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Original article

# Antibacterial potential and synergistic interaction between natural polyphenolic extracts and synthetic antibiotic on clinical isolates

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ABSTRACT

Emergence of antimicrobial resistance complicates treatment of infections by antibiotics. This has driven research on novel and combination antibacterial therapies. The present study evaluated synergistic antimicrobial activity of plant extracts and cefixime in resistant clinical isolates. Preliminary susceptibility profiling of antibiotics and antibacterial activity of extracts was done by disc diffusion and microbroth dilution assays. Checker-board, time-kill kinetics and protein content studies were performed to validate synergistic antibacterial activity. Results showed noteworthy quantities of gallic acid (0.24–19.7 μg/mg), quercetin (1.57–18.44 μg/mg) and cinnamic acid (0.02–5.93 μg/mg) in extracts of plants assessed by reverse-phase high performance liquid chromatography (RP-HPLC). Gram-positive (4/6) and Gram-negative (13/16) clinical isolates were intermediately susceptible or resistant to cefixime, which was used for synergistic studies. EA and M extracts of plants exhibited total synergy, partial synergy and indifferent characteristics whereas aqueous extracts did not show synergistic patterns. Time-kill kinetic studies showed that synergism was both time and concentration-dependent (2–8-fold decrease in concentration). Bacterial isolates treated with combinations at fractional inhibitory concentration index (FICI) showed significantly reduced bacterial growth, as well as protein content (5–62 %) as compared to extracts/cefixime alone treated isolates. This study acknowledges the selected crude extracts as adjuvants to antibiotics to treat resistant bacterial infections.



## 1. Introduction

Bacterial infections are generally encountered by people of every age (Akilandeswari and Ruckmani 2016). These infections range from minor sore throat to life threatening tuberculosis and meningitis. Antibiotics are the "revolutionized medicines" that fight against microbes and their therapeutic applications are considered as miracle in the history of medicine (Uddin et al. 2021). Antibiotics including penicillin, cephalosporin, macrolides and fluoroquinolones are mostly prescribed to combat mild to serious bacterial infection (Hamad 2010). Despite their benefit, the irrational use of antibiotics have led to the emergence of antimicrobial resistance (AMR), where infections by resistant bacterial strains are not effectively treated by the current antibiotics (Hutchings et al. 2019). Bacteria can develop resistance to one or multiple antibiotics making it difficult to treat common infections. Approximately, 70 % of the community or hospital-acquired pathogenic bacteria have become resistant to one type of antibiotic (Economou and Gousia 2015). Methicillin-resistant *Staphylococcus aureus* (*MRSA*), vancomycin-resistant *Enterococcus* (*VRE*), multidrug-resistant *Mycobacterium tuberculosis* (*MDR-TB*), multidrug-resistant *Escherichia coli* and *Klebsiella pneumoniae* are posing serious health concerns (Frieri et al. 2017). According to the report of Center for Disease Control (CDC), 2.8 million cases of antibiotic resistant infections emerge per year in US resulting in about 35,000 deaths (Prevention and Control 2020). Another

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report from Asia claimed that a child dies every 9 min because of antibiotic resistant microbial infections and approximately 50,000 newborn babies died from sepsis of resistant microbes (Subramaniam and Girish 2020). European Antimicrobial Resistance Surveillance Network (EARS-Net) during the period of 2015–2019, classified AMR based on antibiotic class, bacterial species and geographical region (Control 2020) pertaining to the seriousness of the issue.

AMR can be intrinsic or acquired depicting the microorganisms' capability to withstand the impact of an antibiotic to which they were susceptible, allowing the microbes to grow and remain alive (Zaman 2017). Bacteria can develop resistance by producing inactivating enzymes (β-lactamases), altering antibiotic targets, reducing cell wall permeability and increasing energy-driven efflux of antibiotics (Alekshun and Levy 2007). Considering the impact of AMR, it is mandatory to either augment the susceptibility of bacteria to current antibiotics or introduce new drugs with excellent efficacy against resistant microorganism.

Medicinal plants are an abundant source of phytochemicals that can be processed into safe and effective antibacterial drugs. This stems from the traditional use of multiple medicinal plants to treat infections. Literature showed significant antibacterial activities of Adiantum capillus-veneris, *Curcuma longa*, *Allium sativum*, Berberis lyceum, Artemisia absinthium, Nerium oleander and Swertia chirata that are also traditionally used to treat infections (Khan et al. 2018). Research on the antibacterial effects of herbal medicines using clinical isolates showed that crude extracts are a viable source of resistance-modifying factors (Abreu et al. 2012). The phytochemicals such as volatile oils, alkaloids, polyphenols, and tannins have well established antibacterial and resistance-modifying potentials (Ayaz et al. 2019). These phytochemicals can alter protein–protein interactions within bacterial cells as well as modulate host-related immunological response consequently interfering with bacterial signal transduction, division and augmenting apoptosis (Gupta and Birdi 2017).

A combination of crude extracts with antibiotics can serve as an alternative approach to overcome bacterial resistance. It is postulated that phytochemicals in crude extracts can synergize or potentiate the bacteriostatic or bactericidal effects of antibiotics. There are chances of the emergence of resistance against herbal products when used alone (Vadhana et al. 2015). Therefore, synergism is preferred to treat those infections where there is multidrug-resistance (MDR) or there are chances of treatment failure with a single drug. It has been reported that ellagic acid glycoside-rich blackberry extract enhanced the antibacterial capacity of several antibiotics against resistant biofilm-associated Staphylococcus aureus (Khan et al. 2018). Thus, crude extracts are the promising reservoir of antibacterial adjuvants for MDR infections (Ayaz et al. 2019).

Following these lines, the aim of the current study was to evaluate the impact of the combination of crude extracts and antibiotics on selected resistant clinical isolates. The plants were selected based on their reported anti-inflammatory, antioxidant, immunomodulatory and antimicrobial properties (Ahmad et al. 2016, Amalraj and Gopi 2017, Karalija et al. 2021, Mahadevi and Kavitha 2020, Majeed et al. 2021, Mancuso 2020). A battery of tests was used to evaluate the individual efficacy as well as the synergism between antibiotics and test extracts. Here, we report the polyphenol content of test extracts by reverse-phase high performance liquid chromatography (RP-HPLC) analysis and potential benefit of extracts as an adjuvant in treating MDR infections. This study provides valuable insight into the potential use of readily available natural products in inhibiting growth of MDR bacteria and potentiating the efficacy of the standard antibiotics. It is significant addition to the emerging research on using medicinal plants for MDR infections.

## 2. Material and methods

### 2.1. Chemicals

Methanol (M), ethyl Acetate (EA), myricetin, gallic acid, quercetin, syringic acid, rutin, catechin, gentisic acid, cinnamic acid, kaempferol, luteolin, coumarin and apigenin were purchased from Merck (Darmstadt, Germany). Cefixime, ciprofloxacin dimethyl sulfoxide (DMSO), nutrient broth, nutrient agar, Coomassie brilliant blue and fetal bovine serum were obtained from Sigma-Aldrich, USA.

### 2.2. Plant collection, identification and extraction

Locally used plants *Mentha (M) longifolia* (PHM-527), *Gentian (G) lutea* (PHM-528), *Nigella (N) sativa* (PHM-529), *Terminalia (T) arjuna* (PHM-530), *Chamomilla (C) recutita* (PHM-531), *Momordica (M) charantia* (PHM-532), *Murraya (M) koenigii* (PHM-533), *Curcuma (C) amada* (PHM-534) and *Terminalia (T) chebula* (PHM-535) were collected from herbalists, Islamabad, Pakistan. Plants were verified by Prof. Dr. Rizwana Aleem Qureshi, Department of Plant Sciences, Quaid-i-Azam University, Islamabad and voucher specimens were submitted to the herbarium of Quaid-i-Azam University under specific deposit numbers (PHM-527–535). All procedures followed the institutional policy. Moreover, the subject plants are commonly used in traditional medicine, are widely available and are not endangered species.

Plants were shade dried, powdered and extracted (750 g in 2250 ml; 24 h) by maceration in methanol, water (A) and ethyl acetate at room temperature in air tight containers. The marc was extracted twice and filtered with Whatmann filter paper No. 1. The filtrate was condensed (rotary evaporator, Buchi, Switzerland) and dried in a vacuum oven (Yamato, Japan) at 45 °C. All extracts were stored at − 20 °C until further use.

#### 2.2.1. Percent extract recovery

The percentage recovery of the dried extract was estimated by using the formula.

$$\text{Extract recovery}(\%) = C/D \times 100$$

C = weight (g) of dried extract and D = weight (g) of powdered plant.

### 2.3. Quantification of polyphenols

Polyphenols in crude extracts were quantified by RP-HPLC by previously reported protocols (Jafri et al. 2014, Nasir et al. 2017) using Agilent Chem Station and Zorbex C8 analytical column (4.6 × 250 nm and 5 µm particle size) equipped with DAD detector (Agilent technologies, Germany). Mobile phases A & B contained acetonitrile: methanol: water: acetic acid in 5:10:85:1 and 40:60:0:1 ratios, respectively. Mobile phases were run at a flow rate of 1 ml/min. The mobile phase was eluted at different gradients. In terms of mobile phase B, the concentration was changed from 0 to 50 % B over a period of 0–20 min. it was followed by gradient of 50–100 % B for, 20–25 min and lastly, 100 % B was run for 25–30 min. Extracts (20 µl; 10 mg/ml) were filtered using 0.45-µm membrane filters and injected into the column with 10 min reconditioning phase between the two samples. Phenolic standards including myricetin, gallic acid, quercetin, syringic acid, rutin, catechin, gentisic acid, cinnamic acid, kaempferol, luteolin, coumarin and apigenin were prepared in concentrations of 10, 20, 50, 100, 200 µg/ml in methanol. The UV absorption spectra of samples were recorded at 368 nm (myricetin, kaempferol and quercetin and), 325 nm (gentisic acid, ferulic acid, cinnamic acid, apigenin, caffeic

acid), 279 nm (coumaric acid, emodin, catechin, gallic acid) and 257 nm (rutin, vanillic acid). Polyphenols were quantified as μg/mg of sample from the calibration curve. The detection limits were calculated by formula 3.3 × (σ/b) where σ and b stand for the standard deviation of response and slope of the calibration curve, respectively.

