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Original Communication

Inhibitory activity of Fuzhuan tea fractions against *Salmonella enterica* and other enteric pathogens

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ABSTRACT

Salmonella enterica is one of the most common causal agents of food-related illness hospitalizations in the United States. Several multi-antibiotic resistant strains of *Salmonella* are prevalent, making salmonellosis difficult to treat and creating a need for novel antibiotics and treatment strategies. This study investigates the antimicrobial properties of Fuzhuan tea, a microbially fermented tea with numerous reported bioactivities. Water-soluble tea extracts were previously reported to have antimicrobial activity against several enteric pathogens, including *Salmonella*. Investigation of polar extracts of the tea, separated via column chromatography, has led to the isolation and identification of a number of antimicrobially active compounds.

KEYWORDS: Fuzhuan tea, antimicrobial, enteric pathogens

INTRODUCTION

Originating from the Hunan Province in China, Fuzhuan tea is a dark tea, which undergoes a microbial fermentation process during its production [1]. The tea is fermented by a damp piling method (a technique similar to composting) followed by a temperature-controlled incubation to encourage the growth of the fungus, *Eurotium cristatum* [1]. This fermentation process drastically changes the chemical composition of the tea leaves and the brewed tea. Fuzhuan tea has lower levels of catechins, caffeine and amino acids [1], and increased organic acids [2] as compared to green tea, the closest non-fermented cousin of Fuzhuan tea [3]. In addition, several novel compounds have been identified in Fuzhuan tea, including atypical peaks of theaflavins [4], several previously unidentified triterpenoids [1, 5, 6], and anthraquinone pigments [1]. Fermentation of tea leaves by *E. cristatum* is thought to lead to the production of these unique compounds found in Fuzhuan tea [1, 7]. This is further indicated through antimicrobial testing. With increase in the length of fermentation time, the antimicrobial properties of Fuzhuan tea and extracts increase [5, 6, 8], especially against enteric pathogens [8].

Historically, the tea has been consumed as a treatment for dysentery, food-borne illnesses, and to facilitate digestion and nutrition [2]. Several studies from China indicate that Fuzhuan tea is effective in promoting weight loss [1], aiding in digestion through stimulation of pancreatic amylase and protease [9, 10], inhibiting the growth of gastric tumors when tested *in vitro* [11], and reducing the diarrhea index of several known diuretics [12]. In addition, Fuzhuan tea is shown to have a modulatory effect on blood cholesterol levels [1, 3, 13]. The tea decreases low-density lipoprotein levels, HbA1c [1, 3], triglycerides, and total cholesterol levels, while increasing high-density lipoprotein levels [3, 13].

The antimicrobial effects of Fuzhuan tea have been indicated through extensive research. Whole tea [8], hot water extracts [2] (similar in composition to what is consumed), and individual compounds derived from Fuzhuan tea [5, 6] have antimicrobial activity against enteric pathogens. Hot water extracts

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demonstrate inhibitory activity against *Staphylococcus aureus* NCTC 8325, *Pseudomonas aeruginosa* PA01, *Escherichia coli* Op50, *Shigella sonnei*, and *Salmonella enterica* Typhimurium, with significant activity against *S. sonnei* and *S. aureus* [2], indicating that Fuzhuan tea contains compounds active against both Gram-negative and Gram-positive bacterial species, with high efficacy against *Salmonella* spp. Antimicrobial testing of specific compounds isolated from Fuzhuan tea has not been as conclusive. Luo *et al*. showed that a novel norisoprenoid had no antimicrobial activity against *Escherichia coli* EPEC, *S. aureus*, *Shigella dysenteriae*, and *Salmonella typhi* [6], at a concentration of 0.8 mg/mL. However, the novel triterpenoid isolated by Ling *et al.* demonstrated only weak inhibitory activity against *E. coli* EPEC and *S. typhi*, but showed robust activity against *S. dysenteriae* [5].

Fuzhuan tea has specifically been shown to have antimicrobial activity against enteric pathogens [2, 5, 6, 8]. The current study focuses on the antimicrobial activity of Fuzhuan tea against *Salmonella enterica. S. enterica* infection is currently associated with 1.2 million food-borne illnesses in the United States, and is the leading cause of hospitalization and death due to food infection [14], with 35% of hospitalizations and 28% of food-borne illness-related deaths [15]. In addition, antibiotic resistance in *Salmonella* spp. has shown an increase globally over the past several years [16], creating a need for alternate antibiotic methods to combat illnesses from *Salmonella* infection. This study also identifies several compounds isolated from Fuzhuan tea which have antibiotic activity against enteric pathogens. Additionally, this study employs a 96-well plate inhibition assay instead of the traditionally used hole-plate method [2, 5, 6, 8], providing quantitative changes in the growth of each species as compared to a control, and allowing for the calculation of IC_{50} values (the concentration of active compound that reduces the optic density of the microbe by half) of each compound against the tested isolates.

