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Original Research

Synergistic antibacterial effect of apigenin with β-lactam antibiotics and modulation of bacterial resistance by a possible membrane effect against methicillin resistant *Staphylococcus aureus*

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Abstract: Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are easily spread among infected patients, where resistance has dramatically increased resulted in serious health issues. Therefore, there is a need to develop alternative natural or combination drug therapies. Apigenin (AP) is a natural poly phenolic flavonoid has been found to possess many beneficial biological actions. The aim of this study was to investigate the anti-MRSA efficacy and synergistic effect of apigenin (AP) and in combination with ampicillin (AM) and ceftriaxone (CEF). The antibacterial activity of apigenin was assessed by the broth macro dilution, checkerboard micro dilution method and time-kill assay. The mode of action was studied by outer and inner membrane permeabilisation assays, scanning electron microscopy and transmission electron microscopy. The minimum inhibitory concentration (MIC) of apigenin against gram positive and gram negative strain ranged from 32.5 to 62.5µg/ml. In checkerboard method apigenin markedly reduced the MIC of the antibiotics ampicillin 800 µg/ml shifted to 107 µg/ml (AM+AP) and ceftriaxone 58 µg/ml shifted to 2.6 µg/ml (CEF+AP) against MRSA. The synergistic activity of ampicillin and ceftriaxone plus apigenin combinations with FIC indices (CI) between 0.18-0.47. The modulation of methicillin-resistance by apigenin significantly enhanced the activities of ampicillin and ceftriaxone. The result of time-kill assays of the two drug combinations AM +AP and CEF+AP against MRSA showed significant inhibitory effect and reduced the colony count by approximately 99% after 8 h The results for outer membrane (OM) and inner membrane (IM) permeabilization showed that ampicillin and ceftriaxone in combination with apigenin damaged MRSA cytoplasmic membrane and caused subsequent leakage of intracellular constituents. Electron microscopy clearly showed that the above said combination also caused marked morphological damage of cell wall, cell shape and plasma membrane of this strain. From these results, it can be concluded that apigenin has the synergistic effect with ampicillin and ceftriaxone to reverse bacterial resistance against MRSA.

Key words: MRSA, Microbial resistance, Synergistic activity, Apigenin, Ampicillin, Ceftriaxone, Inner membrane permeability and Outer membrane permeability assay.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major pathogen causing nosocomial infection, commonly found in nature as well as in normal skin, the nasal cavity, and oral cavity, which remains a frequent cause of morbidity and mortality in hospital-acquired infections (1). MRSA causes bloodstream infections, pneumonia, skin infections and wound suppuration that eventually lead to sepsis and fatalities. Clinical survey from the U.S Center for Disease Control and Prevention, were reported severe MRSA infection cases 80,461 annually with 11,285 deaths per year in United States (CDC 2013) the threat report by in Malaysian hospitals. Infection cases reported between the year 2002 and 2007 were showing an increasing trend with annual MRSA prevalence over 40% (2-4). In general, MRSA is sensitive to antibiotics, however, recent reports in the food industry have suggested an increasing resistance to antibiotics in MRSA, *Salmonella* and *Escherichia coli* bacterium. The antibiotic-resistant bacteria have developed in the past decades leading to various difficulties in treating infectious diseases. Many no of antibiotics were used in the management of *S.aureus* infections specifically β-lactam antibiotics penicillin and ampicillin but rapidly developing resistant throughout continuous therapy. This is due to the β-lactamase enzyme inactivates β-lactam ring of penicillin and ampicillin structure and also due to the presence of mec A gene (5-9). In that harm situation, recent emergence of multi drug resistant (MDR) strains threatens the susceptible humans throughout the world; therefore researchers continue to search for novel antibacterial compounds in various ways. Many plant extracts and spices are reported to possess phenolic phytochemicals such as phenolic acids, ellagic acid, flavonoids, and proanthocyanidins. These compounds showed positive health benefits, especially antimicrobial potential, against diverse microorganisms. Recently, many scientific data have suggested that these polyphenolic compounds in combination with antibiotics have antibacterial action. Plant-derived compounds are potential sources for combinations. Flavonoids are known to be

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one of the sources of beneficial compounds for many infectious diseases, including antibacterial agents. These compounds in combination with antibiotics have been reported to have antibacterial action against bacterial resistance (5-9).

Therefore, novel flavonoids or new group of phyto pharmaceutical approaches that show synergistic effect with antibacterial agents. In this study, we have investigated the *in vitro* activity of naturally occurring plant flavone Apigenin (AP) chemically it is 4, 5, 7,-trihydroxyflavone, which is richly present in common fruits such as grapefruit, plant-derived beverages, medicinal plants and vegetables. AP has been shown to possess remarkable anti-bacterial, anti-inflammatory, anti-oxidant and anti-carcinogenic properties (10). However, no studies have reported any synergistic effects of Apigenin (AP) with Ampicillin (AM) and Ceftriaxone (CEF) and the reputed mechanism of antibacterial effects of AP against MRSA. Therefore, our study was focused on the evaluation of *in vitro* antibacterial activity of phenolic flavonoid AP alone and in combination with antibiotic AM and CEF determined by means of combination index (CI) value.