### 2.4. Antimicrobial assessment

#### 2.4.1. Cultures and bacterial isolates

Clinical isolates of resistant Gram-negative [*K. pneumonia, E. coli, Acinetobacter, Pseudomonas aeruginosa*] and Gram-positive (*MRSA, S. aureus (S.A), Staphylococcus haemolyticus (Rsh)]* bacteria were cultured on nutrient agar at 37 °C and stored at 4 °C in the laboratory. Antimicrobial studies were conducted after approval from the Ethical committee of Quaid-i-Azam University (No. BEC-FBS-QAU2021-259).

#### 2.4.2. Primary resistance characterization of antibiotics by disc diffusion method

The resistance profile of five antibiotics including ciprofloxacin, clarithromycin, lincomycin, cefixime and doxycycline was scrutinized against resistant clinical isolates of Gram-negative and Gram-positive bacteria by disc diffusion method (Fakruddin et al. 2017). The bacterial culture was refreshed by incubating the inoculum for 24 h at 37 °C and turbidity was confirmed as per McFarland 0.5 turbidity standard. Sterile nutrient agar plates were swabbed with bacterial culture. Discs soaked with 5 μl of antibiotic solution (4 mg/ml in DMSO) were placed on the surface of nutrient agar plates and incubated at 37 °C for 24 h. Zone of inhibition (ZOI) around each disc was measured by the vernier caliper. The assay was performed thrice. Antibiotics that showed ≤ 14 or no ZOI against most of the isolates indicated resistance and were selected for further experiments.

### 2.5. Antibacterial activity of crude extracts

#### 2.5.1. Disc diffusion method

Next, the antibacterial activity of crude extracts of selected plants was evaluated by the disc diffusion method (Fakruddin et al. 2017). Extract (5 μl; 20 mg/ml) soaked filter paper discs were placed on sterile agar plates for 24 h at 37 °C and ZOI was measured. Analysis was carried out in triplicate. DMSO and ciprofloxacin (4 mg/ml) served as positive and negative controls, respectively.

#### 2.5.2. Microbroth dilution method

Based on the result from the disc diffusion assay, extracts exhibiting ZOI ≥ 12 mm were further screened to find minimum inhibitory concentration (MIC) by microbroth dilution method (Malik et al. 2022). Bacterial inoculum ($5 \times 10^4$ CFU/ml; 195 μl) was put in each well of 96-well plate containing twofold serial dilutions of extracts (100, 50, 25, and 12.5 μg/ml) and antibiotics (10, 5, 2.25 and 1.125 μg/ml). A zero-hour reading was taken by measuring the absorbance (600 nm) after 30 min of incubation. Later, the plate was incubated for 24 h at 37 °C and absorbance was recorded again.

### 2.6. Assessment of synergism between test extracts and antibiotics

The integrated antibacterial effect of selected extracts and an antibiotic (cefixime) was determined by the checkerboard microdilution method as described earlier with minor modifications (Dra et al. 2017). In a 96-well plate, all the extracts were diluted (2-fold) horizontally and antibiotics were diluted (2-fold) vertically in such a way that individual columns and rows represented different proportions of the two samples. MIC value of each sample was considered as its initial concentration. An aliquot of 5 μl of sample (2.5 μl of antibiotic and 2.5 μl of extract) and 195 μl of inoculum ($4 \times 10^4$ CFU/ml) were pipetted in 96-well plate and incubated for 24 h at 37℃. The plate was examined visually for the presence of bacterial growth. The concentration of combination (extract and cefixime) in the checkerboard with no visual growth was regarded as MIC (Singh and Katoch 2020). The assay was performed thrice. The value of fractional inhibitory concentration index (FICI) was calculated by applying the given formula (Dra et al. 2017).

$$\mathrm{FICI} = \frac{\mathrm{MICI\ A/B}}{\mathrm{MIC\ A}} + \frac{\mathrm{MICI\ B/A}}{\mathrm{MIC\ B}}$$

Where MIC A and MIC B are MIC of extract and antibiotic, respectively when used alone.

MICI A/B = MIC of extract in combination with antibiotic and MICI B/A = MIC of antibiotic in combination with extract. The results were interpreted as given in Supplementary Table 1.

### 2.7. Time-kill kinetics

Time-kill kinetics was evaluated using the reported protocol (Das et al. 2017). All resistant bacterial isolates were grown and diluted ($4 \times 10^4$ CFU/ml) into mid-logarithmic phase. Then, the diluted bacterial suspension was incubated at 37℃ with MIC, 2MIC, FICI and 2FICI concentrations of test extract alone and in combination with cefixime. Absorbance was measured (600 nm) at the time intervals of 0, 3, 6, 9, 12 and 24 h. Growth of bacteria was observed by plotting a graph between the absorbance value and time interval as per procedure described by Wani *et al* (Wani et al. 2017).

### 2.8. Estimation of bacterial protein

Protein content of bacterial samples was quantified by the Bradford method after treatment with extracts alone or in combination with cefixime to assess the possible mechanism of bacterial growth inhibition (Nouroozi et al. 2015). Bacterial inoculum was treated with MIC, 2MIC, FICI and 2FICI values of the extract alone or in combination cefixime and incubated at 37 °C for 24 h. Each sample was centrifuged for 5 min at 3000 rpm and the bacterial pellet was separated and placed at −4°C for 48 h. Phosphate buffer, bovine serum albumin (BSA; 1 mg/ml; 0–50 μg/ml), distilled water and bacterial inoculum were used as diluent, positive control, negative control and blank, respectively.

Bacterial pellet of all isolates was washed thrice with phosphate buffer, suspended in 20 μl of the buffer and sonicated for 10 min. An aliquot of 5 μl of suspension and 195 μl of the Bradford reagent (1:4) were added into 96-well plate. Change in the color of the reagent to blue or purplish-blue is proportional to protein content. The plate was incubated at room temperature for 5 min with continuous sonication and absorbance was measured. Protein content of all samples was determined from the calibration curve by applying the given formula:

$$\text{Absorbance of unknown sample}(x) = (\text{Absorbance} - \mathrm{b})/\mathrm{m}$$

The whole process was repeated thrice.

### 2.9. Statistical analysis

Statistical analysis was performed using Graph-Pad Prism 9 where data are presented as mean ± SD (n = 3) of respective values. ANOVA was applied to analyzed results that were considered significant at $p < 0.05$.

## 3. Results

### 3.1. Percent extract recovery

Overall, extraction yield was variable in different polarity solvents and ranged from 1.94 to 15 %. Maximum extract recovery was observed in M extract of *G. lutea* (15.03 ± 0.42 % w/w), whereas *M. charantia* A and *C. recutita* A extracts exhibited percentage recovery of 8.06 ± 0.30 % w/w and 9.68 ± 0.52 %, respectively. On the contrary, EA showed least extract recovery with values of 7.2 66 ± 0.38 % and 1.94 ± 0.22 % w/w in *G. lutea* and *M. charantia* extracts, respectively.

### 3.2. Quantification of polyphenols

Polyphenols in test extracts were quantified by RP-HPLC procedure by comparing UV and retention time of 16 reference standards with extracts (Supplementary Table 2). Results of HPLC-DAD analysis (Supplementary Table 3) showed significant quantities of gallic acid, quercetin and luteolin in analyzed extracts. *C. amada* (A) exhibited maximum gallic acid (19.97 µg/mg). *M. koenigii* A and *C. amada* M extracts have 5.93 and 4.78 µg/mg, respectively of cinnamic acid. Chromatograms of detected phenols and standards are presented in Fig. 1(a-f). This indicates that variable polarity solvents affect the extracted quantities of polyphenols from each plant.

### 3.3. Evaluation of antibacterial potential

#### 3.3.1. Primary resistance characterization of antibiotics

Initially, five antibiotics including cefixime, ciprofloxacin, clarithromycin, lincomycin and doxycycline were screened against clinical isolates of Gram-positive (*MRSA, S. aureus and S. haemolyticus*) and Gram-negative bacteria *(E. coli, K. pneumoniae, Acineto bacter and P. aeruginosa*). According to CLSI guidelines, bacterial susceptibility is categorized as susceptible, intermediate and resistant with ZOI $\geq$ 20, =15–19, $\leq$14, respectively (Khan et al. 2021). Results showed that all clinical isolates were mostly susceptible to antibiotics except cefixime. Except for a few, most clinical isolates were either intermediately susceptible or resistant to cefixime (Supplementary Tables 4 & 5) with ZOI ranging between 0 and 17 mm and 0–19 mm for Gram-positive (4/6) and Gram-negative bacteria, respectively (13/16). Thus, cefixime was used for further evaluation of synergism with plant extracts.

#### 3.3.2. Antibacterial activity of crude extracts

The disc diffusion method was used to explore the antibacterial potential of crude extracts against several bacterial isolates. It was observed that the crude extracts (100 µg/disc) showed mild to intermediate activity against some bacterial isolates with zero activity against others (Supplementary Tables 6 & 7). Antibacterial profile is variable depending on the plant, extraction solvent and nature of clinical isolate. EA extracts of *N sativa* (20 ± 1.4 mm) and *M. charantia* (15 ± 1.1 mm) depicted significant ($p < 0.05$) antibacterial activity against *S.A* (3,3). *S.A* (3,4) has intermediate susceptibility to EA and M extracts of *C. amada* with ZOI values of 15 ± 0.6 mm and 16 ± 0.2 mm, respectively. Likewise, *S.A* (3,1) is susceptible to *M. charantia* M and *C. amada* M extracts (Supplementary Table 6).

