The synergistic effects of green tea polyphenols and antibiotics against potential pathogens

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ABSTRACT

Green tea leaves contain many polyphenolic compounds such as (−)-epicatechin (EC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin-3-gallate (EGCG). These polyphenol compounds have been implicated to have distinct properties that combat the harmful effects of cell proliferation. They contain certain anti-viral and anti-bacterial properties that inhibit growth. In this study, 1% green tea and modified lipophilic green tea polyphenols (GTP and LTP) were used in combination with the most commonly prescribed antibiotics to study their effects on gram-positive, gram-negative, and acid-fast bacteria. The results indicated that 1% GTP and 1% LTP provided different synergistic effects on several antibiotics in various bacteria. It was found that 1% GTP works the best synergistically against *Enterobacter aerogenes*, making the resistant strain susceptible to 8 out of 12 antibiotics used. 1% LTP worked the best on *Escherichia coli* and was able to convert 7 antibiotic resistant categories to susceptible. In addition, 1% LTP was also able to inhibit the growth of *Serratia marcescens* synergistically with 3 antibiotics. These results suggest that 1% GTP and 1% LTP provide beneficial effects on selected antibiotics against microbial growth and are able to reverse the antibiotic resistance to susceptible. Green tea polyphenols could serve as natural alternatives to combat against antibiotic resistance pathogens.

Keywords: GTP; LTP; Green Tea Polyphenols; Pathogenic Microorganisms; Kirby-Bauer Disk Diffusion Method; Antibiotic Resistance

1. INTRODUCTION

Chinese green tea is an orally ingested beverage popular in Asian and Western communities. The tea is derived from an herbal plant *Camellia sinensis* [1]. The greatest cultivation of this plant is mainly in Mainland China and the areas that surrounding it. South and Southeast Asia recorded green tea as the second most popular beverage consumed worldwide after water [2]. Green tea has been observed to have several medical benefits, some of which include reduction in cholesterol level, protection against cardio-vascular diseases, cancer, etc. [3]. Polyphenolic compounds found exclusively in green tea have been implicated to have distinct properties that combat the harmful effects of many potentially pathogenic bacteria [4,5].

The polyphenolic compound that has been attributed to the positive claims of green tea is known as Epigallocatechin-3-gallate (EGCG). This pure compound is relatively expensive and unstable. It can be oxidized rapidly in normal atmospheric conditions and is not lipid-soluble. Since, EGCG has poor membrane permeability, low chemical stability and is usually metabolized rapidly [6], it loses its abilities long before one would be able to apply it. Most of the studies with EGCG have to be conducted with freshly prepared EGCG otherwise it loses its potent antimicrobial activity [7]. This poses an issue since EGCG has been reported to have many medical benefits. The green tea polyphenols need to be modified to form a lipophilic tea polyphenol (LTP), which is soluble in any lipid medium [8]. This would allow for increased utilization to be employed as a topical application in solution.

Chinese green tea extract has also been found to strongly inhibit the growth of major food-borne pathogens, *Escherichia coli* O157:H7, *Salmonella typhimurium* DT104, *Listeria monocytogenes*, *Staphylococcus aureus*, and a diarrhea food poisoning bacteria *Bacillus cereus* in varied levels of effectiveness [9]. This is of particular interest since EGCG has been suggested to be highly effective against *S. aureus* and also methicillin

resistant *S. aureus* (MRSA). *S. aureus* is of major concern to the dairy industry worldwide since it has been associated with bovine mastitis in high rates causing tremendous economic losses. This pathogen has become an increasingly problematic infection with the response in evolutionary resistance to a broad spectrum of antibiotics. As a positive marker of a recent study, it was concluded that after about 5 to 6 hours of incubation under assay conditions, 500 µg/ml of green tea extract was able to completely inhibit the growth of both susceptible and resistant strains of this bacteria [9].

In this study, green tea polyphenols (GTP) and lipophilic tea polyphenols (LTP) were used in combination with twelve of the antibiotic disks to evaluate and establish a profile for their antimicrobial activities in different groups of microorganisms. GTP is a crude extract of the polyphenols found in green tea and is a cheaper alternative to using EGCG. It has a marked 70% or higher purity. LTP is an esterified version of EGCG and is lipid-soluble. This makes it an ideal candidate to be utilized in topical solutions or ointments to enhance treatment. The evaluation gives insight into understanding the effects of novel tea compounds and its efficacy in conjunction with antibiotics *in vitro*. This may be used as an alternative therapeutic agent or synergistic agent to battle antibiotic resistant bacterial infections in the future.

