Characterisation and evaluation of antibacterial potential of guava extract loaded poly-3-hydroxybutyrate-co-3-hydroxyvalerate nanoparticles against multidrug-resistant bacteria

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In this work, the antibacterial potential of different plant's leaves, i.e. *Psidium guajava* (guava), *Raphanus sativus* (radish), *Solanum pseudocapsicum* (winter cherry), *Mentha royaleana* (mint) and *Calotropis procera* (rubber tree) was evaluated against the multidrug-resistant (MDR) bacteria. Methicillin-resistant *Staphylococcus aureus* (MRSA), *Vibrio cholerae* O1 El Tor (VC O1 ET), *V. cholerae* Non-O1/Non-O139 (VC N-O1/N-O139), enteroaggregative *Escherichia coli* (EAEC) and enteropathogenic *Escherichia coli* (EPEC) were included among the MDR bacterial strains. The significantly high ($P < 0.001$) antibacterial activities were observed against the MDR bacteria in case of the methanol extract of the *P. guajava* (GUV) leaves. The characterisation studies of GUV extract were done by thin layer chromatography, wide angle X-diffraction and Fourier transform infrared techniques. The GUV extract was encapsulated in poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) nanoparticles using the nanoprecipitation method. Blank PHBV nanoparticles demonstrated no antibacterial activity against the selected MDR strains. The GUV extract loaded PHBV nanoparticles produced significantly large ($P < 0.001$) zones of inhibition, i.e. MRSA (24 mm), EPEC (23 mm), EAEC (23 mm), VC O1 ET (21 mm) and VC N-O1/N-O139 (20 mm) against the MDR bacterial strains as compared with the GUV extract alone. The results have demonstrated a great potential of PHBV nanoparticles to become an efficient carrier for delivery of the potent bioactive molecules.

1. Introduction: Multidrug resistance is defined as the insensitivity or resistance of bacteria to the administered antimicrobial drugs despite of having an earlier sensitivity to it. Serious apprehensions are associated with emerging multidrug-resistant (MDR) bacterial pathogens, especially those causing the waterborne diseases and nosocomial infections [1, 2]. Waterborne diseases such as diarrhoea and/or cholera are generally transmitted by drinking the contaminated water. Diarrhoea is the leading cause of morbidity and mortality among the children younger than 5 years in many low- and middle-income countries [3–6]. Enteroaggregative *Escherichia coli* (EAEC) and enteropathogenic *Escherichia coli* (EPEC) are responsible for causing the diarrhoeal outbreaks. Cholera (a severe diarrhoea) is caused by *Vibrio cholerae*. *V. cholerae* belongs to genetically versatile bacterial species. More than 200 serogroups of *V. cholerae* have been identified, among which only O1 and O139 are epidemic in nature [7]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is involved in the spread of nosocomial infections [8].

Plants, in particular, have formed the basis of sophisticated traditional medicine systems. Plants are a rich source of bioactive compounds with antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory and anticancer properties [9]. *Raphanus sativus* is a member of the *Brassicaceae* family. The seed extracts of *R. sativus* displayed antimicrobial activity against *Hafnia alvei, Paecilomyces variotii, Enterobacter agglomerans, Penicillium lilacinum, Lactobacillus*, *Bacillus thuringiensis, Penicillium funiculosum, Spadicoides stoveri* [10]. *Psidium guajava* or guava (in English) belongs to family *Myrtaceae* and is found commonly in hot environmental conditions. Quercetin isolated from guava leaves was found to be the most active antioxidant compound [11]. *Calotropis procera* (Family *Asclepiadaceae*) is a shrub or small tree up to 2.5 m height. *C. procera* has been used in the treatment of wound infections, expectorant, an antidote for snake poison, ulcers, asthma, dysentery, piles, tumours, leprosy and so on. The genus *Mentha* has high pharmacological importance for the treatment of nausea, anorexia, ulcerative colitis, bronchitis, flatulence and liver ailment [12]. *Solanum pseudocapsicum* (family *Solanaceae*) is reported for its antiviral, cytotoxic, hepatoprotective and antitumor properties [13]. Polymeric nanoparticle (PNP) is considered as an ideal carrier for delivery of bioactive molecules or therapeutic agents for treatment of human diseases [14, 15]. The high solubility of constituents, decrease in the therapeutic dose and enhanced absorption of the bioactive components are among the main advantages of PNP-based delivery system. The poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) is a natural aliphatic polyester. The PHBV is synthesised by many microorganisms as inclusion bodies during the growth limiting conditions [16]. The PHBV has a potential application as a drug carrier in the biomedical field due to its biodegradability, non-toxicity and biocompatibility [17].

