Citrus limonum* extract. The addition of *Citrus limonum* extract and bee honey together had a greater impact on the synergistic effect on both TPC and TEAC compared to that of bee honey alone. The mathematical sum of the TFC of individual components (green tea, bee honey, and *Citrus limonum* extract) is far greater than the observed TFC of the resulting infusions,

indicating an antagonistic activity. Moreover, the impact of adding *Citrus limonum* extract on TPC, TFC, and TEAC was considerably higher than that of bee honey alone.

As far as the impact of the temperature at which the green tea brew held, on its resultant TPC, TFC and TEAC of binary and ternary infusions was concerned, chilling temperatures exhibited elevated TPC and TEAC of the resulting infusions. TFC at room temperature was evaluated to be high compared to the TFC at chilling temperature.

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Authors' contributions

Saummi Uduwana: Designing experiments and research, performing the analysis, statistical analysis, interpretation of data, and writing the manuscript to present the findings, Navindu Abeynayake: Assisted in developing the research concept, designing experiments, research plan and writing the manuscript, Prof. Indira Wickramasinghe: Co-author and supervisor. Assisted in developing the concept. Constant supervision of the project. Correction of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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