Among all the tested isolates of Gram-negative bacteria, greatest antibacterial activity was demonstrated against *E.C* (7C) by M and EA extract of *C. amada* with ZOI of 22 ± 0.9 and 18 ± 1.2 mm, respectively. *C. amada* M extract also exhibited intermediate activity against *K.P* (6) and *K.P* (K) with ZOI of 16 mm. *N. sativa* M and EA extracts showed highest antibacterial activity against *A.B* (29) (19 ± 0.4 mm) and *RPA* (18 ± 0.8 mm). *E.C* (80-A) growth was inhibited by *M. charantia* EA (13 ± 0.2 mm) and *T. chebula* EA (18 ± 0.9) and M (15 ± 0.3) extracts (Supplementary Table 7). Cefixime showed either intermediate susceptibility or resistance towards clinical isolates with few exceptions.

#### 3.3.3. Determination of MIC

After the evaluation of the antibacterial potential of the test extracts, the MIC of antibiotics and extracts was determined by microbroth dilution method. The extracts that showed ZOI $\geq$ 12 mm were subjected to MIC evaluation using twofold serial dilutions (12.5–100 µg/ml). Assay (n = 3) was interpreted based on turbidity of the medium (Table 1). Results indicated that most of the test extracts demonstrated a MIC value of 50 µg/ml against selected Gram-positive bacteria. Against *S.A* (3,3), the MIC value of 50 µg/ml was recorded for *N. Sativa* EA and *C. recutita* M extracts, whereas *M. Charantia* EA displayed a MIC value of 100 µg/ml. Similarly, M extracts of *T. Chebula* and *M. Charantia* exhibited a MIC of 50 µg/ml against *S.A* (3,4) and *S.A* (3,1), respectively. On the contrary, most of the plant extracts showed a MIC value of 100 µg/ml against Gram-negative isolates except for *C. amada* M, *N. sativa* M and *C. amada* EA extracts that displayed the MIC value of 50 µg/ml when tested against *A.B* (29) and *E.C* (7C), respectively.

#### 3.3.4. Assessment of synergism between test extracts and antibiotics

Next, the antibacterial activity of the combination of active extracts and cefixime was evaluated to measure potential synergism between the samples. It was done to assess the possible reduction of AMR as an additional strategy to oppose the infections of multidrug-resistant bacteria. Checkerboard method was applied to examine the synergistic interactions with twofold serial dilutions of samples and the FICI value for each sample was determined.

Comprehensive results of synergistic interaction are given in Table 2. It has been observed that M extract of *C. amada* and *T. chebula* manifested total synergism with FICI value of 0.5 against Gram-positive isolates *S.A* (3,1) and *S.A* (3,4). Moreover, M extract of *C. amada* presented total synergism when tested against *A.B* (29) and *K.P* (K), whereas *N. sativa* M extract revealed total synergism with FICI value of 0.375 in the case of *A.B* (29). Among the EA extracts, *M. charantia* showed synergism with the 4-fold and 8-fold reduction in extract and cefixime concentrations, respectively, against *E.C* (80-A), while *N. sativa* exhibited a 4-fold reduction both in cefixime and extract concentrations in response to *E.C* (7C). Similarly, EA extract of *N. sativa* also presented total synergism (FICI = 0.375) against *RPA*. Furthermore, A extract of *T. chebula* showed total synergy (FICI = 0.375) against *S.A* (3,1) and partial synergism (FICI = 0.75) against *A.B* (29).

### 3.4. Time-kill kinetics

Subsequently, time-kill kinetics was evaluated to see whether the antibacterial impact is dependent on treatment duration or concentration of test samples. Antibacterial activity of extracts with a 4-fold reduction of the MIC values was further estimated at MIC, 2MIC, FICI, and 2FICI concentrations for up to 24 h and the results were plotted exhibiting time-kill kinetics.

#### 3.4.1. Time-kill kinetics of Gram-positive resistant S. aureus (3,1, 3,3 and 3,4) isolates

Results showed that there is overall decline in the bacterial growth over a period of 24 h in extracts or cefixime treated isolates when compared with DMSO (negative control) treated isolates (Fig. 2). *S.A* (3,1) isolates displayed similar pattern of growth reduction when treated with A extract of *T. chebula* (Fig. 2a) and M

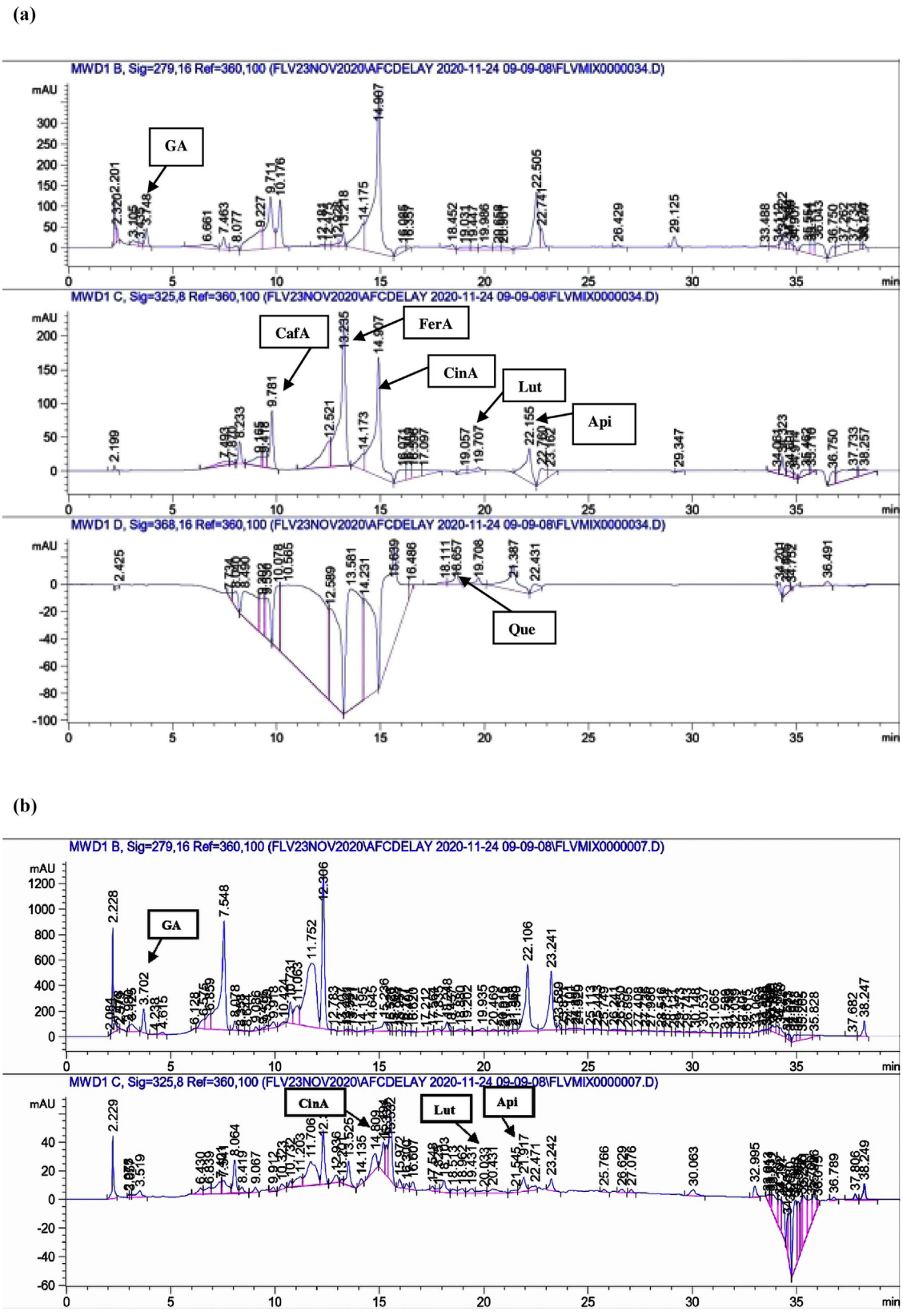



**Fig. 1.** Chromatograms of polyphenols. The figure represents chromatograms obtained from reverse-phase high performance liquid chromatography analysis for **[a]** polyphenolic standards, **[b]** *Curcuma amada* aqueous extract, **[c]** *Murraya koenigii* aqueous extract, **[d]** *Mentha longifolia* methanol extract, **[e]** *Nigella sativa* ethyl acetate extract and **[f]** *Terminalia arjuna* methanol extract.

extract of *C. amada* (Fig. 2b) at MIC concentrations. *S.A* (3,1) exhibited an increase in bacteria growth from 0 to 3 h, with rapid decline at 6 h of treatment. Later, there is a slow increase in growth as compared to DMSO treated isolates. After 9 h of treatment, the bac-