The objective of this Letter was to evaluate the antibacterial potential of the methanol extract of the local plant's leaves, i.e. guava, radish, winter cherry, mint and rubber tree against the selected MDR bacteria. The crude GUV extract demonstrated potent antibacterial activity and was characterised by thin layer chromatography (TLC), wide angle X-ray diffraction (WAXD) analysis and Fourier transform infrared (FTIR) spectroscopy techniques. The GUV extract was encapsulated in the PHBV nanoparticles. The GUV extract loaded PHBV nanoparticles were characterised in term of their physical properties. Additionally, the antibacterial potential of the guava extract loaded PHBV (GUV-PHBV)

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Table 1 Antibiotic-resistant profile of the selected MDR bacterial strains

Strains	MRSA	EAEC	EPEC	VC O1 ET	VC (Non-O1/ Non0139)
tetracycline	R	R	R	R	S
nalidixic acid	S	R	R	R	S
chloramphenicol	S	S	S	S	S
amoxicillin	R	S	S	R	R
erythromycin	R	R	R	S	R
ciprofloxacin	R	R	R	S	R
ampicillin	R	R	S	R	R
sulphamethoxazole	S	R	R	R	S

R indicates the resistance against a particular antibiotic. S indicates the sensitivity against a particular antibiotic. ND means not determined.

nanoparticle was determined against the selected MDR bacterial pathogens. This is the first report, which describes the use of PHBV nanoparticles for delivery of plant based bioactive compounds for the treatment of infections caused by the MDR bacteria.

2. Material methods

2.1. Bacterial strains: Both MDR gram positive (ATCC-25923) and gram negative (ExPEC, EAEC, EPEC) bacterial strains are used in this Letter. Except MRSA all strains were clinical isolates and were obtained from the Microbiology & Public Health lab, Department of Biosciences, COMSATS Institute of Information Technology (CIIT), Islamabad, Pakistan. The antibiotic-resistant profiles of above-mentioned strains are given in Table 1.

2.2. Collection and identification of plant samples: Leaves of mature plants, including guava, radish, winter cherry, mint and rubber tree were collected from District Rawalpindi, Punjab, Pakistan during July 2013 to October 2013. The collected plants were also identified by the experts of the Herbarium at Quaid-i-Azam University, Islamabad, Pakistan.

2.3. Preparation of plant extract: Leaves of all plants were cleaned and shade dried at a room temperature. Dried leaves were ground into powder with the help of an electronic blender (Sonashi, SB-119). Methanol was used as an organic solvent for extraction of the bioactive compounds due to its capability to dissolve a variety of polar compounds. About 25 g of leaf powder of each of the plants was soaked in 250 ml of methanol and kept for 10 days. The methanol extract was filtered with the help of Whatman filter paper and dried at a room temperature.

2.4. Antibacterial studies: Agar-well diffusion method was used to evaluate the antibacterial potential of methanol extract of the selected plants [18]. Briefly, 100 mg of a dried methanol extract of each plant was dissolved in 5 ml of dimethyl sulphoxide. An inoculum of each strain with colony-forming units (0.5×10^8/ml) was spread onto Mueller–Hinton agar (MHA) plates using sterile swabs. The wells were filled with 100 µl of dilution (1, 5, 10, 15, 20 mg/ml) of each plant extract. The MHA plates were incubated for 24 h at 37°C. The methanol extract of the *P. guajava* plant showed significantly higher zones of inhibition therefore it was selected for further studies. Vancomycin was used as a positive control in this study.

2.5. Characterisation of guava extract

2.5.1. Thin layer chromatography: The methanol extract of *P. guajava* was subjected to the TLC as per conventional one-dimensional ascending method. Silica gel G (Merck Millipore, Darmstadt, Germany) was used as a stationary phase. The mobile phase was comprised of N-butanol (BuOH), ethyl acetate (EtOAc), propanol (PrOH), acetic acid (AcOH) and distilled H_2O in the ratio of 7:20:12:7:6. The obtained spots were visualised using Portable UV Lamp (Spectronics, New York) at 365 and 254 nm wavelengths and Rf values were calculated.