Materials and Methods

Preparation of chemicals and media

Ampicillin(AM), ciprofloxacin (CP), ceftriaxone (CEF), apigenin(AP) and crystal violet were obtained from sigma aldrich, Germani stock solution were prepared with suitable solvents at the required concentration. Nutrient broth (NB), Nutrient agar (NA), Muller Hinton broth (MHB), Muller Hinton agar (MHA), Brain heart infusion broth (BHI), Brain heart infusion agar (BHA) were obtained from himedia laboratories, Mumbai.

Bacterial strains

Staphylococcus aureus NCIM 2079, *Klebsiella pneumonia* NCIM 2719*, Enterobacter cloacae* NCIM 2164 were obtained from National collection of industrial microorganisms (NCIM), Pune, MRSA*,* clinical strains *Enterococcus faecalis* and *Staphylococcus aureus* were obtained from KAP Viswanathan medical college, Tiruchirappalli. All microbial strains were stored in refrigerator and frozen at −70°C before use. The stored bacterial strains were suspended in MHB and incubated at 37°C for 24 h (11).

Determination of minimum inhibitory concentration for apigenin and selected antibiotics

The selected test agents initially underwent efficacy determination specifically minimum inhibitory concentration against different strains. Our study specifically focused on MRSA, moreover gram positive and gram negative organisms were also included to determine the efficacy of apigenin against other strains too. Twenty four hours culture of *S. aureus* NCIM 2079, *K. pneumonia* NCIM 2719& *E. cloacae* NCIM 2164 and clinical strains *E. faecalis* & clinical strain MRSA were taken to analyse the inhibitory effect of the antibiotics AM & CEF and AP. Different concentrations of 1000 µg/ml stock solution of antibiotics and AP were prepared. The prepared stock solutions were used for MIC determination as per the standard procedure in tube dilution method (11). All the tubes containing test agents were incubated for 24 hours at 37°C. The test tubes were then observed for turbidity with a naked eye and the impact was calibrated by determining optical density by measuring absorbance at 600 nm in a UV-visible spectrophotometer (UV -Thermo Scientific BIOMATE 35). The lowest concentration of drugs in a tube or plate that failed to show any visible macroscopic growth was considered as its MIC. The experiments were performed in triplicates for each bacterial species and were repeated wherever necessary (11).

Checker board method- Combination index determination for AM,CEF, AP, AM+AP and CEF+AP against MRSA

According to the results obtained from MIC the interactions of two drugs AM or CEF with AP (AM+AP and CEF+AP) against MRSA was determined, the most frequently used procedure is the checker-board method. In this technique, aliquots of log-phase bacterial cultures (0.5 McFarland standard) are transferred to microtiter plates containing different concentrations of drugs- AM at 12.5 to 800 µg/mL; CEF at 0.125 to 32 µg/mL; AP at 1 to 256 µg/mL, and in the ratio of 1: 25 combination of AM+AP, 0.48 – 15.36 µg/mL AP with 12 to 384 µg/mL of AM, and in ratio of 1:3 combination of CEF+AP 0.125 to 16 µg/mL CEF with 0.375 to 48 µg/mL AP. Synergistic combination index was calculated in isobologram software. The two combination test samples; AM with AP and CEF with AP were graphically represented by dose effect curve, combination index value, dose reduction index and isobole method which was interpreted in isobologram software (10, 12,13,14). Preparation of micro plates –

The petriplates were prepared and inoculated on the same day, as certain drugs tend to inactivate each other if stored together.

i. Microplate organization:

- column 1 is antibiotics AM or CEF alone
- column 2 – 7 contain combinations
- column 8 is organism control
- column 9 is medium control
- row 1 is AP alone

ii. Dilution of antibiotic and drugs

The standard concentration of antibiotics AM, CEF and AP were thawed after storage. Micro wells were dispensed with 100 µl of MHB, then carry out required dilutions with 50 µl of stock solution of antibiotics AM or CEF and AP.

iii. Inoculation

An overnight culture of MRSA was diluted in MHB to achieve OD based inoculum of $1x\ 10^5 – 5\ x\ 10^5$. Then all the wells were inoculated (except column 12 which is considered as blank) with 50µl of the diluted organism (the volume in each well should be 200 µl).

The combination index is defined as synergy when the CI index ranges ≤ 0.5, indifference is defined when CI index is > 0.5, but when the CI index value is ≤ 1 – 4.0 it is defined as antagonism (10, 12, 13). The growth inhibition was measured by determining the absorbance at 600 nm in a multi-mode microplate reader (Enspire,

Perkin Elmer, USA). From these readings, combinations index values were determined and the synergistic, additive interactions were calculated.
Dose effect was calculated from the following formula,

$$\frac{\text{Absorbance of positive control (without drug) - Absorbance of test solution}}{\text{Absorbance of positive control}}$$

The percentage inhibition of each drug and its combination was obtained from dose effect value multiplied with 100. The combination index value was determined from the following formula

$$CI = \frac{\text{MIC of substance A in combination}}{\text{MIC of substance A alone}} + \frac{\text{MIC of substance B in combination}}{\text{MIC of substance B alone}}$$

Where CI – Combination index; Substance A denotes MIC of AM or CEF; substance B denotes MIC of AP.

