

Drug delivery applications of exopolysaccharides from endophytic bacteria *Pseudomonas otitidis* from *Tribulus terrestris* L

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Abstract

The present study aimed to produce a novel biocompatible, stable and biodegradable exopolysaccharide (EPS) from endophytic bacteria to deliver drugs into malignant cells effectively. EPS-producing endophytic bacteria, *Pseudomonas otitidis* was isolated and identified from the roots of the medicinal plant *Tribulus terrestris* L. The ice-cold ethanol precipitation method was used to isolate EPS from an endophytic bacterial culture. The isolated EPS was partially characterized by FTIR, NMR spectroscopy, and the presence of reducing sugar and protein contents were also measured. The isolated EPS contained $26.665 \pm 1.302\%$ of reducing sugar and $0.912 \pm 0.023\%$ of proteins. Further, EPS-based quercetin-loaded nanoparticles (NPs) were formulated through the precipitation method to deliver quercetin into breast cancer cells. The fabricated nanoparticles size, shape, physical nature, drug release and release kinetics properties were studied and confirmed. EPS-based nanoparticles could cause the highest release of quercetin in an acidic medium at pH 5.0, and they had high biocompatibility in a physiological medium. In addition, the NPs showed significant free radicals (DPPH* and ABTS*) scavenging and ferric ion reducing potentials and concentration-dependent cytotoxic effects against breast cancer cell lines (MCF-7 cells) after 24 h treatment with IC₅₀ value of $14.313 \mu\text{g ml}^{-1}$. The obtained results demonstrated that the isolated EPS from endophytic bacteria can positively applied as a drug delivery vehicle and enhanced the activity of the loaded drug.

Introduction

For the last two decades biotechnological, healthcare, and pharmaceutical industries have tried to develop drug delivery systems to effectively delivering drugs to the target site without disturbing other healthy tissues/organs (Coelho et al. 2010). The identification of efficient drug delivery systems is an essential part of the design and development of newer medicines (Patra et al. 2018). The drug delivery systems protect the loaded or attached drugs by early degradation of body fluids and keep the blood drug concentrations from fluctuating, and reduce the dosage frequency (Jantzen and Robinson 2002). However, a thorough understanding of drug properties, biocompatibility, and route of drug administration is previously necessary in order to develop a drug delivery system effectively. Therefore, researcher and scientists are continuously looking for new strategies to deliver drugs in a safe and well-controlled release over a long period. Many drug delivery systems have been developed using exopolysaccharides (EPS) without altering the chemical structure of drugs and disintegrating them inside the body with normal biological processes (Saha and Datta 2022). These EPS eliminate the need to remove a drug delivery system once the active ingredient has been delivered (Kumari et al. 2010). Moreover, EPS increase the bioavailability of drugs at the target site in a steady and controlled release manner and maintains the plasma concentration for the entire period of treatment with maximize therapeutic efficacy and minimal adverse effects (Kaur et al. 2018).

Exopolysaccharides are extracellular carbohydrate biopolymers that produce a substantial component of the extracellular polymer surrounding most microbial cells (Nichols et al. 2005). Although, the EPS of various microorganisms (bacteria and fungus) have monomeric compositions similar to well-known

plant or animal products, they exhibit a wide range of structural combinations, which mediates their unique properties (Mahapatra and Banerjee 2013). Over plant- or macroalgae-derived EPS, microbial production has a number of advantages, such as specified and reproducible production parameters to avoid environmental impacts and produce high-quality final products (Kumar et al. 2023). Compared to EPS that are isolated from plants, substantially more significant production titers may be made from bacteria. Some of the most studied EPS produced by microorganisms are xanthan, dextran, alginate, gellan, levan, cellulose, curdlan, pullulan, succinoglycan, and hyaluronic acid (Öner 2013). Majority of the EPS are polyanionic in nature and can attach to positively charged molecules either by polyelectrolyte complexation or ionotropic gelation (Raveendran et al. 2013b). In order to make nanoparticles and nano/microspheres for the delivery of chemotherapies, peptides, and genes, they are widely used in nanotechnology applications (Vanavil et al. 2020). Endophytes are one of the cost-effective producers of EPS, and well-known therapeutically essential compounds (Kalimuthu et al. 2022b).