2.5.2. FTIR spectroscopy: The structural analysis of functional groups of the GUV extract was done by the FTIR (Nicolet model 6700; Thermo Electron Corp, Marietta, OH) spectrophotometer in the wavelength region from 4000 to 400 cm^{-1}. The sample was placed in a sample holder. The spectrum was recorded using an attenuated reflectance technique involving a diamond crystal. The results were averaged over 200 scans.

2.5.3. WAXD analysis: The WAXD spectrum of the GUV extract was recorded using the STOE powder diffraction system (STOE & Cie GmbH) with tube anode Cu over the interval 5–70°/2θ. The operational data were as follows: generator tension (voltage) 40 kV, generator current 40 mA and scanning speed 2°/min.

2.6. Preparation of nanoparticles: PHBV was obtained by fermentation from bacterial strain *Bacillus cereus* S10 [19]. The crude PHBV was purified with hot chloroform using soxhlet apparatus for 72 h. The PHBV was precipitated by cold chilled methanol. The characterisation of purified PHBV was done by FTIR and WAXD techniques. This copolymer was used for preparation of nanoparticles by the nanoprecipitation method. Briefly, PHBV (50 mg) and guava (30 mg) were dissolved in 3 ml acetone. This mixture was added to a 12 mM sodium deoxycholate solution (50 ml) and the mixture was gently stirred at room temperature for 8 h. The GUV-PHBV nanoparticles were separated by centrifugation at 14,000 rpm for 15 min and then washed twice with Milli-Q water (EMD Millipore Bedford, MA, USA). Blank PHBV nanoparticles were also prepared by the same method without taking a GUV extract in an organic phase.

2.7. Characterisation of nanoparticles

2.7.1. Scanning electron microscopy (SEM): SEM was performed to evaluate the surface morphology of blank PHBV and GUV-PHBV nanoparticles. The sample was prepared by uniformly dispersing the nanoparticles in Milli-Q water and diluted to 1 mg/ml. A drop of the nanoparticles suspension was placed on a cover glass and left for air drying at a room temperature. The cover glass containing dried nanoparticle suspension was mounted on the aluminium stumps and was coated with gold in JFC1500 ion sputtering device for 1.5 min at 290°A.The samples were then examined under SEM (JS-6490A, JEOL, Japan).

2.7.2. FTIR spectroscopy: Both blank PHBV and GUV-PHBV loaded nanoparticles were subjected to FTIR analysis, in the wavelength region from 4000 to 400 cm^{-1} as described above.

2.7.3. Antibacterial studies: The antibacterial potential of blank and GUV-PHBV nanoparticles was evaluated against the selected MDR bacterial strains by agar-well diffusion method [18]. Minimum inhibitory concentration (MIC) of the designed delivery system was also determined against the selected MDR strains using different concentrations (1000, 750, 500, 250 and 100 µg/ml) of GUV-PHBV nanoparticles.

2.8. Statistical analysis: All the experiments were carried out in triplicate. The obtained data were analysed using two-way analysis of variance. The results were expressed as mean ± standard error (SE).

3. Results and discussion: The recent alarming rise in the incidence of new types of MDR bacterial infections is a real crisis in public health systems worldwide. Plant-based bioactive molecules are considered as an excellent alternative for the treatment of MDR infections. However, to date, there is no report

using plant-based bioactive molecule loaded PHBV nanoparticles for the treatment of MDR bacterial strains.