2. METHODS

2.1. Culture Strains

There were six microorganisms in stock, constantly maintained and utilized in the experiment. The organisms were gram positive bacteria: *Staphylococcus epidermidis*, *Bacillus megaterium*; gram negative bacteria: *Escherichia coli*, *Serratia marcescens*, *Enterobacter aerogenes*; and an acid-fast bacterium *Mycobacterium smegmatis*. They were used to screen and establish profile in this study. All the cultures were maintained in nutrient broth, nutrient agar plate, Muller-Hinton agar or Muller-Hinton broth. They were grown at 37˚C incubation with consistent shaking at 250 rpm except *Serratia marcescens* which is kept at room temp. The overnight cultures were used in this study. Fresh stocks were prepared and stored at 4˚C and permanent stocks were kept in −80˚C. The abbreviations for the microorganisms are, in the same order as mentioned above, as noted to be the following: *S. epidermidis*, *B. megaterium*, *E. coli*, *S. marcescens*, *E. aerogenes*, and *M. smegmatis*.

2.2. Preparation of GTP and LTP

The preparation of GTP and modified LTP was utilized by creating stock solution from fine tea powder. The GTP was dissolved in water at the final concentration of 1% and the LTP was dissolved in 100% ethanol at the final concentration of 1%. The solution then went through a 0.45 µm filter unit to obtain a sterile solution.

2.3. Disk Diffusion Method

The experiment utilized the Kirby-Bauer disk diffusion method. After inoculation and stamping of the antibiotics, 25 µl of 1% GTP or LTP was directly infused with the filter disk at room temperature. The antibiotics alone were used as control for each microorganism. The plates were incubated at 24 and 48 hours at 37˚C. The zones of inhibition were measured in millimeters across their diameter of their clear region. This region included the antibiotic disk itself and utilized minimum-detection limit (MDL) as shown in **Figure 1**. The zones of inhibition (ZOI) were categorized into three unique categories that effectively informed the status of the effectiveness against the microorganism. The three categories are susceptible (S), intermediate (I), and resistant (R) which are defined in **Table 1** with its respective abbreviation [10, 11]. The antibiotic disks included, in alphabetical order, are: Ampicillin (AM10), Bacitracin (B10), Cephalothin (CF30), Chloramphenicol (C30), Doxycycline (D10), Erythromycin (E15), Gentamicin (GM10), Penicillin (P10), Polymyxin (PB300), Rifampin (RA5), Streptomycin (S10), and Tetracycline (TE30). Three repeating with or without tea polyphenols (GTP or LTP) of each experiment were carried out and the results were shown by the mean and standard deviation. The profiling of antibiotics on each microorganism in the presence or absence of GTP or LTP was established.

The percentage of increase/decrease of combination treatment was calculated as **Eq.1** where A is zone of inhibition of combined treatment and B represents the zone of inhibition of respective antibiotics [12]:

$$\% = \frac{A - B}{B} \times 100 \qquad (1)$$

A: ZOI of combined treatment; B: ZOI of respective antibiotics.

Table 1 is the reference chart derived from Clinical Laboratory Standards Institute to determine the classification of the antibiotics per given microorganism [11]. Variation occurs among microorganisms, however, it provides a good consensus to establish a quick, efficient profiling schematic to further determine if any effects have occurred (+/− in percentage). Afterwards, closer examination and research can be done to determine positive versus negative impacts in our research of combinative polyphenol antibiotic supplementation.

3. RESULTS

The effects of 12 antibiotics, alone or in combination, with one of the tea polyphenols (GTP or LTP) on 6 dif-
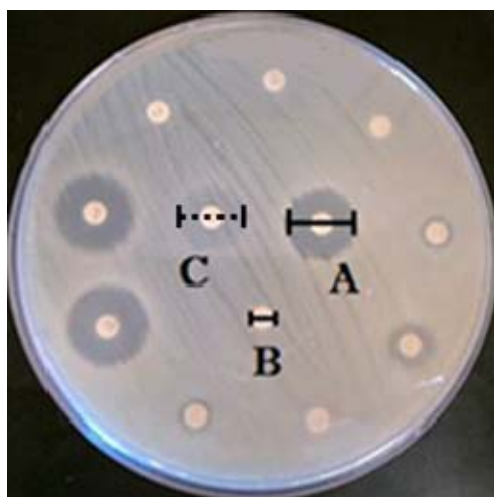
Figure 1. Disk diffusion susceptibility test used for the Kirby-Bauer testing with antibiotic disks infused with the compound of interest. A: Complete zone of inhibition (ZOI); B: No ZOI; C: Partial/weak ZOI.

Table 1. Kirby-Bauer disk diffusion categories of susceptible (S), intermediate (I) and resistant (R).

