

ORIGINAL RESEARCH

Black and green teas may have selective synergistic or antagonistic effects on certain antibiotics against *Streptococcus pyogenes* in vitro

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Abstract

Background. Black tea, the most popular drink in Iran, has several polyphenolic compounds with possible antibacterial effects. Although the antimicrobial properties of green tea have already been reported, the microbiological effects of black tea have been less widely investigated. In this study, the anti-streptococcal effects of black tea extract were evaluated and compared with those of green tea. **Design.** In vitro evaluation of antibacterial effects.

Methods. Both black and green tea extracts were analysed using high-performance liquid chromatography to compare their major polyphenol profiles. Different concentrations of the extracts or gallic acid, the abundant phenolic compound found in black tea, were used for bacterial sensitivity tests in both pour plate and disc diffusion methods. Disc diffusion was then used to evaluate the interactions between the extracts and certain anti-streptococcal antibiotics.

Results. Both black and green teas, at a concentration of 12.5 mg ml⁻¹ after 7 hours and at 25 mg ml⁻¹ after 3 hours, completely inhibited *Streptococcus pyogenes* growth. However, gallic acid at a concentration as low as 5 µg ml⁻¹ after 5 hours and at 10 µg ml⁻¹ after 2 hours inhibited streptococcal growth. Both black tea and green tea extracts were found to have either synergistic or antagonistic effects at different concentrations on the selected antibiotics, whereas gallic acid strengthened the antibacterial effects of all antibiotics in a dose-dependent manner.

Conclusion. Both black tea and green tea extracts may have synergistic or antagonistic effects on certain anti-streptococcal antibiotics. These effects are more prominent with black tea.

Key words: Tea extract, microbiological effects, *Streptococcus pyogenes*

Introduction

Streptococcus pyogenes (group A), a Gram-positive extracellular species mostly colonized on the skin and in the throat, may cause various purulent infections and the related non-purulent outcomes [1]. Bacterial pharyngitis [2], scarlet fever and impetigo [3] are among the most prevalent streptococcal infections. The importance of *S. pyogenes* lies not only in the related severe suppurative infections, but in such post-streptococcal infection sequelae

as rheumatic fever [4], acute glomerulonephritis [5] and reactive arthritis [6]. The infection has been recently associated with Tourette's syndrome, tics and attention deficit hyperactivity disorders [7]. Although antibiotic therapy has been, and continues to be, the core of fighting against bacterial infections, the emergence of bacterial resistance and several drug side-effects have drawn researchers', as well as clinicians', attention to the various herbal medicines as an adjunct therapy against many infections [8].

Black tea, *Camellia sinensis*, one of the most popular drinks throughout the world, and especially in Iran, contains several polyphenolic compounds, including flavins and thearubigins [9]. Although the antimicrobial properties of green tea have already been reported [10], the microbiological effects of black tea have been less widely investigated. In this study, the microbiological effects of black tea extract, alone and in conjunction with some selected antibiotics, were evaluated and compared with those of green tea extract.

Materials and methods

Materials

Bacterial strain ATCC 1447 was purchased from the Iranian Organization of Scientific and Industrial Research. All culture media were obtained from Merck. Polyphenol standards, including Polyphenon 60, were obtained from Sigma Aldrich. The solutions used for chromatographic analyses were all high-performance liquid chromatography (HPLC) grade and were obtained from Romil, UK. Antibiotic discs, including ampicillin, amoxicillin and cephallexin, were purchased from Padtan Teb, Iran.

Preparation of tea extract

To prepare the extract, the percolation method was used. A total of 100 g of dried tea leaves (green or black) was soaked in 2 l of 70% ethanol and incubated at 60°C for 48 hours, after which the extract was filtered using Whatman paper. The extraction step was repeated for more efficiency. The extract was concentrated to 20 ml using an evaporator and was then dried at 50°C. The dried extract was scraped and ground to a fine powder. The extract was then reconstituted in dimethyl sulphoxide at 1000 mg ml⁻¹ concentration.