3.1. Antibacterial studies: Totally, five plants were investigated in the present study: *Mentha royleana, C. procera, S. pseudocapsicum, P. guajava* and *R. sativus*. Figs. 1*a–e* shows the comparison of antibacterial activities of methanol extract of *M. royleana*, *S. pseudocapsicum*, *C. procera, R. sativus* and *P. guajava* against selected MDR strains. It is observed that the methanol extract of *M. royleana* was significantly effective ($P < 0.001$) only against the VC O1 ET (14 mm) and VC N-O1/N-O139 (12 mm) bacterial strains at a concentration of 20 mg/ml (Fig. 1*a*). The plant extract of *Mentha spicata* suppressed the growth of *Staphylococcus aureus, Salmonella typhi* and *Pseudomonas aeruginosa* [20]. The methanol extract of *C. procera* at a concentration of 20 mg/ml significantly ($P < 0.001$) inhibited the growth of the EAEC (12 mm), VC O1 ET (12 mm) and VC N-O1/N-O139 (12 mm) strains (Fig. 1*b*). The methanol extract of *C. procera* demonstrated moderate antibacterial activity against *Pseudomonas marginalis* and *Streptococcus mutans* [21]. The methanol extract of *R. sativus* at a concentration of 20 mg/ml exhibited potent antibacterial activity (14, 13.5, 14, 14, 14.5 mm) against the MRSA, EPEC, EAEC, VC O1 ET and VC N-O1/N-O139 strains (Fig. 1*c*). On the other hand, the methanol extract of *S. pseudocapsicum* (at a concentration of 20 mg/ml) showed significantly higher ($P < 0.001$) antibacterial properties (12, 12.5, 12.5 and 11.5 mm) against four MDR bacterial strains, i.e. MRSA, VC O1 ET, VC N-O1/N-O139 and EPEC (Fig. 1*d*). Ethyl acetate, acetone and methanol extracts of *S. pseudocapsicum* showed antibacterial activity against *Aspergillus niger, Penicillium notatum* and *Fusarium oxysporum* [22]. The methanol extract of *P. guajava* at a concentration of 20 mg/ml was observed to be the most potent of all tested extracts and inhibited the growth of all MDR strains (Fig. 1*e*). The zone of inhibition was found to be 16 mm against EAEC strain. While, 15 mm zone of inhibition was observed against the MRSA, VC O1 ET, VC N-O1/N-O139 and EPEC bacterial strains, respectively. Guava extract is useful to control the foodborne pathogens and spoilage organisms due to the presence of antibacterial compounds in it [23]. The zone of inhibition for vancomycin (a positive control) was found to be 16 and 10 mm, respectively, against MRSA and EPEC strains. However, no zone of inhibition of vancomycin was observed against EAEC, VC O1 ET, VC N-O1/N-O139 bacterial strains.

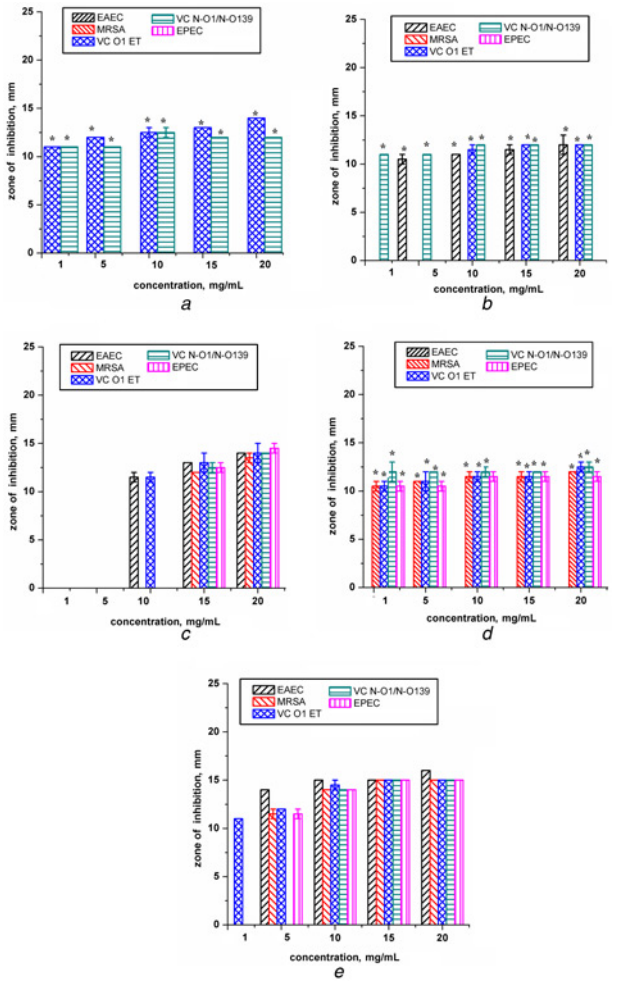

Fig. 1 *Comparison of antibacterial activities of methanol extract of*
a M. royleana
b C. procera
c R. sativus
d S. pseudocapsicum
e P. guajava against the selected MDR bacterial strains
(* represents $P < 0.001$; I represents SE and data shown as mean ± SE) ($n = 3$). (EAEC represents enteroaggregative Escherichia coli; EPEC represents enteropathogenic Escherichia coli; VC O1 ET represents Vibrio cholerae O1 El Tor; VC N-O1/N-O139 represents Vibrio cholerae Non-O1/Non-O139; MRSA represents methicillin-resistant Staphylococcus aureus]

