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In vitro Assessment of Novel Antimicrobial from Methanol Extracts of Matured Seed Kernel and Leaf of *Mangifera indica* L. (Kesar Mango) for Inhibition of *Pseudomonas* spp. and their Synergistic Potential

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ABSTRACT

This study was done in order to determine the anti *Pseudomonas* potential of methanolic extracts and their fractions of matured seed kernel and leaf of *Mangifera indica*. The antibacterial susceptibility test was determined by agar well diffusion assay against five *Pseudomonas* spp. Thereafter, synergistic effect was determined by the minimal inhibitory concentration and the minimal bactericidal concentration using the micro broth dilution technique and synergistic effect was determined by calculating FIC index. Further, time kill assay was performed for the potential synergistic combination ($\Sigma FICI \leq 0.1$) at 1×MIC concentrations. The highest zone of inhibition was in crude extracts. The results of MIC and MBC showed potential anti *Pseudomonas* activity in the range of 9.75 to >250 and 156 to >1250 $\mu g\ mL^{-1}$, respectively which was better than the standard drug used. Amongst all studied synergistic effects, RSM combined with LM showed better synergistic and bactericidal effects against *P. syrigae*. The results of this study indicate that it should be relatively easy to utilize antimicrobial extracts in future treatments for multi drug resistance strains.

Key words: *Mangifera indica*, MIC index, chloramphenicol, ceftazidime, *Pseudomonas* spp.

INTRODUCTION

Bacterial resistance to currently available antibiotics has rapidly emerged to a global problem and posing a growing public health risk. Further more new antibiotics against Gram-positive pathogens have entered the market recently but the pipeline against Gram negative bacteria appears to be dry. Novel anti-*Pseudomonas* activity is of particular interest as it is the leading cause of nosocomial infections and has developed mechanisms of resistance to common classes of antibiotics (Marzouk *et al*., 2009; Chanda *et al*., 2010a, 2013a; Yao *et al*., 2012). Moreover, *Pseudomonas* spp. can cause food spoilage and an opportunistic, nosocomial pathogen which typically infects the pulmonary, urinary tracts, burns and wounds, blood infections in immuno-compromised individuals (Chanda *et al*., 2011a, 2013b; Shu *et al*., 2012; Rakholiya *et al*., 2014a).

The resistance of bacteria and other microorganisms to antimicrobial agents has become a wide-spread medical problem. To reduce health hazards and economic losses as a result of food

borne microorganisms, the use of natural product as antimicrobial compounds is gaining importance. Several plants have been reported to be used in treating and managing the infectious diseases (Chanda *et al.*, 2011b; Kaneria and Chanda, 2012, 2013a, b). However, it is necessary to establish the scientific basis for the therapeutic actions of traditional plant medicines. Furthermore, a promising trend is combination between standard antibiotics and natural extracts as well as within extracts, it enhances activity as well as reduces the amount of drug concentration to kill pathogens (Chanda and Kaneria, 2011b; Rakholiya and Chanda, 2012a).

In this study, the *in vitro* anti *Pseudomonas* activity of *Mangifera indica* L. (matured seed kernel and leaf) was investigated. Mango is one of the important tropical fruits in the world and India and considered as a king of fruits in Indian delicacy.

MATERIALS AND METHODS

Chemicals and reagents: Mueller Hinton No. 2, Nutrient broth, 2-(4-Iodo phenyl)-3-(4-nitro phenyl)-5-phenyltetrazolium chloride (INT), Chloramphenicol (CH) and ceftazidime (CF) antibiotics were purchased from Hi-Media Laboratory Pvt. Ltd., (Mumbai, India). Dimethyl sulfoxide (DMSO), methanol, ethyl acetate, chloroform and petroleum ether were purchased from Merck (Mumbai, India). Water was purified with a Milli-Q system (Millipore, Bedford, USA). All solvents and chemicals used were of analytical grade.

Collection and sample preparation: The matured seed and leaf of *Mangifera indica* L. var. 'Kesar' (SU/BIO/514/Thakrar) were collected in May, 2010 from Gujarat, India and identified by comparison with specimens available at the Herbarium of the Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India. The materials were washed thoroughly with tap water. The kernels were removed from the seeds manually, shade dried, homogenized to fine powder and stored in air tight bottle.