Total antioxidant capacity assay

Total antioxidant capacity was determined using 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonate) (ABTS) as the reagent. Potassium persulphate converts ABTS to ABTS cation radical (ABTS^{•+}), which is green-blue with a maximum absorption at 734 nm. Adding antioxidant solution to this in a given time decreases colour intensity depending on the antioxidant activity and concentration. Therefore, decolorization will be expressed as a percentage of ABTS^{•+} inhibition based on the difference between the primary and secondary absorbance divided by the primary absorbance multiplied by 100 [11]. The inhibition percentage was compared with the antioxidant activity of bovine serum albumin (BSA) as the standard. Green and black tea extracts at a concentration of 1 mg ml⁻¹ were tested for total antioxidant capacity and the results were expressed as mmol l⁻¹ of BSA.

Chromatographic analysis of black tea polyphenols

Both green and black tea extracts were analysed using a HPLC system equipped with an ultrasonic degassing system (Young Lin, South Korea) and a photodiode array detector

2800 with Chromgate software (Knauer, Germany). A 2.5 g tea bag (Noushine Chaai, Iran) was infused in deionized hot water for 15 min. After cooling, the extract was filtered (0.45 μm cartridge) and injected into the column. Polyphenon 60, a standard green tea extract, was also analysed for polyphenolic compounds. The chromatographic conditions were as follows: column: C18 Nova-Pak, 4 \times 150 mm, 4 μm (Waters, USA); flow rate: 1.5 ml min⁻¹; pressure: 2700 psi; temperature: 30°C; mobile phase: water: methanol: ortho-phosphoric acid, 90:10:0.1, v:v:v); λ max: 210 nm; run time: 25 min.

Bacterial sensitivity tests

Two methods were used for the bacterial sensitivity tests: pour plate and disc diffusion.

Pour plate. Different concentrations of black or green tea extracts (6.25, 12.5, 25, 50 and 100 mg ml⁻¹) in brain heart broth (final volume 10 ml) were prepared. Of the bacterial suspension, 1 ml equivalent to 0.5 MacFarland was added to the suspensions and incubated at 37°C. After 1, 2, 3, 5, 7 and 24 hours, 1 ml of the suspension was transferred to a sterile Petri dish containing liquid blood agar at 45°C and dispersed evenly. All plates incubated at 37°C were checked after 24 hours.

To evaluate the antibacterial effects of gallic acid, the same procedure was used with the concentrations 2.5, 5, 10, 25, 50, 100 and 1000 $\mu\text{g ml}^{-1}$.

Disc diffusion. Twenty-five microlitres of black or green tea extract as well as gallic acid (10, 50 and 100 mg ml⁻¹) was inoculated to the blank or antibiotic discs. The discs were dried by incubating at 37°C. The antibiotics used were ampicillin (30 $\mu\text{g disc}^{-1}$), amoxicillin (25 $\mu\text{g disc}^{-1}$) and cephallexin (30 $\mu\text{g disc}^{-1}$). The bacterial sensitivity disc diffusion test was repeated eight to 11 times on different days. The mean diameter of growth inhibition was calculated and used for further statistical analyses.

Statistical analyses

The normality of the data distribution was evaluated using Kolmogorov–Smirnov. A comparison of means was carried out with Student's t-test or, when the distribution was not normal, Mann–Whitney U–Wilcoxon. The predetermined upper limit of significance throughout this investigation was $p < 0.05$. All statistical analyses were performed with Windows/SPSS version 11.5 package.