MATERIALS AND METHODS

Separation of fractions from whole tea

Fractions of whole tea were separated via column chromatography, using the Teledyne ISCO CombiFlash® R_f 200 system, following the manufacturer-established protocol. 4 g of whole tea powder (Naturalin Biosciences, Hunan, China) was mixed with 12 g of silica and loaded into an empty 25 mL column. The column was loaded into the system, along with a Silica 4 g RediSep Column. Flow rate was 18 mL/min, with an equilibration volume of 33.6 mL. The compounds were measured with two wavelengths, 254 nm and 280 nm. The tea was separated using a hexane/ethyl acetate concentration gradient. Separation of peaks began at 80% ethyl acetate and continued until the completion of the run after 20 minutes of runtime. The fractions corresponding to peaks on the chromatogram (generated by the CombiFlash® R_f system) were collected, transferred into clean, massed tubes, and dried by nitrogen stream. The dried fractions were then massed and dissolved in water to a concentration of 5 mg/mL. Fractions were labeled based on relative separation time.

Fractions 11, 12, and 15 demonstrated antimicrobial activity and were further separated by column chromatography using a water/methanol gradient. Separation of the peaks largely occurred at 50% water. The subfractions were dried using a nitrogen stream. The dried subfractions were massed and dissolved in water to a concentration of 2.5 mg/mL or 1 mg/mL depending on the mass of the compound. Subfractions from fraction 11 were labeled N, subfractions from 12 were labeled P, and subfractions from 15 were labeled Q. Subfractions were identified after inhibition testing.

Culture conditions

All bacterial strains were taken from frozen 10% glycerol/brain heart infusion (BHI) stocks and grown at 37 °C as follows:

Candida albicans was grown in yeast peptone dextrose (YPD) broth for 24 hours, *Listeria monocytogenes* was grown in Luria Broth (LB) for 48 hours, *Shigella sonnei* was grown in LB for 48 hours, *Salmonella enterica* Typhimurium 14028s was grown in a Columbia broth for 24 hours, *Escherichia coli* 0157 was grown in a LB for 24 hours and *Staphylococcus aureus* NCTC 8325 was grown in a Columbia broth for 48 hours. Samples were inoculated to the inhibition assay plates directly following incubation.

96-well optical density inhibition assay

Initial fractions were tested against *C. albicans*, *L. monocytogenes*, *S. sonnei*, *S. enterica*, *E. coli*, and *S. aureus*. Subfractions were tested against *S. enterica* due to inhibitory action against *S. enterica* in the initial fraction set.

The optical density of the incubated broths was measured using spectroscopy on a multi-mode plate reader (BioTek Synergy 2). Bacteria were diluted to an optical density (OD_{600}) of 0.02 using the appropriate bacterial media. In a 96-well plate, each well was filled with 100 µL of the appropriate medium. In the top row of the plate, the treatment fractions were put into three wells each, two replicates and one blank, for a total of three treatments per plate. The final well was reserved for a water control. The treatments and water control were diluted in a 1:2 serial dilution down the plate, with a final volume of 100 µL in each well. The final concentrations of the treatments on the plate, in descending order, were 2.5 mg/mL, 1.25 mg/mL, 0.625 mg/mL, 0.313 mg/mL, 0.156 mg/mL and 0.078 mg/mL. In the subfraction plates, the concentrations were 1.25 mg/mL, 0.625 mg/mL, 0.313 mg/mL, 0.156 mg/mL, 0.078 mg/mL and 0.039 mg/mL for subfractions with an initial concentration of 2.5 mg/mL (7N, 11N, 13N, and 11P). For subfractions with an initial concentration of 1 mg/mL (7P, 8P, and 2Q), the plate concentrations were 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.063 mg/mL, 0.031 mg/mL and 0.016 mg/mL.

Five microliters of bacterial cultures diluted to OD_{600} = 0.02 were inoculated in each treatment and control well. The treatment blanks were excluded. The plate was then sealed with Parafilm and incubated at 37 °C. After 24 hours, the plate was read on the BioTek plate reader at an absorbance of 600 nm. The plate was then incubated for an additional 24 hours (48 hours in total) and the absorbance was read again at 600 nm.

Calculating percent change in growth

The percent change in growth relative to growth of untreated controls was calculated using the following formula:

$$\%\ Change = \frac{(treatment - control)}{control} \text{ x } 100,$$

where the control is equal to the average of the absorbance of the control wells, and the treatment is equal to the average of the absorbance of the treatment replicates minus the treatment blank with the corresponding concentration.

GC analysis

Active fractions were prepared for Gas Chromatography (GC) coupled with Mass Spectrometry (MS) by derivatization with trimethylsilyl. GC-MS chromatograms were compared against standards in the National Institute of Standards and Technology (NIST) Atomic Spectra Database to give candidate identifications.

IC_{50} values

Relative IC_{50} values for each fraction and subfraction were calculated using GraphPad Prism. Data was fitted using a non-linear, 4 parameter regression of log (inhibitor) vs. response. For bacteria species that underwent growth with whole tea treatment, rather than inhibition, IC_{50} values were marked as ‘N/A’.

