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Mechanistic insight into the synergistic antimicrobial potential of *Fagonia indica* Burm.f. extracts with cefixime

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ARTICLE INFO

ABSTRACT

Fagonia indica Burm.f. is known for its anti-infective character and has been studied in the present work as a synergistic remedy against resistant bacterial strains. Initially, phytochemicals were quantified in n-Hexane (n-Hex), ethyl acetate (E.A), methanol (MeOH), and aqueous (Aq.) extracts by Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and Reverse Phase High Performance Liquid Chromatography (RP-HPLC) analysis. Later, after establishing an antibacterial resistance profile for extracts and antibiotics against gram-positive and gram-negative strains, synergism was evaluated in combination with cefixime through time-kill kinetics and bacterial protein estimation studies. Topographic images depicting synergism were obtained by scanning electron microscopy for Methicilin-resistant *Staphylococcus aureus* (MRSA) and Resistant *Escherichia coli* (R.E. coli). Results showed the presence of maximum phenolic (28.4 ± 0.67 µg GAE/mg extract) and flavonoid (11 ± 0.42 µg QE/mg extract) contents in MeOH extract. RP-HPLC results also displayed maximum polyphenols in MeOH extract followed by E.A extract. Clinical strains were resistant to cefixime whereas these were moderately inhibited by all extracts (MIC 150–300 µg/ml) except Aq. extract. E.A and n-Hex extracts demonstrated maximum synergism (Fractional inhibitory concentration index (FICI) 0.31) against *R.E. coli*. The n-Hex extract displayed total synergism against *R.P. a* with a 4-fold reduction in cefixime dose. Time-kill kinetics showed maximum inhibition of gram-negative bacterial growth from 3 to 12 h when treated at FICI and 2FICI values with > 10-fold reduction of the extracts' dose. All combinations demonstrate > 70 % protein content inhibition with bacterial cell wall disruption in SEM images. Fortunately, FICI concentrations have low hemolytic potential (<5%). Conclusively, *F. indica* extracts can mitigate antimicrobial resistance against cefixime and can be investigated in detail by *in vivo* and mechanistic studies.

1. Introduction

The ethnopharmacological characteristics of medicinal plants have been employed as the main source for early drug development. To counteract environmental exposure, plants, bacteria, fungi, and insects eventually develop secondary metabolites with unique and varied structural characteristics (Najmi et al., 2022). These secondary metabolites are made up of a variety of chemical substances that have been shown to possess several medical benefits (Anand et al., 2019). Despite the advancements seen around the globe, many pathological illnesses are still treated using plant-based medicines, or they are utilized as an alternative to contemporary pharmaceuticals. The majority of the time, these herbal remedies or natural products are utilized as unpurified extracts (Dzobo, 2022).

Antibiotics are one of the most commonly prescribed medicines worldwide (Theuretzbacher et al., 2019). Their overuse is associated with easy availability and irrational prescribing (Widdifield et al., 2013), which causes antimicrobial resistance (AMR) against implicating life-threatening infections (El-Sayed Ahmed et al., 2020; Ibrahim et al., 2021). The multifactorial challenge of antimicrobial resistance poses a

severe risk due to treatment failures and mortality related to bacterial infections. This stemmed since current antibiotics have lost their effectiveness. (Zhu et al., 2022). This calls for evaluating and bringing more medicines that can either act solely or in combination with current antibiotics to improve their efficacy. Hence, the scientific community is interested in medicinal plants as a potential source of antibacterial compounds. The application of phytochemicals with low toxicity profiles and effective antimicrobial properties is becoming more popular worldwide (Ballester-Costa et al., 2013). Scientists are concentrating a lot of their efforts on investigating phytochemicals to aid antibiotics in combating AMR (Li et al., 2013). A synergistic effect between two antibacterial drugs is seen when a combination of both is utilized to provide more efficacy than individual use. Clinically, this synergism is significant as it offers more therapy choices for resistant infections (Cassone and Otvos Jr, 2010) by acting on multiple bacterial targets that greatly enhance the antibacterial activity and prevent the emergence of drug resistance (Wang et al., 2008). For this purpose, the checkerboard test is most frequently used to assess the synergy that depicts the fractional inhibitory concentration (FIC) index for a drug combination (Gorityala et al., 2016).

There are various plants with proven antimicrobial activity against multidrug-resistant (MDR) strains such as *Caesalpinia coriaria* methanol extract against MDR *Staphylococcus aureus; Commiphora molmol* and *Boswellia papyrifera* methanol extract against methicillin-resistant *Staphylococcus aureus* (MRSA)*; Centratherum punctatum* methanol and ethyl acetate extracts against MDR-*Acinetobacter; Thonningia sanguinea* aqueous extract against ESBL-producing *E. coli* and lastly, *Acacia nilotica, Cinnamomum zeylanicum*, and *Syzygium aromaticum* ethanol extracts are active against *Escherichia coli*, *Klebsiella pneumoniae* and *Candida albicans* (Abdallah, 2011). The genus *Fagonia* is one of the numerous plant species in Pakistan that are significant for ethnomedicine and is constituted of 240 species (Qureshi et al., 2016). Most of the species of *Fagonia* are herbs, shrubs, and bushes that are 60–100 cm in height and 100 cm in width. It has needle-like purple or pink color petals (Farheen et al., 2017). *F. indica* (Family: *Zygophyllaceae*) is commonly known as Sachchi Buti (true herb) and Dhamasa Buti in the Indian subcontinent (Khan et al., 2016). It is widely found in the deserts of Asia and Africa. In Pakistan, it is usually present in Multan, Dera Ghazi Khan, and Khyber Pakhtunkhwa (KPK) (Naz and Manzoor; Shehab et al., 2011). This herb is used by the practitioners of Unani medicine as a blood purifier and to supplement cancer treatment (Farheen et al., 2017). Numerous bioactive flavonoids, sterols, and triterpenoids are present in *F. indica* (Almilaibary et al., 2022). Pharmacological testing has confirmed the plant's antibacterial (Mustafa et al., 2016), anti-inflammatory (Saleh et al., 2011), thrombolytic, antidiabetic (Saleem et al., 2014), hepatoprotective (Rasool et al., 2014), antioxidant, wound healing (Rasool et al., 2014), and anticancer properties (Waheed et al., 2012). It is claimed to be used for the treatment of vomiting, dysentery, stomach and liver problems, fever, thirst, asthma, typhoid, urinary discharges, toothache, and to reduce swellings of the neck and tumors (Ali and Khan, 2021; Mohamed et al.). Considering the pharmacological profile of the plant, particularly as an antimicrobial agent, the present study was designed to investigate the antibacterial capability of *F. indica* extracts against resistant bacterial strains and to perform a synergistic analysis in combination with a standard antibiotic. This study is a good addition to the literature as it contributes to the ongoing search for remedies against resistant bacterial infections. Here, we have reported significant synergism between an antibiotic cefixime and different extracts of *F. indica* in reducing the growth of resistant bacteria.

2. Materials & methods

2.1. Chemicals and biological samples

Methanol (MeOH), ethyl acetate (E.A), n-hexane (n-Hex), quercetin, gallic acid, Folin-Ciocalteu (FC) reagent, and sea salt were procured from Sigma-Aldrich (Germany). Reference antibiotics (Ciprofloxacin and Cefixime) were purchased from Sigma-Aldrich. Doxorubicin, nutrient agar, nutrient broth and fetal bovine serum were acquired from Merck (Germany). All chemicals were bought from Sigma-Aldrich unless stated otherwise. Four resistant bacterial strains were used to evaluate the antimicrobial potential of extracts, which include Resistant *Streptococcus haemolyticus* (R.*S. h.*; MIC-101), Resistant *Escherichia coli* (R. *E. coli*; MIC-102), Resistant *Pseudomonas aeruginosa* (R.*P. a.*; MIC-103), and Methicillin-Resistant *Staphylococcus aureus* (MRSA; MIC-104). *Artemia salina* (Ocean 90, USA) and freshly isolated human red blood cells (RBCs) were used for cytotoxicity and hemolytic assays.

2.2. Ethical approval

The study was conducted after the approval of the Bioethical Committee of Quaid-i-Azam University, Islamabad, Pakistan. Experiments conducted on human blood were approved under protocol No. #BEC-FBS-QAU2021-355 and followed the WHO recommendations for blood sampling (2010). All volunteers for blood collection provided their informed consent.

2.3. Collection and preparation of plant samples

The aerial parts of a plant (flower, stem and leaves) were acquired from Mardan, Khyber Pakhtunkhwa (KP), Pakistan in June 2017. The plant was recognized as *F. indica* Burm. f. by Professor Dr. Mir Ajab Khan, Faculty of Biological Sciences, Quaid-i-Azam University, and a voucher was submitted to the Herbarium of Medicinal Plants (Number PHM-520), Quaid-i-Azam University. The plant material was washed with water and dried in the shade for four weeks with enough airflow. Then it was crushed into the coarse powder and extraction was performed.

2.4. Maceration and extraction

Serial extraction was performed with ultrasonication-assisted maceration using n-Hex, E.A, MeOH, and water (Aq.). Powdered plant material was first seeped in n-Hex (1:4), a non-polar solvent, for 3 days in 1000 ml Erlenmeyer flasks and regularly sonicated at 25 kHz. Utilizing a vacuum rotary evaporator, the solvent was removed from the filtrate. The marc was re-extracted for another day. The second solvent (E.A) was then added to the marc, and steps identical to the first extraction were followed. A similar process was used for the subsequent extraction of marc using MeOH and Aq. (Ali et al., 2022).

2.5. Extract recovery

The weight of the dried extracts was obtained, and the percent recovery of the crude extracts was calculated using the formula below:

$$Percent\ extract\ recovery(\%w/w) = (x/y) \times 100$$

Where x is the "total dried weight of the crude extract" and y is the "total dried weight of the powdered plant material used in extraction".

2.6. Phytochemical analysis

2.6.1. Total phenolics content (TPC)

The Folin-Ciocalteu reagent was used to determine the TPC following a previously described methodology (Ul-Haq et al., 2012). In the respective wells of a microplate, a test sample (20 µl) from 4 mg/ml stock of extracts and 90 µl of FC reagent were added. They were then incubated for 5 min at room temperature. About 90 µl of sodium carbonate (6 % w/v) was added to the wells containing the mixture. Using a microplate reader (Biotech USA, microplate reader Elx 800), the absorbance was measured at 630 nm after 30 min of incubation at 37 °C. Gallic acid (3.125, 6.25, 12.5, and 25 µg/ml) was utilized as a positive

control and DMSO as a negative control. TPC was calculated in micrograms of gallic acid equivalent per milligrams of extract (µg GAE/mgE). The assay was done three times.