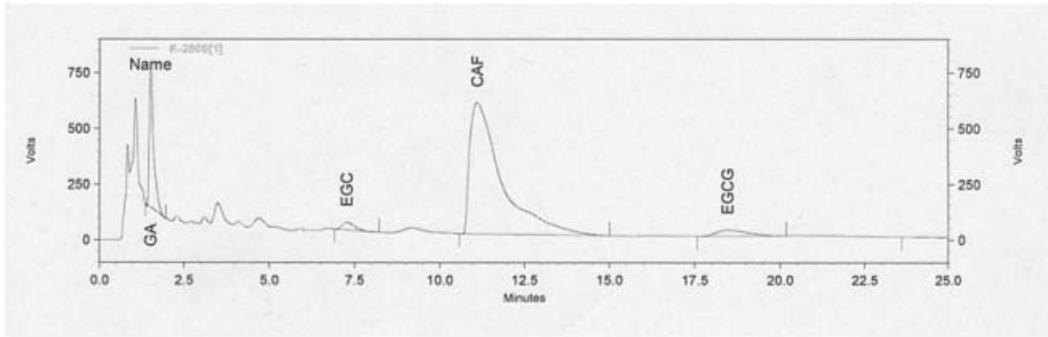
Results

Chromatographic analysis of green and black tea polyphenols

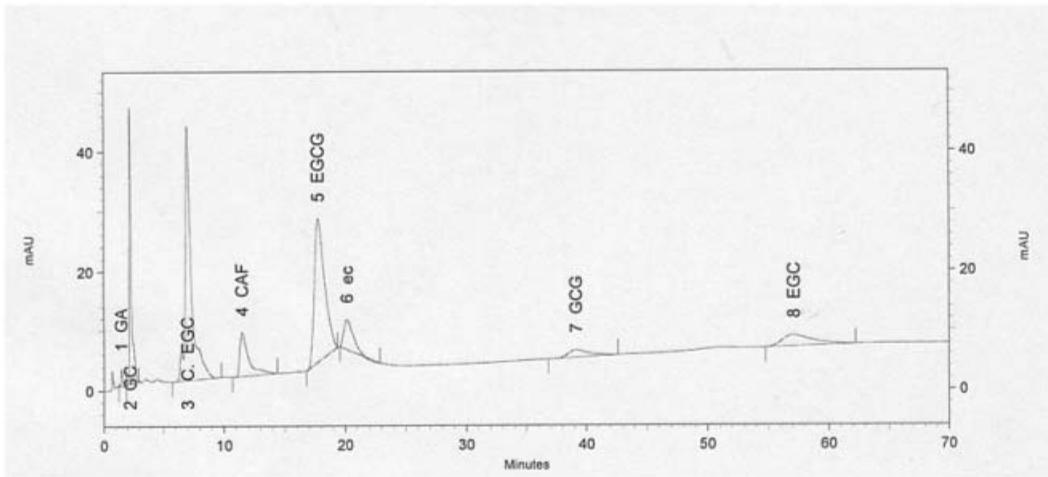
Gallic acid and caffeine were the most abundant constituents of black tea, whereas epigallocatechin (EGC) and epigallocatechin gallate (EGCG) were the prominent polyphenols in green tea (Figure 1). Table I shows the retention times of caffeine and major polyphenols in tea.

Total antioxidant capacity

The antioxidant capacity of green and black tea extracts at a concentration of 1 mg ml⁻¹ were 2.68 ± 0.11 and 1.22 ± 0.10 mM of BSA, respectively ($p < 0.001$).



(a)



(b)

Figure 1. High-performance liquid chromatography analysis of (a) black tea extract and (b) green tea extract (Polyphenon 60). Peaks 7 and 8 are of the isomers of the corresponding polyphenols. GA, gallic acid; EGC, epigallocatechin gallate; CAF, caffeine; EGCG, epigallocatechin gallate.

The antibacterial effect of black and green teas

Both black and green teas at a concentration of 12.5 mg ml⁻¹ after 7 hours and at 25 mg ml⁻¹ after 3 hours completely inhibited *S. pyogenes* growth. Bacterial growth was absolutely inhibited after a 1 hour incubation with higher concentrations of the extract

Table I. Retention times of caffeine and three major polyphenolic compounds of tea extract with the concentrations in the black tea extract (mean ± standard deviation).