Endophytic bacteria are beneficial to plant bacteria living inside the plant tissues (Kalimuthu et al. 2022a). They can increase the ability of plants to absorb nutrients and minerals from soil and without causing any apparent harm to their host (Gerdemann 1968). Amazingly, the ability of endophytic bacteria to produce novel bioactive compounds and EPS biopolymers with enhanced therapeutic potential has attracted great attention in recent years (Angelin and Kavitha 2020). EPS are water-soluble gums with unique and novel properties (Sutherland 1998). EPS have a wide range of applications in industry and research due to their structural, physical, and rheological properties, making them effective emulsifying, gelling, thickening, stabilizing, suspending, film coating, coagulating, and viscosifying agents (Shanmugam and Abirami 2019; Prajapati et al. 2013). EPS have also been said to be Generally Recognized as Safe (GRAS) substances, which means that using them poses no health risks (Badel et al. 2011). As a result, endophytic bacterial EPS are widely used for pharmaceutical excipients and drug delivery applications.

Tribulus terrestris L. (caltrop family: Zygophyllaceae) is a plant that has been used for various potential health benefits for many years (Hashim et al. 2014). It is widely distributed around the world. *T. terrestris* L. has been used for centuries in Ayurveda to treat impotence, venereal diseases, and sexual debility (Chhatre et al. 2014). In Bulgaria, the plant is used as a folk medicine for treating impotence (Chhatre et al. 2014). EPS from endophytic bacteria is a renewable source and protect themselves as well as plant against pathogens (Moscovici 2015). Because some of the bacterial exopolysaccharides also possess medicinal values. The present work aimed to investigate the exopolysaccharides from endophytic bacteria isolated from the root of *T. terrestris* and their potential application as a drug delivery carrier.

Experimental Section

Materials and reagents

The chemicals, quercetin, Dulbecco's Modified Eagles medium (DMEM), rhodamine-123, fetal bovine serum (FBS), Tris-EDTA buffer, propidium iodide (PI), ethidium bromide (EtBr), annexin-v, and 4',6'-diamino-2-phenylindole (DAPI), fluorescein isothiocyanate (FITC), amphotericin B, phosphate-buffered saline (PBS), 2',7'-dichlorofluorescein diacetate (H₂DCF-DA), penicillin G sodium, streptomycin sulphate, lactophenol, 2,2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Himedia Laboratories, Pvt., Ltd., Mumbai, and Sigma-Aldrich Chemicals, Ltd., Bengaluru, India. Merck and SD Fine Chemicals in Mumbai, India supplied all other reagents, solvents and various chemicals (analytical grade).

Cell lines and culture media

MCF-7 cell line (human breast epithelial cancer cells) was procured from the National Centre for Cell Science (NCCS), Pune, India. The MCF-7 cells were grown in T 25 flasks (Falcon, USA) containing a complete DMEM medium composed of 20% heat-inactivated FBS, 1% penicillin, streptomycin, and amphotericin B at 37 °C and 5% CO₂ in a humidified atmosphere.

Plant sample collection and isolation of endophytic bacteria

The healthy plant, *T. terrestris* L. was harvested from Sundarapandiam village, Virudhunagar Dist., Tamil Nadu, India (latitude 9.59721°N, longitude 77.67405°E). The region has a large variety of plants and a high level of vegetative growth, with diverse flora. The harvested plant was brought to the lab under sterile conditions. On the same day, the plant sample was processed for the isolation of the endophytic bacteria from the root. The root part of the plant sample was incised separately into small pieces and washed with tap water. The root was then surface sterilized with deionized water for 3 min. Later, the root was soaked in 80% ethanol for 5 min and then washed in sodium hypochlorite (1%) for 2 min. Finally, the root sample was again washed with deionized water for 1 min. The externally sterilized root sample was finely crushed using a sterile mortar and pestle and serially diluted. The serial dilutions (10⁻² to 10⁻⁶) were inoculated in the nutrient agar plates. The plates were maintained at 27 °C for 24 h. The control plate was also incubated to check the contamination. After the incubation period, the plates were observed for the growth of colonies. The bacterial colony which was observed to produce exudate (exopolysaccharides) was Gram's stained and observed microscopically.