2.6.2. Total flavonoids content (TFC)

A colorimetric approach involving aluminum chloride was employed to determine TFC (Ali et al., 2022). From the test extracts (4 mg/ml), an aliquot of 20 µl was transferred to each well followed by potassium acetate (10 µl), aluminum chloride (10 µl), and distilled water (160 µl). The resultant mixture was left at room temperature for 30 min and absorbance was then assessed at 415 nm using a UV–visible DAD spectrophotometer (Agilent Technologies). The positive control to attain the calibration curve for this assay was quercetin (2.5, 5, 10, 20, and 40 µg/ml) while DMSO was employed as a negative control. Results were presented as µg quercetin equivalent per mg of the extract (µg QE/mgE).

2.7. Reverse-phase high-performance liquid chromatography (RP-HPLC)

To identify and measure the number of polyphenols present in *F. indica* crude extracts, RP-HPLC was performed according to the standard protocol (Jafri et al., 2017; Malik et al., 2022). A Zorbex-C8 analytical column (5 µm particle size, 4.6 cm, and 250 nm) connected with a 3D-PDA detector and LC solution software on an HPLC system (Shimadzu, Japan) was used. A binary gradient system with mobile phase A (methanol: water: acetic acid: acetonitrile in 10:85:1:5 ratio) and mobile phase B (acetonitrile: methanol: acetic acid in 40:60:1 ratio) was used to accomplish the polyphenols' detection. The column was injected with 50 µl of the sample solution prepared in methanol, and the flow rate was set to 1 ml/min. The column was conditioned using methanol for 10 min before injecting each sample. The gradient of mobile phase B was changed from 0 to 50 % in the first 0–20 min to 50–100 % in the next 20–25 min, and lastly, to 100 % in the final 25–30 min. Samples were centrifuged and filtered before inserting into the HPLC system, while standard solutions and mobile phases were first degassed and then filtered using 0.45 m membrane filters (Millex-HV). A calibration curve was created for each standard (10, 20, 50, 100, and 200 mg/ml in methanol) using the peak area and final concentration. By comparing the UV–Vis spectra and retention time of chromatographic peaks to reference standards, polyphenols were identified at 257 nm for plumbagin, vanillic acid, and thymoquinone; 279 nm for coumaric acid, catechin, syringic acid, and gallic acid; 325 nm for apigenin, gentisic acid, caffeic acid, luteolin, and ferulic acid and 368 nm for quercetin, myricetin, and kaempferol. Results were quantified in terms of µg/mg extract (µg/mgE).

2.8. Resistant profiling of antibiotics

The resistance profile of bacterial strains was established using ciprofloxacin, doxycycline, cefixime, clarithromycin, and lincomycin through the disc diffusion method (Ngamsurach and Praipipat, 2022). Bacterial inoculum, adjusted to the 0.5 McFarland standard, was spread on sterile Petri plates. Filter paper discs coated with each antibiotic solution (4 mg/ml stock) were placed with care on inoculated agar plates and incubated at 37 °C for 24 h. Using a Vernier caliper, the dimensions of the zone of inhibition (ZOI) surrounding each disc were determined. The assay was performed in triplicate.

2.9. Antibacterial susceptibility assay

Antimicrobial activity of test extracts was performed using the micro broth dilution method (Singh et al., 2017; Wani et al., 2017). Each strain's inoculum was prepared at the seeding density of 5×10^4 CFU/ml. An aliquot of 195 µl of the inoculum and 5 µl of the test sample/standard was mixed in the 96-well plate. Fresh solutions of test extracts (100–500 µg/ml in DMSO) and antibiotics ciprofloxacin (10–0.33 µg/ml) and cefixime (400–100 µg/ml) were used in the study. After that, the

plate was incubated for 24 h at 37 °C. The absorbance of the plate was measured at 600 nm after 30 min of incubation for a zero-hour reading and after 24 h of incubation. The minimum inhibitory concentration (MIC) was calculated for each extract.

2.10. Determination of synergy using the checkerboard method

The microdilution checkerboard method is one of the conventional methods for calculating antibiotic synergy (Li et al., 2018). *In vitro* synergy test was performed against resistant bacterial strains using *F. indica* extracts and cefixime (Fadli et al., 2012). Extracts and antibiotics were serially diluted 2-fold in a 96-well plate. Cefixime was diluted vertically while extracts were diluted horizontally so that every row and column had a constant amount of the first sample and a decreasing amount of the latter. The first well in each column and row contains MICs of the respective extract and antibiotic. Each well received an aliquot of 5 µl of the sample (2.5 µl of extract + 2.5 µl of antibiotic) and 195 µl of inoculum (4×10^4 CFU/ml). After 24 h of incubation at 37 °C, the plates were examined visually to determine bacterial growth. Combinatorial MIC was considered in the well that had no visible bacterial growth. Furthermore, absorbance was measured immediately after sample addition and 24 h later to determine the fractional inhibitory concentration index (FICI) as follows:

$$FICofextract = MICofextract(combination)/MICofextract(alone)$$

$$FICofantibiotic = MICofantibiotic(combination)/MICofantibiotic(alone)$$

Whereas, the FIC index is calculated as the sum of the FIC of both i.e. extract and antibiotic.

$$FICI = FICofextract + FICofantibiotic$$

Where, FIC = Fractional inhibitory concentration, FICI = Fractional inhibitory concentration index, MIC = Minimum inhibitory concentration.

Results were interpreted as synergism, partial synergism, additive, and antagonism based on FICI values of ≤ 0.5, $0.5 < FICI \leq 0.75$, $= 1$, and > 4, respectively (Singh and Katoch, 2020).

2.11. Time-kill kinetics study

Time-kill kinetics study was performed using the previously published method (Singh and Katoch, 2020). Resistant bacterial strains were grown to the mid-logarithmic phase. The diluted bacterial suspension (1×10^4 CFU/ml) was incubated with MIC and 2MIC concentrations of extracts and with FICI and 2FICI concentrations of their combination with cefixime for 0, 3, 6, 9 and 12 h. Plates were incubated at 37 °C for a maximum of 24 h and absorbance was measured at 600 nm. A graph between absorbance and incubation time was drawn to analyze the results.

2.12. Scanning electron microscopy (SEM)

Field emission scanning electron microscopy was conducted as previously described (Lv et al., 2011) to observe changes in the morphology of bacterial cells treated with antimicrobial agents. Untreated and extract/cefixime-treated gram-positive strain (MRSA) and gram-negative bacteria (*R.E. coli)* were selected for this procedure. Based on the results of the previous experiment, bacterial cells were either treated with active extracts (2MIC) or cefixime or a combination of both. Then cells were centrifuged, and pellets were washed three times with sterile PBS. Cells were then fixed with 2.5 % glutaraldehyde in phosphate buffer (pH 7.4) and incubated overnight at 4 °C. Cells were washed and dehydrated in different concentrations of ethanol (30, 50, 70, 80 and 90 % for 10 min each and then in 100 % ethanol for 1 h). A dehydrated bacterial smear was mounted on a slide and observed under SEM (MIRA TESCAN).

2.13. Estimation of bacterial protein

To understand the effect of antimicrobial treatment on resistant bacteria, their overall protein content was estimated before and after the treatment (Nouroozi et al., 2015). Bacteria were propagated up to the mid-logarithmic phase and were treated with MIC, 2MIC, FICI, and 2FICI of extracts alone and in combination with cefixime. The protein content of bacterial samples was determined using the Bradford reagent to check the possible mechanism of action of inhibition of bacterial growth. After the treatment of bacteria with extract/cefixime for 24 h, an aliquot of 5 µl of treated inoculum was incubated with 195 µl of Bradford reagent for 5 mins at 25 °C with continuous sonication. The test was run thrice and absorbance was recorded at 595 nm. The protein content of samples was calculated using the formula:

$$Absorbance\,of\,unknown\,sample\,(x) = (Absorbance - b)/m$$

Samples were diluted with phosphate buffer. Bacterial inoculum, bovine albumin serum (0–50 µg/ml), and distilled water served as a negative control, positive control and blank, respectively.

2.14. Hemolytic activity of samples

Using human RBCs, the hemolytic activity of *F. indica* extracts alone and in combination with cefixime was assessed (Conlon et al., 2003). The blood sample was obtained from healthy volunteers after informed consent and centrifuged at 13000 rpm for 10 min to separate RBCs. After that, RBCs were washed carefully with normal saline three times and were suspended in phosphate buffer to prepare a 5 % solution. In an Eppendorf tube, 250 µl of RBC suspension and 750 µl of the sample were incubated for 30 min at 37 °C. Later, the supernatant was separated after 20 min of centrifuging at 2000 rpm. A spectrophotometer was used to measure the absorbance of the supernatant at 360 nm. The experiment was repeated thrice.

2.15. Brine shrimp cytotoxicity assay

Using laboratory-grown *Artemia salina* (Ocean 90, USA), the brine shrimp cytotoxicity experiment was carried out in triplicate with few changes (Fatima et al., 2015). Eggs were maintained for hatching in sterile seawater in a two-compartment tray under a steady oxygen supply and lighting for 24–48 h at 30–32 °C. Mature phototropic nauplii were drawn to the light and collected in a beaker using a pasture pipette. Samples were added to each well of the 96-well plate along with 10 shrimps and incubated for 24 h. The MICs for the samples were recorded. Doxorubicin (4 mg/ml) and DMSO were used as positive and negative controls, respectively. To prevent DMSO's hazardous effects on shrimps, its concentration was kept at less than 1 %. After 24 h incubation period, the percentage mortality of each sample was calculated by counting the dead shrimps. Using table curve software, the median lethal dosage was estimated for samples with $\geq$ 50 % deaths (LC_{50}) (Fatima et al., 2015).

2.16. Statistical analysis

Data were expressed as the mean ± standard error of triplicate analysis. The IBM SPSS Statistics version 23 was used for statistical analysis of data while Origin Pro was used for graphical representation. Graph Pad Prism Software® version 5 was used to calculate LC_{50} values. The significance level was set at p < 0.05.

3. Results

3.1. Percent extract recovery

Efficient extraction is an essential step to yield secondary metabolites that can be distributed into the solvent of variable polarity. In the present study, four solvents with increasing polarity were used successively and the extraction efficiency was measured in terms of percent extract recovery (Supplementary Table 1). The highest quantity of the dried extract was obtained when distilled water (Aq.) was used as a solvent for extraction (4.04 % w/w) with a yield of 202 gm, probably pertaining to the presence of more polar phytoconstituents in the plant material. Subsequently, MeOH (1.94 % w/w) and E.A (0.59 % w/w) solvents provided sufficient yields of 97 gm and 29.5 gm respectively of the extracts. With n-Hex, the least extract yield was collected (0.14 % w/w) with 7 gm. The influence of polarity on extraction efficiency was confirmed by a change in extract recovery when the solvent was changed. Nevertheless, a higher extract yield does not indicate a higher level of biological activity. For large-scale extraction optimization, this information is crucial.