Constituent	Retention time (min)	Concentration (mg dl ⁻¹)
Gallic acid	1.5	3.5 ± 0.08
EGC	7.3	0.43 ± 0.03
Caffeine	11.5	30.9 ± 0.4
EGCG	18.58	0.25 ± 0.01

EGC, epigallocatechin; EGCG, epigallocatechin gallate.

Table II. The effect of black tea extract on the growth of *Streptococcus pyogenes* at different times and concentrations.

Incubation time (hours)	Concentration (mg ml ⁻¹)				
	6.25	12.5	25.0	50.0	100.0
1	>10 ⁴	2 × 10 ³	8 × 10 ²	–	–
2	8 × 10 ³	10 ³	10 ²	–	–
3	6 × 10 ³	5 × 10 ²	–	–	–
5	2 × 10 ³	10 ²	–	–	–
7	5 × 10 ²	–	–	–	–
24	–	–	–	–	–

Table III. The effect of green tea extract on the growth of *Streptococcus pyogenes* at different times and concentrations.

Incubation time (hours)	Concentration (mg ml ⁻¹)				
	6.25	12.5	25.0	50.0	100.0
1	>10 ⁴	10 ³	2 × 10 ²	–	–
2	2 × 10 ⁴	8 × 10 ²	50	–	–
3	6 × 10 ²	2 × 10 ²	–	–	–
5	2 × 10 ²	50	–	–	–
7	80	–	–	–	–
24	–	–	–	–	–

(Tables II and III). However, gallic acid at a concentration as low as 5 µg ml⁻¹ after 5 hours and at 10 µg ml⁻¹ after 2 hours inhibited streptococcal growth. At higher concentrations no growth was observed even after a 1 hour incubation (Table IV).

Evaluation of tea extract interactions with some antibiotics

Adding 1.25 mg of green tea extract enhanced the antibacterial effect of a standard ampicillin disc, as judged by the diameter of bacterial growth inhibition ($p=0.02$), whereas it showed no effect on amoxicillin and affected cephallexin antagonistically ($p<0.001$). Increasing the amount of green tea extract to 2.5 mg resolved the antagonistic effect on cephallexin and affected amoxicillin synergistically so that the diameter of bacterial growth inhibition became significantly more than that of the standard disc ($p=0.02$).

Adding black tea extract to the standard ampicillin discs enhanced the antibacterial effect and this was statistically significant at 2.5 mg ($p<0.001$). Although black tea extract at

Table IV. The effect of gallic acid on the growth of *Streptococcus pyogenes* at different times and concentrations.

Incubation time (hours)	Concentration (µg ml ⁻¹)						
	2.5	5.0	10.0	25.0	50.0	100.0	1000.0
1	>10 ⁴	5 × 10 ³	2 × 10 ³	–	–	–	–
2	>10 ⁴	5 × 10 ²	50	–	–	–	–
3	>10 ⁴	30	–	–	–	–	–
5	>10 ⁴	–	–	–	–	–	–
7	>10 ⁴	–	–	–	–	–	–
24	–	–	–	–	–	–	–

1.25 mg showed an inhibitory effect on both amoxicillin and cephallexin ($p < 0.001$), the inhibition was attenuated with increasing the amount of black tea extract to 2.5 mg ($p < 0.001$) although the diameter of bacterial growth inhibition was still lower than that of baseline ($p < 0.001$).

Gallic acid showed a synergistic effect with all the antibiotics tested in a dose-dependent manner ($p < 0.001$). This effect was more prominent with ampicillin. Our results showed that 2.5 mg of black tea extract had the most synergistic effect with ampicillin, but with the other antibiotics and in lower amounts may have antagonistic effects. Meanwhile, gallic acid, the most abundant phenolic of black tea in our HPLC analyses, strengthened the antibacterial effects of all antibiotics in a dose-dependent manner (Figure 2).