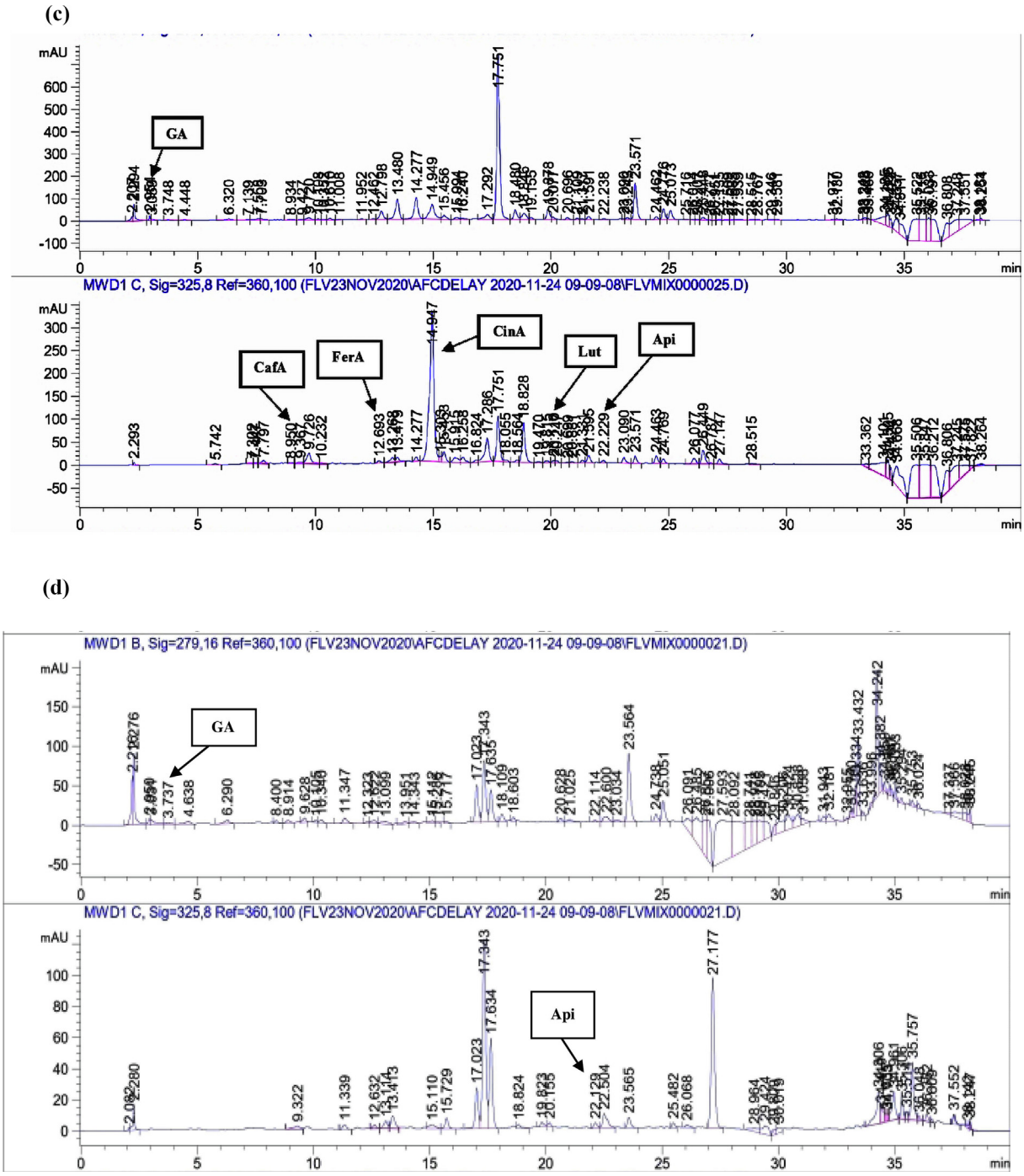



**Fig. 1** (*continued*)

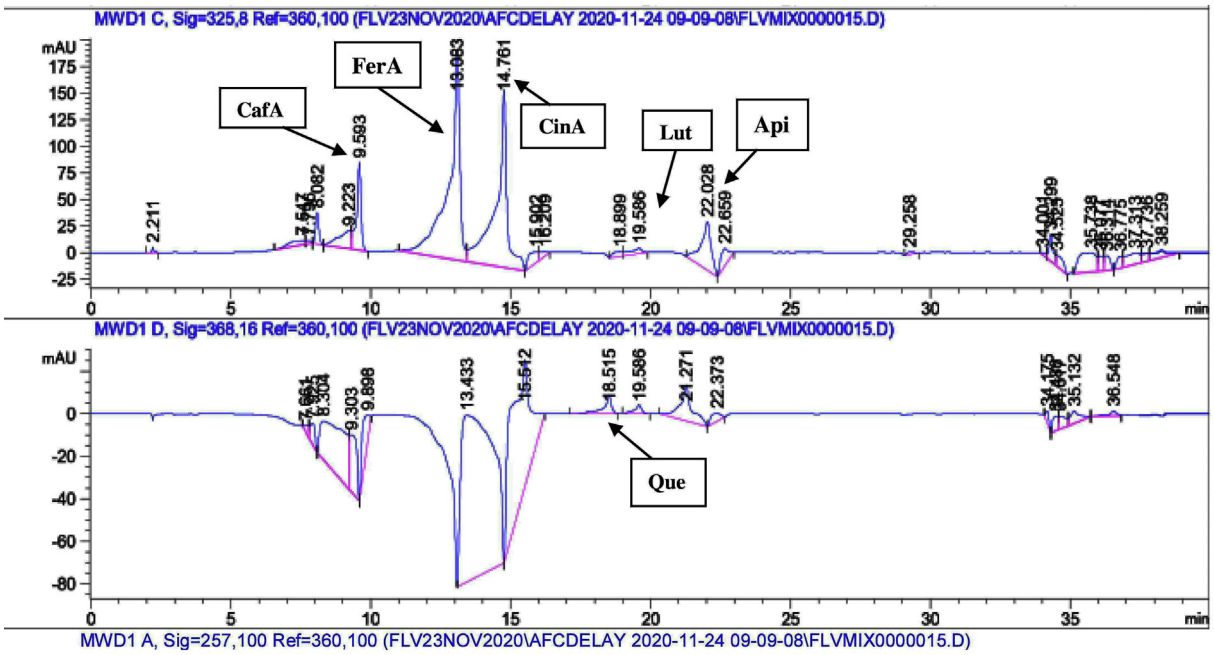
teria can be identified to be present in stationary phase. Likewise, *N. sativa* EA extract tested against *RSA* (3,3) showed significant ($p < 0.05$) decrease in bacterial growth at 6 h when tested alone at MIC (Fig. 2c). A steady increase in growth was observed up to 24 h; yet, it was significantly ($p < 0.05$) lower than DMSO treated isolates. Similar pattern of growth reduction was observed in *C. amada* (Fig. 2d) and *T. chebula* (Fig. 2e) treated *RSA* (3–4) isolates.

Overall, the 2MIC concertation was more effective than MIC. The combination of extracts with cefixime was more effective than extract or cefixime alone. It was observed that bacterial growth persistently declined from 3 to 12 h showing synergism between cefixime and plant extracts. The 2FICI showed almost similar activity as compared to ciprofloxacin (10 µg; positive control) where there was maximum inhibition of bacterial growth as compared to DMSO treated or extract/cefixime alone treated isolates.

*3.4.2. Time-kill kinetics of resistant Gram-negative isolates*

*3.4.2.1. Time-kill kinetics of resistant E. Coli (7C and 80-A) strain.* Time-kill values of *E.C* (7C) strain showed that isolates treated with FICI and 2FICI of combination of *N. Sativa* EA extract and cefixime were more active as compared to isolates treated with extract or cefixime alone. Overall, treatments significantly ($p < 0.05$) inhibited bacterial growth as compared to DMSO treated isolates. Furthermore, growth inhibition pattern of 2FICI treated *E.C* (7C) was

(e)




(f)

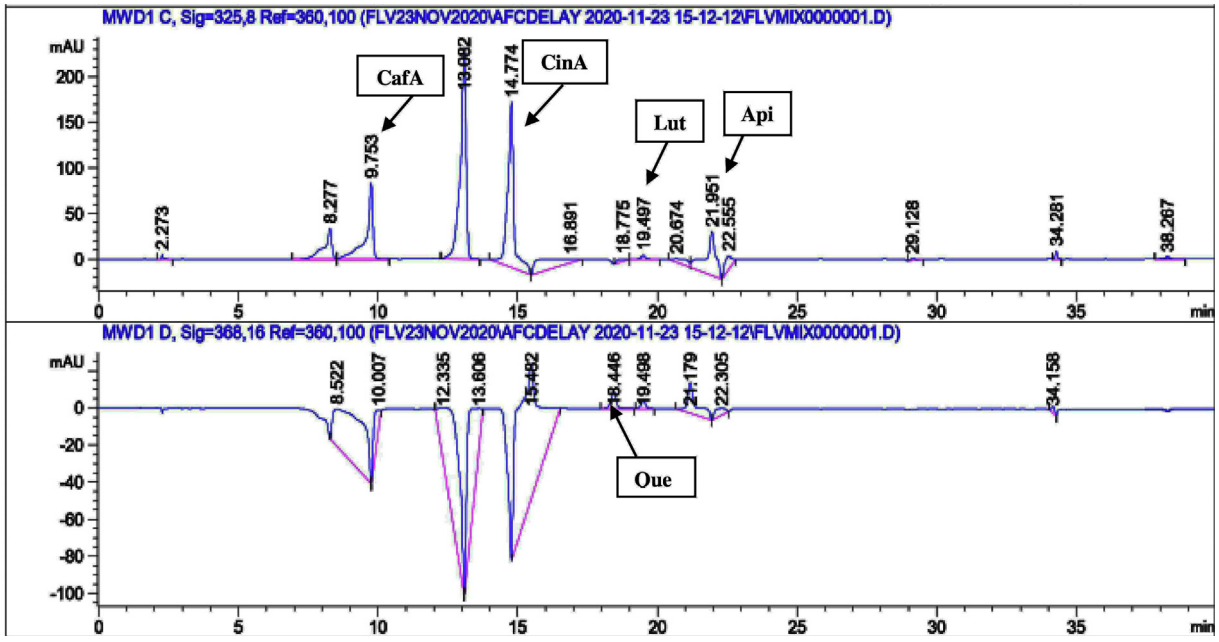



**Fig. 1** (*continued*)

comparable with ciprofloxacin (10 μg) (Fig. 3a). Thus, it can be concluded that due to synergism between extract and cefixime, the bacteria are lodged in the stationary phase.

