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In-vitro evaluation of the interactions between herbal drug (*Gardenia latifolia* Ait) and antibiotic (Streptomycin)

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Abstract
The effect of combinations of the ethanol extract of *Gardnia latifolia* leaf and antibiotics was investigated by means of fractional inhibitory concentration (FIC) indices as well as by the use of time kill assays. The activity of streptomycin was increased up to 10-fold when the combined mixture of *G. latifolia* and antibiotic was used against microbial pathogens. The herb-drug interactions tested by checkerboard method and expressed as fractional inhibitory concentration (FIC) index, showed synergistic effects. The synergism is a new concept of developing drug molecule for treating drug resistant bacteria and prevent emergence of new drug resistant bacteria. We conclude that the ethanol extract of *G. latifolia* can be a potential source of broad spectrum antibiotics resistance modifying compounds.

Keywords: MIC, FIC, FICI, ethanol, antibiotics, medicinal plant

Introduction
The discovery of antibiotics is one of the novel parts in combating bacterial infections that once ravaged humankind. Different antibiotics exercise their inhibitory activity on different pathogenic organisms. The broad use of antibiotics in the treatment of microbial infections has led to the emergence and spread of resistant strains. Infections due to *Staphylococus aureus* are presently resistant to beta-lactams (Cook, 1998), while *Enterococcus* strains are resistant to vancomycin, ampicillin, gentamycin and streptomycin (Montecalvo *et al*., 1994).In recent days, clinically important microbial pathogens are not only characterized by single drug resistance and also by multidrug resistance. These drug resistance pathogens are difficult to treat and are accountable for a variety of infectious diseases. In other hand, the speed of development of new antimicrobial drugs has slowed down while the prevalence of resistance has grown at high rate. That is, the rate of emergence of antibiotic resistant bacteria is not matched by the rate of development of new antibiotics to combat them.
For the few years, there has been enormous scientific interest in pharmacological investigations of the biological properties of medicinal plants. Medicinal plants have been the source of many medications that are now applied in clinical practice. The use of extracts as antimicrobial agents shows allow risk of increasing resistance to their action, because they are complex mixtures, making microbial adaptability very difficult and it is also reported to have minimal side effects. *Gardenia latifolia* (Rubiaceae) is commonly known as Indian boxwood or Ceylon boxwood, is a densely foliaceous small tree that occurs throughout the greater parts of Indian common in deciduous forests along the streams. The stem bark and fruits are reported to be used in the treatment of various ailments such as snake bite, skin diseases, stomach pais, caries in humans and ephemeral fever in live stocks [7, 8, 9]. Fruits are used for making perfumes [10]. The purpose of the present work was to determine the antimicrobial activity of ethanol extract of *G.latifolia* and to investigate the synergistic effects of chosen plant combined with streptomycin against *Staphylococcus aureus*, thereby throwing light on the potential role of plants in increasing the effectiveness of antibiotics.

Materials and methods
Plant collection
The fresh aerial plant parts were collected from Kolli hills, Namakkal District, Tamil Nadu, India. The collected plant is identified by Botanical Survey of India (BSI/SRC/5/23/2013/Tech-795 & Serial No. 1), Coimbatore and the voucher specimens were deposited at the herbarium of Department of Botany, National College (Autonomous), Tiruchirappalli-1.

Preparation of extracts

Plant material

Fresh and health leaves were collected from Kolli hills, Tamilnadu, India. The leaves were washed thoroughly in distilled water and the surface water was removed by air drying under shade. The leaves were powdered with the help of mechanical blender and used for extraction.

Microorganism

The bacterial strain was obtained from Microbial Type Culture Collection, Chandigarh, India and the cultures were maintained on Nutrient agar until further studies.

Ethanol extract

Air dried powder of 10 g was placed in a conical flask containing 100 ml of ethanol plugged with cotton and then kept on a rotary shaker at 200 rpm for 24 hrs. Later, it was filtered through 8 layers of muslin cloth and centrifuged at 5000 rpm for 15 min. The supernatant was collected and the solvent was evaporated to make volume one fourth of its original volume.

Determination of Minimal Inhibitory Concentration (MIC) of Synergistic Mixture

Minimal concentration of synergistic mixture required to inhibit the growth of bacterial culture can be determined. To 5ml of Muller Hinton broth 100µl of antibiotic of various concentrations, plant extract of various concentrations and synergistic mixture of various concentrations were added in separate tubes. 20µl of 3 x 10^4 CFU of bacterial culture was inoculated in each tube respectively. A control was kept without any addition of plant extract or antibiotics. The tubes were kept for incubation at 37^0C for 24 hours. The absorbance was taken at 600nm before and after the incubation. The MIC is defined as the lowest concentration of antimicrobial agents in combination at which visible bacterial growth was inhibited.