3.2. Quantitative evaluation of phytochemicals
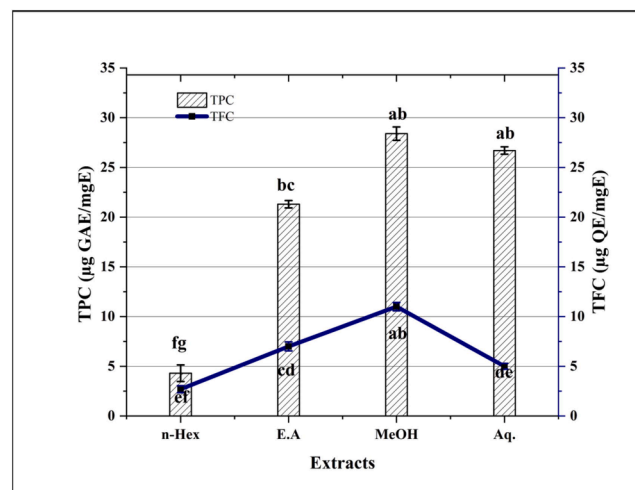
3.2.1. Total phenolic content

Initially, extracts were tested for phenolic content (Fig. 1A). MeOH extract (28.4 ± 0.67 µg GAE/mgE; y = 0.0852x + 0.6804, R^2 = 0.9471) showed the maximum amount of TPC. Extracts including Aq. and E.A also exhibited significant phenolic content, i.e., 26.7 ± 0.17 and 21.3 ± 0.37 µg GAE/mgE, respectively. Minimum phenolic content was quantified in n-Hex extract with the value of 4.3 ± 0.84 µg GAE/mgE. The phenolic content was observed to decrease in the subsequent order: MeOH > Aq. > E.A > n-Hex (Figs. 1A, 1B).

3.2.2. Total flavonoid content

Relative assessment of TFC showed that the maximum TFC was found in MeOH extract followed by Aq. and E.A extracts with values of 11 ± 0.42, 7 ± 0.42, and 5 ± 0.291 µg QE/mgE (0.0406x + 0.0223 R^2 = 0.9951), respectively (Figs. 1A, 1B). The least TFC was reckoned in n-Hex extract (2.7 ± 0.35 µg QE/mgE).

3.3. RP-HPLC analysis

The quantification of various polyphenols was performed using the RP-HPLC method by comparing the UV spectra and retention times of the standard with those of test extracts (Table 1, Figs. 1 B-D). Polyphenols in four extracts of *F. indica* were quantified using 14 standards. The n-Hex extract showed maximum concentrations of emodin and rutin

Fig. 1A. Total phenolic content (TPC) and total flavonoid content (TFC). Note; n-Hex = n-hexane, E.A = ethyl acetate, MeOH = methanol, Aq. = aqueous extract. Values are presented as mean ± standard deviation from the triplicate investigation. The columns with different superscript (a-g) letters show significant values (P < 0.05).
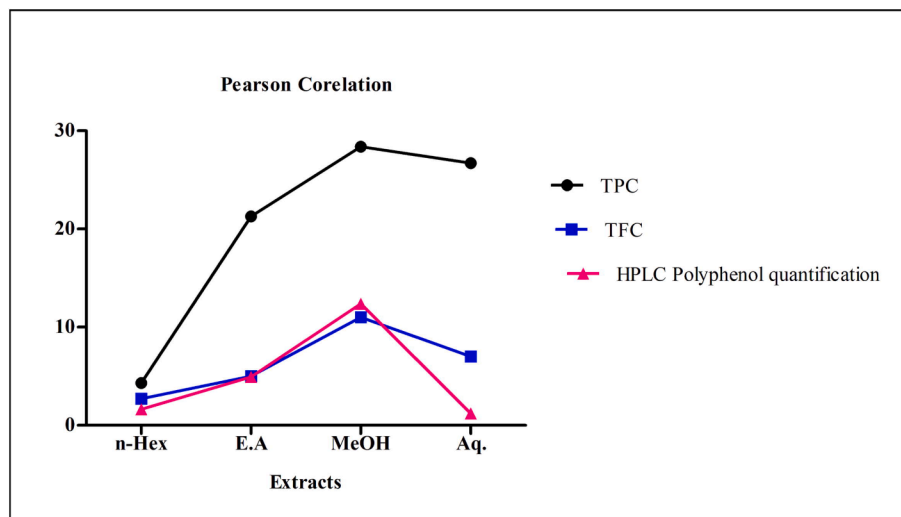
Fig. 1B. Pearson Corelation between TPC, TFC and Cumulative HPLC polyphenols quantified, Note; From graph it is shown, the graphical representation of the results clearly indicates a linear correlation among all three components with non-significant p-values (P > 0.05).

Table 1
Quantification of polyphenols in *F. indica* extracts using reverse-phase high-performance liquid chromatography.

Phenolic standard compound	Signal wavelength (Gordon and Roedig-Penman)	Calibration curve/R^2	Extracts (μg/mgE)			
			n-Hex	E.A	MeOH	Aq.
Vanillic acid	257	$y = 6.357x + 12.113$, $R^2 = 0.998$	–	0.758 ± 0.01	0.677 ± 0.08	0.040 ± 0.02
Rutin	257	$y = 9.547x + 22.217$, $R^2 = 0.997$	0.189 ± 0.02	0.137 ± 0.05	0.873 ± 0.02	0.162 ± 0.07
Catechin	279	$y = 7.878x - 19.532$, $R^2 = 0.996$	0.006 ± 0.001	0.118 ± 0.08	0.035 ± 0.06	0.058 ± 0.001
Gallic acid	279	$y = 23.573x - 43.167$, $R^2 = 0.995$	0.023 ± 0.001	0.434 ± 0.001	0.119 ± 0.01	0.019 ± 0.003
Syringic acid	279	$y = 9.532x + 13.754$, $R^2 = 0.998$	0.021 ± 0.002	0.420 ± 0.0032	0.198 ± 0.02	0.126 ± 0.0012
Coumaric acid	279	$y = 9.7644x + 14.281$, $R^2 = 0.999$	0.031 ± 0.0022	0.277 ± 0.012	0.199 ± 0.0012	0.173 ± 0.012
Emodin	279	$y = 9.7815x + 18.167$, $R^2 = 0.994$	1.365 ± 0.012	1.320 ± 0.16	2.347 ± 0.03	0.097 ± 0.001
Gentisic acid	325	$y = 12.21x - 20.348$, $R^2 = 0.996$	–	0.006 ± 0.0007	0.003 ± 0.00	0.002 ± 0.05
Caffeic acid	325	$y = 25.093x + 92.465$, $R^2 = 0.995$	0.001 ± 0.006	0.001 ± 0.02	0.007 ± 0.00	0.007 ± 0.00
Ferulic acid	325	$y = 19.51x - 16.67$, $R^2 = 0.998$	0.001 ± 0.00	0.051 ± 0.00	0.110 ± 0.005	0.012 ± 0.009
Apigenin	325	$y = 18.111x + 25.565$, $R^2 = 0.997$	0.011 ± 0.0002	1.391 ± 0.07	4.500 ± 0.08	0.431 ± 0.012
Myricetin	368	$y = 5.2278x - 6.3043$, $R^2 = 0.998$	0.006 ± 0.00	0.006 ± 0.0009	2.706 ± 0.012	0.081 ± 0.02
Quercetin	368	$y = 10.386x - 46.458$, $R^2 = 0.997$	–	0.001 ± 0.0001	0.270 ± 0.02	0.006 ± 0.06
Kaempferol	368	$y = 63.551x + 47.208$, $R^2 = 0.998$	–	0.011 ± 0.001	0.335 ± 0.006	0.001 ± 0.00
Cumulative Results						

Note; – = Not detected, n-Hex = n-hexane, E.A = ethyl acetate, MeOH = methanol, Aq. = aqueous extract, Data values shown represent mean ± SD per mg of extract (n = 3).

(1.36 ± 0.12 and 0.189 ± 0.02 μg/mgE, respectively) while quercitin was not detected. The E.A extract was found to have apigenin (1.39 ± 0.07 μg/mgE) and emodin (1.32 ± 0.16 μg/mgE). Apigenin (4.50 ± 0.08 μg/mgE), myrcitin (2.70 ± 0.12 μg/mgE), and emodin (2.34 ± 0.03 μg/mgE) were detected highest in MeOH extract among all other extracts whereas Aq. extracts showed all polyphenols in fewer quantities with noticeable amounts of apigenin (0.43 ± 0.02 μg/mgE) and cumaric acid (0.17 ± 0.02 μg/mgE).