RESULTS

Inhibition of *S. enterica* by initial fraction set

Seven fractions were isolated from the first column chromatography run for testing against *S. enterica.* Fractions were distinguished by their relative time of separation during column chromatography (Supplemental Figure 1). Of the seven fractions tested, three were chosen for subfractioning based on their activity in the initial inhibition screening. Figure 1 shows the percent change in *S. enterica* growth after treatment with each fraction as well as with the crude tea. Negative percentages represent decreased growth in the treated wells, indicating greater *S. enterica* growth inhibition in those wells.

Significant reduction in growth of *S. enterica* was observed at 24 and 48 hours when treated with 2.5 mg/mL of each fraction. Additionally, significant reduction in growth of *S. enterica* was evident after 24 hours when treated with 1.25 mg/mL of each fraction. Fraction 15 showed significant reduction in *S. enterica* growth at 24 hours at concentrations of 0.625 mg/mL and 0.313 mg/mL and significant reduction at 48 hours at a concentration of 1.25 mg/mL. Furthermore, all fractions showed greater reduction in bacterial growth at the highest

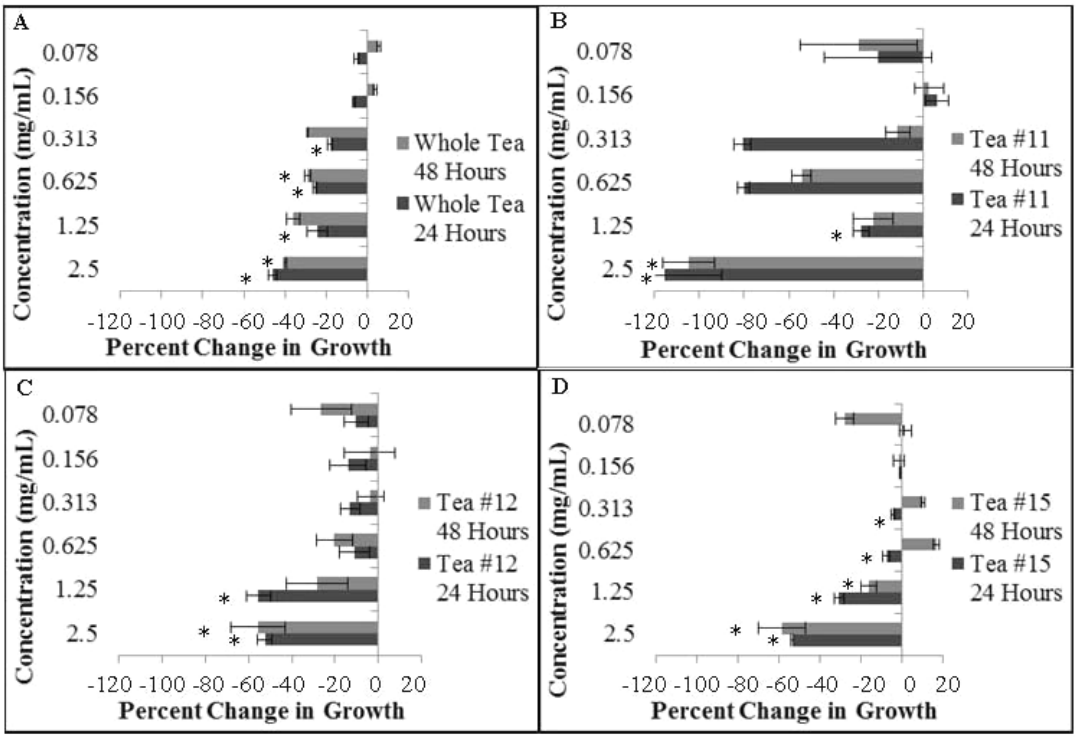

Figure 1. Percent change in growth of *S. enterica* after treatment with whole tea (A) and the initial fraction sets 11 (B), 12 (C), and 15 (D), notated by the time of separation during chromatography- at varying concentrations of each fraction. Fractions represented here were chosen for subfractioning. Negative values indicate decreased growth in treated wells, as compared to control. (*) indicates significance at $p \leq 0.05$.

concentration point than when treated with crude tea. Several concentration points show greater inhibition after 24 hours, with recovery of *S. enterica* growth at 48 hours. This can largely be attributed to the presence of several compounds in each fraction set. Some of these compounds could be acting as inhibitory agents, while others could be promoting bacterial growth. Subfractioning of the three active treatments was performed to further isolate specific compounds.

Inhibition of *S. enterica* by subfraction set

In total, twenty subfractions were separated from the second round of column chromatography - nine subfractions from fraction 11 (marked N, Supplemental Figure 2), seven subfractions from fraction 12 (marked P, Supplemental Figure 3) and four subfractions from 15 (marked Q, Supplemental Figure 4). Of these, seven were identified as having inhibitory activity toward *S. enterica*. Figure 2 shows the percent change in growth of the seven active subfractions, as compared to the untreated controls. Based on similar patterns in inhibitory activity, two pairs of fractions, 13N and 11N, and 7P and 8P, were determined to be containing the same compounds.