Determination of Fractional Inhibitory Concentration Index (FICI)

The synergistic interactions were evaluated by Fraction Inhibitory Concentration Index (FICI). Various dilutions of antibiotic and plant extract concentration and synergistic mixture were prepared. 20 different combinations of synergistic mixture were tested against the target bacteria. To 5ml of Mueller-Hinton broth 100µl of each dilutions of extract and antibiotic were added. The broth was inoculated with 20µl of bacteria and incubated at 37^0C for 24h. Each test included growth control, and sterility control. In vitro interactions between antimicrobial agents were determined and quantified by calculating the fractional inhibitory concentration (FIC) index using the following formula

FICI = MIC of plant extract in combination/MIC of plant extract alone + MIC of antibiotic in combination/MIC of antibiotic alone

Time kill assay

The time kill assay of extracts in combination with antibiotics against microbial pathogens were performed in sterile microtitre plate containing 96 wells contained 0.04 ml Muller hinton broth (MHB). The combined tested agents (extract – antibiotic) were inoculated with 0.05 ml test organism to a density of 1 x 10^8 CFU/ml in a final volume f 0.1 ml. Then, the plates were incubated at 37ºC and viable count was calculated at 4 hr after addition of treatment agent. At each hour, 0.01 ml of the sample was drawn from the well to be serially diluted in normal saline (0.9% NaCl) solution prior to being plated in triplicates on MHA plate using sterile wire lop for determination of viable count. Total bacterial CFU/ml was counted after 24 hr of incubation at 37ºC. A well of inoculated MHB with antibiotic free serve as growth control. For each plate, a colony count of surviving bacteria between 30 to 300 were taken in to plot the time mortality curve with log 10 CFU/ml on the y-axis and time (hour) on the x-axis. The interaction was interpreted as synergistic if there is a decrease > 2 log10 CFU/ml in colony counts between the combinations and the most active single drug after 24 hr (Jacquline *et al*., 2003). Additive or indifference effect was described as a < 2 log10 CFU/ml reduction in viable count after 2 hr. for the combination, in comparison to the most active single agent (Aiyegoro *et al*., 2011). Antagonism was defined as an increase in the colony count of > 2 log10 CFU/ml by the combination compared to the count obtained by the most active single agent alone after 24 hr (Lee *et al*., 2006). The combination was defined as a bactericidal if it produced a > 3 log10 CFU/ml reduction in colony counts during incubation period denoting > 99.9% killing compared to the size of the starting inoculums (Jacqueline *et al*., 2005; Lee and Burgess, 2013) [8].

Interference

Synergistic - decrease > 2 $\log_{10}$ CFU/ml (2 hr incubation period & combination with active single drug)

Additive - < 2 $\log_{10}$ CFU/ml (2 hr incubation period & active single drug)

Antagonism - > 2 $\log_{10}$ CFU/ml (24 hr incubation period & combination with active single drug)

Results and Discussion

The increase of microbial resistance to antibiotics threatens public health on a global scale as it reduces the effectiveness of treatments and increases morbidity, mortality and health care costs. Evolution of highly resistant bacterial strains has compromised the use of newer generations of antibiotics. The antibacterial results, *S.aures* (MTCC) is taken for synergistic studies. The synergistic mixture used in study of antibacterial of antibiotics and *G.latifolia* leaf extracts is a combination of antibiotics at different concentrations (80 – 0.312 µg/ml & 10 -100mg/ml) and *G.latifolia* leaf extracts at concentration 20 mg/ml. Tables 1- 4 showed that there is an improvement in the antibacterial activity of the antibiotics when combined with the *G.latifolia* extract. The combination of streptomycin with ethanol extract of *G.latifolia* showed high antibacterial activity.

The minimal concentration of streptomycin required for inhibition of *Staphylococcus aureus* growth was 20µg/ml which is inferred from the table 1; whereas the usage of large quantity of antibiotic to treat pathogens may cause the evolution of new drug resistant microorganism. The minimal concentration of streptomycin required to inhibit the growth of *S.aureus* in combination with *G.latifolia* was 1.25 µg/ml. The concentration of streptomycin required was low when compared with the activity of antibiotic alone. The growth of *S.aureus* was not inhibited by *G.latifolia* at low concentration (10-30 mg/ml). The inhibition in growth of *S.aureus* was seen only at high concentration of plant extract. The minimal concentration required to inhibit the growth of *S.aureus* by *G.latifolia* extract was 80 mg/ml which is shown in table 1. From Table 3 shows that the minimal concentration of ethanol

leaf extract of *G.latifolia* required to inhibit the growth of *S.aureus* in combination with antibiotic was 1.25 µg/ ml.