Subsequently, M extract of *T. chebula* M inhibited the growth of *E.C* (80-A) at FICI and 2FICI concentrations where maximum inhibitory response was displayed at 9 h. Although, cefixime alone showed maximum inhibition at 6 h; however, *E.C* (80-A) proliferated more after 6 h in cefixime alone treated samples as compared to 2FICI treated samples (Fig. 3c).

Furthermore, the time-kill kinetic curves of *M. charantia* EA extract at MIC/2MIC demonstrated a decline in growth at 6–12 h, with a steady growth phase from after 12 h, which was still significantly ($p < 0.05$) lower than DMSO treated isolates (Fig. 3b). Yet, the rate of growth in FICI/2FICI treated isolates was significantly ($p < 0.05$) lower as compared to extract or cefixime alone treated *E.C* (80-A) isolates (Fig. 3b). Concisely, there is both time and concentration-dependent synergism between extracts and cefixime that has more effectively inhibited bacterial growth.

**Table 1**
MIC values of extracts against resistant clinical isolates.

| Sr. no | Extracts | MICs of the extracts and drugs against Gram positive isolates (µg/ml) | | | MICs of the extracts and drugs against Gram negative isolates (µg/ml) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *S.A* (3,1) | *S.A* (3,3) | *S.A* (3,4) | *A.B* (29) | *E.C* (80-A) | *E.C* (7C) | *K.P* (6) | *K.P* (K) | *RPA* |
| 1 | **NS(EA)** | – | 50 | – | – | – | 100 | – | – | 100 |
| 2 | **NS(M)** | – | – | – | 50 | – | – | – | – | – |
| 3 | **CR(M)** | – | 50 | – | – | – | – | – | – | – |
| 4 | **MC(EA)** | – | 100 | – | – | 100 | – | – | – | – |
| 5 | **MC(M)** | 50 | – | – | – | – | – | – | – | – |
| 6 | **CA(EA)** | – | – | 100 | – | – | 50 | 100 | – | – |
| 7 | **CA(M)** | 100 | – | 100 | 50 | – | 100 | 100 | 100 | – |
| 8 | **TC(EA)** | | – | – | – | 100 | – | – | – | – |
| 9 | **TC(M)** | 100 | – | 50 | – | 100 | – | – | – | 100 |
| 10 | **TC(A)** | 100 | – | – | 100 | – | – | – | – | – |
| 11 | **Cefixime** | 25 | 25 | 25 | 25 | 30 | 30 | 50 | 50 | 100 |
| 12 | **Ciprofloxacin** | 2.5 | 2.5 | 1.25 | 2.5 | 1.25 | 1.25 | 5.00 | 1.25 | 1.25 |

Values (mean ± SD) are average of triplicate analysis of each plant extract ($n$ = 3). EA = ethyl acetate, M = methanol. A = aqueous and – = No Activity. *E.C* = *E. coli, K. P* = *K. pneumonia*, *A. B*= *Acineto bacter* and *RPA* = resistant *P. aeruginosa*.

**Table 2**
MIC values of extract/cefixime alone and combination.

| Sr. no | Clinical isolates | Samples | MIC | MICI Combination (ug/ml) | Red. Fold | FICI | Result |
|---|---|---|---|---|---|---|---|
| 1. | ***S.A* (3,1)** | TC(A) | 100 | 25 | 4 | 0.375 | Synergy |
| | | Cefixime | 25 | 3.125 | 8 | | |
| | | TC(M) | 100 | 50 | 2 | 0.75 | Partial synergy |
| | | Cefixime | 25 | 6.25 | 4 | | |
| | | CA(M) | 100 | 25 | 4 | 0.375 | Synergy |
| | | Cefixime | 25 | 3.125 | 8 | | |
| | | MC(M) | 50 | 25 | 2 | 0.75 | Partial synergy |
| | | Cefixime | 25 | 6.25 | 4 | | |
| 2. | ***S.A* (3,3)** | CR(M) | 50 | 6.25 | 8 | 0.625 | Partial synergy |
| | | Cefixime | 25 | 12.5 | 2 | | |
| | | NS(EA) | 50 | 12.5 | 4 | 0.375 | Synergy |
| | | Cefixime | 25 | 3.125 | 8 | | |
| | | MC(EA) | 100 | 50 | 2 | 0.75 | Partial synergy |
| | | Cefixime | 25 | 6.25 | 4 | | |
| 3. | ***S.A* (3,4)** | CA(M) | 100 | 25 | 4 | 0.5 | Synergy |
| | | Cefixime | 25 | 6.25 | 4 | | |
| | | CA(EA) | 100 | 50 | 2 | 0.75 | Partial synergy |
| | | Cefixime | 25 | 6.25 | 4 | | |
| | | TC(M) | 50 | 12.5 | 4 | 0.5 | Synergy |
| | | Cefixime | 25 | 6.25 | 4 | | |
| 4. | ***A.B* (29)** | NS(M) | 50 | 12.5 | 4 | 0.375 | Synergy |
| | | Cefixime | 25 | 3.125 | 8 | | |
| | | CA(M) | 50 | 12.5 | 4 | 0.5 | Synergy |
| | | Cefixime | 25 | 6.25 | 4 | | |
| | | TC(A) | 100 | 50 | 2 | 0.75 | Partial Synergy |
| | | Cefixime | 25 | 6.25 | 4 | | |
| 5. | ***E.C* (80-A)** | TC(EA) | 100 | 50 | 2 | 1 | In different |
| | | Cefixime | 30 | 15 | 2 | | |
| | | TC(M) | 100 | 25 | 4 | 0.5 | Synergy |
| | | Cefixime | 30 | 7.5 | 4 | | |
| | | MC(EA) | 100 | 25 | 4 | 0.375 | Synergy |
| | | Cefixime | 30 | 3.75 | 8 | | |
| 6. | ***E.C* (7C)** | NS(EA) | 100 | 25 | 4 | 0.5 | Synergy |
| | | Cefixime | 30 | 7.5 | 4 | | |
| | | CA(EA) | 50 | 25 | 2 | 0.625 | Partial synergy |
| | | Cefixime | 30 | 3.75 | 8 | | |
| | | CA(M) | 100 | 25 | 4 | 0.75 | Partial synergy |
| | | Cefixime | 30 | 15 | 2 | | |
| 7. | ***K.P* (6)** | CA(EA) | 100 | 50 | 2 | 1 | In different |
| | | Cefixime | 50 | 25 | 2 | | |
| | | CA(M) | 100 | 50 | 2 | 0.75 | Partial synergy |
| | | Cefixime | 50 | 12.5 | 4 | | |
| 8. | ***K.P* (K)** | CA(M) | 100 | 12.5 | 8 | 0.25 | Synergy |
| | | Cefixime | 50 | 6.25 | 8 | | |
| 9. | ***RPA*** | NS(EA) | 100 | 25 | 4 | 0.375 | Synergy |
| | | Cefixime | 100 | 12.5 | 8 | | |
| | | TC(M) | 100 | 50 | 2 | 0.75 | Partial synergy |
| | | Cefixime | 100 | 25 | 4 | | |

MIC= Minimum inhibitory concentrations, FICI= Fractional inhibitory concentration index, EA = ethyl acetate, M = methanol, DW = distilled water. FICI $\leq$ 0.5 = Total Synergism, 0.5 < FICI $\leq$ 1 = additive, 1 < FICI $\leq$ 2 = No Effect, FICI > 2 = Antagonism.