All subfractions isolated from fractions 11 and 12 showed significant reduction in bacterial growth at the highest concentration at 24 hours. Of the subfractions isolated from fraction 11, 7N showed significant reduction at the 1.25 mg/mL concentration. 11N and 13N, most likely the same compound, showed significant reduction in *S. enterica* growth at concentrations of 1.25 mg/mL and 0.313 mg/mL, especially at 24 hours. Of the subfractions isolated from fraction 12, subfraction 11P showed significant reduction in *S. enterica* growth at concentrations of 1.25 mg/mL (at 24 and 48 hours), and 0.625 mg/mL at 48 hours. Subfractions 7P and 8P were determined to be the same compound and showed significant reduction in bacterial growth at all concentrations at 24 hours. Subfraction 2Q, the only active subfraction isolated from fraction 15, showed reduction in *S. enterica* growth, especially when

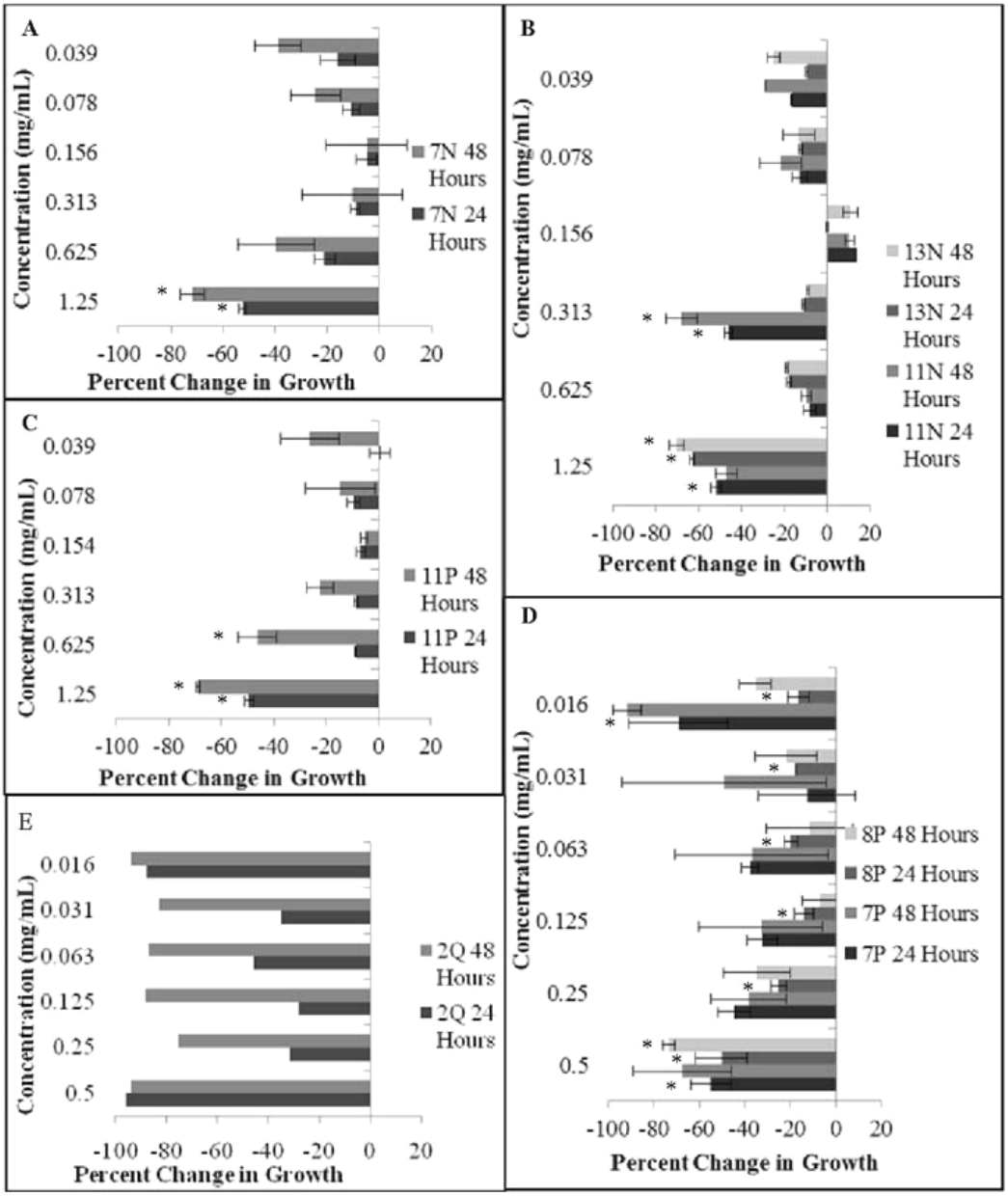

Figure 2. Percent change in growth of *S. enterica* when treated with subfractioned samples 7N (A), 11N/13N (B), 11P (C), 7P/8P (D), and 2Q (E), as compared to growth in the control, at varying concentrations of fraction. Negative values indicate decreased growth in treated wells as compared to control. Fractions marked 'N' are separated from fraction 11. Fractions marked 'P' are separated from fraction 12. Fractions marked 'Q' are separated from fraction 15. Statistics were not calculated for 2Q, as the sample size was too low for multiple replicates. (*) indicates significance at $p \leq 0.05$.

treated at concentrations of 0.5 mg/mL and 0.016 mg/mL at 24 and 48 hours. However, statistical significance could not be established for this subfraction, as the concentration of the compound was too low to test multiple replicates.

