

Antimicrobial activity of phenolic compounds isolated from *Caesalpinia pulcherrima*

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Abstract

Introduction

Multidrug resistance is an alarming issue worldwide. Herbal plants are being used as therapeutic agents since ages. One such plant is *Caesalpinia pulcherrima*, known for its beneficial effects. Therefore, this study was planned to isolate pure compounds and check its antimicrobial activity along with synergism.

Methodology

Antimicrobial susceptibility pattern of MDR clinical bacterial strains against phenolic compounds was performed by Agar well diffusion method. Minimum inhibitory concentration (MIC) was determined by tube dilution and micro broth dilution methods. Synergistic combinations of Methyl gallate with antibiotics were assessed by checkerboard titration method. Scanning Electron Microscopy was conducted to analyse the changes in cell morphology of MDR *E. coli* and Methicillin-Resistant *Staphylococcus aureus* after treatment with Methyl gallate

Results

Methyl gallate showed promising antimicrobial activity (250–500 µg/ml), whereas, Ethyl gallate, Syringic acid and Gallic acid trimethyl ether showed MIC (500–2500 µg/ml). Time kill kinetics assays of Methyl gallate showed bactericidal activity. Methyl gallate showed good synergistic combinations with Amoxicillin and Ciprofloxacin. Scanning electron microscopy of Methyl gallate depicted profound disruption in the cell wall, incomplete cell division and distorted structure of MDR bacteria.

Conclusions

Phenolic compounds were found to be a potential source of therapeutic substances as compared to the conventional antibiotics. Further studies are required to explore their mechanism of action.

Introduction

The discovery of antibiotics is one of the greatest achievements in the history of medical sciences. Antibiotics are not only involved in the treatment of infections but also for the assurance of successful surgical interventions [1]. Despite all the facts regarding their effectiveness, the evolution of resistance against antibiotics is a distressing issue. Natural resistance was found in some bacteria even before the invention of antibiotics. There are many factors associated with the emergence of resistance such as mutations in microbes, inappropriate use and over the counter sale of antibiotics. Herbal medicine has been in use in the treatment of various diseases since ages [2]. It has been reported that more than 80% of world's population depends on herbal medicine for various illnesses [3]. There is a rich history of utilization of herbal medication and transfer of knowledge from generation to generation. The WHO has been involved in collecting and documenting the benefits of herbal medicine from the natives of different cultural backgrounds [4]. Many studies have been and are conducted on plants to authenticate the claim of their use in herbal medicine. Health benefits of medicinal plants are due to the presence of secondary metabolites called phenolic compounds [5]. The benefits of these compounds are related to their consumption as food and their bioavailability [6]. *Caesalpinia pulcherrima* is an ornamental plant, inhabitant to Central America but also found in Africa, Asia and Australia [7]. Extracts from different parts of the plant have been used as anti-convulsant, anti-inflammatory and immuno-stimulating agents [8]. The stem was found to be useful as an abortifacient and antiulcer agent [9]. The idea of synergy between herbal drugs and antibiotics is a new approach to treating multidrug-resistant bacteria. Antimicrobial activity of extracts of different parts of this plant has been explored in our previous study [10]. After obtaining promising results, we planned this study and isolated pure compounds from fresh pods and performed antimicrobial activity against MDR (multi-drug resistant) bacteria alone and in combination with antimicrobials and also checked its effect on bacterial cell morphology.

Materials And Methods

Preparation of plant extracts and isolation of Pure Compounds

The methanol extract of pods (CP-Pods-M 292gms) was subjected to solvent separation which gave hexane (P.E), ethyl acetate (EA), butanol (But) and aqueous (Aq) phases separately. A small quantity (1.6 g) of EA phase (35.6g) was fractionated using different ratios of PE:EA mixture, which led to the isolation of a pure compound in one of the above fractions (PE:EA 1:1), identified as methyl gallate through spectral studies. Major quantity of EA phase (34.0 g) was subjected to VLC, which afforded 23 fractions. The fraction VLC-5 afforded Methyl-4-hydroxy benzoic acid, Fraction VLC-6 afforded Ethyl gallate, Fraction VLC- 7 and VLC-8 afforded methyl gallate in large quantity (1.4 % wet weight base), while fraction 9 was found to be a mixture of methyl gallate and gallic acid. Based on this work, phenolic compounds (Dodecyl gallate, Propylgallate, Vanillic acid, Syringic acid, Ethyl-4 hydroxy benzoic acid, Gallic acid trimethyl ether) obtained commercially were used in the present study.