Calculation of the median-effect concentration (IC_{50}) or the median –effect dose (ED_{50}) is a usual method in medical sciences . The old arbitrary method was to draw the empirical best-fit curve and then predict IC_{50} or ED_{50} value from the empirical curve. The goodness of fit to the straight line can be determined by the linear correlation coefficient (r value) calculated from x and y intercept which represent y = ax+b.
A plot x= log (D) Vs y=log(fa/fu).
fa + fu = 1, fu = 1-fa where fa-fraction affected, fu-fraction unaffected
Dose reduction index (DRI) was calculated from the following equation where $(Dx)_1$ is the dose of drug 1 alone that inhibits x% .
DRI can be really obtained from the reciprocal of each term of the CI equation thus,
$(DRI)_1=(D_x)_1/(D)_1$
$(DRI)_2=(D_x)_2/(D)_2$
DRI >1 and <1 indicate favorable and not favorable dose reduction; DRI=1 indicates no dose reduction.

Time kill analysis for apigenin with ampicillin and ceftriaxone

The rate of antibacterial action and synergistic effect of AM + AP and CEF + AP were determined by using time kill assay. The bacterial culture was inoculated in BHI and incubated at 37°C for 24 h after which the culture was diluted with saline solution at a concentration of $1x10^6$cfu/ml. The test samples AM, CEF, AP, AM + AP and CEF + AP were added to the bacterial suspensions at different concentrations and then incubated at 37°C. The final concentration was $5x10^5$cfu/ml. DMSO was used as a negative control. 0.1 ml of the incubated culture was removed at time interval of two hours ranging from 0 – 18h from the time of incubation and was subjected to 10-fold dilution using normal saline. Then 20µl of each dilution was cultured on MHA and the number of viable colonies was calculated at 18 h incubation. Colony counts were performed on plates and 30 – 300 colonies were enumerated. The antimicrobial agents used were considered bactericidal at the lowest concentration that reduced the original inoculum by 3 log10 CFU/ml (99.9%) for each of the indicated times. On the other hand, they were considered bacteriostatic if the inoculum was reduced by 0–3 log10 CFU/ml. The antibacterial synergistic effect was considered as when the CFU/ml is reduced to >2log 10 (15).

Outer membrane permeabilisation assay-crystal Violet assay

The mode of antibacterial action of AM, CEF, AP, AM + AP and CEF + AP were determined by studying the alteration of membrane permeability by the crystal violet assay (10). To the one day old MRSA suspension at 0.5 McFarland standard, the test agents were added at a concentration as follows, individual test agents: 800µg/ml of AM, 35µg/ml of CEF, 60µg/ml of AP and combinations, 100µg/ml of AM with 5µg/ml of AP, 3µg/ml of CEF with 8µg/ml of AP. Control samples were prepared similarly without treatment (10,13). The cells were harvested at 9300 × g for 5 min after which they were resuspended in PBS containing 10µg/ml of crystal violet. The cell suspension was then incubated for 10 min at 37°C. The suspension was then centrifuged at 13,400 × g for 15 min and the OD at 590nm of the supernatant was measured in UV–VIS spectrophotometer (UV-Thermo Scientific BIOMATE 35). The OD value of the crystal violet solution was taken and it was considered as 100%. The percentage of crystal violet uptake of all the samples was calculated using the following formula:

$$= \frac{\text{OD value of the sample}}{\text{OD value of crystal violet solution}} \times 100$$

Inner-membrane permeabilization assay-UV at 260nm

The UV-absorbing material releases in the microbial cells' were calculated by UV–Visible spectrophotometer. To the one day old MRSA suspension at 0.5 McFarland standard the test agents were added at a concentration as follows, individual test agents: 800µg/ml of AM, 35µg/ml of CEF, 60µg/ml of AP and under combinations, 100 µg/ml of AM with 5 µg/ml of AP, 3 µg/ml of CEF with 8 µg/ml of AP. Control samples were prepared similarly without treatment (10,13). All the samples were incubated at 37°C for 60 min. After treatment, the cell suspension was centrifuged at 13,400 × g for 15 min and the OD260 value of the supernatant was taken as a percentage of the extracellular UV-absorbing materials released by cells. All the measurements were done in triplicate in UV–VIS spectrophotometer (UV-Thermo Scientific BIOMATE 35) (10,13).

Cell wall damage analysis by SEM

SEM observation was carried out on MRSA cells. MRSA cell suspension at the concentration of 0.5 McFarland standard was inoculated on a MHA plate and incubated at 37°C for 12 h. The AM+AP and CEF+AP at the required concentration was added onto the inoculated agar and further incubated for another 36 h at the same temperature. The untreated culture was used as a control. A small block of MRSA containing agar was withdrawn from the inoculated plate at 0 and 36 h, coated with gold, and viewed under SEM. The SEM study was done under the following analytical conditions: L = SE1, WD = 21 mm, and EHT = 10.0 kV (Vega 3, Tescan, USA edx: Bruker Nano, Germany) (23).

Transmission electron microscopy (TEM)

MRSA subculture was prepared to examine the cell wall by TEM FEI, Tecnai G2,T12, Germany and followed the standard protocol (10). The combination of

Table 1. Anti bacterial activity of plant extracts against selected strains determined by MIC.

S.No	Name of the bacterial strains	MIC of Antibiotics and AP in µg/ml		
		AM	CEF	AP
1	*S. aureus* NCIM 2079 (µg/ml)	500	15.6	31.25
2	*Klebsiella pneumoniae* NCIM 2719(µg/ml)	125	7.8	31.25
3	*Enterobactor clocea* NCIM 2164(µg/ml	125	15.6	31.25
4	Clinical strain *Enterococcus faecalis*(µg/ml)	125	15.6	31.25
5	MRSA clinical strain(µg/ml)	1000	62.5	62.5

All the values represented as triplicates.

