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Synergistic antioxidant interactions between green tea and *Ocimum gratissimum*

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ABSTRACT

Objective: To evaluate the antioxidant interactions between aqueous infusions of green tea and *Ocimum gratissimum* at different ratios.

Methods: Antioxidant activities of aqueous infusion of green tea and *Ocimum gratissimum* (leaves) alone or in combination at various proportions (3:1, 2:1, 1:1, 1:2, 1:3) were determined by DPPH, ABTS, NO and *ex−vivo* assays including lipid peroxidation and haemolysis. Total phenolic content and flavonoid content was calculated by Folin-Ciocalteu reagent and aluminum chloride colorimetry method, respectively. A correlation study was also conducted between the antioxidant activity and total phenolic/flavonoid content of various infusions. The interactions were analyzed by combination index applying CompuSyn software.

Results: Green tea exhibited high radical scavenging ability as compared to *Ocimum gratissimum* infusion. Combination of green tea and *Ocimum gratissimum* exhibited moderate antagonism to strong synergistic interaction at various ratios. A strong correlation was found between total phenolic content/total flavonoid content and antioxidant activities of individual infusions (green tea and *Ocimum gratissimum*). For binary mixture at different ratios, a weak to strong correlation was observed between total phenolic content and antioxidant activity and almost no correlation between total flavonoid content and antioxidant potential.

Conclusions: Overall, green tea and *Ocimum gratissimum* combination (1:1) displayed the highest antioxidant potential and maximum synergism.

1. Introduction

Green tea is a refreshing health drink that is becoming popular throughout the world[1]. It is derived from the steamed or pan-fried leaves of the plant *Camellia sinensis*[2]. It has attracted significant attention both from consumer and scientific communities due to its diverse health-promoting properties like antioxidant, anti-lipid peroxidation, anticarcinogenic, antiangiogenic, antimutagenic, antihypertensive and anti-obesity[3−5]. To enhance the green tea flavour and medicinal properties, various green tea combinations are available commercially. The combination of various plant products may exhibit different types of interactions (synergistic, additive and antagonistic) among their different phytochemicals, and may change their biological properties[6]. Although these combinations are flooded in the market, limited scientific data is available that whether these combinations will act in a synergistic, antagonistic or additive manner. It was found that *Osmanthus fragrans* flowers and green tea showed synergistic antioxidant interaction[7]. Whereas, binary combinations of *Potentilla fruticosa* leaves extract (PFE) and

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green tea polyphenols (GTP) demonstrated synergistic to additive interactions[8]. However, a pair of green tea and ascorbic acid demonstrated all three type of interactions in various antioxidant assays[9].

Some studies also pointed out that the ratio of the herbs would influence the antioxidant properties[8,10]. It is important to investigate the modulation of antioxidant properties depending on the proportions of herbs in a mixture that can be used to design functional foods and pharmaceutical products at different concentrations[9]. The ratio of the extracts significantly influenced the antioxidant capacity of a binary mixture and the type of interaction between their bioactive components[9-11]. In our earlier work, we disclosed the different interactions of binary combinations of green tea with medicinal plants [*Ocimum gratissimum* (*O. gratissimum*), *Cymbopogon citratus*, *Cymbopogon flexuosus* and *Hibiscus rosa-sinensis*], which are commonly used as herbal teas or tisanes. Among these studied combinations, green tea and *O. gratissimum* combination showed the highest antioxidant potential and maximum synergistic interaction (unpublished data). *O. gratissimum* commonly called Vana Tulsi in India belongs to family Lamiaceae and is a valuable medicinal plant that has been used since ancient times[12,13]. It is well known due to its medicinal properties such as antioxidant, antifungal, antimicrobial, anti-inflammatory, antiviral and anticancer[12,14]. However, there is no scientific literature about the interaction between green tea and *O. gratissimum* at different ratios. The proportion of individual herb could play an important role in determining the chemopreventive potential or bioefficacy in a health-promoting herbal combination. The aim of present study was to investigate the antioxidant interaction between green tea and *O. gratisssimum* at different proportions.