Antimicrobial Activity of Plants and Phenolic compounds

Antimicrobial activity of phenolic compounds (Gallic acid, Methyl gallate, Ethyl gallate, Propyl gallate, Dodecyl gallate, Syringic acid, Vanillic acid, Para hydroxybenzoic acid, Methyl 4 hydroxy benzoic acid, Ethyl 4 hydroxy benzoic acid, Gallic acid trimethyl ether) was checked against MDR clinical bacterial isolates and ATCC reference strains. Antimicrobial susceptibility pattern of MDR clinical bacterial strains against phenolic compounds was assessed by Agar well diffusion method [11]. Minimum inhibitory concentration (MIC) was determined by tube dilution and micro broth dilution methods [12].

Agar Well Diffusion Method

Antimicrobial activity of phenolic compounds was tested against MDR clinical pathogen by agar well diffusion method. The stock solution of all plant extracts was prepared in sterile DMSO. The overnight grown culture was inoculated in Mueller Hinton Broth, incubated for 3–4 hours in aerobic conditions at 37°C to get a log phase culture. The turbidity of culture was matched with 0.5 Mac Farland index. Mueller Hinton Agar plates were seeded with bacterial culture. 20µl of extracts were added in the wells (diameter = 6mm) made by sterile borer and incubated for 24 hours at 37°C in aerobic conditions. Results were recorded by measuring zones of inhibition around wells. The experiment was run in triplicate on three different occasions

Minimum Inhibitory Concentration By Micro broth Dilution Method

Phenolic compounds as well as conventional antibiotics that lost efficacy were checked to determine their MIC. Two-fold serial dilutions of extracts, fractions, phenolic compounds and conventional antibiotics were prepared in Mueller Hinton broth in 96-well flat bottomed plates. 20 µl of inoculums 10⁶ CFU/ml were added in each well to keep a total volume at 200 µl. Micro titer plates were incubated at 37°C for 24 hours. Dilution with no turbidity was considered as MIC. The experiment was run in triplicate on three different occasions.

Minimum Inhibitory Concentration By Tube Dilution Method

MIC of phenolic compounds as well as conventional antibiotics was reconfirmed by tube dilution method. 1ml MH broth was added in sterile test tubes with inoculums (10⁶ CFU/ml). Tubes without extracts but containing MH broth and inoculum were designated as positive control, whereas negative control had extract and MH broth.

Minimum Bactericidal Activity:

MBC was determined by taking 100 µl of MHB from the wells and tubes containing plant extracts showing no apparent turbidity and subcultured on MHA plates. The lowest concentration without any growth was considered as MBC value [13].

Time Kill Kinetics Assay:

Time Kill Kinetics was performed to study the effect of pure Methyl gallate on the growth of MDR bacterial isolates [14]. MRSA (n = 10), MDR *E. coli* (n = 10) and control ATCC cultures of *S. aureus* and *E. coli* were selected for this study. 20 ml cation adjusted MHB with the absolute concentration of 0.5, 1 and 2× MIC of Methyl gallate. The log phase culture of bacteria with the inoculums 10⁵CFU/ml was transferred to the flask. The flask was incubated for 24 hours at 37°C. 100 µl was taken from the flask and plated on MHA at 2,4,6,8 and 24 hours time interval. The plates were further incubated for 24 hours at 37°C to check CFU/ml. The experiment was run in replicate on three different occasions.

Antimicrobial activity of Methyl gallate with Ciprofloxacin and Amoxycillin:

The emergence of multidrug resistance is on the rise; therefore, there is an urgent need for exploring alternative therapeutic regime, which may include a combination of plant-derived substances with antibiotics that have lost efficacy. In the present study, different combinations of Amoxycillin and Ciprofloxacin with Methyl gallate were studied by checkerboard titration method as demonstrated in earlier study [15]. The assay was performed in flat bottomed sterile 96 well-bottomed plate. Methyl gallate with its two-fold serial dilutions was performed in all rows. Dilutions of antibiotics were made in separate test tubes. Antibiotics with their dilutions were added to all columns with equal quantity. Each well of 96 well plate had same combinations of compound and antibiotics. Inoculum size was adjusted to 10⁶ CFU/ml in each well. The highest concentration of Ciprofloxacin and Amoxycillin was 650µg/ml and 325µg/ml, respectively. Moreover, the highest concentration of Methyl gallate was 500µg/ml. Fractional Inhibitory concentration index (FICI) was measured as the sum of MIC of agent with combination divided by MIC of agent alone. Results of FICI were evaluated as synergy = ≤ 0.5, additive effect = > 1 to 2, no effect or antagonism = > 2.