3.4. Antibiotic resistance profiling of selected bacterial strains

Fluoroquinolones, Tetracycline, Cephalosporin, Macrolides, and Lincosamide were chosen as the reference antibiotics from the major antibiotic classes, and assessment of their resistance profile was carried out on MRSA, *R.S. h., R.E. coli* and *R.P. a.* strains by disc diffusion method. Clinical and Laboratory Standard Institute (CLSI) recommendations state that any antibiotic with a ZOI ≤ 14 mm is resistant at the recommended CLSI dosage (Humphries et al., 2021). The preliminary resistance profile of antibiotics demonstrated that cefixime was resistant to all tested bacteria by exhibiting no zone of inhibition against *R.E. coli,*
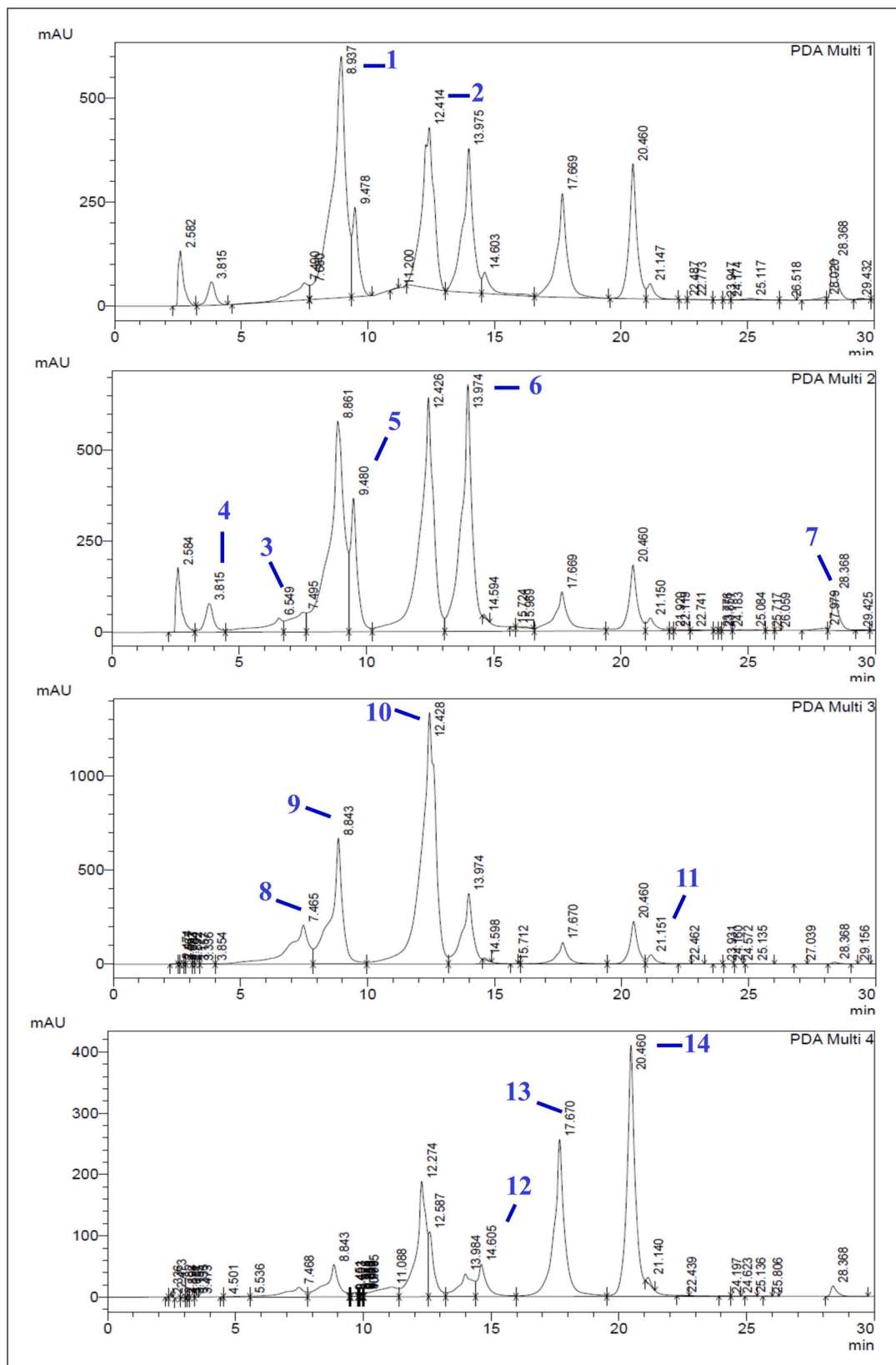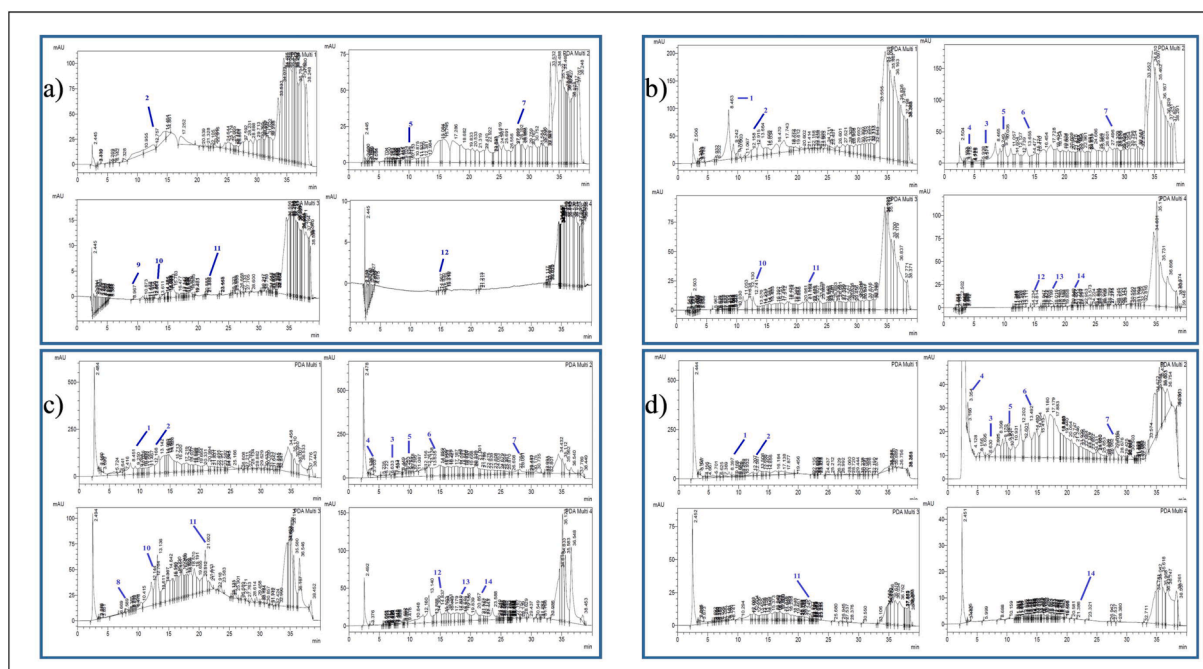
Fig. 1C. HPLC chromatograms of standard polyphenols.

R.P. a., MRSA*,* and *R.S. h.* (Supplementary Table 2). This is in accordance with the CLSI recommendations that define the resistance ZOI value for cefixime as ≤ 15 mm at the 5 µg/disc (Humphries et al., 2021). Hence, cefixime and the four strains listed above were chosen for the synergism assays with *F. indica* extracts.

Fig. 1D. HPLC fingerprints of *F. indica* where each graph presents individual extracts. Note: where n-Hexane (a), ethyl acetate (b), methanol (c) and distilled water (d). 1. Vanillic acid 2. Rutin 3. Catechin 4. Gallic acid 5. Syringic acid 6. Coumaric acid 7. Emodin 8. Gentisic acid 9. Caffeic acid 10. Ferulic acid 11. Apigenin 12. Myricetin 13. Quercitin 14. Kaemferol.

3.5. Bacterial susceptibility and MIC determination by microdilution method

The micro broth dilution technique was used to assess the antimicrobial activity of *F. indica* extracts and selected antibiotics. MIC was obtained by two-fold dilutions of samples from 100 to 500 µg/ml in a 96-well plate (n = 3). Results indicated that the Aq. extract did not show antibacterial activity at any concentration. It can be due to the small amount of polyphenols in Aq. extract as seen from HPLC data.

3.6. Synergistic interaction of different extracts

In the current study, *F. indica* extracts have shown significant partial or total synergism against cefixime-resistant bacteria (Table 3). It was observed that total synergism was shown by n-Hex and E.A extracts on *R.E. coli* and by E.A extract on *R.P. a.* with the 8–16-fold reduction in

Table 2
MIC of *F. indica* extracts and cefixime.

Test Samples	Activity	MIC against selected bacterial strains (µg/ml)			
		R.E. coli	*R.P. a.*	MRSA	*R.S. h.*
n-Hex	++	300 ± 1.9	300 ± 1.8	300 ± 1.8	150 ± 1.19
E.A	++	300 ± 2.7	300 ± 2.2	150 ± 2.91	300 ± 1.16
MeOH	++	150 ± 1.5	200 ± 2.8	300 ± 1.9	300 ± 1.7
Aq.	–	Nd	Nd	Nd	Nd
Cefixime	++	100 ± 1.6	100 ± 3.5	100 ± 1.8	100 ± 1.8
Ciprofloxacin	++	2.5 ± 2.8	1.25 ± 2.2	3 ± 0.193	1.25 ± 2.9

Note;–= no activity, Nd = not detected, n-Hex = n-hexane, E.A = ethyl acetate, MeOH = methanol, Aq. = aqueous extract, Data values shown represent Mean ± SD (n = 3), R.E. coli = Resistant Escherichia coli, R.P. a. = Resistant Pseudomonas aeruginosa, MRSA = Methicillin Resistant Staphylococcus aureus, and R.S. h. = Resistant Staphylococcus haemolyticus, MIC – minimum inhibitory concentration

MIC values of extracts. Interestingly, cefixime's MIC also declined by 2–4-fold. The FICI values, which were ≤ 0.5 indicated complete synergism between the two samples. Partial synergism was noted with MeOH extract against *R. P.a.,* MRSA, and *R.S. h.* with 2–4 fold reduction in MIC of cefixime. The rest of the extracts showed minimum synergistic potential or showed an additive effect. Results were calculated as described previously (Bonapace et al., 2002).

3.7. Time-kill kinetics assay

Time-kill Kinetics assay was performed with each test bacteria receiving four distinct treatments, including MIC, 2MIC, FICI, and 2FICI to determine its antibacterial effects for 12 h (Figs. 2-3). Bacterial samples that were treated with a combination of extracts and cefixime showed declined growth as compared to cefixime or DMSO (negative control) alone treated samples.

It was observed that *F. indica* MeOH extract, when inoculated with gram-positive strain MRSA, showed maximum growth inhibition of 81 % to 85 % from 6 h to 9 h, respectively at 2FICI value compared to 40 % and 41 % inhibition when treated with cefixime alone at the same interval (Fig. 2A). Likewise, the n-Hex extract at 2FICI value showed growth inhibition of 84 % after 9 h of treatment but E.A extract did not show much synergistic potential at all intervals with a maximum of 40 % of growth inhibition at 9 h. This pattern was similar to Cefixime. On the other hand, the treatment of *R.S. h.* strain with a combination of Cefixime and *F. indica* MeOH extract at 2FICI displayed maximum growth inhibition in exponential order from 3 to 12 h where there was 90 % inhibition at 12 h. It was followed by E.A and n-Hex extracts demonstrated 84 % and 85 % growth inhibition, respectively at 12 h as compared to 19 % inhibition by Cefixime alone (Fig. 2B). Treatment at MIC and 2MIC values of extract were still more effective than the MIC of Cefixime alone. Overall, there was a steady growth decline from 3 to 9 h after treatment with n-Hex, E.A, and MeOH extracts that remained constant till 12 h of treatment.

The *F. indica* extracts showed a decrease in the growth of gram-negative bacteria when given in combination with cefixime (Fig. 3). The growth pattern of R.*E. coli* strain showed bacterial growth from 0 to

Table 3
Synergistic interaction of *F. indica* and cefixime.