3.2. Characterisation studies

3.2.1. Thin layer chromatography: TLC profiling of GUV extract was done by mobile phase comprising of BuOH:EtOAc:PrOH:AcOH:H_2O in the ratio of 7:20:12:7:6. The obtained spots had 0.38, 0.40, 0.42 and 0.45 *R*f values, which were consistent with the previous findings [24]. According to Arima and Danno [24], the antibacterial compounds such as morin-3-*O*-lyxoside, morin-3-*O*-arabinoside, quercetin-3-*O*-arabinoside and quercetin, had Rf values, i.e. 0.38, 0.40, 0.42 and 0.45 respectively, using the above-mentioned mobile phase.

3.2.2. FTIR analysis: The FTIR spectrum of the GUV extract showed absorption bands at 2930, 1687, 1453 and 1031 cm^{-1}, respectively (Fig. 2*a*). The absorption bands at 2930 and 1453 cm^{-1} were indicating the presence of the CH stretching vibrations of CH, CH_2 and CH_3 bonds. The band at 1687 cm^{-1} showed the existence of α, β-unsaturated ketone. The band of α, β-unsaturated ketone (1656 cm^{-1}) and aromatic ring (1606 and 1502 cm^{-1}) was due to the presence of antimicrobial compounds in the guava leaves extract [24]. Flavonoids are the hydroxylated phenolic substances and occur as C3–C6 unit linked to an aromatic ring [25]. The absorption band at 1031 cm^{-1} was a characteristic feature of carbohydrates [26].

Fig. 2*b* shows the IR spectrum of purified PHBV. FTIR spectrum of PHBV was characterised by absorption bands at 2975, 2932, 1450 and 1375 cm^{-1}, which were indicating the presence of the

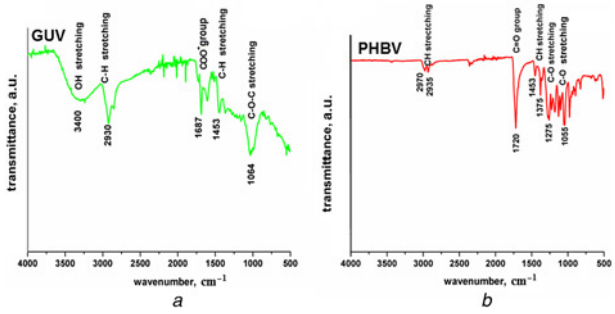

Fig. 2 *FTIR spectrum of*
a Guava (GUV) extract
b Pure PHBV

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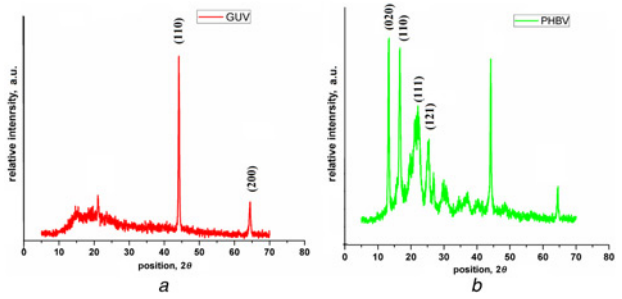

Fig. 3 *WAXD spectrum of*
a Guava (GUV) extract
b Pure PHBV

CH stretching vibrations of CH, CH_2 and CH_3 bonds. The absorption peaks at 1719 and 1275 cm^{-1} were corresponding to the presence of the C=O stretching and C–O stretching, respectively. The absorption bands at 1724 and 1280 cm^{-1} were representing the presence of C=O and C–O groups, respectively, of polyhydroxyalkanoates [27].