Effect of Methyl gallate isolated from C. pulcherrima on Morphology of Bacterial Cells by Scanning Electron Microscopy (SEM):

In order to study the effect of subinhibitory concentrations of Methyl gallate on bacterial cell morphology by SEM, MRSA and MDR, *E. coli* was grown in MH broth with 0.5 MIC of compound for 24 hours at 37 °C. After incubation, the cells were washed thrice at 10000 rpm with sterile deionised water and resuspended in PBS. A drop of culture was fixed with ethanol on 4mm glass slides. Negative staining was performed with 0.2% uranyl acetate for 30 seconds. Results were observed under GOEL JEM 1200 EX II Electron Microscop

Results

Methyl gallate:

Methyl gallate isolated from *C. pulcherrima* was found to be a potent antimicrobial substance against *S. typhi* ATCC 27853, MRSA, *S. aureus* ATCC 29213 MDR *E. coli* and

K. pneumoniae (MIC = 125 µg/ml). However, antimicrobial activities against remaining clinical bacterial isolates were in the range of 250–500 µg/ml as shown in Table 1A, 1B.

Phenolic Compounds:

Gallic acid exhibited lower MIC (500 µg/ml) against MRSA, *S. aureus* ATCC 29213,

S. typhi, *S. typhi* ATCC 27853, *K. pneumoniae*, *E. coli* and *E. coli* ATCC 25922. Ethyl gallate displayed MIC (500 µg/ml) against MDR *E. coli* and *E. coli* ATCC 25922. Syringic acid and Gallic acid trimethyl ether showed MIC (500 µg/ml) against *S. typhi* ATCC 27853. Dodecyl gallate, Propyl gallate, Vanillic acid,

Parahydroxy benzoic acid, Ethyl 4 hydroxy benzoic acid and Methyl 4 hydroxy benzoic acid failed to show antimicrobial activity against MDR clinical bacterial isolates as shown in Table 1A, 1B.

Effects of Methyl gallate on Growth Kinetics of MDR Bacteria:

Time kill kinetics of gram-positive bacteria MRSA and *S. aureus* ATCC 29213 and gram-negative bacteria MDR *E. coli* and *E. coli* ATCC 25922 was carried out to study the effect of Methyl gallate on their growth. Ten strains (n = 10) of each species were included in this study. MRSA and *S. aureus* ATCC 29213 showed a reduction of CFU within 2 hours and complete bactericidal activity was observed at 24 hours and 6 hours, respectively at MIC and 2×MIC. Moreover, at 0.5×MIC, bacterial count was reduced at 5 hours in MRSA. In case of *S. aureus* ATCC 29213, a steady decline in CFU till 6 hours and then an increase in bacterial count were noticed (Fig. 1,2).

Figure 3 shows concentration-dependent fall in CFU of *E. coli* ATCC 25922, less than the MIC showed lag phase for 4 hours and then started to fall between 4 to 6 hrs, and again multiplied. At MIC and 2×MIC, a drastic reduction in growth was recorded after 2 hours and complete bactericidal activity after 6 hours of post-incubation. Figure 4 shows a slight reduction in CFU of MDR *E. coli* was noticed in culture treated with 0.5 MIC to 5 hours. Culture grown in 2×MIC showed complete inhibition of growth after 8 hours. At MIC inhibition, bacterial growth was observed till 8 hours and cidal activity was noticed at 24 hours.

Figure #5 shows prominent structural changes in the cell wall of MDR *E. coli*. The surface of the cell was rough, disrupted with expulsion of cell contents. Arrow indicated the appearance of depression on the surface of the cell (C). Incomplete cell division and septum formation were also noticed as shown by arrows (D). There was a significant malformation, typical long rod shape converted into small rods and cocci. Morphological changes in MRSA after treatment with Methyl gallate are shown in Fig. 6. Overall, the size of cells was bigger with a distorted surface (C and D) as compared to untreated cells (A and B). Septum formation was as indicated by arrow leading to incomplete separation of daughter cells (D and E).

Antimicrobial Activity of New Synergistic Combinations of Methyl gallate:

In order to develop efficient therapeutic combinations against MDR bacterial isolates, Methyl gallate, a phenolic compound isolated from *C. pulcherrima*, in combination with commonly used conventional antibiotics was evaluated for its antimicrobial activity.

Synergistic Combinations of Ciprofloxacin with Methyl gallate:

When synergistic combinations of Methyl gallate and Ciprofloxacin were tested against MDR *E. coli*, there was a significant reduction of MICs of Ciprofloxacin and Methyl gallate from 512 µg/ml to 0.125 µg/ml and 500 µg/ml to 15.6 µg/ml, respectively. However, MICs of Ciprofloxacin were still higher than the breakpoint values. Average FICI (fractional inhibitory concentration index) was (0.137 ± 0.11) suggesting good synergism between the two combinations. MIC of Ciprofloxacin failed to reach its breakpoint, therefore, no zone of inhibition around Ciprofloxacin disc placed in MHA incorporated with (0.2 ×MIC) of Methyl gallate was observed (Table 2).