Strains	Extracts	MIC (alone) µg/ml	MIC (combination) µg/ml	Fold Reduction	FICI	Description
R.E. coli	**n-Hex**	**300**	**18.75**	**16**	**0.31**	**Total synergism**
	Cefixime	**100**	**25**	**4**		
	E.A	**300**	**18.75**	**16**	**0.31**	**Total synergism**
	Cefixime	**100**	**25**	**4**		
	MeOH	**150**	**18.75**	**8**	**0.62**	**Partial synergism**
	Cefixime	**100**	**50**	**2**		
R.P. a.	**n-Hex**	**300**	**37.5**	**8**	**0.37**	**Total synergism**
	Cefixime	**100**	**25**	**4**		
	E.A	**300**	**18.75**	**16**	**0.56**	**Partial synergism**
	Cefixime	**100**	**50**	**2**		
	MeOH	**200**	**37.5**	**5**	**0.68**	**Partial synergism**
	Cefixime	**100**	**50**	**2**		
MRSA	**n-Hex**	**300**	**37.5**	**8**	**1.12**	**Additive**
	Cefixime	**100**	**100**	**0**		
	E.A	**150**	**18.75**	**8**	**1.12**	additive
	Cefixime	**100**	**100**	**0**		
	MeOH	**300**	**150**	**2**	**0.75**	**Partial synergism**
	Cefixime	**100**	**25**	**4**		
R.S. h.	**n-Hex**	**150**	**37.5**	**4**	**0.75**	**Partial synergism**
	Cefixime	**100**	**50**	**2**		
	E.A	**300**	**150**	**2**	**1**	additive
	Cefixime	**100**	**50**	**2**		
	MeOH	**300**	**18.75**	**16**	**0.56**	**Partial synergism**
	Cefixime	**100**	**50**	**2**		

Note; n-Hex = n-hexane, E.A = ethyl acetate, MeOH = methanol extract, R.E. coli = Resistant Escherichia coli, R.P. a. = Resistant Pseudomonas aeruginosa, MRSA = Methicillin Resistant Staphylococcus aureus, and R.S. h. = Resistant Staphylococcus haemolyticus.

3 h. There is inhibition of bacterial growth from 3 to 12 h when treated at FICI and 2FICI values with > 10-fold reduction in the extracts' dose. It resulted in 90 %, 90 %, and 82 % of growth inhibition with n-Hex, MeOH and E.A extracts at their FICIs, respectively. On the contrary, the bacterial growth inhibition at 2MIC values were around 45 %, 33 %, and 31 % for E.A, MeOH, and n-Hex extracts, respectively at 12 h. These outcomes were equivalent to the effect produced by cefixime alone. This means that combination treatment was more effective in inhibiting resistant strains as compared to alone regimens. Similarly, the *R.P. a.* when treated with cefixime alone (Fig. 3B) showed maximum growth inhibition of 39 % at 3 h, and from 6 to 12 h, it demonstrated a stationary pattern with no significant inhibition of bacterial growth. MeOH extract at FICI, 2FICI, MIC, and 2MIC showed drastic inhibition of bacterial growth with values of 89 %, 90 %, 76 %, and 77 %, respectively. The n-Hex extract at MIC displayed the same pattern as cefixime but with a maximum of 60 % growth inhibition at 3 h. It increased to 85 % at 12 h at 2MIC concentration indicating the effectiveness of the extract. The E. A extract at MIC and 2MIC showed that bacterial inhibition was around 60 % at 6 h, which then showed a stationary phase till 12 h. The growth inhibition was pronounced at FICI and 2FICI concentrations from 3 h to 12 h which was almost similar to ciprofloxacin with 87 % growth reduction.

3.8. SEM study

Based on previous results, we selected n-Hex and E.A extracts treated *R.E. coli* samples and MeOH extract treated MRSA samples. Bacteria were exposed to MIC of cefixime, extracts and FICI concentrations to depict the results.

By SEM it was possible to see the control cells (untreated *R.E. coli* cells) as typical rod-shaped organisms with a smooth surface (Fig. 4Aa). Uneven, wrinkled cells were seen after treatment with cefixime alone (Fig. 4Ab). After treatment with the n-Hex extract of *F. indica*, cells became elongated and showed disruption in the cell membrane (Fig. 4Ac). Bacterial cells after treatment with FICI concentration exhibited cell membrane shrinking and cellular damage due to the loss of the cell's distinctive form (Fig. 4Ad). The bacterial cells in Fig. 4Ae-f were treated with E.A. extract of *F. indica* alone and in combination, respectively. It was observed that E.A extract treated cells did not lose

much integrity although the cells appeared swollen and long (Fig. 4Ae) whereas the cell membrane was not smooth and the shape of the cells was disturbed in FICI-treated bacteria (Fig. 4f).

Subsequently, the untreated MRSA cells in the control group exhibited their characteristic structure, displaying a spherical form and a smooth surface (Fig. 4Bg). When cells were treated with cefixime alone (MIC), their form wasn't significantly altered (Fig. 4Bh) while treatment with MeOH extract disrupted their integrity yet cell membrane lysis did not occur (Fig. 4Bi). In contrast, when the strain was treated with a combination of cefixime and MeOH extract (FICI), there was damage to the shape of the cell. Microstructural studies showed that the MeOH extract and cefixime together destroyed the cell's integrity and caused intracellular components to seep out. The shape was seen to be imperfect and distorted, demonstrating membrane lysis and rupture (Fig. 4Bj).

3.9. Protein estimation

Next, in an attempt to further evaluate the possible mechanism of synergism between *F. indica* extracts and cefixime, the effect on bacterial protein content was determined by the Bradford reagent (Kummari et al., 2022). Due to its simplicity, speed, and relative sensitivity, this approach is widely used to measure protein content (Ku et al., 2013).

Bacterial samples treated with MIC and/or FICI concentrations showed a significant ($p < 0.05$) decline in the overall protein content of bacteria. It was observed that cefixime, being a cell wall synthesis inhibitor, moderately decreased the protein content probably due to the leakage or cell wall damage inducing the overall death of bacterial cells (Table 4). On the contrary, *F. indica* extracts at their MICs dominantly reduced the protein concentrations in bacteria indicating that the plant itself can damage bacterial proteins. The effect of synergism at FICI concentrations on bacterial cells was not significantly visible from extracts alone. Yet, it was much higher than cefixime treatment (Table 4).