AM+AP and CEF+AP that dramatically decreased the MIC when added to the MRSA culture was chosen for electron microscopy study.

Results

The MIC of AM, CEF and AP exhibited broad spectrum of antibacterial activity against both gram positive and gram negative organisms against clinical and reference strains in the MIC values between 7.8 to 1000 µg/ml shown in Table 1.

Synergistic effect determined for AM, AP and AM+AP against MRSA

The checkerboard method was used to determine the synergistic activity for the combination of natural flavonoid AP individually and in combination with AM and CEF against MRSA strain. The interactions were interpreted and its growth inhibition was found using this combination index values denoted in Table 2. The observed values from checker board dilution of two-drug combination between AP and in combination with AM and CEF were plotted as dose effect curve, logarithmic combination index value, dose reduction index values and isobologram denoted in figure 1a-d, 2a-d. A predominant synergism was also detected when AP and in combination with AM and CEF and its inhibition was determined by ED 50, ED90 and its corresponding CI values were calculated in table 2.

The bacterial growth inhibitory effect of AM, AP and AM+AP on MRSA was observed and interpreted. From the following formula, fraction affected and fraction unaffected was calculated and the dose effect curve was plotted using isobologram software.

Individual
- AM - 12.5 to 800 µg/ml;
- AP at 1 – 64 µg/ml

Combination
AM + AP (25:1)- 12 – 384 µg/ml of AM with 0.48 – 15.36 µg/ml of AP.

The CI values of the combined drugs AP, AM and AP+AM were calculated using the formula and its results represented in table 2.

Table 2 shows results of the checker board assay for determination of MIC of the antibiotics AP & AM and its combination AP+AM against clinical strain of MRSA. AP and AM exhibited MIC at concentration of 57.35µg/ml and 800.6µg/ml respectively. Whereas, AP + AM as a combination has shown a significant reduction in the required effective concentration of AP and AM which is 5 µg/ml and 107 µg/ml respectively. This shows that AP and AM exhibits a lesser synergistic effect when put together as a combination (AP + AM), thereby, exhibiting only a slight increase in the antibacterial potency against MRSA.

The CI values of the combined drugs were calculated. According to the results less synergistic interactions was derived for AP + AM (0.2 to 0.5).

Graphical representation of dose effect curve, logarithmic combination index, dose reduction index and isobologram for each test sample and its combination has been presented in figure 1 a, b,c & d.

Logarithmic combination index plot for AP+AM in-

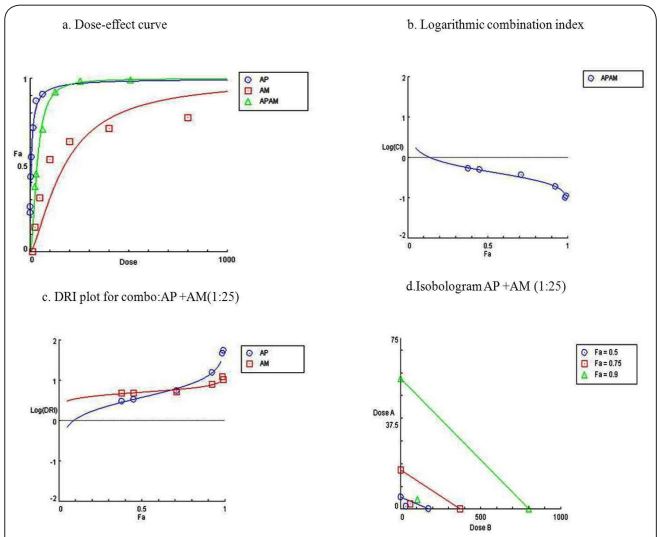

Figure 1. Combination index determination forAP, AM and AP+AM against MRSA. All values are represented as triplicate values. A) Dose-effect curve; b) Logarithmic combination index; c) DRI plot for combo:AP+AM(1:25); d) Isobologram AP +AM (1:25).

Table 2. Combination index value determined for AM, AP and AM+AP against MRSA.

Drug/ Combination against MRSA	Fraction affected 50%			Fraction affected 75%			Fraction affected 90%		
	CI value (FIC)	Drug concentration in µg/ml		CI value (FIC)	Drug concentration in µg/ml		CI value (FIC)	Drug concentration in µg/ml	
AP		5.19			17.6			57.5	
AM			173.99			373.2			800.5
APAM	0.47	1.36	33.96	0.30	2.42	60.4	0.21	4.30	107.4

All the values represent triplicate values.

CI< 1 indicates synergism, CI=1 indicates additive effect, CI > 1 indicates antagonism

AM combines with AP, MIC reduced to 1/7 of the MIC of AM and 1/13 of the MIC of AP (107 µg/ml with 4.3 µg/ml).

Table 3. Combination Index value determined for CEF, AP and CEF+AP against MRSA.