Synergistic Combinations of Amoxicillin with Methyl gallate:

A synergistic combination of Amoxicillin and Methyl gallate was observed against MRSA, with a remarkable fall in MICs of Amoxicillin and Methyl gallate from 256µg/ml to 0.0625µg/ml and 500 µg/ml to 31.25µg/ml, respectively. Sub-inhibitory concentration (0.2 ×MIC) of Methyl gallate was incorporated in MHA plate. 10mm zone of inhibition of Amoxicillin was noted and compared with control MHA plates. Average FICI (0.225 ± 0.162) exhibited potent synergistic antimicrobial combination (Table 3).

Discussion

Multi drug-resistant bacterial infections are responsible for greater portion of infectious disease burden worldwide. For the last twenty years, the emergence of antimicrobial resistance along with the adverse effects of antibiotics has directed the researchers for the exploration of new antimicrobial substances specifically from herbal extracts, which can be able to solve the above problems [1]. Moreover, the therapeutic potential of herbal plants has been studied and identified to use them as a source of new drugs. According to the guidelines of WHO in 1997, beneficial plants can be used as an alternative of drugs [2].

During the course of the study, different clinical samples were collected from patients suffering from a variety of infections. These samples were processed for isolation of MDR human pathogens, which were screened against different antibiotics in order to determine their antibiotic susceptibility pattern. Methyl gallate exhibited remarkable antimicrobial activity against both MDR gram-positive (MIC = 250 µg/ml) and gram-negative bacteria (MICs = 125–250µg/ml). According to one study, phytochemicals with MIC between 100–1000µg/ml are considered as potential antimicrobial agents [3]. Earlier reports stated the antimicrobial activity of Methyl gallate against MRSA with (MIC = 1.25mg/ml) [4, 5]. MRSA is one of the most important pathogens that accounts for respiratory tract, skin, and surgical site infections with high mortality and morbidity [6]. Previous studies indicated the antimicrobial activity of Methyl gallate against non-MDR *E. coli*, *S. typhi* and *Vibrio Cholerae* [7, 8]. Time kill kinetics study of Methyl gallate showed dose-dependent bactericidal activity in both gram-negative and gram-positive bacteria. Profound effects of Methyl gallate on cell morphology of MRSA and MDR *E. coli* were seen by Scanning Electron Microscopy. Disruption in cell wall, incomplete cell division and distorted structure suggested that the site of action of Methyl gallate was cell wall.

Gallic acid showed antimicrobial activity (MIC = 500–1000µg/ml) in the current study, whereas, previous study endorsed our results [9]. Antimicrobial activity of Gallic acid was observed in one study with (MIC = 100µg/ml, 1100µg/ml, 1250µg/ml) against non- MDR *E. coli*, *P. aeruginosa* and *S. aureus*, respectively [10]. Moreover, Ethyl gallate showed antibacterial activity against MDR *E. coli* and *E. coli* ATCC 25922 (MIC = 500µg/ml). Another study supports the findings of our research work [5].

It was reported that the antimicrobial activity of alkyl esters of GA depends on specific lipophilic characteristics, assuming that cytoplasmic membrane could be a possible site of action. There are various modes of actions regarding the antimicrobial activities of phenolic compounds including interaction with including sulfhydryl group, change in permeability of cytoplasmic membrane, inactivation of enzymes, insoluble complexes with amino acids and proteins.

One more study explained the antibacterial activity of Ethyl gallate against *P. aeruginosa* ATCC 27853 [10]. However, Dodecyl gallate and Propyl gallate did not show antimicrobial activity against MDR bacteria in the present study but another study reported good antimicrobial activity of both compounds against MRSA [5]. Propyl gallate performed antibacterial activity against non-MDR *E. coli*, *P. aeruginosa* and *S. typhi* [11]. Gram-negative bacteria have specialized cell wall structure, therefore, they are difficult to kill as compared to gram-positive bacteria. The diffusion of antibiotic and active compounds is obstructed due to the presence of periplasmic space and murein layer. Mutation in active efflux pumps out anti-bacterial agent through the efflux pumps, which further promotes the development of intrinsic resistance for gram-negative bacteria [12]. Chemical structure including saturated chain length position and number in the benzene ring of phenolic compounds plays a key role in determining their anti-microbial activity. Phenolic acids had lower antimicrobial activity compared with their butyl and methyl ester [13]. The antimicrobial effect increased with increasing length of the alkyl chain [14].