Individual cold percolation extraction method: The dried powder of two parts was extracted individually by cold percolation method (Rakholiya *et al.*, 2011; Rakholiya and Chanda, 2012b). The powder was first defatted with petroleum ether and then extracted in methanol. First, 10 g of dried powder was added to 100 mL of petroleum ether in a conical flask which was plugged with cotton wool and kept on a rotary shaker at 120 rpm for 24 h the extracts was filtered with 8 layers of muslin cloth and centrifuged at 5000 rpm (Remi Centrifuge, India) for 10 min. Supernatant was collected and the solvent was evaporated using a rotary vacuum evaporator (Equitron, India) to dryness. Petroleum ether was evaporated from the powder. This dry powder was then taken in 100 mL of methanol and was kept on a rotary shaker at 120 rpm for 24 h, the extract was filtered with 8 layers of muslin cloth and centrifuged at 5000 rpm for 10 min. The supernatant was collected and the solvents were evaporated. The residues were weighed to obtain the extractive yield of both extracts RSM and LM. They were stored in air tight bottles at 4°C.

Isolation of RSM and LM extracts: Fractionation of the methanol extract of matured seed kernel and leaf was done by solvent-solvent partition (Tang *et al.*, 2010). Five grams of methanol extract was dissolved in hot methanol (200 mL). Slight precipitation obtained was discarded as methanol insoluble matter. The methanol soluble fraction was filtered and collected. It was concentrated to about 50 mL volume and ethyl acetate was added to it till faint turbidity was obtained. Then it was allowed to settle down in a refrigerator. The settled gelatinous reddish mass

and supernatant was separated and collected separately. The supernatant was further concentrated and ethyl acetate step was repeated till reddish gelatinous mass obtained. All the settled mass was collected together and dissolved in methanol. It was concentrated further to dryness and designated as RSM I and LM I. The collected supernatant was concentrated further to near dryness and then dissolved in methanol. Then chloroform was added to it and cooled. Light yellow waxy sediment was separated and light buff coloured supernatant was collected. This supernatant was concentrated further to dryness and designated as RSM II and LM II.

Antimicrobial study

Microorganism and growth conditions: The five *Pseudomonas* spp. [*Pseudomonas aeruginosa* ATCC27853 (PA), *Pseudomonas pictorum* NCIB9152 (PPi), *Pseudomonas putida* NCIM2872 (PPu), *Pseudomonas syrigae* NCIM5102 (PS) and *Pseudomonas testosterone* NCIM5098 (PT)] were obtained from National Chemical Laboratory (NCL), Pune, India. They were maintained on nutrient agar medium (Hi Media, India) at 4°C and sub-cultured before use. The *Pseudomonas* spp. are clinically important ones causing several infections, food borne diseases, spoilages, skin infection and it is essential to conquer them through some active therapeutic agents.

Agar well diffusion assay: *In vitro* antibacterial activity of the RSM, LM and their fractions was studied against pathogenic microbial strains by the agar well diffusion method (Rakholiya *et al*., 2014b; Chanda and Rakholiya, 2011a). Mueller Hinton No. 2 (Hi-media) was used for the antibacterial susceptibility test. The extracts and fractions were diluted in 100% DMSO (Dimethyl sulfoxide) to give a concentration of 5 mg mL^{-1}. The Mueller Hinton agar was melted and cooled to 48-50°C and 200 µL standardized inoculum (1.5×10^8 CFU mL^{-1}, 0.5 McFarland) was then added aseptically to the molten agar and poured into sterile Petri dishes, wells (8.5 mm) were prepared in the seeded agar plates. The test compound (100 µL) was introduced in the well. The plates were incubated over night at 37°C for 24 h. DMSO was used as negative control. The bacterial growth was determined by measuring the diameter of the zone of inhibition and the mean values are presented with ±SEM.

Preparation of bacterial inocula and extracts or antibiotics for MIC and MBC study: The inoculum of the test organism was prepared using the colony suspension method (EUCAST., 2003). The extract and fractions dissolved in 100% of DMSO were first diluted to highest concentration (1250 µg mL^{-1}) to be tested and then serial two-fold dilution was made in a concentration range from (0.605-1250 µg mL^{-1}). Chloramphenicol and ceftazidime were used as a positive control (0.0156-32 µg mL^{-1}).