2. Materials and methods

2.1. Plant materials and preparation of green tea and O. gratissimum extracts

The green tea (Darjeeling green classic tea, Batch No. 6011) was purchased in the form of 50 g packet (loose leaf tea) from Bud white Teas Pvt. Ltd., Delhi, India. This whole leaf green tea was obtained from top bud and two leaves of *Camelia sinensis* plant as per the information provided by the manufacturer. The leaves of *O. gratissimum* (Vana Tulsi) were collected from the herbal garden (Lovely Professional University, Punjab, India) in the month of October 2017. This plant species *O. gratissimum* was authenticated by Dr. Sumeet Gairola, Indian Institute of Integrative Medicine and the voucher specimen with accession number RRLH-23391 was submitted to Janaki Ammal herbarium of Indian Institute of Integrative Medicine, Jammu, India. The collected leaves were air-dried under shade at room temperature[8]. 2% aqueous infusion of green tea and *O. gratissimum* individually and in combination at five different ratios (3:1, 2:1, 1:1, 1:2, 1:3) were steeped at 95-100 ℃ for 5 min[4,15]. The selection of dose is on the basis of the amount of green tea or *O. gratissimum* leaves usually used for preparing their hot water infusions. The above-mentioned extracts were filtered using Whatman's filter paper No.1 and stored at 4 ℃ for further investigation.

2.2. Determination of antioxidant capacity, total phenolic and flavonoid content

The scavenging potential of test samples against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined by Mensor's method[16]. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity of the infusions was analyzed by the method described by Re *et al*[17]. Nitric oxide (NO) scavenging activity was studied by following the method described by Shirwaikar *et al*[18]. Lipid peroxidation assay (LPO) was performed according to the method described by Ohkawa *et al*[19]. Erythrocyte haemolysis test was carried out according to the method described by Okoko and Ere[20].

Total phenolic content (TPC) and total flavonoid content (TFC) of different infusions was determined using Folin-Ciocalteu reagent[21] and aluminum chloride colorimetry assay[22], respectively. A linear calibration curve of gallic acid was prepared ranged from 20-120 μg/mL, with a coefficient of determination, R^2 = 0.990 for TPC; while a standard curve of quercetin was prepared ranged from 10-50 μg/mL, with a coefficient of determination, R^2 = 0.995 for TFC. The TPC is represented as gallic acid equivalents (GAE), and TFC as quercetin equivalents (QE). To study the effect of the combination of green tea and *O. gratissimum* (GT+OG) at different proportions on the TPC and TFC, the difference between theoretical value and experimental value was evaluated. Theoretical value is calculated by the sum of phenolic or flavonoid content of green tea and *O. gratissimum* infusion separately, relative to their respective ratio in a binary mixture. Furthermore, the coefficient of determination (R^2) between TPC/TFC and antioxidant activities (DPPH, ABTS, NO, LPO and anti-haemolysis) was also investigated for individual infusions (GT and OG) and their combination.

2.3. Combination index (CI) analysis

Combination index method was used to determine the interactions between components in a mixture. The interactions (synergistic, antagonistic or additive) between green tea and *O. gratissimum* mixture at five different ratios (3:1, 2:1, 1:1, 1:2, 1:3) were tested using effective concentration causing 50% scavenging activity (EC_{50}) and CI method described by Chou[23] (CompuSyn software, version 1.0). The type of interaction is classified based on CI as follows: 0.1-0.3 (strong synergism), 0.3-0.7 (synergism), 0.7-0.85 (moderate synergism), 0.85-0.90 (synergism), 0.90-1.10 (nearly additive), 1.20-1.45 (moderate antagonism).

2.4. Statistical analysis

All assays were carried out in triplicates and data of each result were given as mean ± standard deviation (SD). The statistical analysis was performed by using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference test using SPSS software (version 18). If the *P*-values were 0.05 or less, the results will be considered statistically significant.

3. Results

3.1. Antioxidant potential and TPC/TFC of green tea and O. gratissimum

DPPH, ABTS, NO, anti-lipid peroxidation and haemolysis assays showed that green tea possessed significantly higher antioxidant potential as compared to *O. gratissimum* (Table 1). Ascorbic acid (vitamin C) was used as a standard in DPPH (EC_{50} = 5.42 μg/mL), ABTS (EC_{50} = 6.11 μg/mL), NO (EC_{50} = 6.18 μg/mL) and haemolysis (EC_{50} = 7.82 μg/mL) assays, and butylated hydroxyl toluene (EC_{50} = 20.23 μg/mL) in anti-lipid peroxidation assay. Green tea also demonstrated higher amount of TPC and TFC than *O. gratissimum* (Table 2).