Discussion

Our findings suggest that the microbiological effects of tea extract are selective, i.e. they differ depending on the concentration and type of the extract (black vs. green), and the type of antibiotic. Our similar experiments on other bacterial species have shown that these effects may also differ depending on the bacterial species (unpubl. data) so that they may be either growth inhibitory or stimulatory.

Many studies have reported antimicrobial effects of tea [12,13] and its purified polyphenols [10]. Synergistic effects of tea with some antibiotics have also been reported [14,15]. The synergistic effect of green tea extract and levofloxacin against *Escherichia coli* has been observed [16].

To the best of our knowledge, however, this is the first report of selective and dose-dependent microbiological effects of both green and black teas. A possible interaction between tea constituents and the antibiotics may in part explain this observation. Moreover, the amount of such effective antimicrobial compounds as gallic acid may be too low at lower concentrations of black tea extract. Plant polyphenols, tannins, have been suggested to exert their growth inhibitory effects through auto-oxidation and hydrogen peroxide production, but in certain circumstances some bacterial genes may be induced (like OxyR in *Escherichia coli*) so that strengthening bacterial antioxidant defence mechanisms may overcome tannin inhibitory effects [15]. The concentration of the polyphenolic compounds may have some role in this process. However, the inhibition of bacterial growth after 3–24 hours of incubation with tea extracts and also the attenuation of antibiotics by tea extracts at certain concentrations all strengthen the possibility of selective drug–tea interactions. In accord with this, strong and dose-dependent synergism between gallic acid and all the antibiotics tested showed the strong antibacterial effects of black tea, when the possible interfering constituents were removed.

The most potent antimicrobial polyphenols of green tea, EGC and EGCG, were found in minute amounts in black tea. It is probable that the microbiological effects of black tea, along with its antioxidant capacity, are lowered during the process of oxidation. However, this study has some limitations. The compounds of tea leaves are not just confined to the phenolics examined here. There are more polyphenolics in black tea than in green tea, partly due to the oxidation processes, often called ‘fermentation’. Moreover, when dried tea leaves are infused in hot water and even when a cup of tea is left to stand, further reactions can be happening [13]. Two major groups of compounds thus produced from flavanols during fermentation are theaflavins and thearubigins [17]. These phenolics, with very complex structures and interesting biological effects [17,18], were not examined in the present study. Meanwhile, this study was in vitro and both antibiotics and polyphenolic