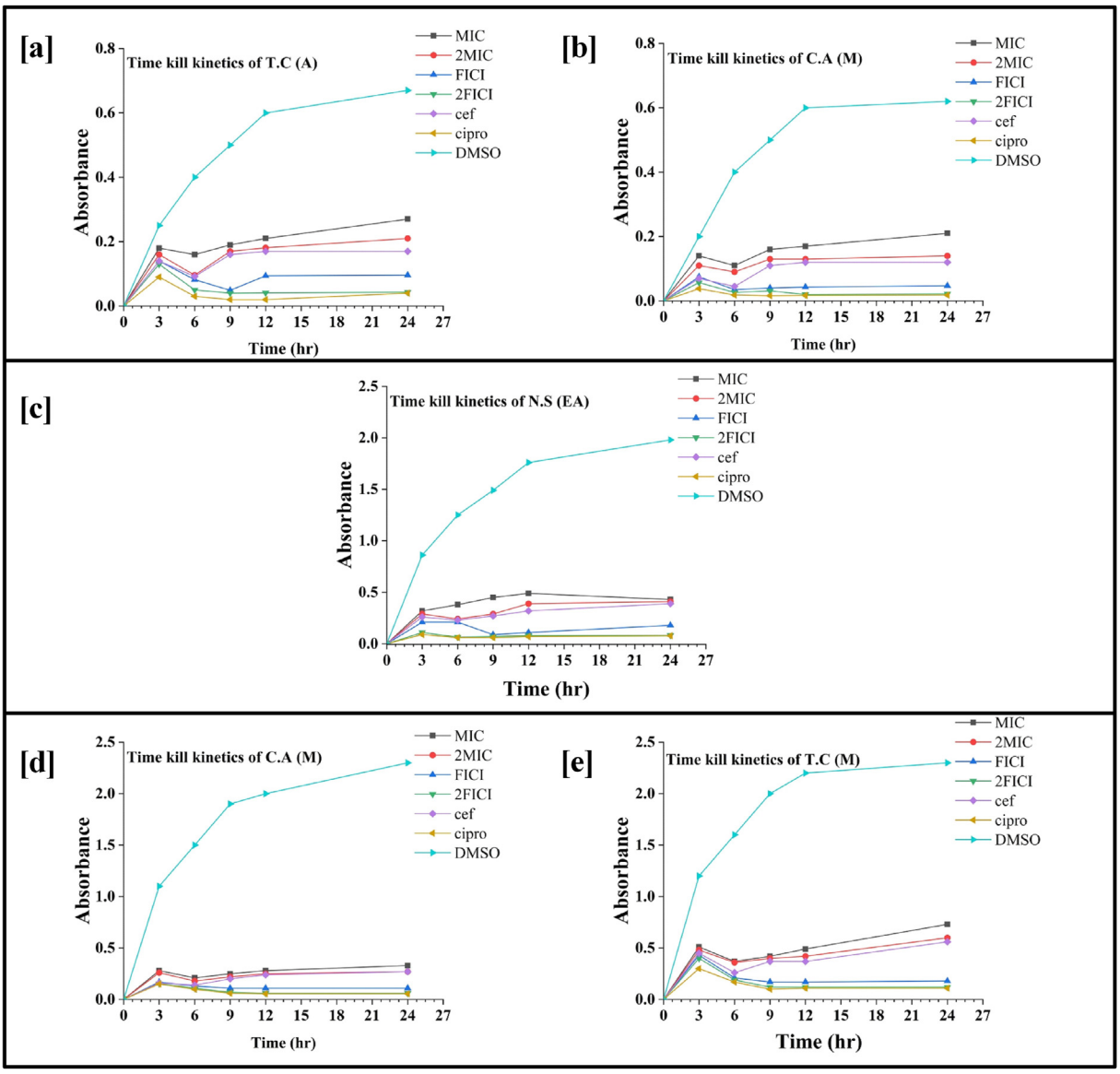



**Fig. 2.** Time-kill kinetic curves for *S. aureus*. Time-kill kinetics curve of **[a]** *T. chebula* (T.C) A and **[b]** *C. amada* (C.A) M extracts against *S.A* (3,1). **[c]** Time-kill kinetics curve of *N. sativa* (N.S) EA extract against *S.A* (3,3). Time-kill kinetics curve of **[d]** *C. amada* (C.A) M and **[d]** *T. chebula* (T.C) M extracts against *S.A* (3,1). FICI (Fractional inhibitory concentration index at single concentration) and 2FICI (Fractional inhibitory concentration index at double concentration), DMSO: dimethyl sulfoxide, MIC and 2MIC are Minimum inhibitory concentration at single and double concentration.

*3.4.2.2. Time-kill kinetics of resistant P. Aeruginosa strain.* All concentrations of *N. Sativa* EA extract, cefixime and combination significantly ($p < 0.05$) inhibited the growth of *RPA* as compared to DMSO treated isolates (Fig. 4). *N. Sativa* EA extract at MIC and 2MIC concentrations inhibited the bacterial growth till 9 h, after which there were either growth increments or a stationary growth phase. Likewise, cefixime alone (at MIC) inhibited bacterial growth till 6 h with gradual increase till 24 h, though less than the extract alone. On the other hand, cefixime when paired with the plant extract (FICI/2FICI), kept the *RPA* isolates in stationary phase with minimum growth that was comparable with ciprofloxacin (10 μg) (Fig. 4).

*3.4.2.3. Time-kill kinetics of K. Pneumoniae (K) strain.* Results of time-kill kinetics of *K.P (K)* shows that M extract of *C. amada* at its MIC and 2MIC significantly ($p < 0.05$) inhibited the bacterial growth as compared to DMSO treated samples (Fig. 5). After 3 h of an initial growth spurt, there is a minimum growth rate in *K.P (K)* isolates as compared to the control. Likewise, cefixime alone inhibited bacteria from 0 to 3 h and growth increased from 3 to 24 h. It was observed that FICI and 2FICI concentrations of extract/cefixime combination displayed synergism and suppressed the bacterial growth from 0 to 6 h with a slight increase up to 24 h (Fig. 5). Yet, the growth rate was much lower than negative control and extract/cefixime alone treatments. In short, the combination of extracts and cefixime has enhanced antibacterial activity against resistant bacterial isolates in this experiment.

*3.4.2.4. Time-kill kinetics of Acinetobacter (29) strain. A.B* (29), a gram-negative strain when treated with the M extract of *N. sativa* alone at MIC and 2MIC concentrations, showed inhibition after 3 h and the maximum result of growth inhibition was observed at of 9 h (Fig. 6a). A similar response was observed in cefixime treated isolates. There was synergistic relationship between cefixime and *N. sativa* extract where both FICI and 2FICI constantly inhibited *A. B* (29) growth for 24 h similar to ciprofloxacin. Furthermore,

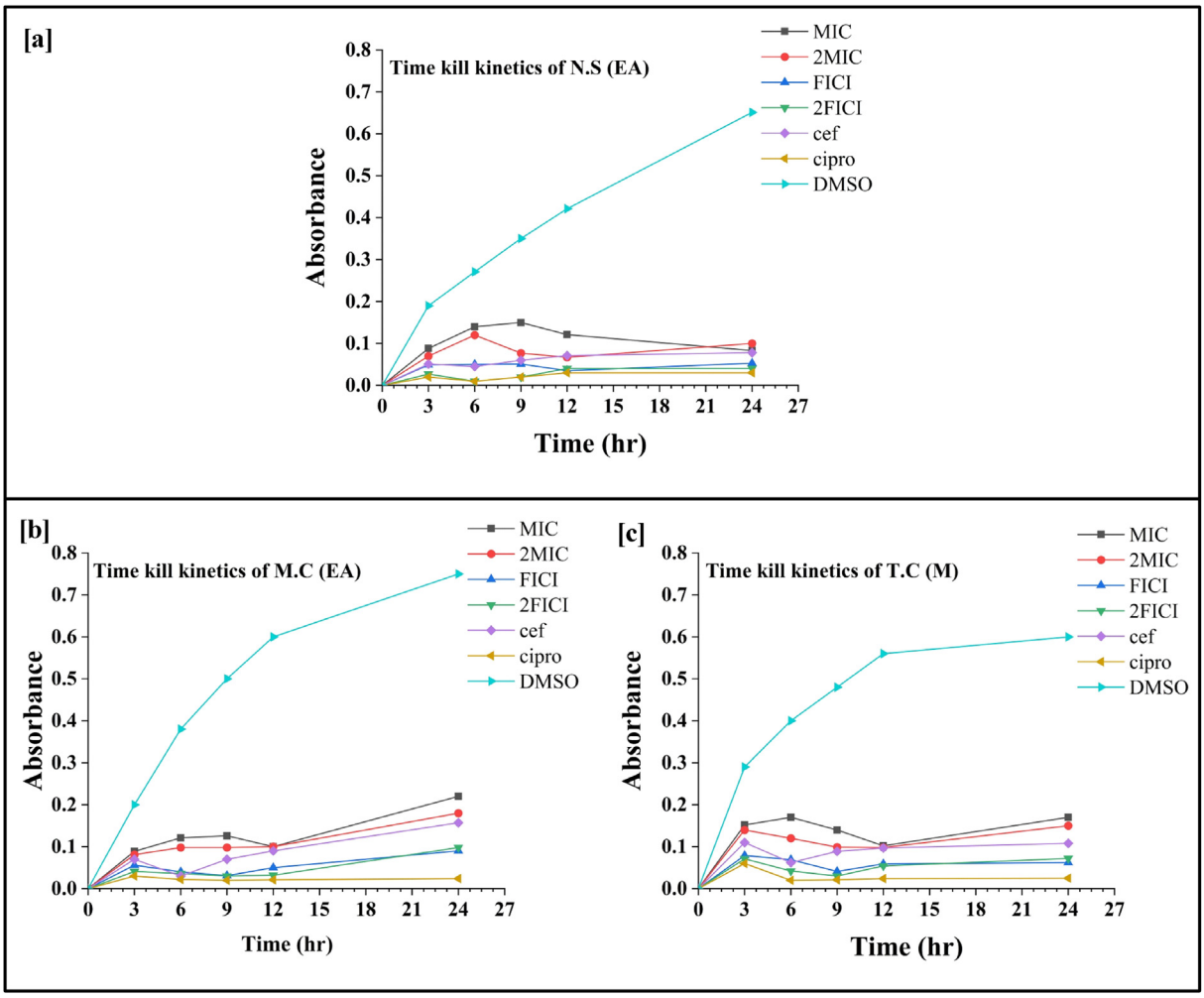



**Fig. 3.** Time-kill kinetic curves for *E. Coli.* **[a]** Time-kill kinetics curve of *N. sativa* (N.S) EA extract against *E.C* (7C). Time-kill kinetics curve of **[b]** *M. charantia* (M.C) EA and **[c]** *T. chebula* (T.C) M extracts against *E.C* (80-A). FICI (Fractional inhibitory concentration index at single concentration) and 2FICI (Fractional inhibitory concentration index at double concentration), DMSO: dimethyl sulfoxide, MIC and 2MIC are Minimum inhibitory concentration at single and double concentration.

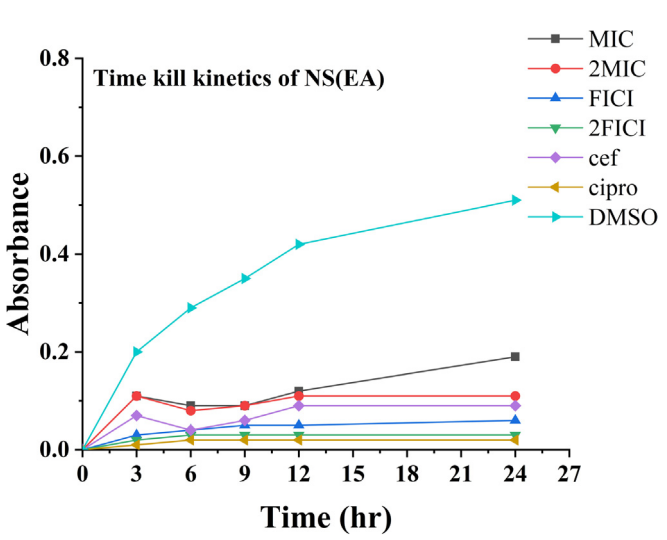



**Fig. 4.** Time-kill kinetics curve of *N. sativa* (N.S) EA extract against *RPA.* FICI (Fractional inhibitory concentration index at single concentration) and 2FICI (Fractional inhibitory concentration index at double concentration), DMSO: dimethyl sulfoxide, MIC and 2MIC are Minimum inhibitory concentration at single and double concentration.

