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# Impact of Altering the Ratio of Black Tea Granules and *Ocimum gratissimum* Leaves in a Binary Infusion on Radical Scavenging Potential Employing Cell Free Models and Ex Vivo Assays

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**Abstract:** Black tea is one of the most popular beverages consumed in the world. It is stronger in taste as well as in flavour compared to other less oxidized teas. It is made from the leaves of the shrub *Camellia sinensis* var. *assamica*. Black tea can be supplemented with other plant parts to enhance its flavour and health-promoting properties. In India, *Ocimum* spp. leaves have been used for their medicinal properties since ancient times. These leaves can be added during black tea preparation to enhance their aroma and healing activities. *O. gratissimum*, known as "Scent Leaf", is traditionally used for the management of many diseases, such as the common cold and cough. This work was designed to evaluate the antioxidant interaction between black tea and *O. gratissimum* (leaves) at five different ratios (1:1, 1:2, 1:3, 2:1, and 3:1). To determine the antioxidant activity, chemical-based methods and ex vivo assays were conducted. The total phenolic and flavonoid contents were calculated by Folin's reagent and aluminium chloride colorimetric assays, respectively. The antioxidant interactions were determined by the combination index (CI), using CompuSyn software. The black tea exhibited higher radical quenching activity (DPPH, ABTS, and NO) and antihaemolytic and anti-lipid peroxidation potential compared to the *Ocimum gratissimum* infusion. Variation in the antioxidant capability was observed for various ratios of the black tea and *O. gratissimum* (BT+OG) combination. The antioxidant interaction between BT and OG ranged from nearly additive to antagonistic. The total phenolic content was higher for *O. gratissimum*, whereas the total flavonoid content was high in black tea. The binary mixture of BT+OG at all ratios (3:1, 2:1, 1:1, 1:2, and 1:3) expressed similar phenolic and flavonoid levels. Overall, black tea and *O. gratissimum* displayed additive antioxidant interaction and the highest free radical scavenging potential at a 3:1 proportion in all the performed parameters.

**Keywords:** antioxidant; black tea; *Ocimum gratissimum*

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## 1. Introduction

Tea is known to have both preventive and therapeutic disease ameliorating abilities. It is rich in phytochemicals that have significant therapeutic effects [1]. The tea plant has two major varieties: the small-leaved China plant (*Camellia sinensis sinensis*) and the large-leaved Assam plant (*Camellia sinensis assamica*). Commercially available teas can be categorized into three types based on their processing method and degree of fermentation: black tea (maximally fermented), oolong tea (partially fermented), and green tea (unfermented), all of which come from the same plant, *C. sinensis* [2–4]. Black tea is one of the most popular refreshing drinks consumed worldwide. It is obtained from the maximally oxidized leaves of *C. sinensis* var. *assamica* [5,6]. It is rich in theaflavins and thearubugins, which are responsible for the distinct red-brown colour, stronger flavour, and astringent taste [7,8]. Both of these phytochemicals are known to have antioxidant, antidiabetic, antidyslipidemic, antihyperglycemic, cardioprotective, and cancer preventive effects and are associated with augmenting beneficial gut microbiota [9,10].

Black tea has health-promoting effects, such as anti-inflammatory, antioxidant, antiviral, antiallergic, and anticarcinogenic effects [11,12]. To enhance the medicinal properties and flavour of black tea. It can be supplemented with ginger, tulsi leaves, green cardamom, and other medicinal herbs.