Drug/Combination against MRSA	Fraction affected 50%			Fraction affected 75%			Fraction affected 90%		
	CI value (FIC)	Drug concentration in µg/ml		CI value (FIC)	Drug concentration in µg/ml		CI valve (FIC)	Drug concentration in µg/ml	
CEF		0.87			7.15			35.68	
AP			5.20			17.26			57.35
CEFAP	0.19	0.11	0.33	0.17	0.53	1.59	0.18	2.57	7.71

All the values represented as triplicate values

CI< 1 indicates synergism, CI=1 indicates additive effect, CI > 1indicates antagonism

The CEF combines with AP, the MIC reduced to 1/23 of the MIC of CEF and 1/7 of the MIC of AP (2.6 with 7.7µg/ml).

dicates combination index point below the diagonal line indicates better synergistic effects and DRI >1 indicates favourable dose reduction.

Isobolograms for 50%, 75% and 90% inhibition are shown in figure 1d. Combination data point lower left of the diagonal line indicates synergism. In the present combination of AP+AM, IC_{90}, IC_{75} and IC_{50} showed the synergistic observations.

Synergistic effect determined for CEF, AP and CEF+AP against MRSA

The bacterial growth inhibitory effect of CEF, AP and CEF+AP on MRSA was observed and interpreted. From the following formula, fraction affected and fraction unaffected was calculated and the dose effect curve was plotted using isobologram software.

Individual
- CEF - 0.125 to 32 µg/ml
- AP - 1 – 256 µg/ml

Combination
- CEF + AP (1:3)- 0.125 – 16 µg/ml of CEF with 0.375 – 48 µg/ml of AP

Table 3 shows results of the Checker Board assay for determination of MIC of the antibiotics CEF & AP and its combination CEF+ AP against clinical strain of MRSA. CEF and AP exhibited MIC at concentration of 35.68µg/ml and 57.35 µg/ml respectively. Whereas, CEF + AP as a combination has shown a significant reduction in the required effective concentration of CEF and AP which is 3µg/ml and 8µg/ml respectively. This shows that CEF and AP exhibits a lesser synergistic effect when put together as a combination (CEF + AP), thereby, exhibiting only a slight increase in the antibacterial potency against MRSA.

The CI values of the combined drugs were calculated. According to the results less synergistic interactions was derived for CEF + AP (0.17 to 0.19).

Graphical representation of dose effect curve, logarithmic combination index, dose reduction index and isobologram for each test sample and its combination has been presented in figure 2 a, b,c & d.

Dose effect curve was plotted using OD value in figure 2a, with help of the curve x intercept, y intercept and median effect of the dose was calculated. Logarithmic combination index plot for CEF+AP in figure 2b indicates combination index point below the diagonal line indicates good synergistic effects and DRI >1 in figure 2c indicates favourable dose reduction.

Isobolograms for 50%, 75% and 90% inhibition are shown in figure 2d. Combination data point on the lower left indicates synergism. In the present combination of

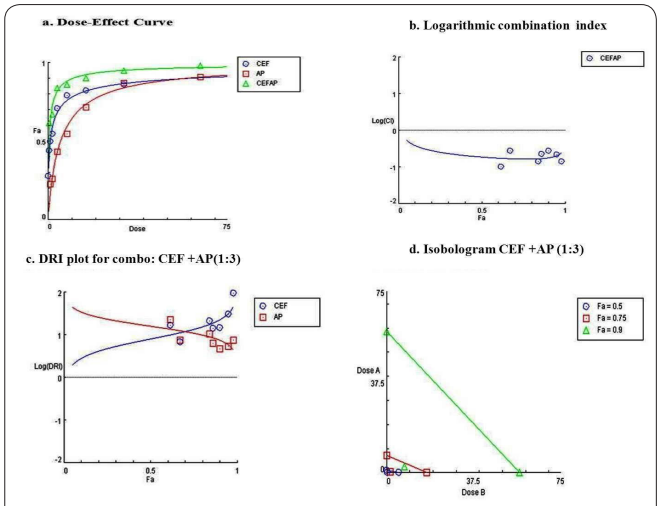

Figure 2. Combination Index determination for CEF with AP against MRSA. All values are represented as triplicate values. a) Dose-effect curve; b) Logarithmic combination index; c) DRI plot for combo: CEF +AP (1:3); d) Isobologram CEF +AP (1:3).

CEF+AP, IC_{90}, IC_{75} and IC_{50} showed synergistic effect.

Bactericidal efficacy determined against MRSA by time-kill curve assay

To confirm the synergistic effect of AP the selected antibiotics (AM & CEF) and their combination AM + AP and CEF + AP on inhibition of MRSA, time-kill assay was performed. Figure 3. show the results of the time-kill assay against clinical MRSA. AM, CEF and AP individually induced bacterial cell death upto 18 h incubation, i.e 2 X MIC of 800µg/ml of AM, 35µg/ml of CEF, 60µg/ml of AP individually, when used together in the proportion the combination of 100µg/ml of

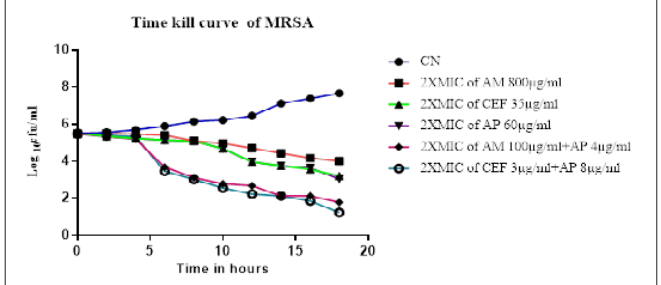

Figure 3. Determination of bactericidal efficacy of AM, CEF, AM + AP and CEF + AP against MRSA by Time-kill curve assay. The effect of ampicillin, ceftriaxone alone and in combination the viable counts of MRSA. 2MIC; 800µg/ml of AM, 35µg/ml of CEF, 60µg/ml of AP individually and the combination of 100µg/ml of AM with 5 µg/ml of AP, 3 µg/ml of CEF with 8 µg/ml of AP the values plotted are the means of 3 observations, standard errors of the means. P<0.001 compared to control group.