3.2. Interaction effects on antioxidant potential of GT+OG at different ratios

Compared to other proportions, green tea and *O. gratissimum* combination at 1:1 showed remarkably higher nitrite radical scavenging and anti-haemolytic activity; whereas GT+OG at 3:1, 2:1 and 1:1 displayed similar antioxidant potential in DPPH, ABTS and LPO assays (Table 1). The binary combination at 1:1 exhibited the lowest CI values and maximum synergism in DPPH, NO, and anti-haemolysis analyses. In ABTS radical scavenging assay, synergism was observed at all ratios except 3:1 (moderate synergism), and similar synergistic interactions were found at all tested ratios for anti-lipid peroxidation potential (Table 1).

3.3. TPC and TFC of GT+OG mixture at different ratios

GT+OG at 1:1 showed the highest TPC whereas GT+OG at 1:2, 3:1, and 1:1 showed similar TFC results. The experimental values of TPC were lower than theoretical values of GT+OG combination for respective ratio, but TFC was similar (3:1, 1:1 and 1:3), and minor differences were observed in case of 2:1 and 1:2 (Table 2).

A strong correlation was noticed between TPC/TFC and antioxidant parameters (TPC/TFC–DPPH: R^2 = 0.994/0.975, TPC/TFC–ABTS: R^2 = 0.991/0.989, TPC/TFC–NO: R^2 =0.965/0.983, TPC/TFC–LPO: R^2 =0.902/0.934 and TPC/TFC–Anti-haemolysis: R^2 = 0.962/0.979) for individual infusions (green tea and *O. gratissimum*), but strength of correlation was strong to weak in GT+OG combination at different ratios (TPC–DPPH: R^2 = 0.793, TPC-ABTS: R^2 = 0.798, TPC–NO: R^2 = 0.449, TPC–LPO: R^2 = 0.826 and TPC–Anti-haemolysis: R^2 = 0.585). However, almost no correlation was found between TFC and antioxidant potential of GT+OG combination (TFC–DPPH: R^2 = 0.060, TFC–ABTS: R^2 = 0.002, TFC–NO: R^2 = 0.013, TFC–LPO: R^2 = 0.006 and TFC–Anti-haemolysis: R^2 = 0.005).

Table 1. Synergistic, additive and antagonistic antioxidant interactions of green tea and *Ocimum gratissimum* combinations at different ratios.

Assay types	Ratio	EC_{50} (μg/mL)	Combination index	Description
DPPH	1:0	11.68±2.24[a]	-	-
	3:1	14.70±4.30[a]	1.006[c]	Nearly additive
	2:1	12.61±3.45[a]	0.791[b]	Moderate synergism
	1:1	12.36±2.19[a]	0.633[a]	Synergism
	1:2	33.93±0.85[c]	1.351[c]	Moderate antagonism
	1:3	23.97±3.08[b]	0.815[b]	Moderate synergism
	0:1	59.21±2.06[d]	-	-
ABTS	1:0	10.88±1.07[a]	-	-
	3:1	10.10±0.59[a]	0.726[c]	Moderate synergism
	2:1	9.27±1.43[a]	0.604[b]	Synergism
	1:1	9.24±0.78[a]	0.480[a]	Synergism
	1:2	16.06±1.43[b]	0.620[b]	Synergism
	1:3	15.56±2.68[b]	0.496[a]	Synergism
	0:1	84.07±1.31[c]	-	-
NO	1:0	39.59±4.37[d]	-	-
	3:1	48.70±5.41[c]	0.993[d]	Nearly additive
	2:1	33.31±3.42[b]	0.626[c]	Synergism
	1:1	14.21±0.97[a]	0.221[a]	Strong synergism
	1:2	48.33±6.04[c]	0.596[c]	Synergism
	1:3	44.21±5.76[c]	0.473[b]	Synergism
	0:1	170.25±4.39[e]	-	-
LPO	1:0	50.40±4.03[c]	-	-
	3:1	37.26±0.60[a]	0.677[a]	Synergism
	2:1	37.67±0.55[a]	0.663[a]	Synergism
	1:1	35.69±0.60[a]	0.589[a]	Synergism
	1:2	40.65±1.41[b]	0.626[a]	Synergism
	1:3	40.22±0.59[b]	0.597[a]	Synergism
	0:1	75.81±6.50[d]	-	-
Haemolysis	1:0	38.52±1.68[c]	-	-
	3:1	31.87±2.66[b]	0.726[d]	Moderate synergism
	2:1	30.29±1.45[b]	0.658[c]	Synergism
	1:1	20.32±1.26[a]	0.398[a]	Synergism
	1:2	39.17±3.69[c]	0.686[c]	Synergism
	1:3	33.57±0.95[b]	0.552[b]	Synergism
	0:1	72.54±3.84[d]	-	-