Molecular identification of the endophytic bacteria

The exopolysaccharides producing endophytic bacteria from *T. terrestris* L. was identified through the molecular gene sequencing (Andrés-Barrao et al. 2017). The monolayer colonies of bacterial cells were grown and subjected to lysis. The lysis buffer (50 mM Tris pH 8.0, 10% glycerol, 0.1% Triton X-100, 100 µg/mL lysozyme, DNase 3U, 1mM PMSF (antiprotease), 2 mM MgCl final concentration) about 500 µL in a 2 mL microcentrifuge tube was mixed with the bacterial culture. The cells were finely lysed by repeated pipetting. 4 µL of RNase and 500 µL of neutralization buffer (3/5 M potassium acetate, pH 6.0) was added to the contents. The contents were vortexed and incubated for 30 min at 65 °C in a water bath. To minimize shearing the DNA molecules, mix DNA solutions by inversion. After the incubation, the contents

were centrifuged at 10,000 rpm for 10 min. Following centrifugation, the resulting viscous supernatant was transferred into a fresh 2 mL microcentrifuge tube without disturbing the pellet. Then, about 600 μL of chloroform isoamyl alcohol was added, and hand mixed vigorously. The contents were then centrifuged for 10 min at 10,000 rpm and 600 μL of the aqueous phase was transferred into a fresh 2 mL microcentrifuge tube. For binding, the 600 μL of binding buffer (6 M guanidine thiocyanate in pH 8.0) was added to the mixture by pipetting and incubated at room temperature for 5 min. Then, about 600 μL of the contents were transferred to a spin column placed in a 2 mL collection tube. The contents were centrifuged for 2 min at 10,000 rpm and discarded flow-through. The spin column was reassembled, and the remaining 600 μL of the lysate was transferred to the collection tube. The contents were centrifuged for 2 min at 10,000 rpm. For the washing process, 500 μL washing buffer I (20 mM NaCl, 2 mM Tris-HCl, 80% ethanol, in pH 7.5) was added to the spin column and centrifuged at 10,000 rpm for 2 min. The spin column was reassembled and 500 μL washing buffer II (50 mM Tris HCl, 10 mM EDTA, RNase A (100 $\mu\text{g}/\text{mL}$), in pH 8.0) was added, centrifuged at 10,000 rpm for 2 min and discard flow-through. 100 μL of elution buffer (0.5 mM EDTA, 10 mM Tris-HCl, in pH 9.0) was added in the middle of the spin column, which contains DNA and incubated at room temperature for 2 min. Single-pass sequencing was performed for bacterial identification. The ITS region of the DNA was amplified using universal 27F (AGAGTTTGATCTGGCTCAG) forward and 1492R (TACGGTACCTTGTTACGACTT reverse primers. The fluorescent-labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. Using the protocols, the products were sequenced in both the forward and reverse directions using a Genetic Analyzer 3500 (Life Technologies Corporation, Applied Biosystems®, California 94404, USA).

Extraction and purification of the bacterial exopolysaccharides

The endophytic bacterial exudate (exopolysaccharides) was separated by the ethanol precipitation method (Wang et al. 2011). Ethanol precipitation is one of the frequently considered methods for the separating EPS from the bacterial culture. The bacterial broth culture was centrifuged at 5,000 rpm for 10 min at 4 °C to remove the pellet containing bacterial cells. The supernatant containing the bacterial EPS fraction was mixed with an equal volume of ice-cold ethanol (96% v/v) and incubated overnight at 20 °C. After the incubation period, the mixture was centrifuged at 1000 rpm for 15 min to separate the settled EPS (pellet) in the broth. The collected EPS was lyophilized and used for further analysis.

Bacterial exopolysaccharides characterization

Fourier Transform Infrared (FTIR) Spectroscopy

The structural characterization of the EPS was examined through FTIR spectroscopy to determine the functional group's presence in the EPS. Approximately 5 μg of dried EPS was finely mixed with 100 μg of KBr powder (FTIR grade) and exposed to the hydraulic press to prepare a round translucent disc (5mm $\varnothing$). The FTIR spectra was recorded in transmittance mode in a range of 4000 to 400 cm^{-1} with an

accumulation of 15 scans, and a resolution was 4 cm^{-1} , using a Shimadzu IR Tracer-100 spectrometer (Chowdhury et al. 2017).