FICI= MIC of plant extract in combination + MIC of antibiotic in combination/ MIC of plant extract alone MIC of antibiotic alone

MIC of *G.latifolia* extract alone = 80 mg/ml

MIC of streptomycin alone = 20 µg/ml

MIC of *G.latifolia* extract in combination with streptomycin = 30 µg/ml

MIC of streptomycin in combination = 1.25 µg/ml

Fraction Inhibitory Concentration Index: 0.4375

Whereas the FICI value was less than 0.5 which proves that the antibacterial activity was due to synergism between streptomycin and *G.latifolia* according to the Fractional inhibitory concentration index.

The synergy detected in this study was not particular to any group of organisms or class of antibiotics. This suggests that crude extracts of this plant could be containing a mixture of compounds that can improve the activity of different antibiotics. The leaves of *G.latifolia* have been known to contain a number of antimicrobial compounds (Iwu *et al*., 1999) [5] such as polyphenols and flavonoids. The antimicrobial and resistance modifying potentials of naturally occurring flavonoids and polyphenolic compounds have been reported in previous reports (Cushnie and Lamb, 2005; Sato *et al*., 2004) [3, 12]. This would propose that, the synergy with antibiotics observed in this study could be attributable to such compounds.

Some of these compounds like polyphenols have been shown to exert their antibacterial action in the course of membrane perturbations. This perturbation of the cell membrane coupled with the action of beta-lactams on the transpeptidation of the cell membrane could lead to an enhanced antimicrobial effect of the combination (Esimone *et al*., 2006) [4]. It has also been shown that some plant derivatives can improve the *in vitro* activity of some peptidoglycan inhibiting antibiotics by directly attacking the same site (i.e. peptidoglycan) in the cell wall (Zhao *et al*., 2001) [14]. Bacterial efflux pumps are responsible f or a significant level of resistance to antibiotics in pathogenic bacteria (Kumar and Schweizer, 2005) [7]. Some plant derived compounds have been observed to enhance the activity of antimicrobial compounds by inhibiting MDR efflux systems in bacteria (Tegos *et al*., 2002) [13]. 5'-methoxyhydnocarpin is an example of an inhibitor of the NorA efflux pump of *S. aureus* isolated from *Berberis fremontii* (Stermitz *et al*., 2000) [11, 13]. It is likely that the ethanol extract of *G.latifolia* could be containing potential efflux pump inhibitors. Such compounds are likely to be broad spectrum efflux inhibitors considering that the synergistic effect of the extract was observed on both gram positive and gram negative organisms as well as in combination with, cell wall inhibiting and protein synthesis inhibiting antibiotics.

Time Kill Assay

The time assay was carried out to determine the antibacterial activity of synergistic mixture of Streptomycin and *G.latifolia* at various time intervals. In increase of time period the antibacterial activity of the synergistic mixture is increased when compared to antibacterial activity of Streptomycin and *G.latifolia* alone as shown in table 5. As an alternative method, the time kill assay was also used to determine the potential of combinations of the crude extracts of leaves of *G.latifolia* and antibiotics. This method was based on a comparison of the killing rate of the combination to that of the individual agents. In the experiment, the extract was incorporated at sub-inhibitory concentrations (1/2× MIC) with the antibiotic at the minimum inhibitory concentration. In contrast to the checkerboard method, the time kill assay detected synergy against both gram positive and gram negative organisms. Strong synergistic interactions with the extract were observed in combinations involving beta-lactams (streptomycin) against *S.aureus*. Combinations involving streptomycin and plant extract against the same gram negative organisms were largely antagonistic. The synergism is a new concept in developing new drug molecules and treatment of drug resistant bacteria. Synergism concept can be used for drug discovery for antibacterial activity. New effective drug molecules can be produced by using the synergism.

Table 1: Minimal Inhibitory Concentration of streptomycin alone

Concentration (µg/ml)	OD at 600 nm (0 hr)	OD at 600 nm (24 hr)
80	0.046	0.041
40	0.018	0.034
20	0.019	0.029
10	0.021	0.021
5	0.029	0.038
2.5	0.035	0.098
1.25	0.030	0.145
0.625	0.020	0.246
0.312	0.018	0.125
Control	0.021	1.243

Table 2: MIC of streptomycin in combination with ethanol extract of *G.latifolia*

Concentration (µg/ml)	OD at 600 nm (0 hr)	OD at 600 nm (24 hr)
80	0.058	0.054
40	0.061	0.063
20	0.054	0.059
10	0.051	0.056
5	0.061	0.060
2.5	0.059	0.061
1.25	0.049	0.062
0.625	0.048	0.048
0.312	0.041	0.043
Control	0.004	0.982