DPPH: 2,2-diphenyl-1-picrylhydrazyl, ABTS: 2, 2' azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), NO: nitric oxide, LPO: lipid peroxidation. EC_{50}: Effective concentration causing 50% scavenging activity; Results are presented as mean value ± SD (n = 3). Different alphabets depict significant difference at P<0.05 within the same column for a particular assay type. The type of interaction is classified based on combination index as follows: 0.1-0.3 (strong synergism), 0.3-0.7 (synergism), 0.7-0.85 (moderate synergism), 0.85-0.90 (synergism), 0.90-1.10 (nearly additive), 1.20-1.45 (moderate antagonism).

Table 2. Total phenolic and flavonoid content of green tea and *Ocimum gratissimum* combinations at different ratios.

Ratio	Theoretical value of TPC (mg GAE/g)	Experimental value of TPC (mg GAE/g)	Theoretical value of TFC (mg QE/g)	Experimental value of TFC (mg QE/g)
1:0	-	212.91±2.52[a]	-	29.21±0.54[a]
3:1	204.73±1.85[*]	161.51±2.03[e]	27.18±0.52	26.90±0.30[b]
2:1	202.00±1.63[*]	167.58±2.44[d]	26.51±0.52[*]	24.73±0.97[c]
1:1	196.54±1.22[*]	176.32±1.11[c]	25.16±0.53	26.54±0.58[b]
1:2	191.54±1.22[*]	112.91±2.60[e]	23.81±0.56[*]	27.88±0.41[b]
1:3	188.36±0.73[*]	115.28±4.00[e]	23.13±0.57	23.75±0.42[c]
0:1	-	180.17±0.76[b]	-	21.11±0.63[d]

Data are shown as mean ± SD, n=3. The asterisk [*] indicates a significant difference (P<0.05) between theoretical value and experimental value. Different letters indicate significant difference in experimental value of TPC and TFC at different ratios.

4. Discussion

In present study, the binary combination of whole leaf aqueous extracts of green tea and *O. gratissimum* at different ratios was evaluated, simulating the household method of tea preparation. Green tea has shown higher antioxidant potential, TPC, and TFC as compared to *O. gratissimum*. Earlier studies also reported remarkably higher antioxidant activity and TPC/TFC of green tea in comparison to other teas and herbs[8,15,24,25]. A significant difference was observed in the antioxidant potential and TPC among different extracts of green tea cultivars (Kashmir, Uttarakhand, and Assam)[26]. It was found that 19 samples of green tea from 5 subtypes (dragonwell, gunpowder, jasmine pearl, sencha, and gykuro) demonstrated wide variation in their phytochemical profile and the DPPH radical scavenging activities[27]. The difference in antioxidant potential was influenced by many factors such as manufacturing processes, cultivar, grade, geographical area, age of the leaves, collection season and the climate[28,29]. A study showed that aqueous extract of whole leaf white tea exhibited a similar antioxidant capacity as compared to its active compound epigallocatechin gallate[30].