Identification of active compounds through GC-MS

Active subfractions were identified by matches in the NIST Atomic Spectra database after GC-MS analysis. Subfraction identifications are reported

in Table 1. Subfractions 11N and 13N, as well as 7P and 8P, were grouped together based on similarity in activity during inhibition testing and were considered to contain the same compounds within each set. Identifications were considered based on match and reverse match values greater than 500 and relative abundance within each fraction. Although subfractions were processed to isolate individual compounds, it is likely that each subfraction contains a few compounds.

Benzocaine was found in both subfractions 7N and 11N/13N, as both were subfractions of fraction 11. With the exception of 2,4-dimethyl benzaldehyde, a flavoring additive, the pesticide 2,4-dimethyl-formanalide, and the unknown compound, subfractions were identified as either being of plant origin, derived from the *C. sinensis* plant, or bacterial and fungal origin, derived from the microbes involved in the fermentation of tea during processing. Subfraction 7P/8P had no suitable matches within the NIST database, and is possibly a previously unidentified compound arising from fermentation with the fungus *E. cristatum* during processing of the tea. 2,4-dimethyl benzaldehyde was one of three possible isomers that were identified as potential compounds in subfraction 2Q. The other two isomers contained different arrangements of the methyl groups, and all three had the same match and reverse match values. However, because 2,4-dimethyl benzaldehyde is a flavoring agent, it is more likely to be found in the processed tea, and is the most likely match.

Of the compounds isolated, three are derived from fermentation by fungal or bacterial species, pyrazine (7N), diphenyl sulfone (11N/13N) and the unknown compound (7P/8P). These compounds are likely unique to Fuzhuan tea and other microbially fermented teas. As the compounds in Table 1 exhibit antibacterial activity against *S. enterica*, they are likely to be involved in the overall antimicrobial activity of Fuzhuan tea demonstrated in Table 2.

Inhibition of enteric pathogens by initial fraction set

The seven fractions (Fractions 9-15) isolated from the first separation of the crude tea by column chromatography were tested for activity against an additional six microbial species. Relative IC_{50} values for each bacterial species when tested against each fraction and against whole tea are

Table 1. Possible identities of compounds from the subfraction set detailed in Figure 2. Identifications were based on comparison with standards in the NIST database. Identifications were considered based on match and reverse match values greater than 500 (greater than 600 if possible). *Level of identification is based on standardized criteria established by Sumner, *et al.* [25].

Fraction	Compound name	Match value	Reverse match value	Origin	Level of identification*
7N	Ethyl 4-aminobenzoate (Benzocaine)	872	999	Plant origin [17]	2
	Pyrazine	570	805	Fungal and bacterial origin [18]	2
11N/13N	Ethyl 4-aminobenzoate (Benzocaine)	872	999	Plant origin [17]	2
	Diphenyl sulfone	574	647	Bacterial origin [19]	2
11P	1,3,7-trimethylxanthine (Caffeine)	936	936	Plant origin [20]	2
	2,6-dimethyl-6-trifluoroacetoxyoctane	816	855	Plant origin [21]	2
7P/8P	Unknown compound	N/A	N/A	Unknown	4
2Q	2,4-dimethylformanilide	657	711	Pesticide [22]	2
	2,4-dimethyl benzaldehyde	856	880	Flavoring additive [23]	2
	Isothiazole	895	999	Plant origin [24]	2

Table 2. Relative IC_{50} values of initial fractions against all bacterial species and subfractions against *S. enterica*, calculated with non-linear regression of data.

Candida albicans								
Fraction	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**Whole tea**
24 hours	2.691	1.978	0.6217	1.463	1.767e - 006	1.573	1.535	N/A
48 hours	0.05898	0.05788	1.294	1.429	2.656	2.634	0.1503	1.19
Escherichia coli								
Fraction	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**Whole tea**
24 hours	0.4520	0.4799	0.3438	1.16	0.1475	0.1549	1.309	1735
48 hours	0.3497	0.6046	0.3727	1.232	0.189	7.176e - 013	1.370	3.537
Listeria monocytogenes								
Fraction	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**Whole tea**
24 hours	1.165	1.131	0.2932	8.248e - 005	0.5114	0.6069	0.6231	1.291
48 hours	2.697	0.6527	0.8150	0.8908	0.4688	0.3873	0.6192	0.3115
Salmonella enterica								
Fraction	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**Whole tea**
24 hours	1.137	3.120	0.2476	0.8122	956.9	5.748	1.304	1.539e + 007
48 hours	0.9779	2.575	2.857	1.847	1.498	3.043	1.323	0.2404
Shigella sonnei								
Fraction	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**Whole tea**
24 hours	0.3063	1.02	1.12	0.709	1.777e - 005	3.299e - 006	1.330	0.01993
48 hours	0.3008	0.5237	0.4412	1.634	0.9749	135747	1.577	N/A
Staphylococcus aureus								
Fraction	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**Whole tea**
24 hours	2.793	0.1496	0.1563	0.6250	1.454	0.5278	1.25	0.2961
48 hours	1.264	0.1853	0.2870	0.6927	2.32	0.04726	1.466	0.3860
Further fractioning (*Salmonella enterica*)								
Fraction	**7N**	**11N**	**13N**	**7P**	**8P**	**11P**	**2Q**	
24 hours	1.316	1.348	1.378	0.2539	0.2647	4.559	0.6627	
48 hours	1.281	1.857	0.7502	0.02713	0.2629	0.5919	0.01164	