Antimicrobial agents	Disk potency	Resistant (mm)	Intermediate (mm)	Susceptible (mm)
Ampicillin (AM10)	10 µg	<12	12 - 13	>13
Bacitracin (B10)	10 µg	<9	9 - 12	>12
Cephalothin (CF30)	30 µg	<15	15 - 17	>17
Chloramphenicol (C30)	30 µg	<13	13 - 17	>17
Doxycycline (D30)	30 µg	<15	15 - 16	>16
Erythromycin (E15)	15 µg	<14	14 - 17	>17
Gentamicin (GM10)	10 µg	<15	14 - 17	>17
Penicillin (P10)	10 µg	<12	12 - 21	>21
Polymyxin (PB300)	300 µg	<9	9 - 11	>11
Rifampin (RA5)	5 µg	<12	12 - 14	>14
Streptomycin (S10)	10 µg	<12	12 - 14	>14
Tetracycline (TE30)	30 µg	<15	15 - 18	>18

ferent bacteria were evaluated. Profiling of these studies was generated from zone of inhibition, % of increase/decrease, and comparative analysis in determining inhibition efficiency versus the antibiotics alone. The results also indicate whether the synergism of GTP or LTP possesses the possibility to convert original antibiotic resistant to antibiotic sensitive.

3.1. Gram-Positive Microorganisms

Two gram-positive bacteria, *S. epidermidis* and *B. megaterium*, were used in profiling the combination effect of green tea polyphenol with different antibiotics. *S. epidermidis* is a normal flora on our skin, however, given certain conditions can become a potential pathogen. *B. megaterium* is a gram-positive bacterium and endospore producer. These spores produced by *Bacillus* are a main concern for hospitals in the medical community and the food industry due to its difficulty in eradication [13]. The results from this profiling study are shown in **Figure 2(a)** and the % of increase or decrease with GTP or LTP on the effect of antibiotics are shown in **Figure 2(b)**. One percent of GTP and LTP increased antibiotic efficacy ranging from 17% to 213% and 11% - 183% respectively. Rifampin is noted to have the most significant increase in polyphenol/antibiotic inhibitive efficacy being 213% and 183% for RA5 for GTP and LTP respectively. Combination of GTP with AM10, B10, C30 and RA5 and combination of LTP with C30 and RA5 increased the efficacy of these antibiotics more than 100%. The results indicate that both GTP and LTP have synergistic effect on all the antibiotics except PB300 for *S. epidermidis*. 1% LTP is able to convert *S. epidermidis* resistant to C30 and RA5 into susceptible. 1% GTP can work with AM10, B10, C30, P10 and RA5 to convert antibiotics that are not effective to having an impact against this microorganism.

In the study of *B. megaterium*, shown in **Figures 3(a)** and **(b)**, GTP was found to increase antibiotic efficiency from 5% to 112% except on doxycycline (D30). LTP had a range from 3% to 55% with many negative impacts. The result revealed that GTP had an inhibition of 112% with B10. Some negative impacts of adding GTP or LTP on the antibiotics that were observed may be due to interaction of the tea polyphenols with antibiotics, which reduce the activities of antibiotics. The results suggest that GTP has a significant synergistic effect on antibiotics for *Bacillus* than LTP. Although GTP and LTP were able to increase the inhibition of some antibiotics, they were not able to convert the status from antibiotic resistant to sensitive for all the antibiotics used in this study.

3.2. Acid-Fast Microorganism

The results of profiling the effect of 1% of GTP and LTP with antibiotics on *M. smegmatis* are shown in **Figures 4(a)** and **(b)**. The effects of GTP and LTP had ranges of 9% to 89% and 83% to 125% respectively. The highest percentage of inhibition was observed with 1% LTP in combination with P10 and AM10.