Green tea and *O. gratissimum* (GT+OG) at different ratios influenced the radical scavenging ability in various assays. The proportion of the individual extracts alters the antioxidant ability of the binary combination in various *in vitro* models such as DPPH, ABTS, Trolox equivalent antioxidant capacity assay, and ferric reducing antioxidant power[8-11]. Interestingly, it was found that increasing the proportion of green tea (active extract) in a binary mixture leads to an increase/decrease or almost no change in the antioxidant capacity in various assays. Green tea-ascorbic acid pair, black tea-ascorbic acid pair, and cocoa-cinnamon pair demonstrated changes in antioxidant potential with an increase in the active component, but no alteration was noted at certain fractions[9,10]. It was also observed that in PFE and GTP combination, with enhancement in the GTP (active extract) from 7:1 to 1:7, an increase in radical scavenging activity was detected, but decrease or no variation was also noticed at certain ratios[8]. These studies suggested that an increase in the proportion of active extract will not always enhance the activity. The single infusion demonstrating higher activity as compared to other individual extracts may illustrate higher/lower/similar activity as that of the most effective ratio of mixed extract[9,10]. Green tea displayed either lower (NO, LPO and haemolysis assays) or similar potential (DPPH and ABTS assays) in comparison to GT+OG (1:1). The interaction between green tea and *O. gratissimum* ranged from moderate antagonism to strong synergism. *Prunus fruticosa* and green tea polyphenols combination demonstrated mostly a synergistic interaction, but the additive effect was also observed in a few cases. However, for cocoa and cinnamon combination, all types of interactions were detected in DPPH and FRAP assays[8,10]. These synergistic interactions may originate due to various mechanisms such as regeneration, spatial distribution, sacrificial oxidation, mutual protection and metal chelation[31-35]. Moderate antagonism was detected only in the DPPH assay at 1:2. This interaction may be ascribed to the regeneration of the less active antioxidant *via* a more effective compound, competition between the production of antioxidant radical adducts and regeneration of the antioxidant, and modification of the microenvironment of one antioxidant by another[36,37]. GT+OG (1:1) manifested the highest activities for NO and anti-haemolysis assays, and was comparable with 2:1 and 3:1 in DPPH, ABTS and LPO tests. The extent of synergism was maximum at 1:1 in all antioxidant models except LPO where similar synergistic interaction was observed. The antioxidant potentials and antioxidant interactions depend correspondingly on the nature of different radicals, structural characteristics of the paired compounds, the ratio of the bioactive compounds and the reaction mechanism of assays (DPPH, ABTS, NO, LPO and haemolysis)[11,38,39].

The therapeutic effects of many medicinal plants have been attributed to the presence of phenolic compounds[40]. A positive correlation was observed between antioxidant capacity and TPC/TFC of green and white tea extracts[41]. It was expected that increasing the proportion of an infusion with high phenolic content in a binary mixture would enhance the TPC of the combination. In our study, it was noticed that by increasing the proportion of green tea, no change was noticed in TPC initially, after that an increase and then decrease in TPC was denoted. The scientific data about the effect of the proportion of individual extracts on the phenolic content of the binary mixture is limited. A previous study reported that increasing the fraction of GTP, which has a higher contribution to phenolic content as compared to PFE in a binary combination leads to an increase in phenolic content from 1:7 to 7:1[8]. In the

current study, the TPC was significantly lower than the theoretical value at different ratios for the GT+OG combination. Earlier it was demonstrated that in a binary combination of PFE and GTP, the observed and expected value showed similar phenolic content, but at certain ratios, a significant difference was detected[8]. The flavonoid content was similar for most of the ratios with a slight variation in certain fractions. The individual infusions (green tea and *O. gratissimum*) showed a strong correlation between antioxidant capacity and TPC/TFC, whereas a weak to strong correlation was observed between antioxidant capacity and TPC, and almost no correlation was detected in case of TFC for GT+OG combinations. This may be due to various mechanisms involved during the interaction of different components of individual infusions that may alter the structural characteristic of polyphenols and may also lead the polymerization of monomer to dimer[42,43]. These changes may possibly affect the estimation of TPC with Folin-Ciocalteu reagent.

Green tea and *O. gratissimum* combinations displayed significant antioxidant potential, and 1:1 is the best proportion, showing the highest radical quenching ability and strongest synergism in both *in vitro* and *ex vivo* models. Thus, this study provides the scientific basis for combining aqueous infusions at a particular ratio to attain maximum antioxidant potential.

Conflict of interest statement

Authors declare that there are no competing interests.

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