AM with 5 µg/ml of AP, 3 µg/ml of CEF with 8 µg/ml of AP a reduction of over 2 log10-fold in the bacterial count occurred after 6 h onwards. CEF + AP effectively inhibited the growth of MRSA. The results of the time-kill assays of the two drug combinations AM +AP and CEF+AP against MRSA showed significant inhibitory effect and reduced the colony count by approximately 99% after 6 h. The low bactericidal effect of AM and CEF induced us to combine it with other antibacterial agents. AP dramatically enhanced the activity of AM and CEF and lowered the number of CFUs. The two drug combinations AM + AP and CEF + AP used were considered bactericidal at the lowest concentration that reduced the original inoculum by 2 log10 CFU/ml (99.9%) for each of the indicated times.

Inner Membrane Permeability Assay of AM and CEF with AP Against MRSA

The IM permeability assay result for AM, CEF, AP, AM+AP, CEF+AP shows in Figure.4 where cell lysis was measured by UV-absorption of released cellular contents post lysis from MRSA cells at 260 nm. After treatment with 800µg/ml of AM, 35µg/ml of CEF, 50µg/ml of AP individually and the combination of 100µg/ml of AM with 5 µg/ml of AP, 3 µg/ml of CEF with 8 µg/ml of AP the OD value was increased considerably compared to control and CP. The inner membrane of MRSA was permeabilized more rapidly by the CEF + AP combination compared to other groups. CP, which is highly active against the inner membrane, showed inner membrane permeability slightly lower than CEF + AP combination, produce significant difference ($p < 0.001$). This effect was due to the leakage of intracellular contents that was released more rapidly, thereby absorption at 260nm was increased. This effect was a significant difference compared to control.

Outer Membrane Permeablitiy assay for AM, CEF with AP treated MRSA cells

The percentage of crystal violet uptake into the MRSA cell membrane post lysis has been determined by

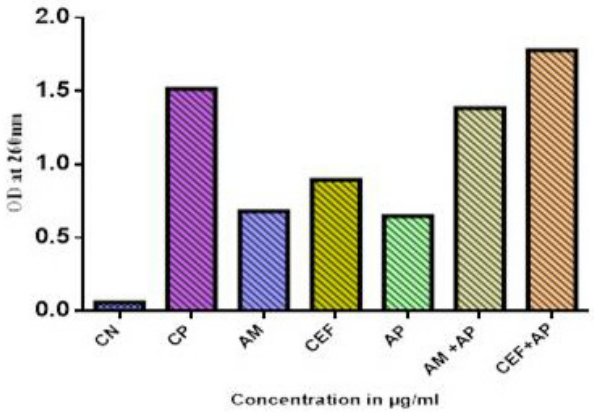

Figure 4. Inner membrane permeability assay for AM, CEF, AP, AM+AP, CEF+AP. 800µg/ml of AM, 35µg/ml of CEF, 50µg/ml of AP individually and the combination of 100µg/ml of AM with 5µg/ml of AP, 3 µg/ml of CEF with 8 µg/ml of AP treated against MRSA, CP-Ciprofloxacin at 2 µg/ml was used as positive control and untreated cells were used as negative control. The mean ± SE for three replicates are illustrated. Means sharing the same superscript are not significantly different from each other ($p < 0.001$).

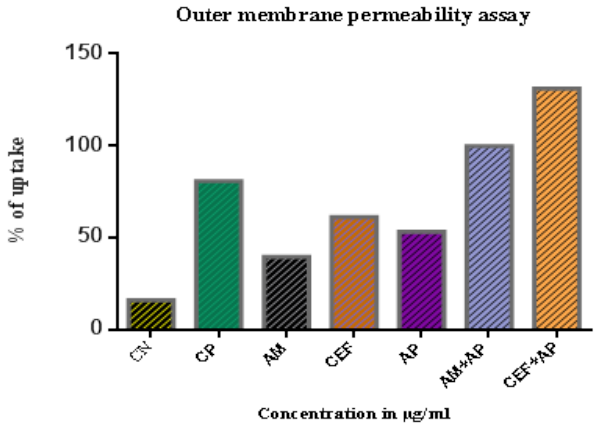

Figure 5. Outer membrane permeablitiy assay for AM, CEF with AP treated MRSA cells. AM- MIC; 800µg/ml of AM, 35µg/ml of CEF, 50µg/ml of AP individually and the combination of 100µg/ml of AM with 5 µg/ml of AP, 3 µg/ml of CEF with 8 µg/ml of AP treated against MRSA. Crystal violet uptake of untreated cells used as control. Ciprofloxacin at 2 µg/ml was used as positive control and untreated cells were used as negative control. The mean ± SD for three replicates are illustrated. The graph shows % of crystal violet uptake of each treatment at 60 min.