GTP was shown to have a synergistic effect on broader-antibiotics but only changed resistant acid-fast mi-
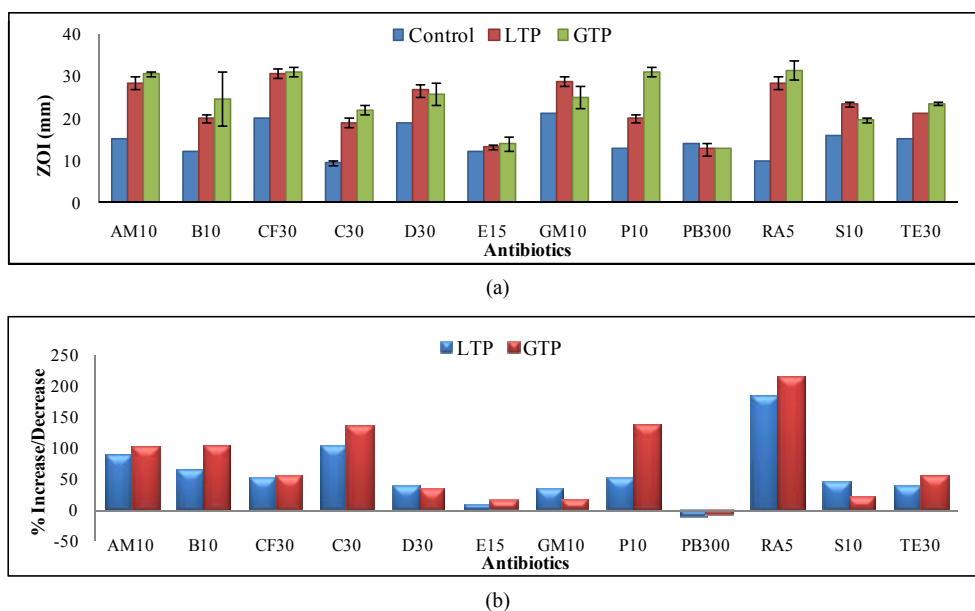
Figure 2. Profiling of *Staphylococcus epidermidis* of antibiotics alone and in combination with either 1% LTP or 1% GTP. (a) Zone of inhibition measured in mm; (b) Percentage increase/decrease of antibiotics with LTP and GTP.
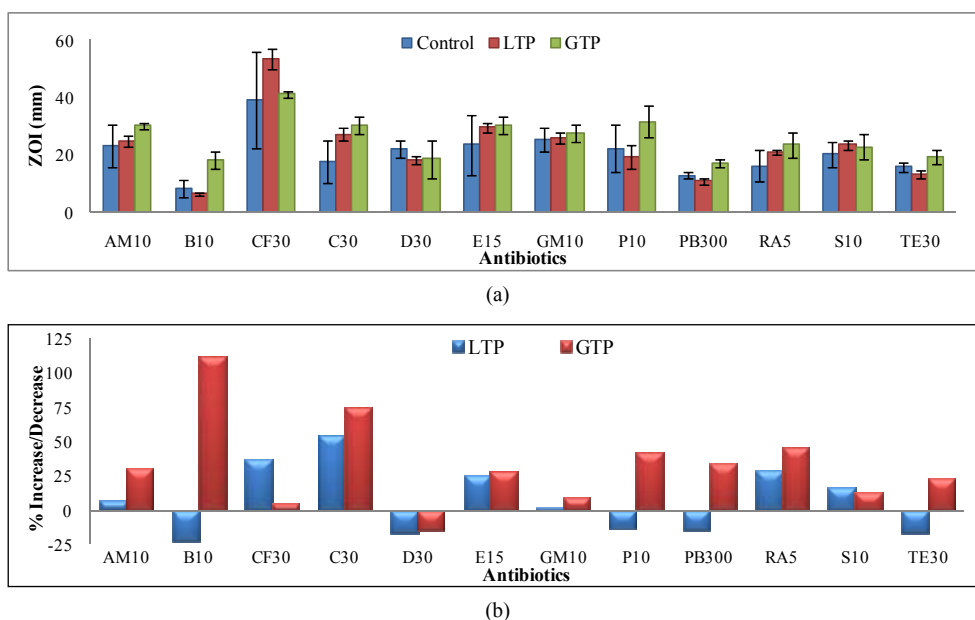

Figure 3. Profiling of *Bacillus megaterium* on antibiotics alone and in combination with either 1% LTP or 1% GTP. (a) Zone of inhibition measured in mm; (b) Percentage increase/decrease of antibiotics with LTP and GTP.

croorganisms to susceptible when used in combination with E15. LTP showed a better infusion with targeted antibiotics AM10, B10, CF30, and P10 as well as converted the status of antibiotic resistant to susceptible.