NMR Spectroscopy

The purified EPS was subjected to NMR analysis. Approximately 30 mg of the purified EPS sample was solubilized in D_2O to exchange deuterium two times by lyophilization. The sample was then put into 4 mm NMR tube with redissolved D_2O (0.5 mL). The experiment was performed by ^1H spectra on Bruker Advance III HD 400 MHz spectrometer (da Silva et al. 2005).

Total reducing sugar determination

By using the phenol-sulfuric acid calorimetric method, the total reducing sugar content of the EPS was calculated by ethanol precipitation (Rühmann et al. 2015). A 200 μL of EPS solution (10 mg/mL) was mixed with 200 μL of phenol solution (5%) and 1 mL of pure H_2SO_4 . After 30 min, the reaction mixture was cooled to room temperature. UV-Visible spectrophotometry (UV-1800 series, UV Probe 2.62 software, Shimadzu, Japan) was used to measure the absorbance of samples and standard sugar (10 to 100 g mL^{-1} ribose) at 492 nm.

Protein content measurement

Bradford's method was used to measure the protein content present in the bacterial EPS (Orhan-Yanikan et al. 2020). 20 μL of the EPS (10 mg/mL) were combined with 1 mL of Bradford's reagent (Himedia Laboratories, Mumbai, India), which was then incubated for 5 min at 37°C . The OD of the sample as well as standard protein (bovine serum albumin (BSA) 0 to 0.75 mg/mL) were both measured at 595 nm (UV-1800 series, UV Probe 2.62 software, Shimadzu, Japan).

Exopolysaccharides as a drug delivery vehicle

In the present investigation, EPS from endophytic bacteria is used as a drug delivery vehicle to deliver the loaded chemotherapeutic agents into cancer cells. Here, quercetin was loaded into EPS (vehicle) as a model drug. Quercetin is a plant-derived pigment (flavonoid), it's mainly present in the human diet (David et al. 2016). It has a wide range of pharmacological actions, including maintaining free radicals, eliminating cancer cells, reducing swelling, regulating blood sugar levels, and helping to prevent cardiovascular diseases (Jan et al. 2010).

Quercetin-loaded nanoparticles

Quercetin-loaded EPS nanoparticles (NPs) was formulated according to our previously published work (Kunjiappan et al. 2018b). In short, 2 mg of quercetin was dissolved in 5 mL of cell culture grade methanol and added drop-wise manner into deionized water (50 mL) containing 10 mg of EPS and 0.1 mL glutaraldehyde (cross-linking agent). The contents were kept in a magnetic stirring at room temperature ($28 \pm 2^\circ\text{C}$). The absence of precipitate in the stirring mixture serves as a visual indicator of

the self-assembled nanoparticles, and UV visible spectrophotometric examination for further confirmation of nanoparticles formation.

Characterization of the quercetin-loaded nanoparticles

The functional groups and their interaction between the EPS and quercetin in the nanoparticles were employed through FTIR spectroscopy (Kunjiappan et al. 2020b). The physical nature of the formulated quercetin-loaded EPS NPs was determined by X-ray diffractometer (BRUKER D 8 Advance ECO XRD system equipped with SSD160 1 D Detector). The XRD pattern was collected in 2θ at the range from 10 to 80° at the intensity between 2 to 12 (Kunjiappan et al. 2020a). The step counting of about 0.78 s/step was used for the analysis. The average particle size and zeta potential of the formulated quercetin-loaded EPS NPs was analyzed through Nano ZS ZEN 3600, Malvern Instrument, UK. The DLS analysis evaluates the presence of nano sized particles of the sample with respect to the stability (Mohan et al. 2020). Thus, 300 μL of sample are dispersedly placed onto the cell and exposed to laser irradiation. The morphological analysis of the formulated quercetin-loaded EPS NPs was performed in Scanning Electron Microscope (CARL ZEISS EVO 18). The dried sample was mounted on the carbon strip and dried thoroughly under mercury lamp for 5 min. In high resolution scanning electron microscope at 15kv voltage the images of the encapsulated material were taken (Baskararaj et al. 2020).