UV absorption at 590 nm. According to the final graph, all the test samples AM, CEF, AM + AP and CEF + AP exhibited cell wall damage to an extent as shown in Figure 5. With CEF + AP, the crystal violet uptake was considerably higher than control and CP. Percentage of crystal violet uptake into the MRSA cell membrane for all other test samples i.e. AM, CEF and AM + AP were significantly high compared to that of the standard CP. From the above results it can be corrugated that the outer membrane permeability was high compared to inner membrane permeability of all the tested combinations. This OM permeabilisation might responsible for antibacterial activity.

Scanning electron micrographs of MRSA treated with test agents AM + AP and CEF + AP

SEM observation was carried out in order to evaluate the percentage of cell wall damage caused due to test agents. SEM of control MRSA and those treated with AM + AP and CEF + AP are shown in Figure 6 a–f. Visualization of the ultrastructure in general revealed marked morphological changes in MRSA treated with AM + AP and CEF + AP.

TEM for MRSA cells treated with AM + AP and CEF + AP

Figure 7 a-f shows the appearance of normal log phase cells of MRSA with cell division. The outer membrane and the cytoplasmic membrane can be distinguished. The TEM image of MRSA after exposure to CEF with AP shows some of these bacterial cells exhibited gap between outer membrane and cytoplasmic membrane (Figure. 7 a-f). The MRSA cells treatment with 3 µg/ml CEF with AP 8µg/ml are shown in Figure 7c. Most of the bacterial cells were damaged after treatment with AM + AP and CEF + AP. The result showed that CEF 3 µg/ml in combination with 8µg/ml of AP also caused marked morphological damage, cell wall, cell shape and losing most organelles in MRSA as

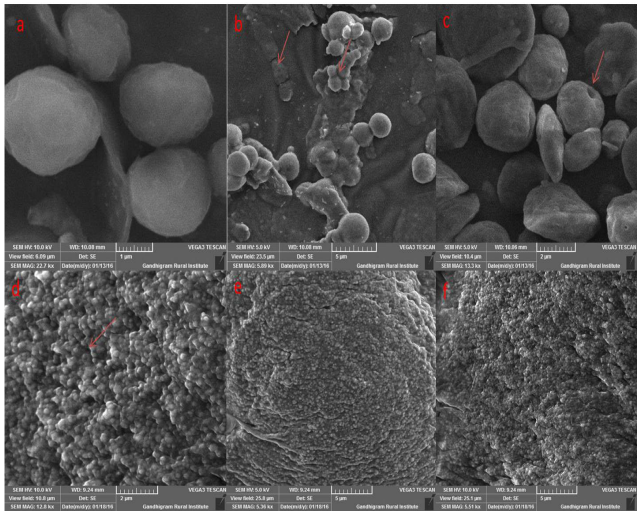

Figure 6. Scanning electron micrographs showing reduction of MRSA growth after 36 h incubation with AM + AP and CEF + AP. a) Control-Untreated cells; (b & c) Cell disruption by Pore formation in AM + AP treated cells; (d, e & f) deformation of cell structure in CEF + AP treated cells.

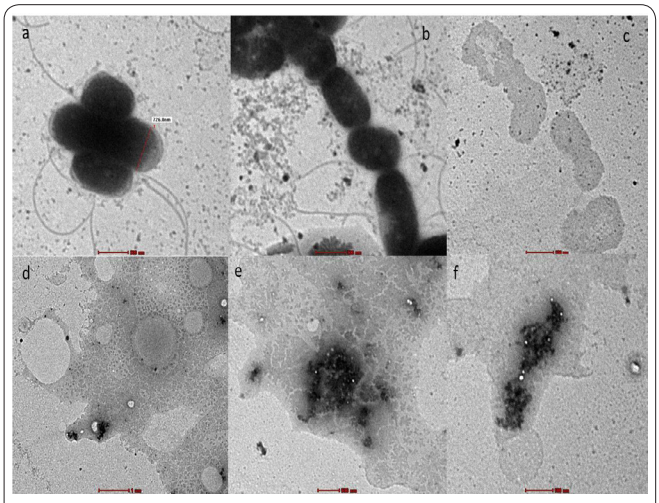

Figure 7. a-f TEM image of AM+AP and CEF+AP treated MRSA cells. a,b- Untreated MRSA cells; c,d- Ampicillin with Apigenin treated cells; e,f- Ceftriaxone with apigenin treated cells.

shown in Figure 7a-f.

Discussion

Bacterial infections are a worldwide burden and treatment of patient with traditional antibiotics is becoming increasingly challenging or ineffective. Natural products can be used as raw materials in the development of new antibacterial substances. Antibacterial screening of medicinal compounds has been used as therapeutic agents. In earlier literature reported that curcumin was obtained from *c.longa* which was shown synergistic activity against gram (+) ve and gram (–) ve especially against MRSA (16).

The experiments in summary deals with 1) evaluation of antibacterial effects of phenolic flavonoid AP 2) investigation of possible synergistic action of AP in combination with β-lactam antibiotics AM and CEF 3) study of membrane permeabilisation caused by the test agents on MRSA using IM and OM assay.

In the present study, antibacterial activity of AP against gram (+) ve and gram (-) ve organism was 32.5-250µg/ml. Earlier reports of apigenin, naringenin, thymoquinone have great antimicrobial activity and their mechanism of activity also studied in detail (17). The antibacterial activity of flavone Apigenin was due in part to their inhibition of DNA gyrase explained in TP.Tim cushnie et al 2005 (18).