3.3. Gram-Negative Microorganism

E. coli is part of the normal flora in our intestine; however, it can cause infection in invasive wounds, urinary tract infections, abdominal cavity ailments, diarrhea and many other diseases [9]. *E. coli* showed great affinity for the usage of LTP over GTP as seen in **Figures 5(a)** and **(b)**. *E. coli* has been shown to have resistance to AM10, B10, C30, D30, E15, P10, PB300 and TE30. The effects of LTP remarkably reversed all these resistance, and enabled an increase in the zone of inhibition ranging from 28% to 161%. It increased the inhibition of AM10, B10,
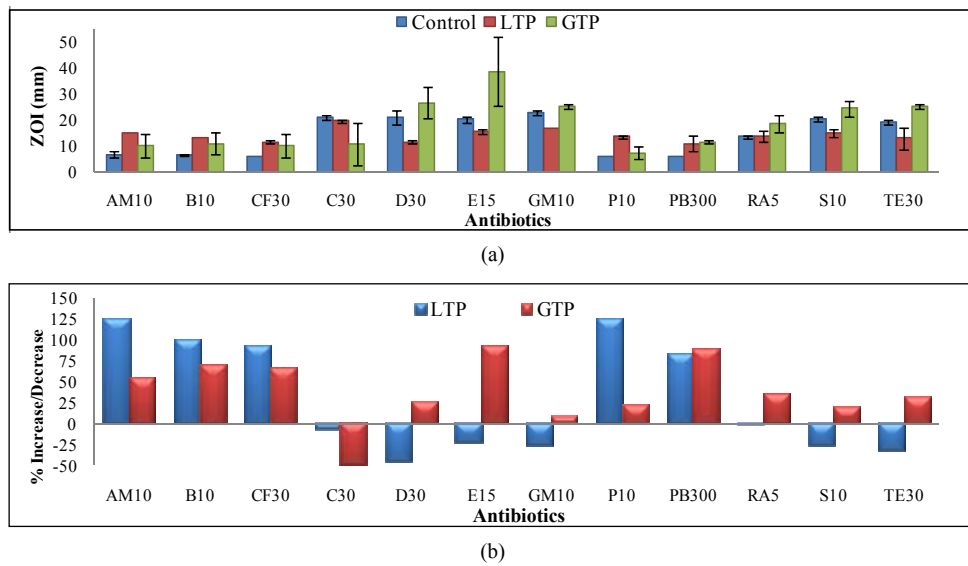
(a)

(b)

Figure 4. Profiling of *Mycobacterium smegmatis* of antibiotics alone and in combination with either 1% LTP or 1% GTP. (a) Zone of inhibition measured in mm; (b) Percentage increase/decrease of antibiotics with LTP and GTP.

(a)

(b)

Figure 5. Profiling of *Escherichia coli* of antibiotics alone and in combination with either 1% LTP or 1% GTP. (a) Zone of inhibition measured in mm; (b) Percentage increase/decrease of antibiotics with LTP and GTP.
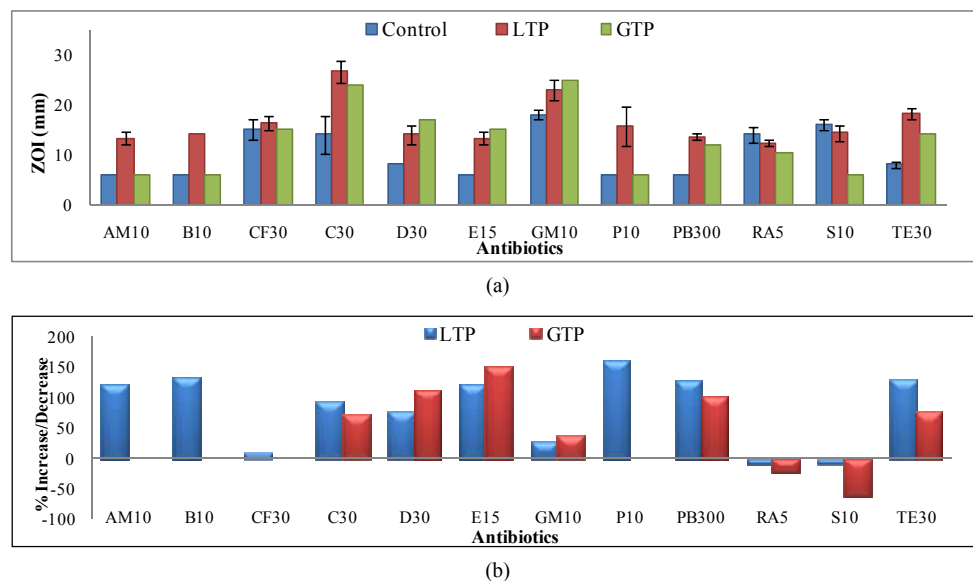
E15, P10, PB300 and TE30 more than 100%. GTP did not have an impact on such a broad spectrum of antibiotics, as aforementioned antibiotics, but did have more than 100% on D10, E15, and PB300. Therefore, it was shown that 1% LTP converted the effect of AM10, B10, C30 E15, P10, PB300, and TE30 on *E. coli* from resistant to susceptible, while GTP had synergistic effects with C30, D30, E15, and PB300.