Determination of the Minimum Inhibitory Concentrations (MIC): The MICs were determined by micro broth dilution method performed in sterile flat bottom 96 well micro test plates (Tarsons Products Pvt. Ltd.) (Edziri *et al*., 2012). This test was performed in sterile flat bottom 96 well micro test plates (Tarsons Products Pvt. Ltd.). One hundred and fifty microlitres of Mueller-Hinton broth was introduced into all the 96 wells and 20 µL of varying concentrations of the extract was added in increasing order along with 30 µL of the test organism suspension. A final volume of 200 µL was achieved in each well. Three control wells were maintained for each test batch. The positive control (antibiotic, Mueller-Hinton broth and test organism) and sterility control (Mueller-Hinton broth and DMSO) and organism control (Mueller-Hinton broth, test organism and

DMSO). Plates were then incubated at 37°C for 24 h overnight. Experiments were performed in triplicate. After incubation, 40 μL of 2-(4-iodophenyl)-3-(4-nitrophenyl) 5-phenyltetrazolium chloride (INT, Himedia, India) solution (200 μg mL^{-1}) dissolved in sterile distilled water was added to each well (Rakholiya *et al.*, 2014c). The plates were incubated for further 30 min and estimated visually for any change in colour to pink indicating reduction of the dye due to bacterial growth. The highest dilution (lowest concentration) that remained clear corresponded to the MIC.

Determination of the Minimum Bactericidal Concentration (MBC): Minimum Bactericidal Concentration was determined from all wells showing no growth as well as from the lowest concentration showing growth in the MIC assay for all the samples. Bacterial cells from the MIC test plate were sub-cultured on freshly prepared solid nutrient agar by making streaks on the surface of the agar. The plates were incubated at 37°C for 24 h overnight. Plates that did not show growth were considered to be the MBC for the extract or drug used (Akinyemi *et al.*, 2005). The experiment was carried out in triplicate.

Determination of MIC index: The MIC index (MBC/MIC) was calculated for extracts to determine whether an extract had bactericidal (MBC/MIC = 4) or bacteriostatic (>4 MBC/MIC<32) effect on growth of bacteria (Teke *et al.*, 2011).

MICs determination of combinations of extracts and antibiotics/extracts: Mixture of RSM, LM and CH, CF at ratios of 1:1 was tested for MICs which was determined by the micro well dilution method as describe above. The Fractional Inhibitory Concentration (ΣFIC) index was calculated by adding the FIC values of antimicrobial compounds (A) and (B) ($FIC_A + FIC_B$). The FIC_A and FIC_B values represent the lowest concentration of antibiotics and extracts, respectively that caused the inhibition of bacterial growth in the combination tests. Calculations were performed as follows:

- $\Sigma FICI = FIC_A + FIC_B$
- $FIC_A = (MIC_A \text{ combination}/MIC_A \text{ alone})$
- $FIC_B = (MIC_B \text{ combination}/MIC_B \text{ alone})$

For interpretation of the results, ΣFIC = 0.5 was assigned as a synergistic effect, 0.5>ΣFIC = 0.75 represented as a partial synergy, 0.76 to 1.0 represented as an additive effect, >1.0-4.0 represented as an indifferent effect (non-interactive) and ΣFIC >4.0 antagonistic effect between two tested antimicrobial agents (Bassole *et al.*, 2011; Sabate *et al.*, 2012).

Determinations of the time kill curve studies: Time kill assay was performed as described by Ernst *et al.* (2002) with some modifications. Flasks containing 15 mL of Mueller Hinton Broth incorporated with appropriate antimicrobial agent were inoculated with equal volumes of the diluted inoculums of each test organism having optical density 0.2 at 600 nm which is equivalent to 6×10^8 CFU mL^{-1} (EUCAST., 2003) and were exposed to 1×MIC of the individual and combination drug. Control experiment without active compound (i.e., a bacterial growth curve) was conducted simultaneously with the time kill studies. The flasks were incubated at 37°C in an orbital shaking incubator at 150 rpm for 24 h. Aliquots were removed at 0, 1, 2, 3, 4, 5, 6 and 24 h and the Optical Density (OD) was measured at 600 nm. A graph of time verses absorbance (OD at 600 nm) was plotted for the growth curve and the effect of drug on bacterial growth was recorded at 1, 2, 3, 4, 5, 6 and 24 h. The experiment was carried out in triplicate.

Statistical analysis: Each sample was analyzed individually in triplicate and the results are expressed as the mean value (n = 3) ±Standard Error of Mean (SEM).