Earlier studies reported that herbs have various bioactive compounds with antioxidant properties [13,14]. When these compounds are combined, they may show a higher scavenging effect than a single compound. There can be three types of possible interactions (synergistic, additive, or antagonistic) between phytoconstituents that may modulate their biological properties [15,16]. Some studies have reported that the ratio of phytochemicals or plant extracts in the mixture can influence the antioxidant potential of the whole extract [17,18]. The concentration and ratio of the antioxidants in the mixtures influence the interactive effect. Numerous antioxidant interactions (antagonistic, additive, and synergistic) have been identified over the last decades from combinations of diverse bioactive compounds, such as purified compounds (vitamins and phytochemicals), synthesized antioxidants, crude extracts, and enzymes. A study conducted on five different ratios of green tea (GT) and *O. gratissimum* (OG) revealed that the GT+OG combination at 1:1 demonstrated the highest antioxidant potency with maximum synergism [19]. *Ocimum* spp., commonly known as tulsi in India, is used as an additive in black tea preparation. *O. gratissimum* is a herbaceous plant commonly known to have immense therapeutic, chemopreventive, and free radical scavenging abilities [20,21]. Eugenol is one of the important active compounds of *O. gratissimum* and has demonstrated analgesic, antioxidant, and anti-inflammatory effects. It also exhibited ameliorating effects against type 2 diabetes, cancer, and metabolic syndrome [22]. A previous study in our laboratory illustrated that BT combined with OG showed high antioxidant activity compared to other *Ocimum* spp. (*O. sanctum* and *O. canum*) [23]. Black tea bags mainly contain black tea granules, and the tea bags or unbagged tea (black tea granules) are commonly used for black tea preparation in comparison to whole leaf black tea. The current study was undertaken to evaluate the effect of altering the ratios of black tea granules and *O. gratissimum* on the antioxidant potential of the binary mixture.

## 2. Materials and Methods

### *2.1. Collection and Preparation of Black Tea (BT) and O. gratissimum (OG) Aqueous Infusions*

Black tea bags (TajMahal brand, Mumbai, India) were purchased from Brooke Bond, India. *O. gratissimum* (specimen voucher number-23391) samples were collected from Herbal Garden, Lovely Professional University (LPU). After plucking the leaves of *O. gratissimum*, they were washed carefully with tap water, spread on a tray, covered with cotton sheets, and then left to dry in the shade for 72 h. After drying, the leaves were stored in an airtight plastic container [24–26].

### *2.2. Preparation*

An aqueous infusion of black tea (1% $w/v$) and *O. Gratissimum* (1% $w/v$) was prepared alone and in combination with BT+OG (2% $w/v$) at five ratios (1:1, 1:2, 1:3, 2:1, and 3:1) and was steeped for five minutes at 95–100 °C. These infusions were filtered with Whatman's filter paper. After filtration, the supernatant of the extract was stored at 4 °C for further experimental analysis [27].

### *2.3. Determination of Antioxidant Capacity, Hemolysis, Lipid Peroxidation, Total Phenolic, and Total Flavonoid Content*

The radical moping capacity of the aqueous infusions was measured by chemical-based antioxidant assays, such as the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate), ABTS [2,2′-

azino-bis(ethylbenzthiazoline-6-sulfonic acid)], and NO (nitric oxide) scavenging assays, and the haemolysis method [28–32]. The total phenolic compounds (TPC) and the total flavonoid compounds (TFC) of the different infusions were calculated by using the Folin–Ciocalteu and aluminium chloride assays [33,34].

### 2.4. Combination Index (CI) and Interaction Analysis

The effective concentration causing 50% scavenging activity (EC50) values of each sample was calculated for different antioxidant assays, and the combination index used to determine the antioxidant interactions at different proportions (3:1, 2:1, 1:1, 1:2, and 1:3) was determined employing CompuSyn software (version 1.0). The classification of different interaction (synergism, additive, and antagonism) types was graded based on the combination index method [35] as follows: <0.1—very strong synergism, 0.1–0.3—strong synergism, 0.3–0.7—synergism, 0.7–0.85—moderate synergism, 0.85–0.90—slight synergism, 0.90–1.10—nearly additive, 1.10–1.20—slight antagonism, 1.20–1.45—moderate antagonism, 1.45–3.3—antagonism, 3.3–10—strong antagonism, and >10—very strong antagonism.

### 2.5. Statistical Analysis

All the samples were analysed in triplicate. The data obtained are presented as the mean ± standard deviation. The intergroup differences were analysed using analysis of variance and Tukey's honestly significant difference test, employing SPSS (Version 18.0, SPSS Inc., Chicago, IL, USA, IBM) software. A $p$-value below or equal to 0.05 was considered significant.

## 3. Results

The black tea demonstrated significantly lower $EC_{50}$ or higher antiradical activity for the performed antioxidant assays (DPPH, ABTS, NO, LPO, and haemolysis) compared to the *Ocimum gratissimum* aqueous infusion (DPPH, ABTS, LPO, and haemolysis) (Table 1).