S. marcescens, a red-pigmented gram-negative microorganism found in nosocomial infections and is an opportunistic human pathogen, is a very resistant microorganism [14]. This study indicated that *S. marcescens* is very resistant to most of the antibiotics. It was found that GTP did not produce favorable results; LTP had a tremendous impact with B10, CF30, and RA5 with 292%, 119%, and 225% increase inhibition respectively. In the presence of LTP, *S. marcescens* resistance was converted to susceptible on these antibiotics as shown in **Figures 6(a)** and **(b)**.

E. aerogenes, a gram-negative microorganism, is known to cause many hospitals acquired bacterial infections [15]. The results indicated that GTP works with all twelve
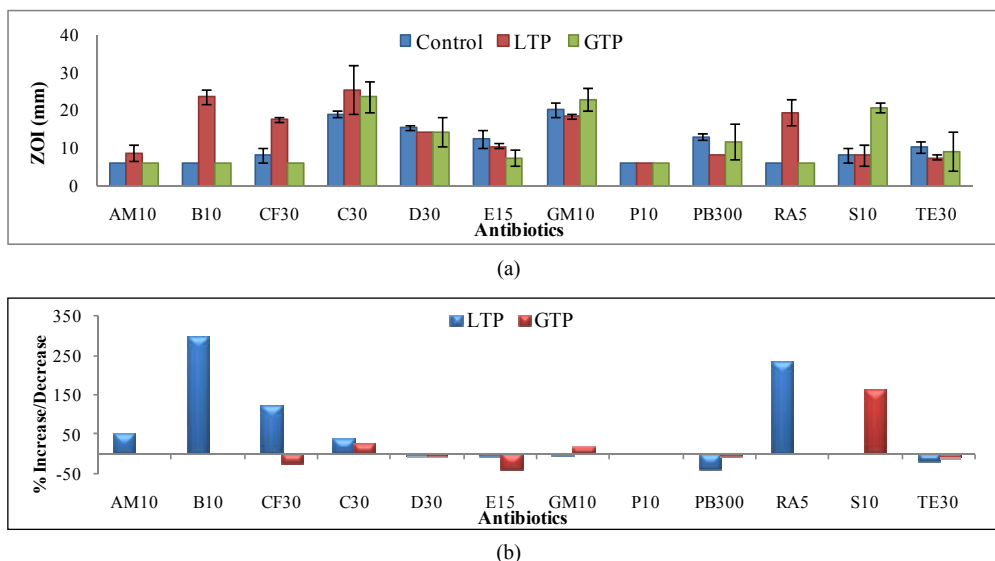
(a)

(b)

Figure 6. Profiling of *Serratia marcescens* of antibiotics alone and in combination with either 1% LTP or 1% GTP. (a) Zone of inhibition measured in mm; (b) Percentage increase/decrease of antibiotics with LTP and GTP.

antibiotics; with a 406% increase inhibition compare with the AM10 alone and 359% increase inhibition for CF30 and over 100% of increase inhibition on B10, D30, E15, P10, PB300, RA5 and TE30. On the other hand, LTP had made negative impacts on many of the antibiotics. It was shown to only works on E15 and TE30 with the increase percentage being 119% and 233% respectively converting resistance to susceptibility (**Figures 7(a)** and **(b)**).

Table 2 showed that 1% GTP worked the best with *E. aerogenes* compared to other microorganisms. It was able to synergistically work with all nine antibiotics with percentages of inhibition above 100%. The greatest percent of inhibition for *E. aerogenes* was 406%. GTP also works very well with *S. epidermidis* where it had percentages greater than 100% for five antibiotics. GTP only had effects on two antibiotics for *S. marcescens*. The data indicates values of percentage against the microorganisms. The highest value is Ampicillin at 359% against the gram-negative microorganism *E. aerogenes*. Any value higher than 100% is favorable. *S. epidermidis* had several antibiotics that showed to have a 100% or greater ZOI in usage with GTP.

Table 3 gave an overview summary of inhibition for each microorganism with 1% LTP in combination with the twelve antibiotics. The data with a numerical value of 100% or greater were the most desired for further analysis and documentation. 1% LTP appeared to inhibit *E. coli*, *E. aerogenes*, *S. marcescens*, and *M. smegmatis* with the most antibiotics. This suggested that 1% LTP in combination with antibiotics seemed to inhibit gram-negative microorganisms preferably; a very significant finding due to gram-negative bacteria's composition and

poorer response to antibiotic treatment. *E. coli* had several antibiotics that showed to have a 100% or greater ZOI in usage with LTP.