reported in Table 2. *C. albicans* at 24 hours and *S. sonnei* at 48 hours showed growth rather than inhibition when treated with whole tea. No IC_{50} value is reported for these two data points. Fractions 11, 12 and 15 were selected for subfractioning, and tested against *S. enterica*. Relative IC_{50} values for subfractions are reported at the bottom column of Table 2.

The majority of IC_{50} values reported are lower than 3 mg/mL of treatment. Furthermore, the actual concentrations of compounds within each fraction may be lower than the relative concentrations used in testing, due to dilution by the fractionation process. Relatively low IC_{50} values indicate potentially relevant pharmacological activity, as inhibitory dosages are reasonable for therapeutic use.

DISCUSSION

Of the nine compounds tentatively identified, six were previously shown to be antimicrobially active [18, 24, 26-29]. Isothiazole derivatives are produced by plants in response to fungal infection, and act as antifungal compounds [24]. The present work also indicates that isothiazole derivatives are effective antibacterial agents. These compounds are likely produced in the tea leaves either by a fungal or bacterial infection prior to harvesting, or by microbes present during the fermentation step. Pyrazines may also be produced in the tea during the fermentation step, as pyrazine-containing compounds are produced by fungal and bacterial species [18]. These compounds are known to have antibacterial activity, and pyrazine-containing

carboxylic acids are commonly used as antibiotics in the medical field [30]. Although pyrazine production by *Eurotium cristatum* has not previously been reported, closely related *Aspergillus* spp. are known pyrazine producers [18]. The isolated pyrazines may be produced by a small population of this genus in the fermenting tea leaves, or by other fungal or bacterial populations present during fermentation. Diphenyl sulfone represents another compound commonly used as an antibiotic. Sulfones, like diphenyl sulfone or the closely related amine-containing compound, dapsone, have been historically used to treat leprosy and dermalogical conditions related to the over activity of eosinophils and neutrophils in the skin [26]. In the twentieth century, these compounds were also identified as anti-malarial agents [31], exhibiting activity against a wide variety of biological agents. Diphenyl sulfone is generally produced by bacteria [19], and may be present in the tea due to bacterial infection prior to harvesting or produced during the microbial fermentation step of tea processing.

Unlike the other compounds identified by the present study, 2,4-dimethylformanilide is a metabolite of amitraz [22], a pesticide, rather than a metabolite of the tea plant itself, or a product of bacterial and fungal activity. This compound is a trace metabolite lingering from pesticide treatment of tea plants prior to harvesting. Beyond their value as pesticides, formamide derivatives are antimicrobially active through inhibition of folic acid production. This class of compounds has been shown to be effective against *C. albicans* in a previous study [27]. This compound possibly contributes to the observed efficacy of Fraction 15 against *C. albicans*.

The two remaining compounds with known antimicrobial activity, 1,3,7-trimethylxanthine (caffeine) and Ethyl 4-aminobenzoate (benzocaine), are derived from the tea plant [17, 20]. The antimicrobial activity of caffeine has been studied extensively [28], and may result from inhibition of DNA repair systems [32]. Caffeine is a known component of *Camellia sinensis* and was isolated in the current study from the leaves of the plant. Benzocaine is also derived from the leaves of plants [17], although no previous study has reported this compound in the leaves of *Camellia sinensis*. Morrow, *et al*., observed antimicrobial activity in dental anesthetics containing benzocaine [29]. However, no previous study has been done to understand the antimicrobial activity of benzocaine. The current study indicates the antimicrobial potential of this compound.

For 2,6-dimethyl-6-trifluoroacetoxyoctane and 2,4-dimethyl benzaldehyde, the current study is the first indication of antimicrobial activity. This study also indicates antimicrobial activity of all nine compounds against enteric pathogens. The unknown compound is likely produced as a result of the fungal fermentation of Fuzhuan tea, and indicates that Fuzhuan tea may be a good source for the identification of novel antimicrobial compounds.

CONCLUSION

Fuzhuan tea has antimicrobial activity against enteric pathogens. Multiple compounds contribute to this bioactivity, either in an additive or independent manner. As Fuzhuan tea has historically been used to treat illnesses [2], further investigation into the health merits of Fuzhuan tea is recommended. The unique fungal fermentation of the tea by *Eurotium cristatum* results in the production of novel antimicrobial compounds. Further investigation is vital to take full advantage of this new antibiotic source.