Drug release studies

In vitro release of quercetin from quercetin-loaded EPS NPs was performed in 0.01M acetate (pH 3.5 and pH 5.0), and 0.01M phosphate (pH 7.2) systems at $36 \pm 1^\circ\text{C}$, according to our previously reported method (Kunjiappan et al. 2020a).

Drug release kinetic studies

Additionally, the above-measured drug release data from quercetin-loaded EPS NPs was computed using the DD solver 1.0 software (Excel-plugin module), and the resulting data was fitted to the zero-order (cumulative% drug release vs time), first-order (log% drug remaining vs time), Higuchi (cumulative% drug release vs square root of time), Hixson-Crowell (cube root of drug% remaining vs time), and Korsmeyer-Peppas (log drug release vs log time) models to verify the kinetics of drug release, based on our previously published research (Kunjiappan et al. 2019a).

Antioxidant potential of the Nanoformulation

DPPH radical scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl solution (prepared by dissolving 8 mg of DPPH in 100 mL of 95% ethanol) was used to determine the DPPH* radical scavenging effect of the quercetin-loaded exopolysaccharides nanoparticles, with minor adjustments, according to a previously reported procedure (Baskararaj et al. 2019). Thus, 1 mL of DPPH reagent was mixed with 3 mL of quercetin-loaded exopolysaccharides nanoparticles with varied concentrations (6.25, 12.5, 25, 50, and $100 \mu\text{g mL}^{-1}$) and

the blank. This arrangement was permitted to incubate for 30 min at room temperature (vortexed in between), following which the absorbance was measured using a UV-visible spectrophotometer at 517 nm against a blank. Ascorbic acid was used as a comparative reference. The scavenging activity was calculated by using the Eq. (1):

$$\text{DPPH scavenging effect (\% inhibition)} = \frac{A_{(\text{Control})} - A_{(\text{Sample})}}{A_{(\text{Control})}} \times 100 \quad (1)$$

ABTS radical scavenging assay

The quercetin-loaded EPS NPs was tested for the ability to scavenge ABTS radical (Ganesan et al. 2018). The reagent was prepared by mixing 5 mL of potassium persulfate (4.9 mM) with 14 mM of 2,2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid) (ABTS) reagent (v/v) about 5 mL and the contents are kept in dark place for 14 to 16 h. The reagent was mixed with 0.1 mL of the quercetin-loaded exopolysaccharides nanoparticles with varied concentrations (6.25, 12.5, 25, 50, and 100 $\mu\text{g mL}^{-1}$), the reagent was diluted by using 0.3 mL ethanol. The whole reaction setup was incubated for 6 min and the absorbance was measured at 734 nm. After the 6 min of incubation, the absorbance at 734 nm was determined and adjusted to 0.700 (0.0020) in UV-visible spectrophotometer. Distilled water was used as blank and the obtained results were compared with the control (only ABTS solution). As given in Eq. (2), the percent ABTS radical scavenging activity was calculated using a standard curve that included Ascorbic acid in 80% of the ethanol:

$$\text{ABTS scavenging effect (\% inhibition)} = \frac{A_{(\text{Sample})} - A_{(\text{Control})}}{A_{(\text{Control})}} \times 100 \quad (2)$$

Ferric reducing antioxidant power (FRAP) assay

According to the standard protocol the 2,4,6-tripyridyl-s-triazine (TPTZ) was treated with the quercetin-loaded EPS NPs for knowing its antioxidant effect (Kalimuthu et al. 2022b). At lower pH, electron-giving antioxidants decrease Fe^{3+} TPTZ (a colorless complex) into Fe^{2+} TPTZ (a blue coloured complex). This mechanism was seen using variations in absorbance at 593 nm. 300 mM acetate buffer (3.1 g sodium acetate, 16 mL acetic acid) at pH 3.6, 10 mM TPTZ solution in 40 mM HCl solution, and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution were used to make the FRAP reagent. The acetate buffer (25 mL) and TPTZ (2.5 mL) were mixed together before adding $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. (2.5 mL). In a dark condition, 40 μL of quercetin-loaded exopolysaccharides nanoparticles with varied concentration (6.25, 12.5, 25, 50, and 100 $\mu\text{g mL}^{-1}$) was allowed to react with newly prepared FRAP reagent for 30 min before measuring the absorbance at 593 nm. The standard was linear at 200 and 1000 M FeSO_4 . M Fe (II) g^{-1} was used to calculate the findings. To compare quercetin-loaded EPS NPs, ascorbic acid was selected as a standard.