In the presence of 1% GTP or 1% LTP, the polyphenols were able to convert some microorganisms from their antibiotic resistant origins to a nature of antibiotic susceptibility as shown in **Table 4**. The results indicated that the 1% LTP worked on *E. coli* and converted seven antibiotics that were categorized as resistant to susceptible. 1% GTP worked the best synergistically against *E. aerogenes*, making the once resistant *E. aerogenes* become susceptible to the eight antibiotics. It is also demonstrated that only 1% LTP were able to inhibit the growth of highly antibiotic resistant *S. marcescens* synergistically with three antibiotics.

4. DISCUSSION

The synergistic effects of GTP and LTP varied depending upon microorganism, strain, classification, and antibiotic used against certain strains. The GTP and LTP compounds were very effective against gram-negative bacteria *E. coli*.

One of the most significant findings for this experiment was that the usage of the LTP was found to have a significant impact and quite successful in producing a therapeutic *in vitro* effect against *S. marcescens*, a highly resistant bacterium. The effectiveness of GTP and LTP were found to work prominently against *E. coli* and *S. epidermidis*, which served as models for severe pathogenic microorganisms that raid clinics worldwide.

It is also notable to understand that it is favorable to use LTP for *E. coli* and GTP for *S. epidermidis*. This suggests that the promotion of LTP could be used for

Table 2. Percentage increase/decrease for individual antibiotic with GTP. A: *E. coli*, B: *S. marcescens*, C: *E. aerogenes*, D: *S. epidermidis*, E: *B. megaterium*, F: *M. smegmatis.*

%	A	B	C	D	E	F
AM10	0	0	406	103	30	55
B10	0	0	162	106	112	69
CF30	0	−25	359	55	5	67
C30	71	26	70	136	74	−49
D30	113	−6	167	36	−15	27
E15	150	−41	237	17	28	92
GM10	39	15	73	19	9	9
P10	0	0	252	138	42	22
PB300	100	−7	189	−7	33	89
RA5	−25	0	137	213	45	37
S10	−63	162.5	92	23	13	20
TE30	75	−10	228	57	23	32

Table 3. Percentage increase/decrease for individual antibiotic with LTP. A: *E. coli*, B: *S. marcescens*, C: *E. aerogenes*, D: *S. epidermidis*, E: *B. megaterium*, F: *M. smegmatis.*

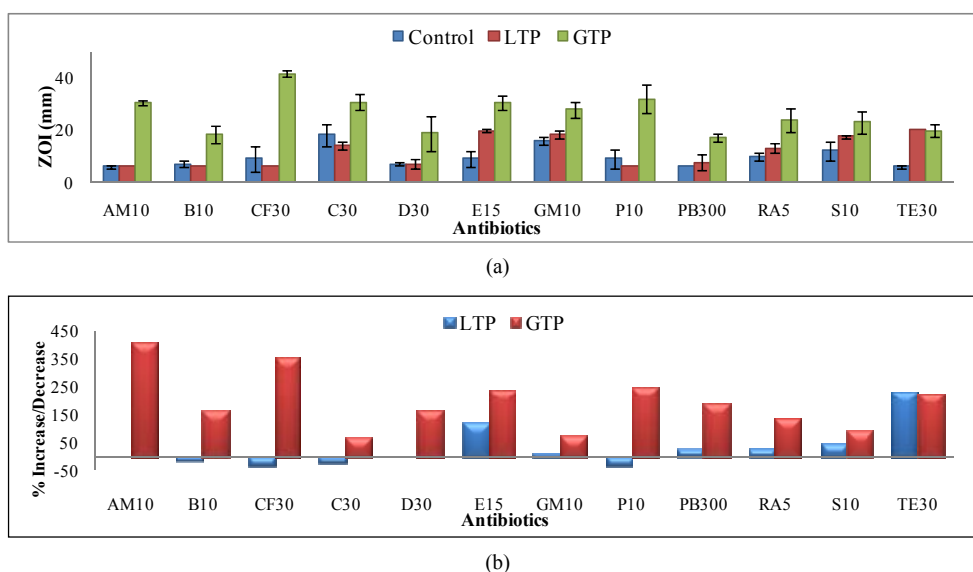| %	A	B	C	D	E	F
AM10	122	42	0	89	7	125
B10	133	292	−14	67	−23	100
CF30	9	119	−33	53	36	92
C30	90	34	−22	104	55	−7
D30	75	−9	0	40	−17	−45
E15	122	−15	119	11	25	−23
GM10	28	−8	15	37	3	−26
P10	161	0	−33	54	−13	125
PB300	128	−38	28	−10	−15	83
RA5	−12	225	30	183	29	−1
S10	−10	0	47	46	16	−26
TE30	129	−27	233	40	−17	−32

(a)

(b)

Figure 7. Profiling of *Enterobacter aerogenes* of antibiotics alone and in combination with either 1% LTP or 1% GTP. (a) Zone of inhibition measured in mm; (b) Percentage (%) of increase/decrease of antibiotics with LTP and GTP.