ACKNOWLEDGEMENTS

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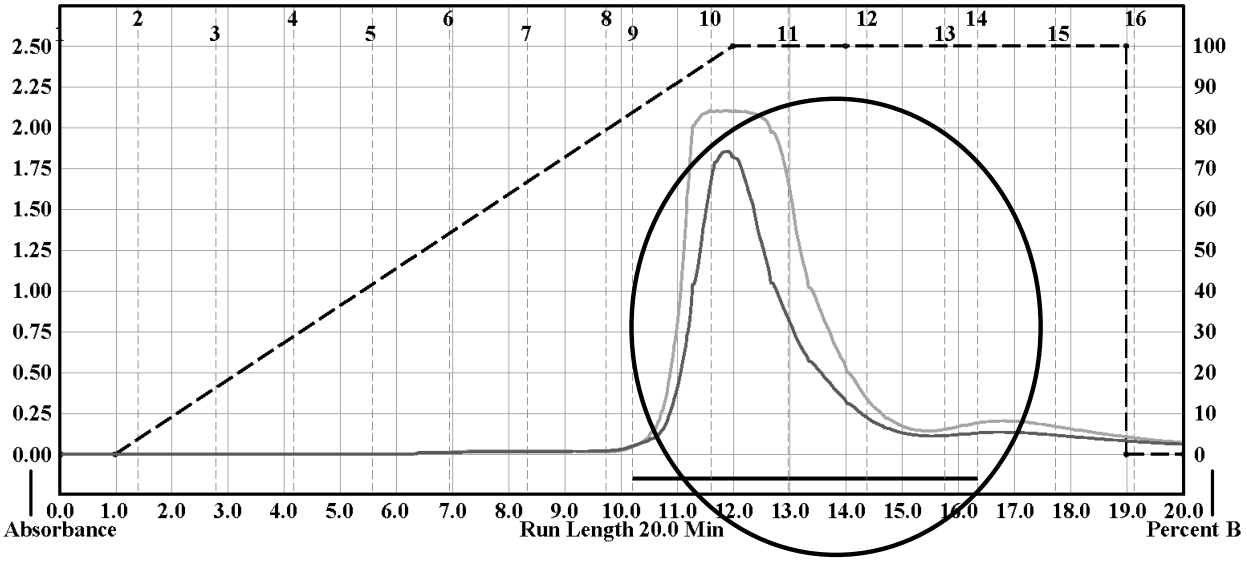
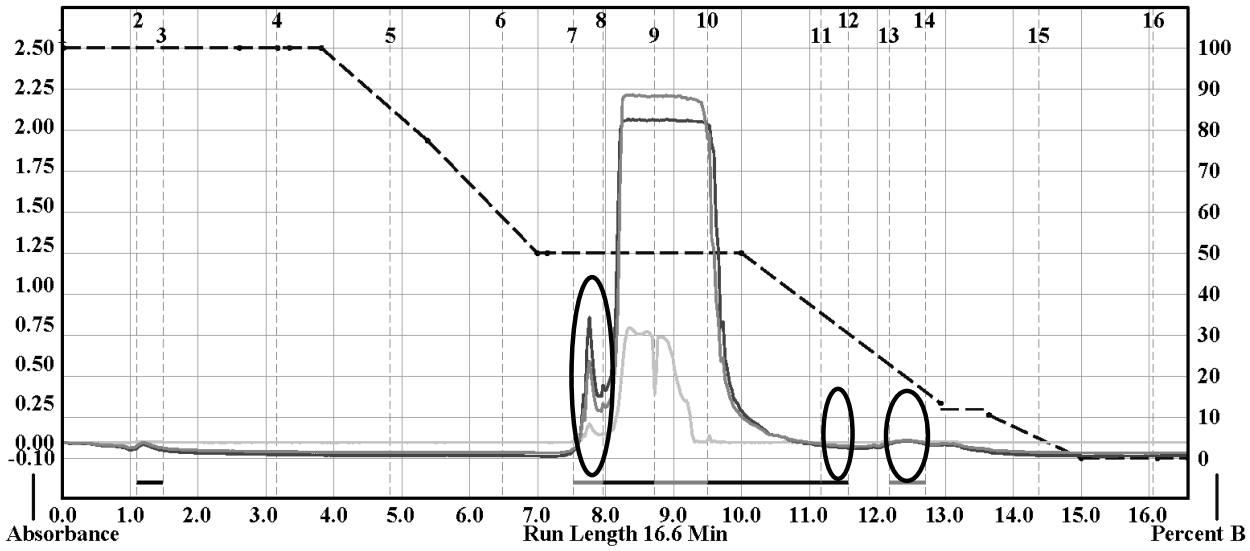
CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