RESULTS AND DISCUSSION

Antimicrobial study: Researchers for many decades have been trying to develop new broad-spectrum antibiotics for treating the infectious diseases caused by pathogenic microorganisms. Prolonged usage of those broad-spectrum antibiotics has led to the emergence of drug resistance. There is an amazing want for novel antimicrobial agents from totally different sources. Another problem is spoilage of food due to the presence of bacterial and fungal infection. This has been a major concern for decades which causes a considerable economic loss worldwide. Plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids, terpenoids, flavonoids, etc. These secondary metabolites have been found *in vitro* to have antimicrobial property and they may serve as alternative, effective, cheap and safe antimicrobials for the treatment of microbial infections (Chanda *et al*., 2010b; Bag *et al*., 2012; Rashid *et al*., 2013; Kaneria *et al*., 2014).

The potencies of RSM, RSM I, RSM II, LM, LM I and LM II as antibacterial agent was evaluated against 5 *Pseudomonas* spp. by agar well diffusion method. The anti *Pseudomonas* activity was determined by measuring the zone of inhibition. The anti *pseudomonas* activity of RSM, RSM I and RSM II against five bacteria is shown in Fig. 1a. All the *Pseudomonas* spp. were susceptible to RSM, RSM I and RSM II. The highest activity was in RSM extract closely followed by RSM II. The anti *Pseudomonas* activity of LM, LM I and LM II against five bacteria is shown in Fig. 1b. All the *Pseudomonas* spp. were susceptible to LM, LM I and LM II except *P. aeruginosa* which was resistant to LM I. The highest activity was in LM extract closely followed by LM II.

Minimum inhibitory concentration refers to the lowest concentration of the antimicrobial agent which is required for the inhibition of visible growth of the tested isolate (Sharma *et al*., 2012;

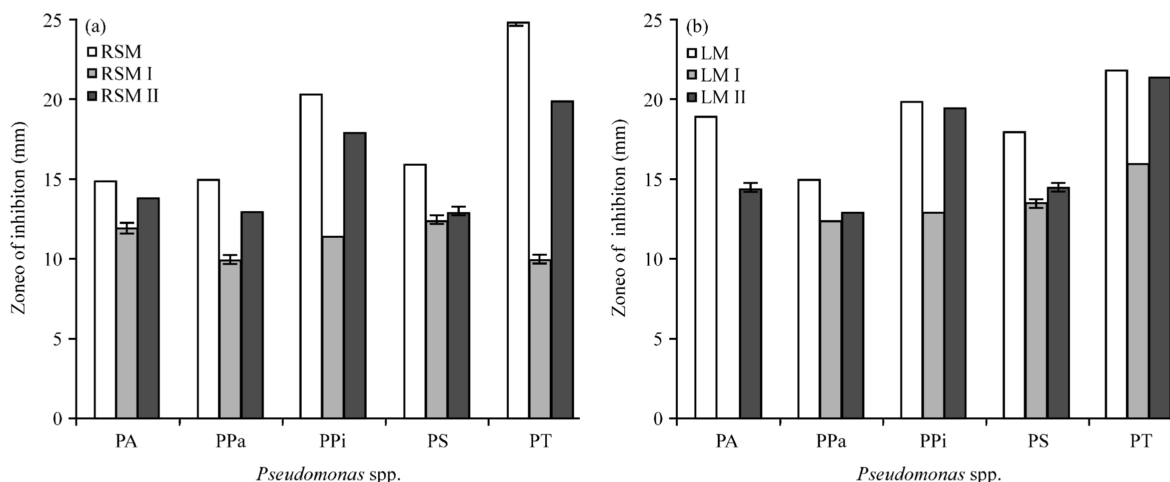

Fig. 1(a-b): Antibacterial activity of (a) RSM, RSM I and RSM II and (b) LM, LM I and LM II against *Pseudomonas* spp. PA: *Pseudomonas aeruginosa* ATCC27853, PPi: *Pseudomonas pictorum* NCIB9152, PPu: *Pseudomonas putida* NCIM2872, PS: *Pseudomonas syrigae* NCIM5102 and PT: *Pseudomonas testosterone* NCIM5098 (The values are Means±SEMs (n = 3))

Table 1: Antibacterial activity and FIC indices values for the independent (RSM, RSM I, RSM II, CH, CF) and equal ratio combinations of RSM with CH and CF