Table 4. Comparison chart of resistant to susceptibility against antibiotics with GTP and LTP.

Microorganisms	1% GTP	1% LTP
E. coli	C30 (71% R→S), D30 (113% R→S), E15 (150% R→S), PB300 (100% R→S)	AM10 (122% R→S), B10 (133% R→S), C30 (90% R→S), E15 (122% R→S), P10 (161% R→S), PB300 (128% R→S), TE30 (129% R→S)
S. marcescens	S10 (R→S)	B10 (292% R→S), CF30 (119% R→S), RA5 (225% R→S)
E. aerogenes	AM10 (406% R→S), B10 (162% R→S), CF30 (359% R→S), D30 (167% R→S), E15 (237% R→S), P10 (252% R→S), PB300 (189% R→S), RA5 (137% R→S), TE30 (228% R→S)	E15 (119% R→S), TE30 (233% R→S)
S. epidermidis	AM10 (103% R→S), B10 (106% R→S), C30 (136% R→S), P10 (138% R→S), RA5 (213% R→S)	C30 (104% R→S), RA5 (183% R→S)
B. megaterium	Impact not significant	Impact not significant
M. smegmatis	E15 (92% R→S)	AM10 (125% R→S), B10 (100% R→S), P10 (125% R→S)

gram-negative bacteria, such as seen in *S. marcescens*, and GTP for gram-positive bacteria. The exception to this statement is found with *E. aerogenes* which seemed to be the most susceptible to GTP. In addition, 1% GTP and 1% LTP provide different synergistic effect on different antibiotics in different bacteria. This suggests that the difference in the molecular structure of GTP and LTP could impact their mode of action in their biochemical mechanisms.

5. CONCLUDING REMARKS

In summary, the percentage inhibition of 1% GTP and 1% LTP with antibiotics against microorganisms are shown in **Tables 2** and **3**. **Table 2** showed that 1% GTP worked the best against *E. aerogenes* compared to the other microorganisms. 1% GTP was able to provide synergistic inhibition percentages above 100% with all 9 antibiotics. The highest synergy was found with Ampicillin and GTP at 406% against *E. aerogenes*. GTP did not show any major synergistic effects for *S. marcescens* except for S10. **Table 3** gave an overview of inhibition for six microorganisms with 1% LTP in combination with the twelve antibiotics. Some of the combination treatments indicated a greater than 100% of inhibition. 1% LTP appeared to inhibit *E. coli*, *S. marcescens* and *M. smegmatis* with the most antibiotics. Any value higher than 100% is noted to be favorable. Several antibiotics showed a 100% or greater ZOI when combined with LTP against *E. coli*. Both GTP and LTP increased inhibition with most antibiotics on *S. epidermidis*, but LTP only worked on C30 and RA5 and GTP worked on AM10, B10, C30, P10 and RA5.

Table 4 summarized that 1% LTP or 1% GTP in combination with different antibiotics resulted in conversion of antibiotic resistant to susceptible. 1% GTP worked the best synergistically against *E. aerogenes*, making the once resistant *E. aerogenes* now susceptible to 8 antibiotics. 1% LTP worked the best on *E. coli* and converted 7 antibiotics resistant to susceptible. It is also demonstrated that only 1% LTP was able to inhibit the growth of *S. marcescens* synergistically with three antibiotics.

This study has established a profile by using 1% GTP and 1% LTP versus controls which have allowed us to evaluate the efficacy of the synergistic combination with these antibiotics. They were able to change some of the antibiotics from resistant to susceptible.

The mechanism of EGCG, the major green tea water-soluble polyphenol, is not yet fully understood. However, there is a definite correlation for EGCG and its affinity to the cell wall composition of bacteria [16]. This suggests that GTP has an effect on bacterial cell wall; meanwhile, LTP provides a very different profiling suggesting perhaps a different mechanism of lipid soluble tea polyphenol on bacteria.

6. FUTURE STUDIES

Pure hydrophilic green tea polyphenol (GTP) such as EGCG and pure lipophilic tea compound EGCG-stearate should be used to further evaluate the efficacy of the synergism of these polyphenols on different antibiotics. The minimum inhibitory concentrations (MIC) with different combinations of tea polyphenols and antibiotics should be determined to obtain the optimal condition for potential application of therapeutic treatment of infection. Furthermore, the synergy mechanisms of GTP, LTP and different antibiotics should also be studied. These findings could prove useful for further *in vitro* studies that potentiate to *in vivo* applications.

7. ACKNOWLEDGEMENTS

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