The synergistic combination of AP with AM and CEF was varied this was much lower than their own MIC. To confirm their CI values against MRSA a time kill assay was conducted. This finding indicated that this CI effect may overcome the problem associated with some MRSA pathogen when used in a combination with commonly used antibiotics AM and CEF. The results of this combination were an agreement with earlier literature artocarpin may be used to enhance the antibacterial activities of the antibiotics AM. Flavonoids are well known to possess antibacterial activity with many possible mechanism of action i.e reduction of membrane fluidity due to interaction of flavonoids with the lipophilic side of cell membrane (15). The antibacterial effect of selected flavonoid has 5,7-OH group of A ring and 1,4-OH group of the B ring in AP are important for synergistic activity with selected antibiotics (10).

The checkerboard analysis showed the synergistic effects of AM + AP and CEF + AP combination against MRSA with CI index between 0.18-0.47. From the results, it can be confirmed that AM + AP as a combination has synergistic action, whereas CEF + AP showed higher synergistic effect. The addition of AP to AM and CEF significantly altered the individual effect of both compounds against MRSA. This activity was compared with an earlier finding that implied that the two additional hydroxyl groups on the structure of quercetin derivative might facilitate its inhibitory activity (19).

Time kill assay of AM + AP and CEF + AP provided a standard platform to observe kinetics of antibacterial action of all the test agents against MRSA. About 99 % MRSA inhibition was observed at 8 h. Therefore, it is assumed that the improved antibacterial efficacy of the tested combinations might be because of the synergistic effect of AP on AM and CEF. This may be attributed to the fact that AP possesses antibacterial properties that contributed to the observed activities either alone or in combination treatment. These antibacterial properties may have several mechanisms of action that affects MRSA growth. The results of this combination were in agreement with earlier literature on artocarpin that was used to enhance the antibacterial efficiency of the antibiotic drug AM. Flavonoids are well known to possess antibacterial activity with many possible mechanism of action for example, reduction of membrane fluidity due to interaction of flavonoids with the lipophilic portion of the cell membrane (15). Investigation on mode of action has shown that these compounds cause cell lysis leading to bacterial death in *S. aureus* species (19). Assessment of MIC values revealed that the selected apigenin and its combination have anti-MRSA activity. The synergistic impact of AP individually combined with AM and CEF has been crucial in suppressing MRSA growth.

The inner membrane permeability results provide evidence that mechanism of action of AP is damage of cytoplasmic membrane thereby increasing its non-specific permeability (13). This hyper permeability may be followed by release of UV-absorbing materials, which is the index of cell lysis and non-selective pore formation that leads to leakage of ions. This was confirmed

with measurement of OD (260 nm) of the resulting samples which consist of deformed cellular organelles, macromolecules and cell membrane. The results seem to be compared with previous findings that *E.coli* outer membrane was significantly changed by amoxicillin plus galangin combination or peptide–peptide nucleic acid conjugate OD at 260nm (10, 20, 21,22).

The outer membrane permeability by the crystal violet assay showed that the membrane of *S.aureus* was significantly altered by the combinations of AM + AP and CEF + AP, compared to control. The effect of AP on outer membrane permeability was evidenced by the percentage uptake of the dye crystal violet. Usually, crystal violet penetration into the outer membrane is very poor, but when the membrane is defective the rate of penetration is higher (13). A significant enhancement in the intake of crystal violet in comparison with the control cells and MRSA treated with AM and CEF alone was observed in MRSA treated AM + AP and CEF + AP. This shows that AM and CEF has low antibacterial efficacy individually, whereas, AP alters membrane permeability and makes the cells, which generally are less permeable, hyper permeable to extracellular components (10, 13). These results seem consistent with reported findings that the outer membrane of *E. coli* was significantly altered by the combination of amoxicillin plus galangin and peptide–peptide nucleic acid conjugate (20, 21, 22). The antibacterial activity of selected combination is mainly due to OM permeability than IM permeability. SEM reports are in agreement with the earlier report determining the morphology of the bacteria after treatment with the *C.longa* extract (23). TEM results of AM+AP and CEF+AP treated cells demonstrated that MRSA cells exhibited marked morphological damage and reduction in average cell areas significantly lesser than control. These results seem consistent with previous findings that the combination of ceftazidime plus galangin caused damage to the ultra-structures of the cells, affected the integrity of the cell walls, and led to an increase in cell size of ceftazidime-resistant *S. aureus* (10).

Hence, AP when used in combination with AM and CEF at this concentration may have a sufficient margin of safety for therapeutic use. In summary, our study provides evidence that AP has the potential to reverse bacterial resistance to originate traditional drug susceptibility of it.

The results of the present study provided scientific explanation for the use of AP. Our findings provide evidence that AP have the synergistic effect with AM, CEF to reverse bacterial resistance. This is the first ever report on the anti-MRSA activity and the mode of bacterial inhibition of AP individually and in combination with AM and CEF. The grade of antibacterial activity indicated that AP is a potential source that could be helpful for the development of new antibacterial agents capable of lessening the burden of drug resistance, cost of management of disease, to allow lower doses of antibiotic to reduce toxicity and without the need of one more antibiotics. Overall, AP offer for the development of a valuable adjunct to AM, CEF against these MRSA strains.

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