**Table 1.** The $EC_{50}$, combination index, and type of antioxidant interactions of black tea and *O. gratissimum* combination for antioxidant parameters.

| TEST | RATIO (BT+OG) | $EC_{50}$ (μg/mL) | CI at $EC_{50}$ | INTERACTION TYPE |
|---|---|---|---|---|
| DPPH | 1:0 | 77.86 ± 1.49 $^{a}$ | - | - |
| | 3:1 | 89.13 ± 1.80 $^{b}$ | 1.09 | Nearly additive |
| | 2:1 | 91.72 ± 2.05 $^{b,c}$ | 1.08 | Nearly additive |
| | 1:1 | 93.24 ± 0.83 $^{c}$ | 1.10 | Slight antagonism |
| | 1:2 | 91.43 ± 1.89 $^{b,c}$ | 1.04 | Nearly additive |
| | 1:3 | 106.96 ± 2.30 $^{d}$ | 1.20 | Antagonism |
| | 0:1 | 93.47 ± 1.33 $^{c}$ | - | - |
| ABTS | 1:0 | 44.57 ± 1.46 $^{a}$ | - | - |
| | 3:1 | 51.13 ± 2.16 $^{b}$ | 1.08 | Nearly additive |
| | 2:1 | 57.59 ± 1.46 $^{c}$ | 1.18 | Antagonism |
| | 1:1 | 59.58 ± 1.74 $^{c,d}$ | 1.20 | Antagonism |
| | 1:2 | 55.11 ± 2.01 $^{c}$ | 1.07 | Nearly additive |
| | 1:3 | 60.51 ± 2.32 $^{e}$ | 1.04 | Nearly additive |
| | 0:1 | 56.70 ± 2.34 $^{c,d}$ | - | - |

**Table 1.** *Cont.*

| TEST | RATIO (BT+OG) | $EC_{50}$ (µg/mL) | CI at $EC_{50}$ | INTERACTION TYPE |
|---|---|---|---|---|
| NO | 1:0 | 48.53 ± 2.57 [a] | - | - |
| | 3:1 | 47.99 ± 2.60 [a] | 0.94 | Nearly additive |
| | 2:1 | 49.96 ± 3.35 [a] | 1.00 | Nearly additive |
| | 1:1 | 55.77 ± 2.74 [b] | 1.17 | Antagonism |
| | 1:2 | 46.36 ± 2.87 [a] | 0.94 | Nearly additive |
| | 1:3 | 84.35 ± 4.10 [c] | 1.69 | Antagonism |
| | 0:1 | 50.61 ± 2.30 [a] | - | - |
| LPO | 1:0 | 43.81 ± 1.20 [a] | - | - |
| | 3:1 | 43.62 ± 0.71 [a] | 0.98 | Nearly additive |
| | 2:1 | 50.13 ± 1.65 [b] | 1.10 | Slight antagonism |
| | 1:1 | 54.64 ± 1.20 [c] | 1.18 | Antagonism |
| | 1:2 | 46.72 ± 1.41 [d] | 0.99 | Nearly additive |
| | 1:3 | 55.35 ± 1.25 [c] | 1.16 | Antagonism |
| | 0:1 | 49.28 ± 1.55 [d] | - | - |
| Haemolysis | 1:0 | 36.03 ± 1.21 [a] | - | - |
| | 3:1 | 36.72 ± 1.40 [a] | 1.00 | Nearly additive |
| | 2:1 | 39.55 ± 0.93 [b] | 1.07 | Nearly additive |
| | 1:1 | 44.30 ± 2.26 [c] | 1.20 | Antagonism |
| | 1:2 | 37.67 ± 1.95 [a] | 0.99 | Nearly additive |
| | 1:3 | 45.06 ± 3.33 [c] | 1.21 | Antagonism |
| | 0:1 | 39.10 ± 2.18 [a] | - | - |

$EC_{50}$ (effective concentration causing 50% scavenging activity); CI (combination index). Data are shown as the mean ± SD ($n$ = 3). The range of CI values and type of interaction as given by Chou (2010) are as follows: <0.1: very strong synergism, 0.1–0.3: strong synergism, 0.3–0.7: synergism, 0.7–0.85: moderate synergism, 0.85–0.90: slight synergism, 0.90–1.10: nearly additive, 1.20–1.45 (moderate antagonism), and 1.45–3.3 (antagonism). BT+OG—black tea + *O. gratissimum*. Different letters represent significant differences at $p \leq 0.05$ within the same column for a particular type of test. Data are shown as the mean ± SD for two independent experiments (each with triplicates for each test point). Different letters show a significant difference ($p \leq 0.05$).