Anticancer activity of nanoformulation

MTT assay

The cytotoxic potential of quercetin-loaded EPS NPs was initially assessed in MCF-7 cells using the MTT method (Kunjiappan et al. 2018a). Thus, MCF-7 cells were seeded in a 96-well microtiter plate and maintained around for 24 h at 37 °C with 5% CO₂. This promotes the exponential development of the cells. The cells were then exposed to quercetin-loaded exopolysaccharides nanoparticles at a range of doses (100, 50, 25, 12.5, 6.25, and 3.125 µg mL⁻¹) for 24 h. After the treatment, the cells undergo a medium change and a 4 h MTT treatment at 37 °C. The excessive MTT stain was removed with DMSO (100 µL). By leaving the plate undisturbed for few minutes, the air bubbles were eliminated. The ELISA plate reader measured at the absorption at 570 nm (Bio-Rad, Model 680, Hercules, USA). The control cells were also used to test the cytotoxicity. The proportion of cell viability is determined from equation (3).

$$\text{Cell viability (\%)}^* = \frac{A(\text{mean absorbance of extract treated cells})}{A(\text{control cells})} \times 100 \quad (3)$$

*n=3, where n is the number of independent experiments.

Apoptotic morphological detection by dual staining assay

The quercetin-loaded EPS NPs was used to treat the MCF7 cells at their IC₅₀ concentration in a 24-well microplate. The treated cells were incubated for 30 min and observed under 40x magnification in a fluorescent microscope. Following treatment, cells were rinsed in ice-cold 1xPBS and stained with two fluorescent DNA-binding dyes [(10 µL mL⁻¹) (Acridine orange and Ethidium bromide)] (Kunjiappan et al. 2019b). Cells that were not treated with NPs, served as the control for the study. The proportion of cell apoptosis was calculated using equation (4).

$$\text{DPPH scavenging effect (\% inhibition)} = \frac{A(\text{Control}) - A(\text{Sample})}{A(\text{Control})} \times 100 \quad (4)$$

Apoptosis analysis by flow cytometer

MCF-7 cells were cultured overnight at 37 °C in a CO₂ incubator after being seeded on a 24-well microplate. The cultured cells were treated to the quercetin-loaded EPS NPs at an IC₅₀ concentration for 48 h. The cells were then centrifuged for 5 min at 500 × g at 4 °C after being incubated and rinsed in PBS. The cell pellets are mixed at 1×10⁵ mL⁻¹ in ice-cold 1X binding buffer after the supernatant is removed. The tubes are placed on ice before being filled with 1 µL of annexin V-fluorescein isothiocyanate reagent and 1 µL of propidium iodide (PI). The tubes were then incubated in ice-cold conditions at dark for about 15 min. As per the protocol, it was then mixed with 400 µL of ice-cold 1X binding buffer, and the sample was injected to a flow cytometer to observe the cell preparations (Kalimuthu et al. 2022b).

Assessment of mitochondrial transmembrane potential

Rhodamine-123, a lipophilic dye, was used to measure the mitochondrial transmembrane potential (Mohan et al. 2020). The technique was carried out in such a way that the MCF-7 cells were

seeded in the 24-well microplate, which was then kept in a CO₂ incubator at 37 °C for 24 h. The half-maximal inhibitory concentration of the quercetin-loaded EPS NPs (IC₅₀) value was measured, and the cells were retained for 48 h. Rhodamine-123 dye was then used to stain the cells for around 30 min. Additionally, they were fixed for 30 min with paraformaldehyde (4%) after being rinsed with PBS. The fluorescent microscope was used to look at the membrane's permeability and morphological changes.