3.10. Hemolytic assay

The hemolytic activity of *F. indica* extracts alone and in combination with cefixime was evaluated using RBCs. It can be depicted from the results that *F. indica* extracts alone showed dose-dependent toxicity on erythrocytes. According to ASTM F756-00 procedures for determination
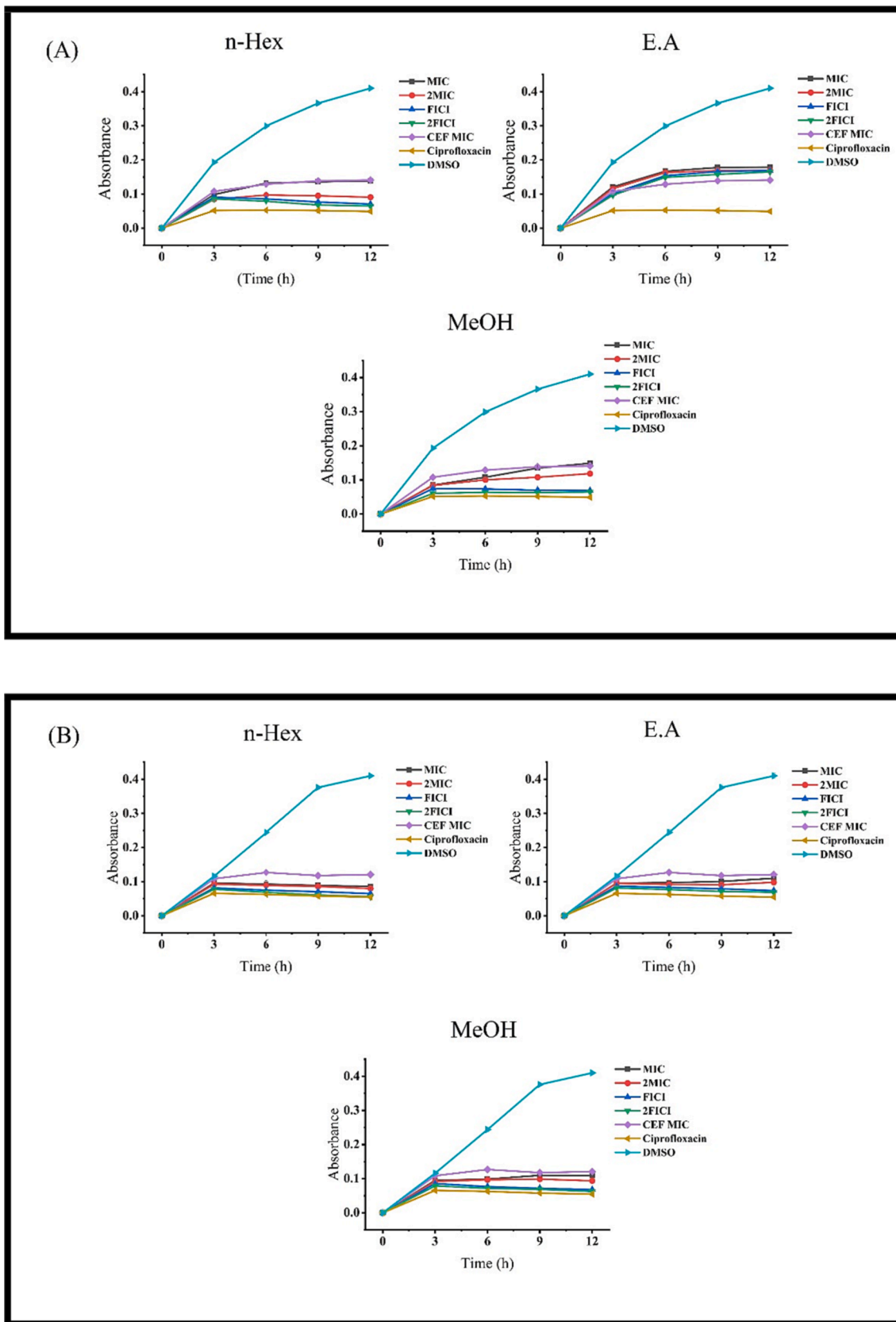
Fig. 2. Time-kill kinetics curves for Gram-positive bacterial strains. Note: where cefixime-resistant gram-positive bacterial strains were (A) MRSA and (B) *R.S. hemolyticus* were treated with *F. indica* extracts, cefixime, and their combination. The bacterial growth was monitored for 0, 3, 6, 9, and 12 h. Colored lines indicate the amount of the treatment used in the experiment where light blue is the untreated control, yellow is a positive control, purple is 1x MIC of cefixime, black is 1x MIC of extract, red is 2x MIC of extract, dark blue is 1x FICI, and green is 2x FICI.
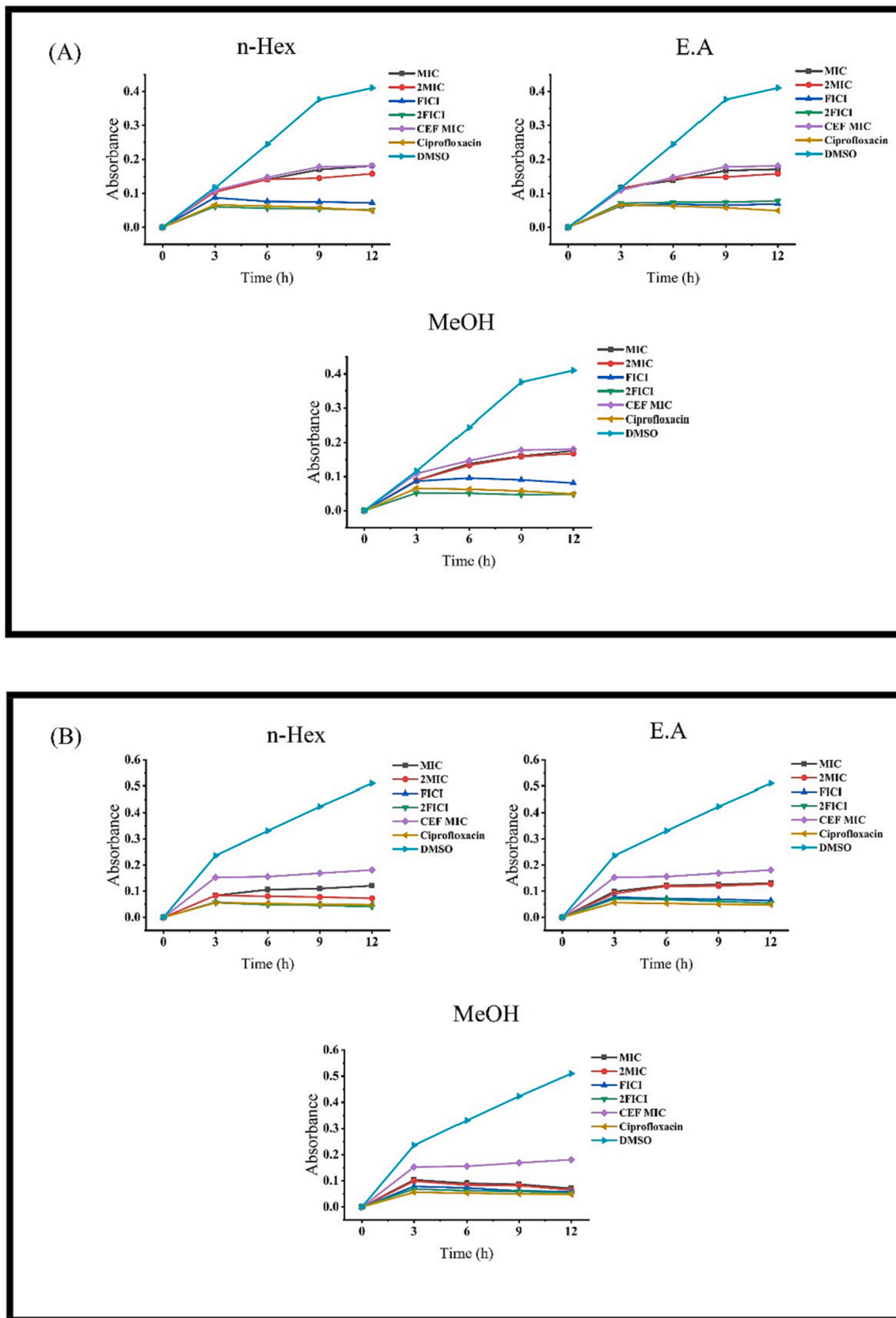
Fig. 3. Time-kill kinetics curves for Gram-negative bacterial strains. Note: Where cefixime-resistant gram-negative bacterial strains (A) *E. coli* (B) *R.P. aeruginosa* were treated with *F. indica* extracts, cefixime, and their combination. The bacterial growth was monitored for 0, 3, 6, 9, and 12 h. Colored lines indicate the amount of the treatment used in the experiment where light blue is untreated control, yellow is positive control, purple is 1x MIC of cefixime, black is 1x MIC of extract, red is 2x MIC of extract, dark blue is 1x FICI, and green is 2x FICI.
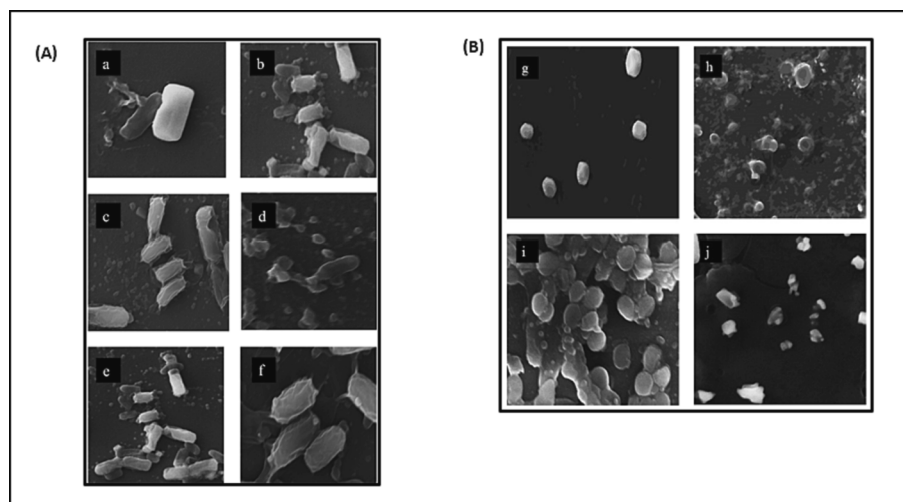
Fig. 4. Scanning electron micrographs of bacterial strains. Note: Where, (A) *R.E. coli* (a) untreated (magnification × 25000x); (b) treated with the cefixime (magnification × 25000x); (c) treated with n-Hex extract alone (magnification × 15000x); (d) treated with combinations of n-Hex and cefixime (magnification × 10000x); (e) treated with E.A extract alone(magnification × 25000x); (f) treated with combinations of E.A and cefixime (magnification × 50000x. (B) MRSA. (g) untreated (magnification × 25000x); (h) treated with the cefixime (magnification × 25000x); (i) treated with MeOH extract alone (magnification × 25000x); (j) treated with combinations of MeOH and cefixime (magnification × 25000x).

of hemolytic properties of samples, constituents with hemolysis percentages of > 5 %, <5%, and < 2 % are regarded as hemolytic, slightly hemolytic, and non-hemolytic, respectively (Elahi et al., 2014). Interestingly, the extracts were hemolytic with > 5 % hemolysis when used alone at their MICs. However, their hemolytic prospective dropped when given in combination with cefixime. All combinations had values ranging between 2 and 3.5 % at FICI and 2.37–3.82 % for ½ FICI. This indicated that synergistic combinations were safer to use at their FICI and ½ FICI concentrations. These results were significantly (p < 0.05) lower than positive control Triton-X 100 (100 % hemolysis).

3.11. Brine shrimp cytotoxicity assay

The brine shrimp cytotoxicity assay is a practical method for figuring out *in vitro* toxicity at a preliminary stage (Abbasi et al., 2021). The nauplii were treated with various concentrations of plant extract for 24 h. The number of motile nauplii was counted to assess the extract's effectiveness. It is simple to use, reasonably priced and requires very little test material (Espíndola et al., 2022). The assay was performed at a MIC value of 300 µg/ml (all extracts) and FICI value of 150 + 100 µg/ml (extract + cefixime) with serial dilutions. Doxorubicin was used as a positive control. At the MIC, the MeOH showed cytotoxic activity (LC$_{50}$ = 150 µg/ml) for the experimental shrimps. The LC$_{50}$ values were 106 µg/ml for E.A extract and 75 µg/ml for n-Hex extract. While at FICI, the MeOH extract's cytotoxicity was decreased (LC$_{50}$ = 120 µg/ml). The LC$_{50}$ value for n-Hex was 73.53 µg/ml, whereas LC$_{50}$ for E.A extract was 74.40 µg/ml at their respective FICIs. Maximum LC$_{50}$ was shown by MeOH extract (150 µg/ml) at MIC that declined when FICI (LC$_{50}$ 120 µg/ml) concentrations were used.

4. Discussion

The polarity of polyphenols ranges from polar to non-polar, a wide variety of solvents are required for their extraction. Thus, polarity plays a crucial role in increasing the solubility of phenols. It can be seen in the results that as compared to nonpolar solvents, polar solvents extracted more phenolic content. Previous studies found a significant correlation between antioxidant capacity and overall phenolic content, pointing to phenolic chemicals as the primary drivers of plants' biological activity (de Carvalho Tavares et al., 2020; Finco et al., 2012). When compared to other bactericides, phenols and phenolic compounds are widely utilized

in treating minor infections and healing wounds (Hussain et al., 2011).

Previous studies have identified various phenolic and flavonoid components, including quercetin, gallic acid, benzoic acid, kaempferol, caffeic acid, myricetin, chlorogenic acid, and Sinopic acid, in *Zingiber zerumbet* preparations. These compounds have demonstrated enhanced antibacterial potential when combined with antibiotics (Ramzan and Zeshan, 2023). Additionally, gallic acid and caffeic acid have been found to reverse the resistance phenotype of *Staphylococcus aureus* and inhibit specific bacterial pumps, while quercetin displayed improved activity against *Staphylococcus aureus* (Dos Santos et al., 2018, Lima et al., 2016, Dos Santos et al., 2021).

In a separate investigation, the methanolic extracts of *Beta vulgaris* and *Carica papaya* were evaluated for antibacterial activity, both individually and in combination with antibiotics. The combined use of methanolic extracts with ampicillin, ciprofloxacin, erythromycin, and tetracycline demonstrated increased effectiveness against *Acinetobacter baumannii* and *Klebsiella pneumonia*. This highlights the potential of antibiotics as a natural source for acquiring therapeutically valuable metabolites against challenging microbial infections, with enhanced efficiency when used in combination (Kousar et al., 2023).

Moreover, our present study has identified phytochemicals consistent with those found in previous research, suggesting their involvement in the observed synergistic antibacterial activity. Specifically, *Fagonia Olivieri* fractions, known for their antibacterial and cytotoxic properties, owe their efficacy to the interactions of flavonoids and tannins with soluble and extracellular proteins, as well as their ability to form complexes with bacterial cell walls (Rashid et al., 2013b).