Hydroxybenzoic and hydroxycinnamic acids occurring in plants exhibit diversity with respect to the number of hydroxyl or methoxy groups. Alkyl gallates disrupt the respiratory mechanism of bacteria by acting as pro-oxidants, which produce free radicles. These ROS cause oxidation of unsaturated fatty acids in the cell membrane, leading to disruption of its structure and function [15]. However, current knowledge on structure–function relationships of the antimicrobial activity of phenolic acids does not account for this diversity of compounds. Para hydroxy benzoic acid was found to be a potent antimicrobial agent against MRSA but the present study exhibited no antimicrobial activity of Parahydroxy benzoic acid and its ethyl and methyl esters against MDR gram-positive and gram-negative bacteria [5]. Methyl 4 hydroxy benzoic acid showed weak antimicrobial activity against non-MDR *P. aeruginosa*, *E. coli* and *S. aureus* [16]. Similarly, Vanillic acid exhibited no antimicrobial activity in the present study. Earlier reports displayed antimicrobial activity against MRSA, MDR *E. coli* and *P. mirabilis*. Antimicrobial activity of Trimethyl gallic acid against non-MDR gram-positive and gram-negative bacteria was reported (350). The previous study explained antimicrobial activity of Syringic acid against MRSA, MDR *E. coli* and *P. mirabilis*, which supports the results of the present study [17].

The absence of antibacterial activity does not mean that there is no potential of inhibiting microbes. It may be possible that the active compound was not in sufficient quantity or diluted with other non-antimicrobial compounds. Sometimes seasonal variations, geographical area, age of plant and method of extraction also influence the activity of the compound [18]. Extraction techniques are usually based on the chemical nature of compounds and their solubility in polar and non-polar solvents [19].

The approach of using antimicrobial substances that suppress the mechanism of antimicrobial resistance, hence increasing the efficiency of antimicrobial product, is known as synergism [20]. Synergistic combinations of different drugs or with herbal formulations play an important role in treating antibiotic resistance. Alkyl gallates are documented to possess resistance modifying action on various antibiotics against MRSA [21]. Therefore, we used different combinations of antibiotics with Methyl gallate. Our study exhibited strong synergistic combinations of Ciprofloxacin and Amoxycillin with Methyl gallate against MDR *E. coli* and MRSA, respectively. An earlier study explained synergistic combination of Ciprofloxacin and Methyl gallate against non-MDR *S. typhi* [22]. There are different mechanisms involved in the augmentation of the antibacterial activity of antibiotics by natural compounds such as interaction on multiple target sites in bacteria; bioavailability and solubility of antibiotics or aimed for a specific bacterial resistance mechanism [23]. These interactions prevent the activity of drugs from enzyme degradation and alterations in transport proteins, thus bypassing the mechanism of antimicrobial resistance [24]. Amoxycillin is a β -lactam antibiotic that inhibits bacterial cell wall synthesis by binding one or more of the penicillin-binding proteins (PBPs). Stimulation of *mecA* gene and gene variants resulted in the formation of PBP2a, which attaches to lower affinity with antibiotics and leads to drug resistance [25]. We assume that methyl gallate interferes with the synthesis of PBP2a in cell wall of bacteria. This could be the explanation of synergistic combinations in the present study.

Conclusions

Our study suggested that phenolic compounds are potential sources of therapeutic substances as compared with the conventional antibiotics that have failed to treat multi drug-resistant infections. Synergistic combinations of Methyl gallate with Ciprofloxacin and Amoxycillin against MDR *E. coli* and MRSA have been found to be effective against MDR bacteria but detailed mode of action and toxicity profiles are needed to be explored.

Declarations

Ethical Declaration:

These studies were conducted after receiving an approval from independent Ethics Committee, ICCBS, UoK No: ICCBS/IEC-008-BC-2015/Protocol/1.0.

Consent for publication:

Not applicable

Availability of data and material:

The datasets generated and/or analysed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Declaration of Interests:

All authors have no conflict of interest.

Funding:

None

Authors's contribution:

FZK has designed plan of work and writing, MY, SJ and AH helped in Conduction of work, SF, SJ and AH performed final editing and statistical analysis and Shahana Urooj Kazmi performed Proof reading. All authors read and approved the final manuscript.

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Tables

Table 1A: Antimicrobial Activity of Phenolic Compounds

S.No	Compounds	MRSA		VRSA		<i>S. aureus</i> ATCC 29213		<i>E. coli</i>		<i>E. coli</i> ATCC 25922		<i>K. pneumoniae</i>	
		MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
1	Gallic acid	500	1000	1000	2000	500	1000	500	1000	500	1000	500	1000
2	Methyl gallate	125	250	250	500	125	500	125	250	250	250	125	125
3	Ethyl gallate	1000	2000	1000	2000	1000	>2000	500	1000	500	1000	1000	2000
4	Dodecyl gallate	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
5	Propyl gallate	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
6	Syringic acid	1000	2000	1000	2000	1500	3000	750	750	1000	1000	1000	2000
7	Vanillic acid	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
8	Parahydroxy benzoic acid	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
9	Ethyl 4 hydroxy benzoic acid	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
10	Methyl 4 hydroxy benzoic acid	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
11	Gallic acid tri methyl ether	1000	2000	1000	2000	1000	1000	500	1000	1000	2000	2000	>4000