Nuclear integrity measurement by DAPI staining

For the preservation of the genetic material and cellular proliferation, nucleus stability is more essential. The MCF7 cells were used to evaluate the nucleus integrity concerning the quercetin-loaded EPS NPs. MCF7 cells that had been exposed to the extract were then rinsed with PBS in a 24-well flat-bottom microplate before being fixed in paraformaldehyde (4%) for 30 min. The cells were then washed twice, once in PBS and once in Triton X100 (0.4%), for approximately 20 min each. The cells were then rinsed after being stained with DAPI (nuclear counterstain) (0.5 g mL⁻¹) for 1 min in a dark environment. The cell images were taken using a fluorescent microscope using the proper filter (Kalimuthu et al. 2022b).

Determination of ROS

Using 2',7'-dichlorofluorescein diacetate, the increase in intracellular ROS was observed. After being exposed to 10% FBS and the IC₅₀ concentration of quercetin-loaded EPS NPs, the MCF-7 cells were cultured for 24 h and 48 h. The cells were then labelled with 10 M H₂DCF-DA on ice after being washed twice with PBS. The plates were shaken for 10 min under the dark condition. Using a fluorescence spectrophotometer, the difference in fluorescence intensity at 475 nm (λ_{ex}) and 525 nm (λ_{em}) was detected (Baskararaj et al. 2020).

Statistical analysis

Statistical Package for Social Science (SPSS) version 20.0 software (SPSS Inc., Chicago, IL, USA) was used to analyse the experimental data. At least three times each study was repeated. The mean and $\pm$ standard deviation was used to express the obtained quantitative data. The significance of the statistical method is investigated using one-way ANOVA and Dunnett's multiple comparison method. A statistically significant difference was determined to exist when the p value was 0.05 or lower. The IC₅₀ was determined for cytotoxicity investigations using the robust curve fit non-linear regression approach from the percentage viable measurements. The paired t-test method was used to assess the ROS production data.

Results

Endophytic bacteria isolation

For the isolation of endophytic bacteria, separate triplicate efforts were made. After the incubation period, the nutrient plates with three different types of endophytic bacterial colonies were observed. The bacterial

colony with white slimy or mucoid morphology which represent the production of bacterial exudate (exopolysaccharides) was taken for the further work. The slimy colony was Gram's stained which shows Gram-negative short rods (Figure 1).

Phylogenetic analysis of endophytic bacteria

The phylogenetic tree was built using character-based and distance-based approaches. The identical topology was found while performing phylogenetic analysis using several methods. The endophytic bacterial sequence was used for the phylogenetic evolution by neighbor-joining technique. The phylogenetic interpretation reveals that the endophytic bacteria was closer to the genus *Pseudomonas*. According to the ideal phylogenetic tree analysis, the isolated endophytic bacteria was found to be *Pseudomonas otitidis* which was classified as distinct branches based on their morphological population (Figure 2). The identified endophytic bacterial sequence was submitted in the GenBank (Accession Number: OM736191).

Characterization of the bacterial exopolysaccharides

The FTIR spectrum (Figure 3(a)) of EPS was studied in the region between 4000 and 400 cm^{-1} and showed numerous peaks from 3433 cm^{-1} to 621 cm^{-1} . The broad absorption peak detected at 3433 cm^{-1} showed the occurrence of intensive hydroxyl groups (O-H) stretching frequency confirming, the polysaccharide nature of the material. The C-H stretching vibration gives signals between 3080 cm^{-1} . The peak detected at 2362 cm^{-1} is attributed to O-H bond groups, which could be explained by their carbohydrate nature. The absorption peak at 1647 cm^{-1} was due to the stretching vibration of carboxyl group (C=O). The absorption at 1452 and 1404 cm^{-1} was due to the symmetric stretching of $-\text{COO}$. There is a peak at 1244 cm^{-1} , indicating that the polysaccharide contained α -pyranose. The intense peak at 1085 cm^{-1} was attributed to the vibration of the glycosidic linkage C-O-C of glucose. $^1\text{H-NMR}$ (DMSO- d_6 , δ ppm): 3.84 (H, Phenolic-OH), 2.36 (H, Alkyl CH_2). A signal at δ 3.84 for phenolic-OH proton in ^1H NMR of exopolysaccharides suggests the presence of phenolic-OH group in the structure. Further a peak at δ 2.36 for alkyl CH_2 proton indicates presence of alkyl CH_2 group in the structure. The observed two peaks for phenolic-OH and alkyl CH_2 clearly indicate the presence of exopolysaccharides in the sample (Figure 3(b)). The dried EPS from the endophytic bacterial colony was determined for the total reducing sugar content by the phenol-sulfuric acid method. The ice-cold ethanol precipitation method of exopolysaccharides recovery from endophytic bacteria is estimated to contain 26.665 ± 1.302 % of the reducing sugar. Further, the endophytic bacterial EPS was analysed for the presence of protein. The total protein content was found to be 0.912 ± 0.023 % in the endophytic bacterial EPS, based on the Bradford's method, as compared with BSA (standard).