The phenolic content of *F. indica* was evaluated to relate the predicted bioactivity of *F. indica* to its phytoconstituents (Dangles, 2012). Due to mounting evidence of the numerous health benefits of flavonoids, there has been an increase in interest in the study of flavonoids derived from dietary sources. Their biological profile includes anti-inflammatory, antioxidant, antiangiogenic, anticancer, free radical scavenging, antihypertensive, prevention of cardiac diseases, and anti-human immunodeficiency virus functions (Xiao et al., 2011). For instance, the availability of electrons to neutralize any free radicals is the basis of antioxidant activity. Additionally, the quantity and type of the hydroxylation pattern on the aromatic ring of flavonoids are associated with an antioxidant action (Gulcin, 2020). This can be beneficial in combating oxidative stress and inflammation associated with infections that impede a patient's recovery. Literature supports the present

Table 4
Effect of *F. indica* extracts and cefixime on bacterial protein content.

Strains	Extracts	MIC or FICI (µg/ml)	Average Protein Concentration (µg/ml)	% Protein inhibition in the extracellular medium
R.E. coli	Control	–	50	–
	Cefixime	100	42.88	14.24
	n-Hex	300	4.24	91.53
	n-Hex with cefixime	18.75 + 25	2.88	94.24
	E.A	300	4.59	90.81
	E.A with cefixime	18.75 + 25	4.24	91.53
	MeOH	150	9.95	80.10
	MeOH with cefixime	18.75 + 50	3.95	92.10
R.P. a.	Control	–	38	–
	Cefixime	100	32.88	13.48
	n-Hex	300	8.24	78.33
	n-Hex with cefixime	37.5 + 25	7.45	80.39
	E.A	300	8.52	77.58
	E.A with cefixime	18.75 + 50	9.02	76.26
	MeOH	200	7.24	80.96
	MeOH with cefixime	37.5 + 50	7.81	79.45
MRSA	Control	–	66	–
	Cefixime	100	46.45	29.62
	n-Hex	300	6.24	90.55
	n-Hex with cefixime	37.5 + 100	14.88	77.46
	E.A	150	9.45	85.68
	E.A with cefixime	18.75 + 100	5.09	92.28
	MeOH	300	14.81	77.56
	MeOH with cefixime	150 + 25	14.88	77.46
R.S. h.	Control	–	43	–
	Cefixime	100	39.31	8.59
	n-Hex	150	3.59	91.64
	n-Hex with cefixime	37.5 + 50	9.52	77.86
	E.A	300	8.66	79.85
	E.A with cefixime	75 + 100	6.88	84.00
	MeOH	300	4.88	88.65
	MeOH with cefixime	18.75 + 50	9.81	77.19

Note; n-Hex = n-hexane, E.A = ethyl acetate, MeOH = methanol extract, R.E. coli = Resistant Escherichia coli, R.P. a. = Resistant Pseudomonas aeruginosa, MRSA = Methicillin Resistant Staphylococcus aureus, and R.S. h. = Resistant Staphylococcus haemolyticus.

results, which showed that *F. indica* has a significant level of flavonoids (Shehab et al., 2015). The current flavonoid analysis of *F. indica* extracts reveals that it would be a useful contender against infectious disorders, once its efficacy is proven.

From above mentioned HPLC analysis it is shown that HPLC analysis confirms the presence of important polyphenols like Apigenin, Emodin, and Myricetin in Fagonia indica, highlighting their medicinal significance (Ibrahim et al., 2008; Miranda et al., 2022; Waheed et al., 2012; Ansari and Kenne, 1987). These compounds, along with ferulic acid and gallic acid, exhibit antibacterial properties by altering bacterial membranes, while phytochemicals such as rutin, cinnamaldehyde, ellagic acid, and resveratrol can block biofilm production, countering antimicrobial resistance (Borges et al., 2013; Brackman et al., 2008; Nazzaro et al., 2013).

Recent research underscores the synergy between polyphenols and antibiotics in combating antibiotic-resistant bacteria. Apigenin,

combined with β-lactam antibiotics, is effective against MRSA, and Gallic acid and caffeic acid enhance antibiotic efficacy by altering bacterial membrane permeability and inhibiting efflux pumps (Atta et al., 2023).As depicted in the results, a higher number of polyphenols were quantified in MeOH extract than E.A extract suggests it as the dominant candidate for bioactivity evaluation. The occurrence of these chemicals can be correlated with the antibacterial potential of extracts studied here. In a prior study, chemical analyses of species extracts from the Plectranthus genus revealed that chlorogenic acid is predominant in EELPa, while caffeic acid is the most prevalent compound in EELPb and EELPo. All these species demonstrated antibacterial efficacy against both Gram-positive and Gram-negative bacteria, indicating their potential for combating bacterial infections due to the presence of these chemicals (Rodrigues et al., 2021).Additionally, other studies found that gallic acid and caffeic acid have the ability to reverse the resistance phenotype of *Staphylococcus aureus,* with caffeic acid efficiently inhibiting bacterial pumps in silico (Dos Santos et al., 2018; Lima et al., 2016). The presence of quercetin also exhibited enhanced antibacterial activity against *Staphylococcus aureus* (Dos Santos et al., 2021).

The irrational and excessive use of antibiotics promotes the development of AMR, which causes the prolonged duration of infections and treatment failures. Infections associated with MDR bacteria are difficult to treat with current antibiotics. Such AMR necessitates the development of remedies that can either enhance the efficacy of current antibiotics or are effective against resistant bacteria (Taylor et al., 2002). Hence, it is presently a critical need to examine natural sources, particularly plants, for the presence of alternative antimicrobials (Ahmad and Beg, 2001). Antibacterial activity of Aq. and MeOH extracts from leaves of *F. indica* has been reported for non-resistant bacteria (Sharma et al., 2009). All extracts (except Aq.) demonstrated significant antibacterial activity with MICs ranging between 150 and 300 µg/ml (Table 2) against selected resistant bacterial isolates. The antibacterial activity of the extracts in this investigation may be attributed to the plant's hydroxylated phenols including emodin, vanillic acid, syringic acid, gallic acid, coumarins, flavonoids, and flavones, which were measured using HPLC. Bacterial cells may suffer structural damage from polyphenols or they may lose the complex shape of their cell walls and intracellular membrane. Phenolic substances have the potential to distort cells, breach cell walls, and induce the condensing of afflicted cells' cytoplasm and membrane debring the cellular material (Othman et al., 2019).

Antibacterial resistance can be prevented and/or mitigated using medicinal plants whose activity has been demonstrated in literature due to the inherent antimicrobial characteristics of phytoconstituents. The best way to deal with AMR is through a combinational strategy that allows for a synergistic interaction between plant extracts and standard antibiotics (Cheesman et al., 2017). Combination therapy has recently become quite popular, particularly in the field of infectious diseases. The WHO states that since combination therapy may target numerous aspects of the disease and reduce resistance, it is preferable over monotherapy in many infectious illnesses that are fatal, including malaria, TB, and HIV/AIDS (Organization, 2013). The checkerboard method is frequently used as the benchmark for synergism studies (Khan et al., 2021). When the MIC of the extract and an antibiotic used in combination is reduced by 4-fold, the combination is said to be synergistic; however, if one MIC drops by 4 and the other by 2-fold, the interaction is said to be partially synergistic (Wang et al., 2014).

Certain plants and antibiotics can impact one other's inhibitory effects when taken together. Due to their alleged antibacterial action, polyphenols and antibiotics used for the treatment of infectious disorders can result in innovative combination therapy regimens. Because of its purported antibacterial properties, ferulic acid exhibits a synergistic effect (Ibitoye and Ajiboye, 2019).

The presence of phytochemicals, including flavonoids and polyphenols like Apigenin, Emodin, and Myricetin, in both our current study and prior research (Ibrahim et al., 2008; Miranda et al., 2022; Waheed

et al., 2012), highlights their significant bioactive potential. It also underscores the medicinal importance of *Fagonia indica* (Ansari and Kenne, 1987).

Notably, compounds like ferulic acid and gallic acid exhibit antibacterial properties through various mechanisms, including alterations in bacterial membrane properties, such as hydrophobicity, negative surface charge reduction, and local membrane rupture or pore formation. These alterations result in the release of essential intracellular components (Borges et al., 2013). Furthermore, phytochemicals like rutin, cinnamaldehyde, ellagic acid, and resveratrol have demonstrated the ability to inhibit bacterial biofilm production, a critical factor in antimicrobial resistance (AMR). This suggests their potential in preventing infections caused by antibiotic-resistant bacteria (Nazzaro et al., 2013).

Our SEM morphological study provides empirical evidence supporting the idea that the combination of cefixime and *Fagonia indica* extracts disrupts the cell membrane of resistant bacteria, leading to the leakage of cellular contents. This observation aligns with another study in which peptides isolated from *Fagonia bruguieri* inhibited bacterial growth by targeting bacterial cell wall components. SEM analysis in that study revealed that these peptides obstructed N-acetyl muramic acid coupling, thereby impeding cell wall synthesis (Al-Dhafri and Ching, 2022). This membrane destabilization exposes bacterial proteins to various stresses (Pag et al., 2008) and results in the leakage of cellular contents (Esfandi et al., 2019a).

Furthermore, compounds like Gallic acid and Caffeic acid are believed to modify the permeability of resistant bacterial membranes, inhibiting efflux pumps and enhancing antibiotic efficacy (Atta et al., 2023).

The detected polyphenols have established bioactivities and further potentiate the medicinal value of F.indica for example, Apigenin was found as the maximum detected polyphenol. The study revealed that apigenin induces apoptosis-like bacterial death in Escherichia coli. Upon administration of apigenin, there was a rapid increase in intracellular calcium levels, as well as elevated levels of reactive nitrogen species (Ngamsurach and Praipipat) and nitric oxide (NO). The compound also triggered the production of reactive oxygen species (ROS) and superoxide anion (O2–), which inhibited the activation of superoxide dismutase in E. coli. Moreover, apigenin disrupted the integrity of the bacterial membrane lipid bilayer, leading to glutathione oxidation and the formation of 8-hydroxy-2′-deoxyguanosine (Kim et al., 2020). Myricetin has significant antioxidant and antimicrobial capabilities (Gordon and Roedig-Penman, 1998). Thus the detected polyphenols being antibacterials exhibit their effect in combination with cefixime.

In summary, the synergy between cefixime and *Fagonia indica* extracts disrupts resistant bacterial cell membranes, causing cellular content leakage, consistent with prior findings on peptides from *Fagonia bruguieri* targeting bacterial cell walls. This collective evidence highlights the potential of natural compounds in combating antimicrobial resistance by targeting various bacterial mechanisms. Further research into these synergistic interactions and mechanisms is essential to advance our understanding in this field.