Table 3: Minimal Inhibitory Concentration of ethanol extract of *G.latifolia* alone

Concentration (mg/ml)	OD at 600 nm (0 hr)	OD at 600 nm (24 hr)
10	0.028	0.621
20	0.029	0.630
30	0.034	0.618
40	0.037	0.591
50	0.039	0.567
60	0.042	0.495
70	0.046	0.368
80	0.047	0.047
90	0.049	0.050
100	0.053	0.051
Control	0.020	0.591

Table 4: MIC of streptomycin in combination with ethanol extract of *G.latifolia*

Concentration (µg/ml)	OD at 600 nm (0 hr)	OD at 600 nm (24 hr)
10	0.004	0.124
20	0.009	0.049
30	0.011	0.011
40	0.026	0.025
50	0.008	0.010
60	0.012	0.014
70	0.024	0.028
80	0.017	0.024
90	0.014	0.019
100	0.017	0.015
Control	0.004	1.283

Table 5: Changes in $\log_{10}$CFU/ml of individual bacterium over 4 h with different antibiotics alone and in combination with ethanolic extract of *G. latifolia*

Antibiotics & Plant extract	*S. aureus* (MTCC)
Streptomycin	1.8
Ethanol	3.21
Strp + Pl.ex	4.28

References

1. Aiyegoro O, Adewusi A, Oyedemi S, Akinpelu D, Okoh A. Interactions of antibiotics and methanolic crude extracts of *Afzelia africana* (Smith.) against drug resistance bacterial isolates. Int J Mol Sci. 2011; 12(7):4477-4487.
2. Chandra Prakash K. Aboriginal uses and management of ethnobotanical spices in deciduous forests of Chhattisgarh state in India. J Ethnobiol Ethnomed. 2009, 5-20.
3. Cushnie TPT, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agen, Dr. Due'sPhytchemical and Ethnbotanical Databases, 2005; 26(5):343-356.
4. Esimone CO, Iroha IR, Ibezim EC, Okeh CO, Okpana EM. *In vitro* evaluation of the interaction between tea extracts and penicillin G against *Staphylococcus aureus.* Afr J Biotech. 2006; 5(11):1082-1086.
5. Iwu MW, Duncan AR, Okunji CO. New antimicrobials of plant origin. Janick J (ed.), Perspectives on new crops and new uses. ASHS Press, Alexandria, VA., 1999; 457-462.
6. Jacqueline C, Caillon J, LeMabecque V, Miègeville AF, Donnio AY, Bugnon D, Potel G. *In vitro* activity of linezolid alone and in combination with gentamicin, vancomycin or rifampicin against methicillin *Staphylococcus aureus* by time–kill curve methods. J Antimicrob Chemothe. 2003; 51(4):857-864.
7. Kumar A, Schweizer HP. Bacterial resistance to antibiotics: Active efflux and reduced uptake. Ad Drug Delivery Rev. 2005; 57:1486-1513.
8. Lee GC, Burgess DS. Polymyxins and doripenem combination against KPC-producing *Klebsiella pneumoniae*. J Clin Med Res. 2013; 5(2):97-100.
9. Madava Chetty K, Sivaji K, Tulasi Ra K. Flowering Plants of Chittor District, First ed., Students Offset Printers, India. 2008, 57-59.
10. Reddy KN, Subbaraju GV, Reddy CS, Raju VS. Ethno veterinary medicine for trating live stck in Eastern Ghats of Andhra Pradesh. Ind J Trad Knowl. 2006; 53:68-372.
11. Stermitz FR, Lorenz P, Tawara JN, Zenewicz LA, Lewis K. Synergy in a medicinal plant: Antimicrobial action of berberine potentiated by 5'-methoxyhydnocarpin, a multidrug pump inhibitor. Appl Biol Sci. 2000; 97(4):1433-1437.
12. Sato Y, Shibata H, Arai T, Yamamoto A, Okimura Y, Arakaki N, Higuti T. Variation in synergistic activity by flavone and its related compounds on the increased susceptibility of various strains of methicillin-resistant *Staphylococcus aureus* to beta-lactam antibiotics. Int J Antimicrob Agen. 2004; 24(3):226-233.
13. Tegos G, Stermitz FR, Lomovskaya O, Lewis K. Multidrug Pump Inhibitors Uncover Remarkable Activity of Plant Antimicrobials. Antimicrob Agen Chemother. 2002; 46(10):3133-3141.
14. Zhao WH, Hu ZQ, Okubo S, Hara Y, Shimamura T. Mechanism of synergy between Epigallochatechin gallate and Lactams againstmethicillin resistant *Staphylococcus aureus*. Antimicrob Agen Chemother. 2001; 45(6):1737-1742.