Table 1B: Antimicrobial Activity of Phenolic Compounds

S.No	Compounds	<i>S. typhi</i>		<i>S. typhi</i> ATCC 27853		<i>P. aeruginosa</i>		<i>Acinetobacter spp</i>		<i>P. mirabilis</i>	
		MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
1	Gallic acid	500	1000	500	1000	1000	1000	1000	>2000	1000	>2000
2	Methyl gallate	250	500	125	250	250	500	250	250	250	>500
3	Ethyl gallate	1000	1000	1000	2000	1000	2000	2500	>5000	2500	5000
4	Dodecyl gallate	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
5	Propyl gallate	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
6	Syringic acid	750	1500	500	1000	1000	1000	2000	2000	2000	2000
7	Vanillic acid	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
8	Parahydroxy benzoic acid	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
9	Ethyl 4 hydroxy benzoic acid	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
10	Methyl 4 hydroxy benzoic acid	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
11	Gallic acid tri methyl ether	1000	2000	500	1000	1000	2000	2500	2500	2000	>5000

Table 2: Synergistic Combinations of Ciprofloxacin with Methyl gallate against MDR *E. coli*

MDR <i>E. coli</i> strains	MICs of Ciprofloxacin		MICs of Methyl gallate		Fractional Inhibitory Concentration Index	
	µg/ml		µg/ml		(FICI)	Average FICI
	In combination	Alone	In combination	Alone		
1	0.125	512	15.6	125	0.125	
2	0.5	512	15.6	125	0.125	
3	0.125	256	31.25	500	0.062	0.137±0.11
4	0.125	512	62.5	250	0.250	
5	0.5	256	31.25	125	0.251	

Table 3: Synergistic Combinations of Amoxicillin with Methyl gallate against MRSA

MRSA Strains	MICs of Amoxicillin		MICs of Methyl gallate		Fractional Inhibitory Concentration Index	
	µg/ml		µg/ml		(FICI)	Average FICI
	In combination	Alone	In combination	Alone		
1	0.125	256	31.25	250	0.125	
2	0.0625	256	31.25	250	0.125	0.225±0.162
3	0.125	128	125	500	0.250	
4	0.125	128	125	250	0.500	
5	0.0625	256	31.25	250	0.125	

Figures

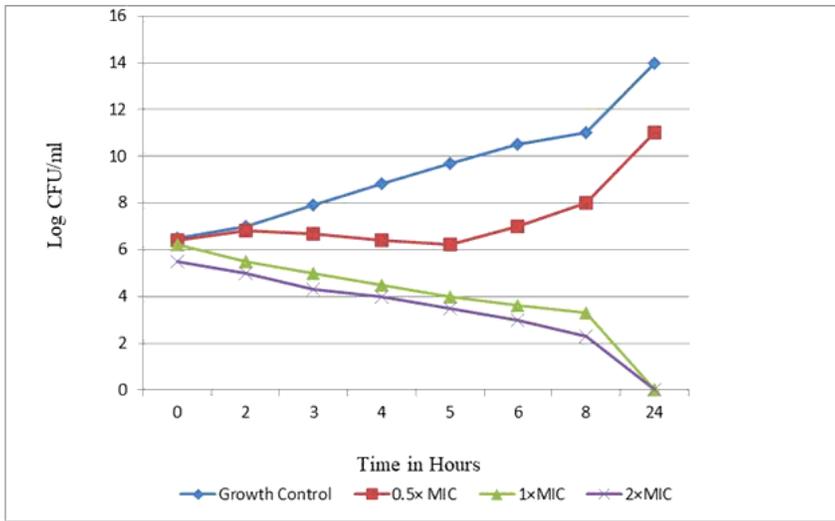


Figure 1

Effects of Methyl gallate on Growth Kinetics of MRSA Growth Kinetics Curve of Methicillin Resistant Staphylococcus aureus (MRSA) with three different concentration of Methyl gallate. Growth control without Methyl gallate was also included for comparison. Decreases in CFU at $2 \times \text{Log}_{10}$ was taken as significant. Results are interpreted as an average and standard deviation of three different sets of experiments. $P < 0.05$ was calculated by one-way ANOVA.