3.2.3. WAXD analysis: Figs. 3*a* and *b* show the WAXD spectra of GUV extract and PHBV. The WAXD spectra indicate the crystalline nature of GUV extract and PHBV. WAXD spectrum of guava extract was characterised by the presence of sharp peaks at 44.14° and 65.18° (2θ) corresponded to the 110 and 200 planes, respectively. WAXD spectrum of PHBV showed peaks at 13.32°, 16.63°, 22.36° and 25.32° (2θ) attributed to the 020, 110, 111 and 121 planes, respectively, as reported previously by Mohamed El-Hadi [28].

3.3. Preparation of nanoparticles: Both blank PHBV and GUV-PHBV nanoparticles were synthesised by the nanoprecipitation method. The nanoparticle formulations were characterised by SEM and FTIR techniques. An average yield and diameter of blank and GUV extract loaded PHBV nanoparticles are given in Table 2.

3.4. Characterisation studies
3.4.1. Scanning electron microscopy: SEM is used to evaluate the surface morphology of nanoparticles. Figs. 4*a* and *b* show the smooth and spherical morphology of blank and GUV extract

Table 2 Yield and average particle diameter of PHBV and GUV-PHBV nanoparticles

Nanoparticles	Nanoparticle yield, %	Particle diameter, nm
PHBV	45.50 ± 1.85	20.00 ± 2.45
GUV-PHBV	40.40 ± 1.43	100.00 ± 2.98

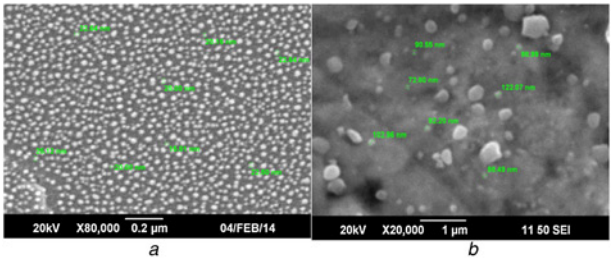

Fig. 4 *Scanning electron micrographs of*
a Blank PHBV
b GUV-PHBV nanoparticles

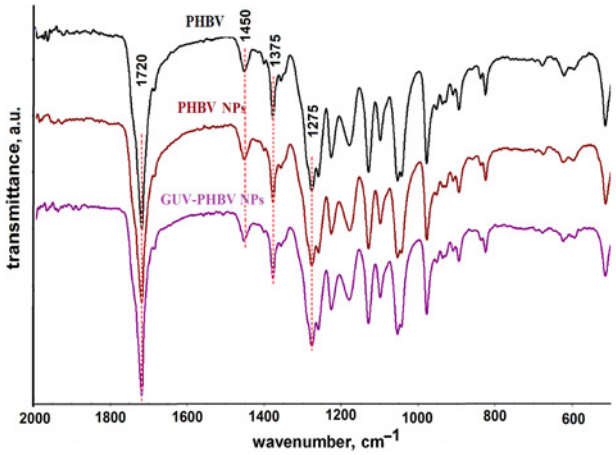

Fig. 5 *FTIR spectra of pure PHBV, blank and GUV extract loaded PHBV nanoparticles in the range of 2000–500 cm^{-1}*

loaded PHBV nanoparticles. The average diameter of the blank PHBV nanoparticle was 20 nm. On the other hand, the average diameter of GUV-PHBV nanoparticle was 100 nm. The larger size of GUV-PHBV nanoparticles was due to absorption and adsorption of the GUV extract on the surface of PHBV nanoparticles. This observation is in agreement with the previous findings [15]. The size of roxithromycin loaded poly-(lactic-co-glycolic acid) nanoparticles was increased due to the encapsulation of roxithromycin within the polymeric matrix of poly-(lactic-co-glycolic acid) [15].