	MIC/MBC ($\mu g\ mL^{-1}$)					MIC value recorded for 1:1 (ΣFICI)	
Microorganisms	CH	CF	RSM	RSM I	RSM II	RSM/CH	RSM/CF
***Pseudomonas* spp.**							
PA	8/>32	32/>32	156/1250	39/625	19.5/156	39/1 (0.4)	39/1 (0.3)
PPu	16/>32	32/>32	1250/>1250	39*/156*	78/625	1250/32 (3.0)	156/4 (0.2)
PPi	32/>32	8/>32	1250/>1250	39/1250	9.75/156	9.75/0.25 (0.02)	19.5/0.5 (0.1)
PS	16/>32	32/>32	625/>1250	1250/>1250	1250/>1250	312/8 (1.0)	312/8 (0.7)
PT	32/>32	8/32	625/>1250	1250/>1250	1250/>1250	1250/32 (3.0)	156/4 (0.7)

ΣFIC index $FIC_A + FIC_B$, FIC_A (MIC_A combination/MIC_A alone), FIC_B (MIC_B combination/MIC_B alone) = 0.5 synergistic, >0.5 to 0.75 partial synergy, 0.76 to 1.0 additive, >1.0 to 4.0 indifferent (non-interactive), >4.0 antagonistic, *MBC/MIC ratio of value = 4 is indicative of a bactericidal nature, PA: *Pseudomonas aeruginosa* ATCC27853, PPi: *Pseudomonas pictorum* NCIB9152, PPu: *Pseudomonas putida* NCIM2872, PS: *Pseudomonas syrigae* NCIM5102 and PT: *Pseudomonas testosterone* NCIM5098

Table 2: Antibacterial activity and FIC indices values for the independent (LM, LM I, LM II, CH, CF) and equal ratio combinations of LM, CH and CF

	MIC/MBC ($\mu g\ mL^{-1}$)					MIC value recorded for 1:1 (ΣFICI)	
Microorganisms	CH	CF	LM	LM I	LM II	LM/CH	LM/CF
***Pseudomonas* spp.**							
PA	8/>32	32/>32	625/>1250	1250/>1250	625/>1250	625/16 (3)	1250/32 (3)
PPu	16/>32	32/>32	156/1250	625/>1250	78*/312*	1250/32 (10)	312/8 (2.3)
PPi	32/>32	8/>32	156/1250	>1250/>1250	78*/312*	39/1 (0.3)	39/1 (0.4)
PS	16/>32	32/>32	1250/>1250	>1250/>1250	>1250/>1250	1250/32 (3)	312/8 (0.5)
PT	32/>32	8/32	625/>1250	625/>1250	625/>1250	1250/32 (3)	312/8 (1.5)

ΣFIC index $FIC_A + FIC_B$, FIC_A (MIC_A combination/MIC_A alone), FIC_B (MIC_B combination/MIC_B alone) = 0.5 synergistic, >0.5 to 0.75 partial synergy, 0.76 to 1.0 additive, >1.0 to 4.0 indifferent (non-interactive), >4.0 antagonistic,*MBC/MIC ratio of value = 4 is indicative of a bactericidal nature

Rakholiya *et al*., 2013a). MIC values were calculated using INT dye on a 96-well micro-titer plate. The MBC is interpreted as the lowest concentration that can completely remove the microorganisms. Synergistic effect was determined by calculating FIC index of plant extracts and antibiotics.

The combination effect of RSM with CH and CF and their individual activity are summarized in Table 1. The activity exhibited concentration dependent inhibition of growth. In CH and CF, MIC and MBC values varied from 8 to >32 $\mu g\ mL^{-1}$ and 156 to >1250 $\mu g\ mL^{-1}$, respectively. In RSM and its fraction, MIC and MBC ranged from 9.75 to 1250 $\mu g\ mL^{-1}$ and 156 to >1250 $\mu g\ mL^{-1}$, respectively. *Pseudomonas pictorum* was found to be the most susceptible bacterial pathogens to RSM II (MIC: 19.5 $\mu g\ mL^{-1}$). RSM I had bactericidal effect against *P. putida*. The FIC indices of combination of RSM and CH ranged from 0.02-3. And this combination manifested synergistic effect on growth of *P. aeruginosa* and *P. pictorum* with FIC indices 0.02 and 0.4 respectively. The FIC indices of combination of RSM and CF ranged from 0.1-0.7. The combination of RSM and CF manifested synergistic effect on growth of three bacteria namely *P. aeruginosa*, *P. pictorum* and *P. putida* with FIC indices between 0.1-0.3. The combination showed partial synergism against *P. syrigae* and *P. testosterone* with 0.7 FIC index.