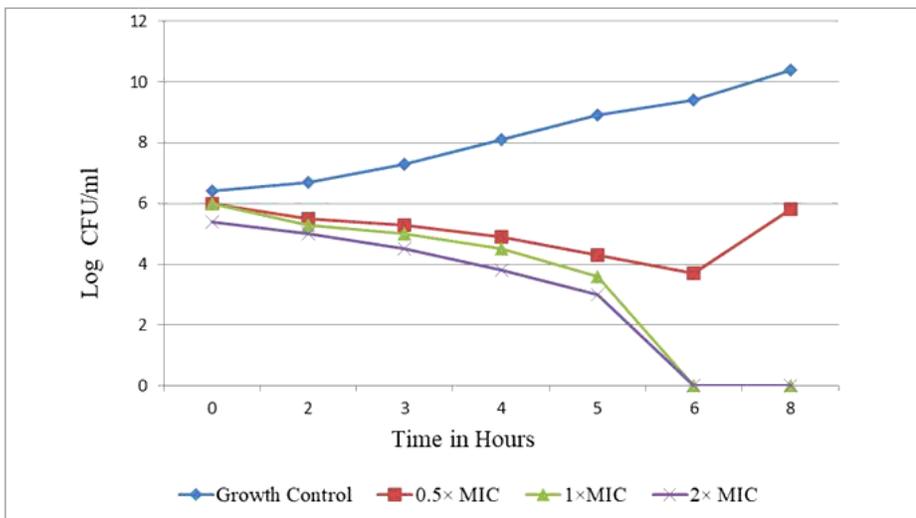


Figure 2

Effects of Methyl gallate on Growth Kinetics of *S. aureus* ATCC 29213 Growth Kinetics Curve of Staphylococcus aureus ATCC 29213 with three different concentration of Methyl gallate. Growth control without Methyl gallate was also included for comparison. Decreases in CFU at $2 \times \text{Log}_{10}$ was taken as significant. Results are interpreted as an average and standard deviation of three different sets of experiments. $P < 0.05$ was calculated by one-way ANOVA.

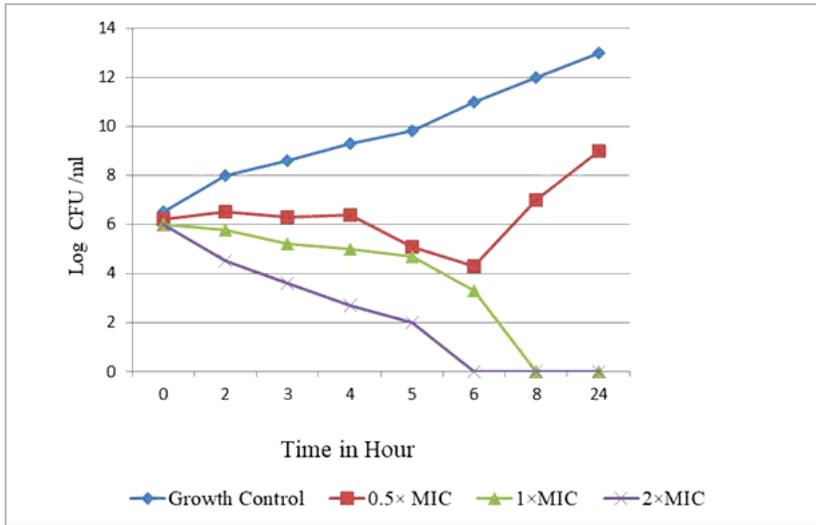


Figure 3
 Effects of Methyl gallate on Growth Kinetics of E. coli ATCC 25922 Growth Kinetics Curve of E. coli ATCC 25922 with three different concentration of Methyl gallate. Growth control without Methyl gallate was also included for comparison. Decreases in CFU at 2xLog10 was taken as significant. Results are interpreted as an average and standard deviation of three different sets of experiments. P<0.05 was calculated by one-way ANOVA