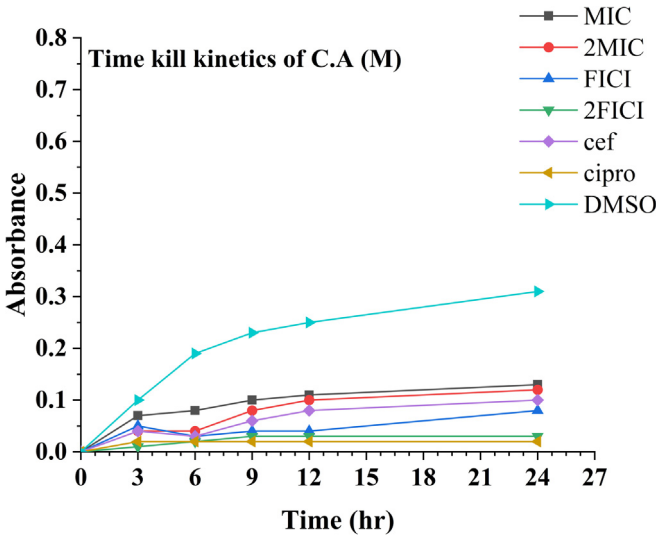



**Fig. 5.** Time-kill kinetics curve of *C. amada* (C.A) M extract against *KP* (K). FICI (Fractional inhibitory concentration index at single concentration) and 2FICI (Fractional inhibitory concentration index at double concentration), DMSO: dimethyl sulfoxide, MIC and 2MIC are Minimum inhibitory concentration at single and double concentration.

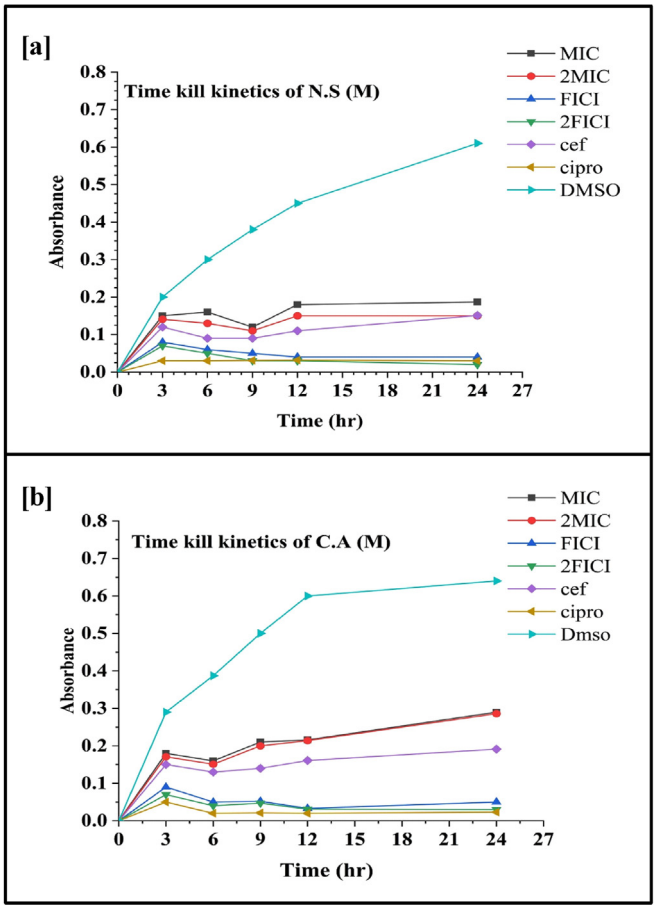



**Fig. 6.** Time-kill kinetics curve of **[a]** *N. sativa* (N.S) M and **[b]** *C. amada* (C.A) M against *A.B* (29). FICI (Fractional inhibitory concentration index at single concentration) and 2FICI (Fractional inhibitory concentration index at double concentration), DMSO: dimethyl sulfoxide, MIC and 2MIC are Minimum inhibitory concentration at single and double concentration.

growth of *A.B* (29) treated by M extract of *C. amada* increased up to 3 h and then gradually declined from 6 to 24 h at FICI and 2FICI (Fig. 6b). The synergism between cefixime and *C. amada* extract was more remarkable with significant ($p < 0.05$) antibacterial activity as compared to alone treatments (Fig. 6).

### 3.5. Protein estimation

Disintegration of the cell membrane causes oozing out of cellular protein during the process of cellular death. Quantification of proteins within an extracellular medium of untreated and treated bacterial isolates represents the damage to cell membrane and is used as an indicator of the mechanism of antibacterial potential. The effect of antibiotics and extracts on bacterial protein was estimated by the Bradford reagent. Proteins were quantified from the calibration curve of bovine serum albumin (positive control) using straight line equation y = 0.0168x + 0.0076, where x" is unknown protein content and "y" is sample absorbance.

#### 3.5.1. Protein estimation for Gram-positive isolates

The results of Gram-positive isolates are given in Table 3. It was observed that there was a reduction in the protein content of *S.A* isolates after treatment with extracts alone or in combination with cefixime. There was 11.54 %, 11.55 % and 54.9 % reduction in protein content of *S. A* (3,1), *S. A* (3,3) and *S. A* (3,4), respectively when treated with cefixime alone. On the contrary, the percentage decline in protein content was more in isolates treated with the combination of extracts and cefixime. Maximum reduction in protein content was observed in *S. A* (3,1), *S. A* (3,3) and *S. A* (3,4) when treated with *T. chebula* A (58.79 %), *N. sativa* EA (54.74 %) and *T. chebula* M (56.59 %) extracts, respectively in combination with cefixime.

**Table 3**
Concentration of protein in samples treated resistant clinical isolates.

| Samples treated with *S.A* | MIC or FICI (µg/ml) | Concentration of protein in µg | %Protein inhibition |
|---|---|---|---|
| **Gram-positive** | | | |
| **TC(A) (3,1)** | 100 | 28.83 | 47.25 |
| **CA(M) (3,1)** | 100 | 36.75 | 32.77 |
| **MC(M) (3,1)** | 50 | 41.15 | 24.71 |
| **TC(A) + cef (3,1)** | 25 + 3.125 | 27.94 | 58.79 |
| **CA(M) + cef (3,1)** | 25 + 3.125 | 22.52 | 48.88 |
| **MC(M) + cef (3,1)** | 25 + 6.25 | 34.13 | 17.18 |
| **Cefixime** | 25 | 48.35 | 11.54 |
| **Control** | – | 54.66 | – |
| **NS(EA) (3,3)** | 50 | 27.58 | 33.07 |
| **NS(EA) + cef (3,3)** | 12.5 + 3.125 | 24.84 | 54.74 |
| **Cefixime** | 25 | 36.45 | 11.55 |
| **Control** | – | 41.21 | – |
| **CA(M) (3,4)** | 100 | 39.48 | 28.07 |
| **TC(M) (3,4)** | 50 | 32.88 | 40.11 |
| **CA(M) + cef (3,4)** | 25 + 6.25 | 29.54 | 46.18 |
| **TC(M) + cef (3,4)** | 12.5 + 6.25 | 23.83 | 56.59 |
| **Cefixime** | 25 | 50.73 | 54.90 |
| **Control** | – | 54.90 | – |
| **Gram-negative** | | | |
| **NS(M) *A.B*(29)** | 50 | 43.47 | 36.45 |
| **CA(M) *A.B*(29)** | 50 | 48.05 | 29.75 |
| **NS(M) + cef *A.B* (29)** | 12.5 + 3.125 | 25.67 | 62.46 |
| **CA(M) + cef *A.B* (29)** | 12.5 + 6.25 | 32.40 | 52.63 |
| **Cefixime** | 25 | 33.53 | 50.98 |
| **Control** | – | 68.41 | – |
| **MC(EA), *E.C* (80-A)** | 100 | 38.23 | 10.94 |
| **TC(M), *E.C* (80-A)** | 100 | 33.89 | 21.06 |
| **MC(EA) + cef *E.C* (80-A)** | 25 + 3.75 | 31.03 | 27.72 |
| **TC(M) + cef *E.C* (80-A)** | 25 + 7.5 | 25.08 | 41.58 |
| **Cefixime** | 30 | 40.02 | 6.79 |
| **Control** | – | 42.94 | – |
| **NS(EA), *E.C* (7C)** | 100 | 45.32 | 41.58 |
| **NS(EA), *E.C* (7C) + cef** | 25 + 7.5 | 34.90 | 55.00 |
| **Cefixime** | 30 | 71.57 | 7.74 |
| **Control** | – | 77.58 | – |
| **CA(M), *K.P* (K)** | 100 | 24.90 | 50.74 |
| **CA(M), + cef *K.P* (K)** | 12.5 + 6.25 | 15.08 | 70.16 |
| **Cefixime** | 50 | 46.92 | 7.18 |
| **Control** | – | 50.55 | – |
| **NS(EA), *RPA*** | 100 | 29.54 | 45.71 |
| **NS(EA), + cef *RPA*** | 25 + 12.5 | 23.95 | 55.99 |
| **Cefixime** | 100 | 51.45 | 5.46 |
| **Control** | – | 54.42 | – |

**Note:** cef; cefixime, MIC; minimum inhibitory concentration, FICI; Fractional inhibitory concentration index, control is untreated bacterial protein concentration and "–" not observed.