The phenolic and flavonoid contents in the BT + OG combinations were found to be similar at all ratios (3:1, 2:1, 1:1, 1:2, and 1:3), as shown in Table 2. The total phenolic content was higher for OG than for BT, whereas the flavonoid content was higher for BT in comparison to OG (Table 2). The results obtained from the combination index illustrated that the BT+OG combination showed antagonism (DPPH: 1:3; ABTS: 2:1, 1:1; NO, LPO, and haemolysis: 1:1, 1:3), slight antagonism (DPPH: 1:1; LPO: 2:1), and nearly additive interaction (DPPH: 3:1, 2:1, 1:2; ABTS: 3:1, 1:2, 1:3; NO: 3:1, 2:1, 1:2; LPO: 3:1, 1:2; and haemolysis: 3:1, 2:1, 1:2) at different ratios. The BT+OG combination demonstrated appreciable radical scavenging potential in all the antioxidant assays. The radical scavenging activity was maximum at the 3:1 combination for ABTS and LPO, while in other tests (DPPH, NO, and haemolysis), the effect was similar to that at the 2:1 ratio, as shown in Table 1.

**Table 2.** The total phenolic content (TPC)/total flavonoid content (TFC) of black tea and *O. gratissimum*, alone or in a binary mixture at five different ratios.

| SAMPLE | TPC (mg/100 mL) GAE | TFC (mg/100 mL) QAE |
|---|---|---|
| BT | 113.6 ± 2.26 [a] | 73.04 ± 0.66 [a] |
| OG | 137.16 ± 1.06 [b] | 63.67 ± 1.53 [b] |
| BT+OG (3:1) | 98.18 ± 0.34 [c] | 86.69 ± 3.45 [c] |
| BT+OG (2:1) | 93.74 ± 0.88 [d] | 84.67 ± 3.34 [c] |
| BT+OG (1:1) | 91.82 ± 0.67 [d] | 81.69 ± 0.43 [c] |
| BT+OG (1:2) | 90.33 ± 0.55 [d] | 80.23 ± 0.60 [c] |
| BT+OG (1:3) | 92.34 ± 0.37 [d] | 83.58 ± 2.06 [c] |

Data are displayed as the mean ± SD ($n$ = 3). BT—black tea, OG—*Ocimum gratissimum* and BT+OG (black tea + *Ocimum gratissimum*). "Different letters show significant differences at $p \leq 0.05$ within the same column for a particular type of test. Data are shown as the mean ± SD for two independent experiments (each with triplicates for each test point). Different letters show a significant difference ($p \leq 0.05$)".

## 4. Discussion

Tea research is currently gaining worldwide attention and importance due to the presence of hundreds of critical therapeutic substances and antioxidants. The flavonoid and polyphenol compounds found in tea leaves have antioxidant properties as well as a wide range of other important biological activities [4,36]. The aqueous infusion of black tea showed a higher scavenging ability and antihaemolytic and anti-lipid peroxidation effects than OG. A similar result was reported in an earlier study [37]. The flavonoid content was higher for BT than OG, whereas higher phenolics were noted for OG than BT. The flavonoid content was greater in different unblended black teas (Kenya, Assam, China, Darjeeling, and Ceylon) than in blended black teas (Earl Gray and English Breakfast), chamomile tea, and fruit juices (apple and orange) [38,39]. In another report, black tea inhibited the copper-induced lipid peroxidation and formation of thiobarbituric acid (TBA) and expressed the dose-dependent protection against the 2,2′-Azobis (2-amidinopropane) (AAPH)-induced erythrocytes oxidative haemolysis [40].The fresh leaf (aqueous, methanolic, and ethanolic) extract of *Ocimum gratissimum* manifested the highest phenolic content in comparison to the other *Ocimum* spp. (*Ocimum americanum, Ocimum minimum, Ocimum citridorum, Ocimum lamlifolium,* and *Ocimum selloi*) [41].