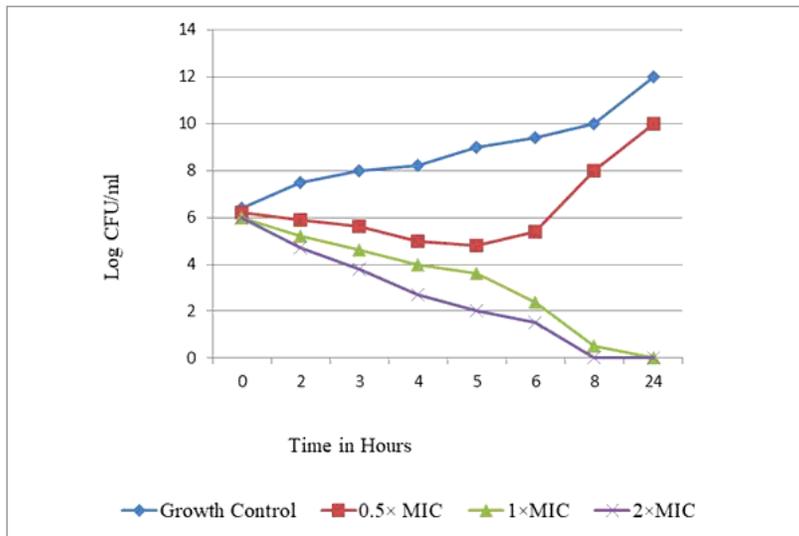
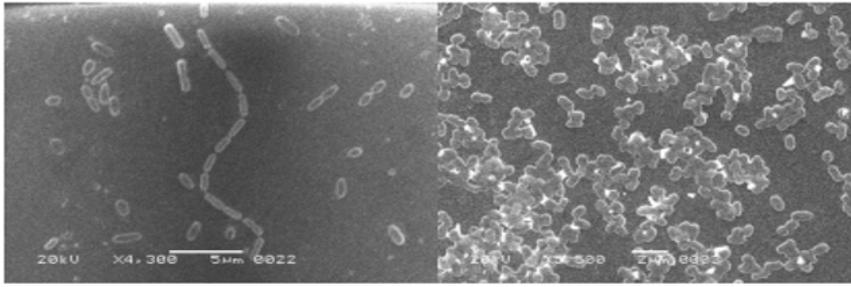
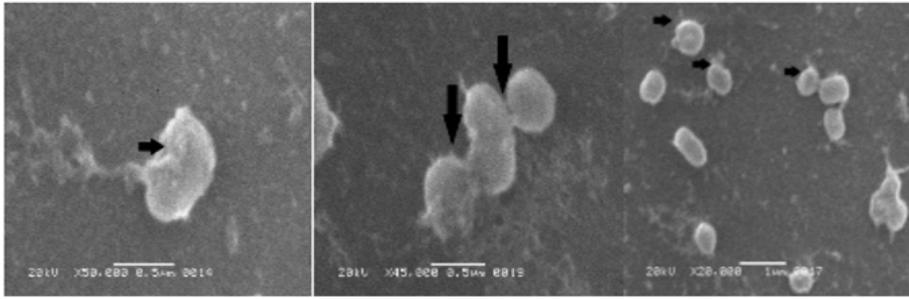


Figure 4
 Effects of Methyl gallate on Growth Kinetics of MDR E. coli Growth Kinetics Curve of MDR E. coli with three different concentration of Methyl gallate. Growth control without Methyl gallate was also included for comparison. Decreases in CFU at 2xLog10 was taken as significant. Results are interpreted as an average and standard deviation of three different sets of experiments. P<0.05 was calculated by one-way ANOVA



A

B

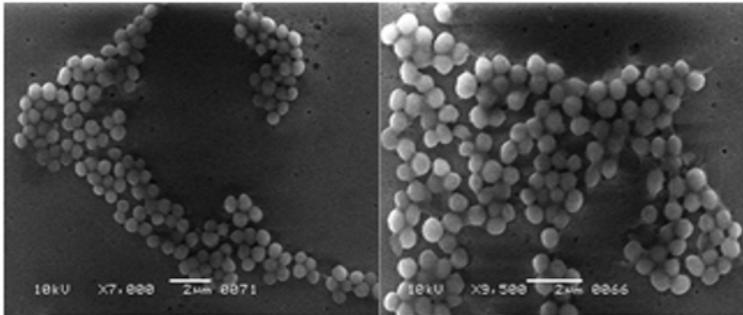


C

D

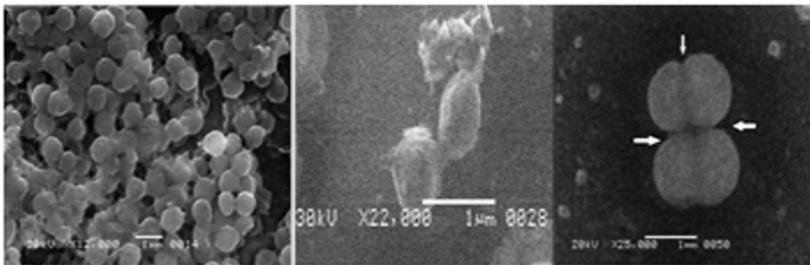
E

Figure 5
 Effect of Methyl gallate on Cell Morphology of MDR E. coli Scanning Electron Microscopy was performed to analyse change in cell morphology of MDR E. coli after treatment with Methyl gallate. A and B (control cells without Methyl gallate). C, D and E showed changes in cell morphology after incubating 24 hours at 37°C with subinhibitory concentration of Methyl gallate.



A

B



C

D

E

Figure 6

Effect of Methyl gallate on Cell Morphology of MRSA Scanning Electron Microscopy was performed to analyse change in cell morphology of Methicillin Resistant Staphylococcus aureus after treatment with Methyl gallate. A and B (control cells without Methyl gallate.). C, D, E and F showed changes in cell morphology after incubating 24 hours at 37°C with subinhibitory concentration of Methyl gallate