Checkerboard studies indicated that almost all of the extracts in combination with the antibiotic showed synergistic potential because of the presence of polyphenols like rutin, vanillic acid, gallic acid, and ferulic acid as confirmed by HPLC. Since, these polyphenols have reported antibacterial activity (Aresta et al., 2010). The n-Hex and E.A extracts shows total synergism whereas MeOH extract shows partial synergism. The phytochemical nature of extracts depends on the polarity of the extraction solvent used, thus the phytochemical profile of n-Hex and E.A would be non-polar as compared to MeOH extract. It was previously reported that non-polar metabolites of *F. indica* extracts were active against bacteria (Mohamed et al., 2021). The presence of non-polar metabolites like triterpenoid and terpenoid saponins has been reported in *F. indica* non-polar extracts (Atiq-ur-Rehman, 2022). Literature also reported that triterpenoids showed antibacterial synergistic

potential (Chung et al., 2011). Therefore the observed antibacterial synergism of non-polar extracts may be attributed to the presence of triterpenoids in polar extract. The time-kill kinetics experiment was carried out to understand the effectiveness of *F.indica* extracts and cefixime against resistant bacterial strains. This assay measures the rate of killing, which could be a more useful parameter for projecting the course of the treatment over time (Doern, 2014). Although time-kill kinetics studies are used to determine bactericidal impact, it is a useful tool to provide data in terms of time or concentration-dependent antibacterial activity (Xedzro et al., 2022). The findings of this study are consistent with those published previously, in which the checkerboard approach revealed a synergistic impact whereas the time-kill kinetics method demonstrated additive results.

The results showed significant synergism between cefixime and *F. indica* extracts against resistant bacterial strains at different time intervals. These results agree with a previous study that reported the noteworthy antibacterial activity of silver nanoparticles of *F. indica* callus extract with antibiotics (Adil et al., 2019). However, we have demonstrated concentration and time-dependent antibacterial activity against resistant strains which is a beneficial addition to the literature. This activity can be correlated with the presence of polyphenols and flavonoids in *F. indica* extracts that have previously reported antibacterial potential. Polyphenols were reported to work by different mechanisms such as inhibition of DNA gyrase, disruption of cell membrane function, and distortion of bacterial energy metabolism in both gram-positive and gram-negative bacteria (Barbieri et al., 2017). A study has shown that hydroalcoholic extracts of *Plectranthus cincinnata* possess antibacterial activity due to phenolic compounds found throughout the plant. These extracts, when combined with conventional antibiotics, can effectively combat inherent bacterial resistance in species like *Staphylococcus aureus* and *Escherichia coli,* enhancing antibiotic efficacy against various bacterial species (Siebra et al., 2018). Hence, bioassay-guided separation of an active natural product is essential to fully understand the synergistic mechanism because extracts may contain hundreds of phytochemicals. When both drugs in combination have equivalent pharmacokinetic characteristics, synergistic combinations may be more efficacious. The right dosing regimen and combination ratio must be improved for greater efficacy and fewer adverse reactions (Ayaz et al., 2019).

Researchers have proposed a variety of mechanisms for how phytochemicals exert their antimicrobial effects (Jayaraman et al., 2010; Omojate Godstime et al., 2014). Generally recognized mechanisms of antimicrobial interaction for synergy are sequential inhibition of a common biochemical pathway, the inhibition of protective enzymes, and the combination of cell wall active agents to improve the uptake of other antimicrobials (Santiesteban-López et al., 2007). For a better understanding of the mechanism of synergistic antimicrobial potential, SEM was performed to evaluate any changes in the integrity of bacterial cell surface (Singh and Katoch, 2020).

In a related study, eleven distinct components were successfully extracted from the methanolic extract of the entire *Fagonia cretica* plant, with subsequent isolation through repeated silica gel column chromatography and preparative TLC of specific soluble fractions. These components, including linoleic acid, β-sitosteryl-3-O-β-D-(6-hexadecanoyl)-glucopyranoside, methyltriacontanoate, teraxerol, β amyrin acetate, oleanolic acid, octacosanoic acid, tetraxerone, arjulonic acid, and 23-hydroxy ursolic acid, displayed substantial antibacterial activity against a variety of bacterial strains (Anjum et al., 2007).

Furthermore, an assessment of the antimicrobial properties of ethanol and aqueous extracts from *Fagonia indica* leaves was conducted. These extracts exhibited significant inhibitory effects on various bacterial strains, including *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Bacillus cereus*. Notably, the ethanol extract exhibited the most pronounced inhibition against *Bacillus cereus* (Puri and Bhandari, 2014).

Importantly, recent research highlighted the antibacterial potential

of *F. indica*, particularly due to the presence of polyphenols like apigenin. Apigenin was found to induce bacterial apoptosis-like death in *Escherichia coli* by increasing intracellular calcium levels, generating reactive nitrogen species (Ngamsurach and Praipipat) and nitric oxide (NO), and disrupting the bacterial membrane lipid bilayer. Additionally, myricetin, another polyphenol, demonstrated significant antioxidant and antimicrobial capabilities (Gordon and Roedig-Penman, 1998; Kim et al., 2020).

Notably, the mechanisms of action of these extracts, either alone or in combination, appear to differ from that of cefixime, a β-lactam antibiotic that interacts with the bacterial cell wall's mucopeptide enzyme (Zhang et al., 2015). These extracts may be acting on multiple targets, including protein synthesis inhibition (Esfandi et al., 2019a) Studies on *Fagonia Olivieri* fractions also indicated remarkable antibacterial activity, attributed to flavonoids and tannins' interactions with soluble and extracellular proteins, as well as their ability to form complexes with bacterial cell walls (Rashid et al., 2013b). This multi-target approach aligns with similar synergistic studies that aim to broaden the antibacterial spectrum, reduce antimicrobial drug toxicity, and minimize the likelihood of resistance development during therapy (Chand et al., 2021).

Additionally, *R.E. coli* treated with FICI showed damage to the cell wall, which could be a result of improved permeability of cefixime promoting its antibacterial effect. On the contrary, bacterial cells treated with MeOH depicted altered cell shape as well as changes in intracellular structures. It is postulated that the MeOH extract might have affected the protein content of bacterial cells, which requires further investigation.

It can be suggested from the results that extract alone and in combination are acting on a different mechanism than cefixime, which is a β-lactam antibiotic that has a mechanism of action by interacting with the mucopeptide enzyme of the bacterial cell wall (Zhang et al., 2015). The test samples might be acting on multi-targets in combination, while alone extracts are involved in protein synthesis inhibition as reported previously (Esfandi et al., 2019b). *Fagonia Olivieri* fractions were assessed for antibacterial and cytotoxic activity and it was reported that plant fractions showed noteworthy antibacterial activity due to the capacity of flavonoids and tannins to interact with soluble and extracellular proteins as well as to form complexes with bacterial cell walls (Rashid et al., 2013a). The multitarget approach has been reported in other synergistic studies too as the combination therapy method is anticipated to provide a wider antibacterial range, reduced toxicity of the antimicrobial drugs, and decreased chance of developing resistance during therapy (Chand et al., 2021). The pharmacological efficacy of other natural polyphenolic extracts with synthetic antibiotic were also evaluated in the literature (Atta et al., 2023).

In the current study, we evaluated the biosafety profile of extracts alone and in combination by performing a hemolytic assay and brine shrimp cytotoxicity assay. The hemolytic assay provides us with information related to the sample's toxicity on blood cells and the Brine shrimp lethality assay helps us assess the sample's preliminary *in vitro* cytotoxic effects. A hemolytic analysis is a quick, affordable, non-destructive, and sensitive test that may quickly examine a substance for biocompatibility. Toxins generated by plants may cause hemolysis since phytochemicals may have an impact on the RBC membrane. Consequently, it is crucial to assess the hemolytic activity of therapeutic herbs (Hansen et al., 2011). This model has been employed in multiple research to assess medication interactions as well as the basic cytotoxicity of plant extracts (Zohra and Fawzia, 2014). It can be seen from the above-mentioned results of the Hemolytic assay that *F. indica* extract's cytotoxicity was higher when extracts were used at MIC concentrations as compared to FICI concentrations. This is an advantage of using a combination in that the doses of the extract and cefixime were reduced, which in turn declined the cytotoxicity, yet, the efficacy was maintained. These results are in a census with literature, which showed that synergistic combinations have reduced cytotoxicity (Chand et al., 2021). This needs to be further validated by *in vivo* investigation of toxicity to

generate the safety profile of the synergistic combination (Singh and Katoch, 2020). Due to the significant buildup of polyunsaturated fatty acids and hemoglobin, erythrocytes are naturally more susceptible to peroxidation. The presence of phytochemicals such as flavonoids and phenolics is responsible for antioxidant potential (Sumaira et al., 2011). The ability of an extract to reduce lipid peroxidation by scavenging hydroxyl radicals is directly connected to its bioactivity and protective effects on RBC (Sumaira et al., 2011) which may have contributed to the hemolytic potential. Whereas, the brine shrimp lethality assay shows that the alone extract was cytotoxic while in combination the cytotoxicity was increased. According to the extensive scientific literature on the genus Fagonia, the saponin glycosides are responsible for the majority of its biological activity (Ibrahim et al., 2008). The mechanism of action of saponins' antibacterial actions may entail disruption to the membrane, which results in the release of cellular materials and ultimately cell death (Mshvildadze et al., 2000). Similar results for antibacterial and cytotoxicity experiments have been reported in other research (Rashid et al., 2013a).

5. Conclusion

According to the study, *F. indica* extracts are antimicrobial, especially when used against resistant bacterial strains. Significant amounts of polyphenols are present in the extracts, which support their antibacterial activity. Studies on synergism revealed that MeOH extract partially synergized with all of the chosen resistant bacteria, while n-Hex and E.A extracts completely synergized with cefixime-resistant *E. coli*. According to time-kill kinetics experiments, the antibacterial effects were dependent on both concentration and time. The extracts and cefixime in combination showed the strongest antibacterial action for 6 to 12 h. The results were further verified by SEM and protein estimation studies, which showed prominent damage to bacterial cell structures and a decrease in bacterial protein content. The combination therapy was found to be less harmful and biocompatible, pointing to the possibility of further study using cefixime and *F. indica* extracts in combination through *in vivo* and molecular testing.

CRediT authorship contribution statement

Anum Abrar: Conceptualization, Methodology, Software, Validation, Investigation, Data curation, Visualization. **Aroosa Zafar:** Methodology, Software, Validation, Formal analysis, Data curation. **Mahvish Fatima:** Formal analysis, Resources, Supervision, Project administration. **Durdana Muntaqua:** Validation, Formal analysis, Writing – review & editing. **Iffat Naz:** Visualization, Project administration. **Humaira Fatima:** Resources, Writing – review & editing, Visualization. **Ihsan ul Haq:** Conceptualization, Validation, Resources, Supervision.

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.

Acknowledgment

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2023.101893.

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