Modulation of the antioxidant potential was observed by altering the ratio of BT and OG in a binary mixture. A study reported that the combination of different herbs in various ratios can also affect the quenching ability [17,18]. The BT+OG combinations displayed antagonism to additive antioxidant interactions in various assays. It was found that variation in the antioxidant potential depends on the reaction rates of the antioxidants, the ratio of the bioactive compounds, and the effective concentration of the antioxidants at the site of oxidation [42,43]. BT+OG showed the maximum antioxidant potential and additive interaction at a 3:1 ratio (Figure 1, Table 1). The antioxidant interaction of a mixture of botanicals (plant-based foods, herbs, or teas) might be influenced by the type of interactions (synergistic, additive, and antagonistic) among their phyto-constituents [13,17,18]. The interaction between the phytochemicals can be ascribed to their structural differences, the regeneration of less active antioxidants, the nature of the different radicals, the competition between the antioxidant regenerations, and the production of radical adducts [44,45]. The ternary combination of black tea with tulsi + ginger + black pepper in the ratio of 1:1:1:1 displayed the highest quenching ability compared to that of a single herb [15]. A study reported that green tea combined with *Ocimum gratissimum* displayed the maximum synergistic interaction and the highest radical quenching effect at a 1:1 ratio, followed by 3:1, 2:1, 1:2, and 1:3 [19]. A study reported that green tea (alone) expressed the highest scavenging activity compared to oak and rosemary, while in the case of a binary mixture, green tea:rosemary:oak (50:50:50) expressed the maximum quenching ability compared to the other ratios (50:50:100, 50:100:50, 100:50:50, 50:100:100, 100:50:100, and 100:100:50). However, the combined extracts behaved differently after being combined, and all three types of interactions (additive, synergistic, and antagonistic) were observed [46]. The antioxidant activities and interactions are influenced by the ratio of bioactive compounds, the nature of the structural properties of the paired compounds, the different radicals, and the reaction mechanisms involved in these assays (LPO, haemolysis, ABTS, and DPPH) [17,18,45].

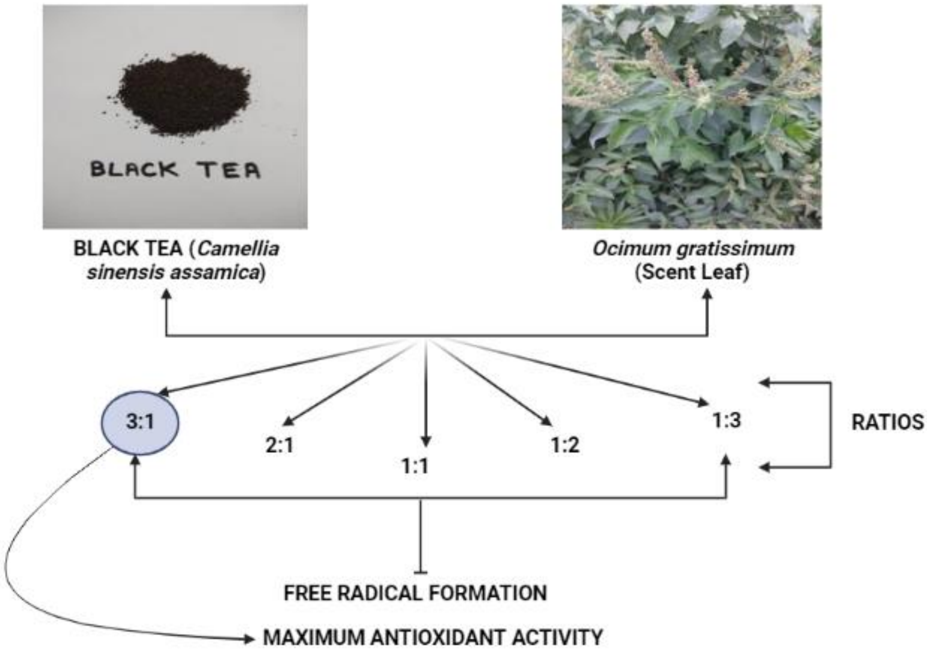


**Figure 1.** Effect of altering the proportion of black tea and *O. gratissimum* at five different ratios.

## 5. Conclusions

Black tea (BT) and *Ocimum gratissimum* (OG) at different ratios alter the radical quenching ability of the binary mixture. Among the ratios, 3:1 showed the maximum radical quenching ability in both the in vitro and the ex vivo assays, and the interaction observed was additive. Thus, the data obtained from this study will help in the future designing or formulation of the binary infusion of black tea with *O. gratissimum*.

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