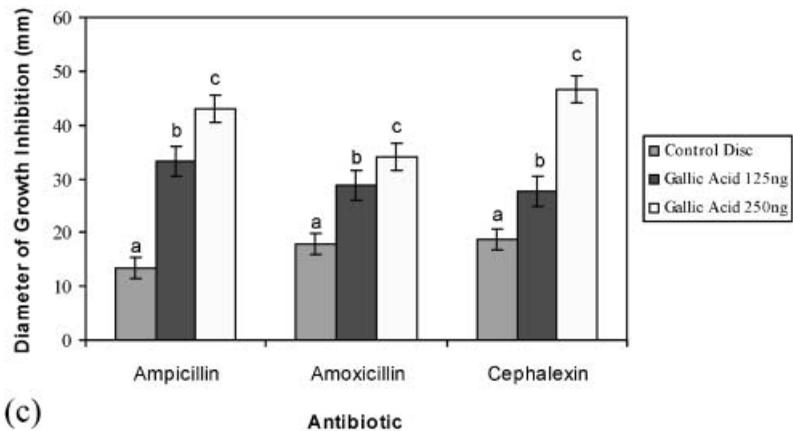
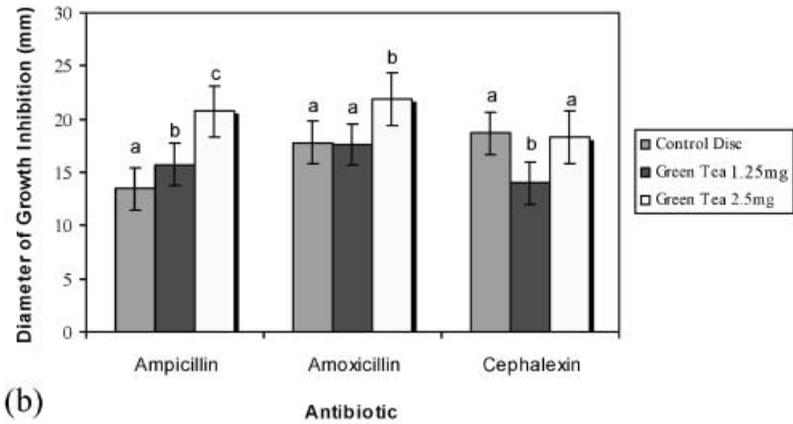
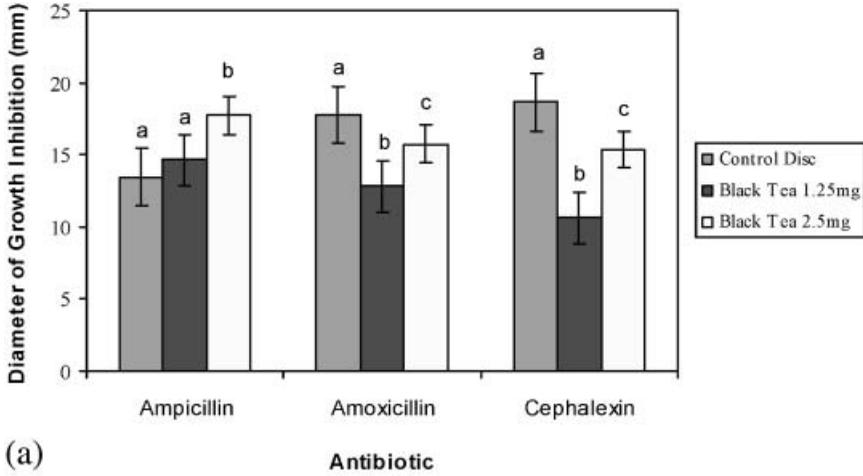


Figure 2. The effect of adding different doses of (a) black tea extract, (b) green tea extract and (c) gallic acid on selected antibiotic discs against *Streptococcus pyogenes* in the disc diffusion test.

compounds undergo metabolic processes in the body and there is less information on the interaction of the related metabolites. Moreover, the tissue distribution of ingested polyphenols is not fully known. The antimicrobial activity of tea may also be affected by both the degree of fermentation and the manufacturing season [19]. Nevertheless, the in vitro bacterial sensitivity test is still a routine procedure for clinical purposes, based on which proper antibiotics are selected to treat the infection [20–22]. Considering that 11 g of dried extract was gained from 100 g of dried black tea leaves, in a rough estimation, 1.25 and 2.5 mg of extract will be equal to 11.4 and 22.7 g of black tea, respectively. As each tea bag was about 2.5 g, the above numbers will be translated to around four to five and nine cups of tea a day, respectively. In the comprehensive food consumption survey during 1999–2001, the per capita consumption of dried tea was estimated at about 3.8 g day⁻¹ (~2 cups a day) [23]. Although the consumption of two to three cups of tea in the patients under chemotherapy with the selected antibiotics does not seem to be problematic, some dietary modifications in terms of having proper amounts of tea may be proven to be helpful. Although the antibacterial effects of gallate derivatives have already been reported [24–26], gallic acid itself was shown to be ineffective against certain micro-organisms [27]. However, our findings demonstrated that the evaluation of the antibacterial effects of gallic acid, as a nutritional supplement in certain infectious diseases, should be the subject of future studies. Finally, from the herbal medicine point of view, the concentration of gallic acid in the different tea varieties may be considered as a measure of its possible curative effects. Our findings necessitate further in vitro and in vivo studies.

Acknowledgements

This work has been funded by the National Nutrition and Food Technology Research Institute. All the laboratory bench works were carried out at the Laboratory of Nutrition Research, National Nutrition and Food Technology Research Institute.

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