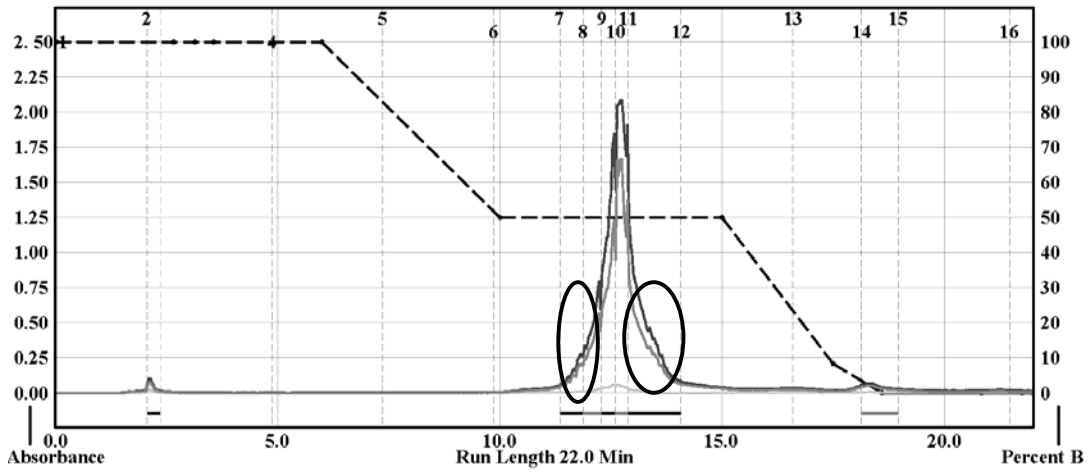
SUPPLEMENTAL DATA



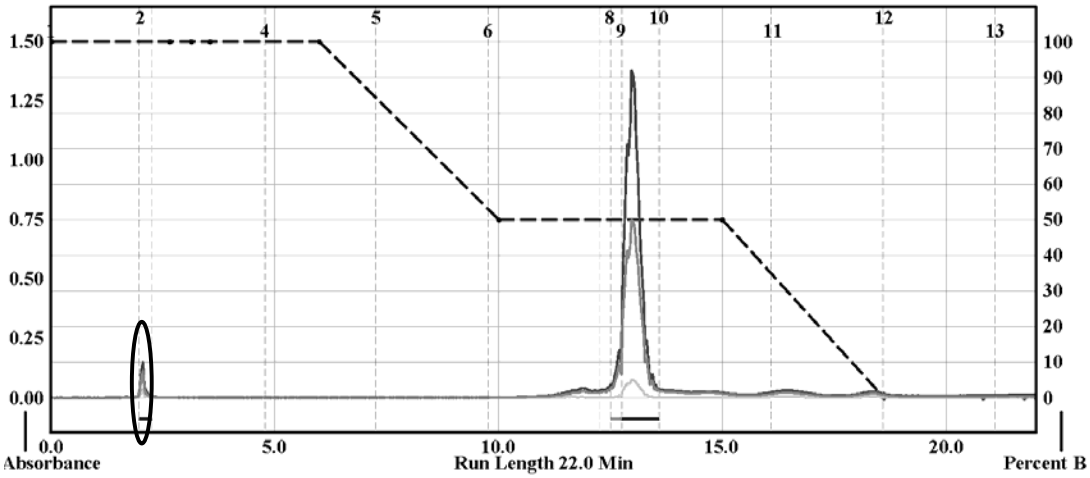
Supplemental Figure 1. Chromatogram from column chromatography separation of Fuzhuan Tea. The dashed line represents the chemical gradient of ethyl acetate (Solvent B) and hexanes (Solvent A) used to elute fractions. Peaks were identified based on absorbance at 254 nm (dark grey), and 280 nm (light grey). Numbers along the top correspond with test tube identification number, and compound-containing fractions are highlighted along the bottom. Active fractions used in this study are circled in black.



Supplemental Figure 2. Chromatogram from subfractioning of Fraction 11. The dashed line represents the chemical gradient of water (Solvent B) and methanol (Solvent A) used to elute subfractions. Peaks were identified based on absorbance at 207 nm (dark grey), 214 nm (medium grey), and over the range of 200-300 nm (light grey). Numbers along the top correspond with test tube identification number, and compound-containing subfractions are highlighted along the bottom. Active subfractions used in this study are circled in black.



Supplemental Figure 3. Chromatogram from subfractioning of Fraction 12. The dashed line represents the chemical gradient of water (Solvent B) and methanol (Solvent A) used to elute subfractions. Peaks were identified based on absorbance at 207 nm (dark grey), 214 nm (medium grey), and over the range of 200-300 nm (light grey). Numbers along the top correspond with test tube identification number, and compound-containing subfractions are highlighted along the bottom. Active subfractions used in this study are circled in black.



Supplemental Figure 4. Chromatogram from subfractioning of Fraction 15. The dashed line represents the chemical gradient of water (Solvent B) and methanol (Solvent A) used to elute subfractions. Peaks were identified based on absorbance at 207 nm (dark grey), 214 nm (medium grey), and over the range of 200-300 nm (light grey). Numbers along the top correspond with test tube identification number, and compound-containing subfractions are highlighted along the bottom. Active subfractions used in this study are circled in black.

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