#### 3.5.2. Protein estimation for Gram-negative isolates

Selected extracts were used to treat clinical isolates of *E.C* and the results are expressed in Table 3. Among tested extracts, EA extract of *N. sativa* in combination with cefixime showed maximum reduction of protein content in *E.C* (7C) (55.00 %) and *RPA* (55.99 %) isolates. Moreover, the combination of cefixime with *C. amada* M extract remarkably reduced proteins in *K.P (K)* (70.16 %) and *A.B* (29) (52.63 %). *N. sativa* M extract with cefixime

displayed maximum reduction in protein content of A.B (29) isolates (62.46 %). In case of *A.B* (29), M extracts of *N. sativa* and *C. amada* exhibited greater protein content reduction in combination with cefixime than extract/cefixime alone indicating that the combination has more effect on cell membrane integrity.

## 4. Discussion

Emergence of multidrug-resistant bacteria is a grave issue that is responsible for prolonged infections and treatment failures. The majority of nosocomial infections now comprise of six resistant pathogenic isolates that have been grouped together as ESKAPE by the Infectious Disease Society of America (Manso et al. 2021). These include Enterococcus faecium, S. aureus, K. pneumoniae, Acinetobacter, P. aeruginosa and Enterobacter species. Bacteria have the genetic potential to transfer and acquire drug resistance with excessive exposure to antibiotics (Antunes et al. 2014). In turn, excessive consumption of antibiotics facilitates modifications in bacteria to develop resistance against a particular class. Hence, it is imperative to develop new treatment strategies either as novel drugs or combination therapies to combat infections of multidrug-resistant bacteria and reduce mortality rates (Tacconelli et al. 2018). Medicinal plants have been used as a remedy to treat several infections (Awuchi 2019). Literature has shown the effectiveness of medicinal plants in reducing resistance due to the presence of secondary metabolites such as tannins, phenols, alkaloids etc (ElSohly et al. 2017). Here we have evaluated and reported synergism between selected crude extracts with cefixime against resistant clinical isolates of some ESKAPE bacteria.

Initially, crude extracts of selected plants were prepared using polar and non-polar solvents. Although, there was a variable extraction profile for each plant; however, overall polar solvents displayed the maximum extraction efficiency. Existence of highly polar water-soluble components along with proteins and simple carbohydrates can be accountable for good extraction efficiency of aqueous extracts (Ajazuddin 2010). Through the extraction process, various secondary products are obtained that are responsible for the activity of plants in various diseases (Gurjar et al. 2012). The presence of polyphenols in ethyl acetate, methanol and aqueous extracts was confirmed by RP-HPLC-DAD analysis. There were significant quantities of caffeic acid, gallic acid, cinnamic acid, luteolin and quercetin in different extracts. These polyphenols have established pharmacological potential. For example, caffeic acid is an antioxidant and reduces aflatoxin production, whereas apigenin can promote autophagy mediated cytotoxicity in leukemia cells (Dai and Mumper 2010). Gallic acid has numerous medicinal applications, particularly as anti-inflammatory, antimicrobial, antiangiogenic and anticancer agents (Choubey et al. 2015). Previous studies have shown synergism between polyphenols and antibiotics to reduce the growth of resistant bacteria. Apigenin demonstrated synergism with β-lactum antibiotics against *MRSA* (Akilandeswari and Ruckmani 2016). Moreover, gallic acid and caffeic acid are reported to inhibit efflux pump and change membrane permeability in resistant bacteria facilitating the action of antibiotics (Khan et al. 2021).

Consequently, in the current study, the antibacterial activity of crude extracts was evaluated and selected extracts were applied in combination with an antibiotic to assess synergism against resistant bacteria. According to the preliminary resistance analysis against Gram-positive and Gram-negative clinical isolates, cefixime was resistant with either no or<14 mm ZOI. Bacteria usually develop resistance against cefixime by inhibiting cell wall permeability, reducing binding to penicillin-binding protein or enhancing efflux (Ramdhani et al. 2021). Most of the tested extracts have the antibacterial capability with ZOI > 12 mm against the resistant isolates. M and EA extracts from most plants exhibited significant antibacterial potential with MIC values of 50–100 μg/ml. It was concluded that EA extracts were distinctly active against *E. coli* and *S. aureus* isolates. The result of EA extract of *N. sativa* was correlated with a previous study (Salman et al. 2016) in which essential oil and seeds extract of *N. sativa* exhibited a well-defined antibacterial potential (Salman et al. 2016). *C. amada* M extract showed a noteworthy zone of inhibition against *S. aureus* isolates. It has been reported that terpenoids and tannins cause disruption of bacterial cell membrane, enzyme inhibition and metal ion complexation (Silva and Fernandes Junior 2010). Steroids, tannins, flavonoids, and polyphenols, i.e., apigenin, luteolin and quercetin exhibited antibacterial action (Silva and Fernandes Junior 2010). Hence, the antibacterial activity of extracts can be due to the quantified polyphenols in the extracts.

Next, results showed that M and EA extracts of most plants inhibited the growth of both Gram-negative and Gram-positive isolates and hence, demonstrated a reduction in resistance to cefixime. It was observed by FICI value that acts as a reference point for the implication of interaction. These extracts were enriched in gallic acid and ferulic acid whose presence could be responsible for the synergistic potential. Furthermore, the checkerboard method indicated that most of the A extracts did not show complete synergy with cefixime. This could be due to the metabolism of phytochemicals in the aqueous extract by the bacteria (Gonelimali et al. 2018). Ferulic acid has reported antibacterial properties (Ibitoye and Ajiboye 2019) as it can accelerate the consumption of ATP, induce cytoplasmic constituents' leakage, and cause morphological variations on the cellular level (Ibitoye and Ajiboye 2019). Gallic acid present in the extracts have evidence of causing irreversible changes within bacterial membrane permeability via hydrophobicity modifications with subsequent leakage of intracellular constituents (Borges et al. 2013). In short, the synergistic interaction can modulate microbial enzymes, biochemical pathways and cell wall mediators to improve the activity of antibacterial agents (Haroun and Al-Kayali 2016).

Subsequent assessment of time-kill kinetics demonstrated that the synergism was both time and concentration-dependent and the most prominent effects were observed at 2FICI concentrations over a period of 24 h. Time-kill assay creates more precise data concerned with the effect of combinations (Broussou et al. 2019). It could be seen in the results that the bacteria went through a time dependent initial growth phase, which was inhibited after 3 h of treatment. Then there was decline in growth rate till 6 h and then bacterial growth rate became stationary dominantly at FICI and 2FICI concentrations. This means that the samples initially induced bacterial cell death then the rate of death became equivalent to rate of deaths keeping the bacterial growth in stationary phase. This also indicates that the synergism between extracts and cefixime has potential bactericidal and bacteriostatic activity, which needs to be investigated further in detail. Additionally, the effect was dependent on the concentration of the samples where 2FICI concentrations were more effective than FICI concentrations. Although, the effect was variable for each clinical isolate; however, extracts remarkably improved the efficacy of cefixime in resistant isolates. Our results are consistent with literature where time-kill kinetics study of methanol extract *Helichrysum pedunculatum* plant showed significant synergy with resistant antibiotic against *S. aureus* (Aiyegoro and Okoh 2009). Similar synergism was observed with acetone extract of *Garcinia kola* and antibiotics (Lacmata et al. 2012). As discussed earlier, the synergism can be credited to the presence of polyphenols in extracts where catechin has been reported to reduce the MIC of antibiotics in *Bacillus subtilis* (Álvarez-Martínez et al. 2020). Analogous results were displayed when alkaloids were given in combination with cefazolin against clinical isolate of *MRSA* (Zuo et al. 2011).

Afterward, the mechanism of action of cefixime and extracts (alone and in combination) was assessed by measuring the protein content present within an extracellular medium. Although, the assay did not indicate a clear picture about the mechanism of action but provides information about interaction with bacterial proteins. Antibiotics inhibit bacterial growth by targeting proteins involved in nucleic acid synthesis, cell wall synthesis and function of the cell membrane (Gutiérrez-del-Río et al. 2018). Reduction in protein content indirectly relates to reduced activity of protein associated bacterial function. Based on the results, Gram-negative and Gram-positive bacteria treated with extract alone or in combination with cefixime showed reduced protein content that might be due to the building of proteins towards periplasm (Dalbey and Kuhn 2012), or damage to peptidoglycan's cell wall, respectively (Schneewind and Missiakas 2012). There was less protein content in samples treated with the combination as compared to cefixime alone. Similar results were obtained by *Cymbopogan khasianus* extracts that showed synergistic interaction with streptomycin against *E. coli* (Singh and Katoch 2020). It is reasonable to propose that polyphenols have enhanced the activity of cefixime. Literature demonstrated that caffeic acid inhibits the RNA polymerase (Srinivasulu et al. 2018), whereas gallic acid disrupts the cell membrane and inhibits the production of biofilm (Lin et al. 2021). Overall, there was synergism between cefixime and EA/M extracts of selected plants that effectively inhibited growth of resistant clinical isolates.

## 5. Conclusions

In conclusion, effective growth inhibition was recorded in M extracts of *T. chebula*, *M. charantia* and *C. amada* treated resistant *S. aureus* isolates. On the other hand, M and EA extracts of *C. amada* and M extract of *N. sativa* revealed remarkable inhibition of Gram-negative resistant isolates. The EA and M extracts also showed significant synergism with cefixime against Gram-positive and Gram-negative resistant isolates. This was further confirmed by the results of protein estimation where there was a significant reduction in protein content of bacterial samples treated with cefixime/extracts combination as compared to individual treatments and negative control. This study provides evidence of the capacity of selected plant extracts as an adjuvant to cefixime in treating infection of resistant *S. aureus, K. pneumoniae* and *E. coli*. It highlights the importance of phytochemicals in managing antimicrobial resistance.

## CRediT authorship contribution statement

**Sania Atta:** Writing – original draft, Methodology. **Durdana Waseem:** Writing – review & editing. **Humaira Fatima:** Supervision, Investigation. **Iffat Naz:** Writing – review & editing. **Faisal Rasheed:** Project administration. **Nosheen Kanwal:** Data curation, Visualization.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2023.103576.

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