The combination effect of LM with CH and CF and their individual activity are summarized in Table 2. The MIC and MBC values of LM, LM I and LM II are presented in Table 2. MIC and MBC

Table 3: Antibacterial activity and FIC indices values for the independent (LM, RSM) and equal ratio combinations of LM and RSM

	MIC/MBC (μg mL^{-1})		MIC and MBC value recorded for 1:1	
Microorganisms	RSM	LM	RSM/LM	ΣFICI
***Pseudomonas* spp.**				
PA	156/1250	625/>1250	312/>1250	2.50
PPu	1250/>1250	156/1250	19.5/312	0.10
PPi	1250/>1250	156/1250	39/312	0.28
PS	625/>1250	1250/>1250	2.43/39	0.01
PT	625/>1250	625/>1250	625/>1250	2.00

ΣFIC index $FIC_A + FIC_B$, FIC_A (MIC_A combination/MIC_A alone), FIC_B (MIC_B combination/MIC_B alone) = 0.5 synergistic, >0.5 to 0.75 partial synergy, 0.76 to 1.0 additive, >1.0 to 4.0 indifferent (non-interactive), >4.0 antagonistic

ranged from 78 to >1250 μg mL^{-1} and 312 to >1250 μg mL^{-1}, respectively. *Pseudomonas pictorum* and *P. putida* were found to be the most susceptible bacterial pathogens to LM II (MIC: 78 μg mL^{-1}) and this fraction showed bactericidal effect. The FIC indices of combination of LM and CH ranged from 0.3-10. The combination of LM and CH manifested synergistic effect on growth of *P. pictorum* with FIC index 0.3. FIC indices of combination of LM and CF ranged from 2.3-0.4. The combination of LM and CF manifested synergistic effect on growth of *P. pictorum* and *P. syrigae* with FIC indices 0.4 and 0.5, respectively.

The combination effect of RSM, LM and their individual activity are summarized in Table 3. The FIC indices of combination of RSM and LM ranged from 0.01-2.5. The combination of RSM and LM manifested synergistic effect on growth of *P. pictorum P. putida* and *P. syrigae* with FIC index between 0.01-0.28. Amongst all the studied synergistic effects, RSM combined with LM showed better synergistic effect and its FIC index was 0.01 and 0.02 against *P. syrigae* and *P. pictorum*, respectively. De Oliveira *et al.* (2011) investigated the synergistic activity of norfloxacin, tetracycline and erythromycin with ethanol extract of *M. indica* peel against *S. aureus* strains. Individual extract did not display significant antibacterial activity (MIC = 2048 μg mL^{-1}) but it modulated the activity of antibiotics (MIC = 512 μg mL^{-1}), i.e., in combination with antibiotics, a four-fold reduction in the MIC values for tetracycline and erythromycin was found.

Time kill curve: The rapidity of the bactericidal effect or the duration of a bacteriostatic effect can be determined by time kill analysis. The pharmacodynamics of an antibiotic may be investigated in several ways, including the study of time kill kinetics. Therefore, we have investigated time kill assays for the potential synergistic combination (ΣFICI≤0.1) on selected bacterial strains at 1×MIC concentrations. The growth curve pattern was shown in Fig. 2. In the control set only the organism culture was grown in MH Broth. In the sample set the bacterial culture was grown in MH Broth in presence of the either individual extracts/antibiotics or synergistic between them and a negative control set containing DMSO was also maintained. All tested bacteria were susceptible more towards combination extracts as compared to individual extracts or antibiotics. Amongst all combination LM+RSM completely inhibited the growth of *P. putida* at 24 h (Fig. 2d). The pattern of activity also suggests that the individual compound and in combination was bacteriostatic against all the studied strains at 1×MIC except combination of RSM and LM which showed bactericidal effect at 3 h against *P. syrigae* and 24 h against *P. putida*. Other researchers also used