3.4.2. FTIR analysis: FTIR analysis of blank and GUV-PHBV nanoparticles is shown in Figs. 5*a* and *b*. A considerable increase in the peak intensity of C=O group (1720 cm^{-1}) was evident for both blank and GUV-PHBV nanoparticles after nanoparticle formation. Additionally, in case of GUV-PHBV nanoparticles, the absorption peaks of CH group of CH, CH_2 and CH_3 bonds at 1450 and 1375 cm^{-1} were shifted to 1465 and 1385 cm^{-1} after nanoparticle formation. The peak at 1275 cm^{-1} (C–O stretching) was shifted to 1265 cm^{-1} and its intensity was also increased. It is suggested that the shift as well as an increase in the peak

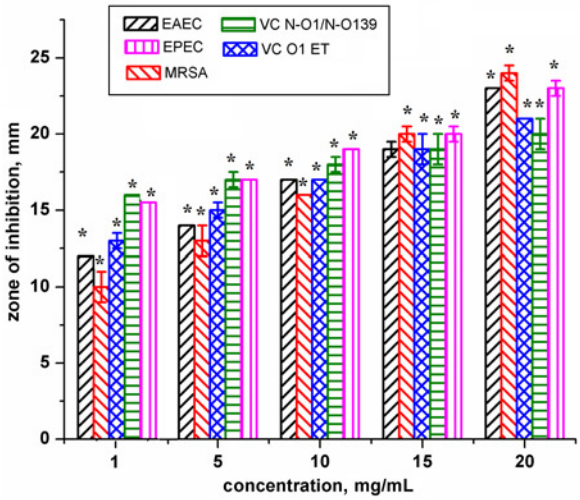

Fig. 6 *Antibacterial activities of GUV-PHBV nanoparticles against selected MDR strains (* represents $P < 0.001$; bars represent SE and data shown as mean ± SE) ($n = 3$). (EAEC represents enteroaggregative Escherichia coli; EPEC represents enteropathogenic Escherichia coli; VC O1 ET represents Vibrio cholerae O1 El Tor; VC Non-O1/Non-O139 represents Vibrio cholerae Non-O1/Non-O139; MRSA represents methicillin-resistant Staphylococcus aureus]*

Table 3 MIC of GUV-PHBV nanoparticles against selected MDR bacterial strains

MDR bacterial strain	MIC, μg/ml
EAEC	50
EPEC	50
VC O1 ET	75
VC NO1/N-O139	50
MRSA	50

intensities was due to the formation of the electrostatic interactions and van der Waals forces between bioactive molecules present in the GUV extract and PHBV after nanoparticle formation.

3.4.3. Antibacterial studies: The antibacterial activity of GUV-PHBV nanoparticles against selected MDR strains is given in Fig. 6. Blank PHBV nanoparticles (20, 15, 10, 5 and 1 mg/ml) showed no antibacterial activity against any of the selected MDR strains. Poly-3-hydroxybutyrate nanoparticles are safe to use in animals according to ISO 10993 [30]. On the other hand, GUV-PHBV nanoparticles produced significantly higher ($P < 0.001$) zones of inhibition against MRSA (24 mm), EPEC (23 mm), EAEC (23 mm), VC O1 ET (21 mm) and VC N-O1/N-O139 (20 mm) bacterial strains at a concentration of 20 mg/ml. The antibacterial activity of GUV-PHBV nanoparticle was due to the presence of the flavonoids. The flavonoids are reported to intricate within the bacterial cell walls and/or extracellular and soluble proteins of the infection causing microorganisms [25]. Flavonoids, especially the morin glycoside, quercetin glycosides and quercetin present in the methanol extract of guava, showed potent antibacterial properties against *B. cereus* and *S. enteritidis* strains [24]. Methanol and ethanol extracts of the guava leaves showed inhibitory activity against the gram-positive bacteria, whereas the gram-negative bacteria were resistant to both solvent extracts [29]. The slow degradation of PHBV chains enabled the release of antimicrobial compounds from nanoparticles in a sustained manner for a prolonged period of time by the surface erosion. The MIC of the designed delivery system against the selected MDR strains is given in Table 3.

4. Conclusion: In the present investigation, leaves extracts of *M. royleana, C. procera, S. pseudocapsicum, P. guajava* and *R. sativus* were prepared with methanol. All plant extracts exhibited the variable antibacterial activities against the selected MDR strains. The GUV extract appeared to be more potent in terms of the antibacterial activity and therefore it was encapsulated in PHBV nanoparticles using the nanoprecipitation method. Blank PHBV nanoparticles showed no antibacterial activity against the selected MDR strains. But, the GUV-PHBV nanoparticles demonstrated a significant increase in the sizes of zone of inhibition against the selected MDR bacterial strains as compared with GUV extract alone. Thus, the PHBV nanoparticles can be used as a safe carrier for the delivery of bioactive molecules.

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