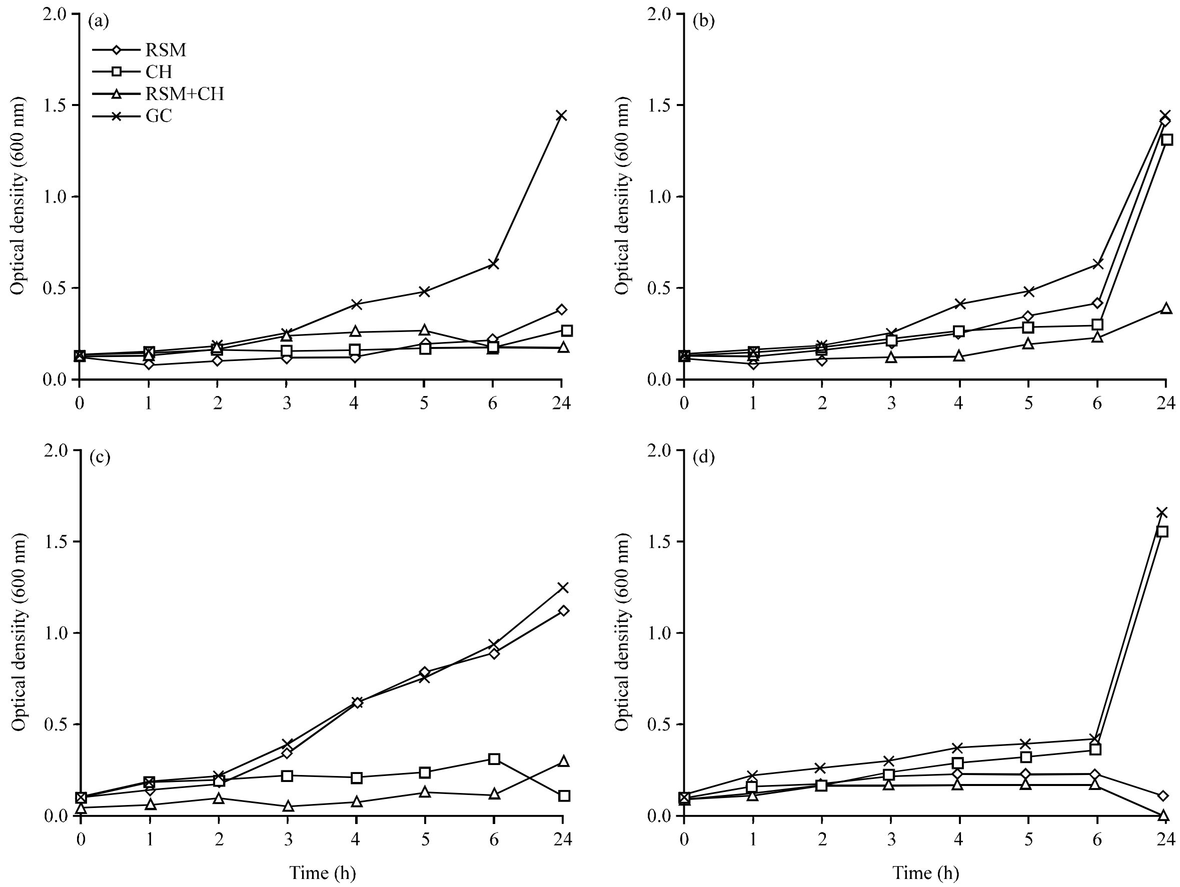

Fig. 2(a-d): Time-kill curve of individual extracts and antibiotics and their combination at 1×MIC concentrations against selected bacteria, PA: *Pseudomonas aeruginosa* ATCC27853, PPi: *Pseudomonas pictorum* NCIB9152, PPu: *Pseudomonas putida* NCIM2872, PS: *Pseudomonas syrigae* NCIM5102 and PT: *Pseudomonas testosterone* NCIM5098

time kill curve to evaluate the antimicrobial activity of medicinal plants, spices and essential oils (Penduka *et al*., 2011; Kurek *et al*., 2012; Lara-Lledo *et al*., 2012; Rakholiya *et al*., 2013b).

CONCLUSION

One way to overcome antibiotic resistance is through the use of new antimicrobial compounds singly or in combination. Amongst all studied synergistic effects, RSM combined with LM showed better synergistic effect and showed bactericidal effect at 3 h against *P. syrigae*. It can be stated that RSM and LM extracts showed better activities and could be considered as one of the sources of natural antibiotics for medicinal use. The study indicated that synergism of RSM and LM may serve as a source of potential adjuvant of antibiotics and food products, to protect them from microorganism spoilage. Additional *in vivo* tests are warranted to fully assess the killing kinetics of extracts in the presence of a competent immune system. The results of this study indicate that it should be relatively easy to utilize antimicrobial extracts in future treatments for multi drug resistance strains.

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