Quercetin-loaded nanoparticles formulation

The quercetin-loaded EPS NPS was formulated by a precipitation method. Following quercetin was added to the exopolysaccharide's solution, the combination initially had a dark yellow colour with suspended

particles, but after continuous stirring, the colour changed to clear with light yellow. The synthesis of quercetin-loaded EPS NPs was validated by the observed colour shift and the elimination of opalescence, which demonstrated that quercetin was completely loaded with EPS. The quercetin-loaded EPS NPS was further examined and used in biological processes. According to measurements, quercetin can be loaded into exopolysaccharides with an encapsulation efficiency of $81.4066 \pm 4.758\%$ and a drug loading capacity of $8.03 \pm 0.018\%$, respectively.

Characterization studies of the nanoparticles

The FTIR spectrum of quercetin-loaded EPS NPs (Figure 4(a)) shows a broad peak at 3250 cm^{-1} signifying the presence of strong O-H bonding of carboxyl groups, and a stretching peak at 2978 cm^{-1} , indicating the presence of $-\text{CH}_2$ group. Peak at 1650 cm^{-1} signifies aromatic conjugation ($-\text{C}=\text{C}-$) between quercetin and exopolysaccharides. The X-ray diffractogram of the quercetin-loaded EPS NPS was presented in Figure 4(b). The broad diffraction peak observed at $2\theta = 22^\circ$ suggested that the formulated nanoparticles are highly amorphous. Figure 4 (c) & 4(d) presented the average particle size, and zeta potential of the quercetin-loaded EPS NPs. The average particle size distribution and zetapotential of the formulated NPs were 780 nm and -20 mV respectively. Further, the formulated quercetin-loaded EPS NPs were found to be non-aggregated, distributed uniformly and spherical shape with particle size were approximately 150-200 nm in diameter when observed under SEM (Figure 5).

Release of quercetin from exopolysaccharides nanoparticles

The therapeutic effectiveness of the loaded drug is primarily regulated by the rate of drug release from EPS NPs. The dialysis bag was used to assess the in vitro release mechanism of quercetin from quercetin-loaded EPS NPs at 37°C in a variety of buffer solutions, including acetate buffer (pH 3.5 and 5.0) and PBS (pH 7.2). The cumulative percentage of quercetin release was calculated after the release data was collected at pre-determined time intervals. The therapeutic effectiveness of the loaded drug is primarily regulated by the rate of drug release from exopolysaccharides nanoparticles. Figure 6 shows the outcome of the proportion of quercetin released from EPS NPs. The findings were gradual, constant, and regulated, at pH 3.2, $4.647 \pm 0.226\%$ of the loaded quercetin was released in 4 h. After 14 h, loaded quercetin from EPS NPs was released with a sharp increase in concentration at pH values of $40.973 \pm 0.302\%$ (pH 3.2), $45.367 \pm 0.77\%$ (pH 5.4), and 43.973 ± 1.015 (pH 7.2). The highest amount of loaded quercetin ($82.523 \pm 0.854\%$) was released from EPS NPs in 25 h at pH 5.0. The loaded quercetin release from EPS NPs is slightly altered by pH variations. Additionally, the experimental results showed that at pH 5.0 compared to the other examined pH ranges, quercetin release from EPS NPs was higher and more effective.

Kinetics of drug release

The results of drug release data were fitted into multiple kinetic models (Zero-order, first-order, Higuchi, Korsmeyer–Peppas, and Hixson–Crowell) to further examine the mechanism of quercetin release from

quercetin-loaded EPS NPs, as shown in Table 1. The regression coefficient (r^2) value and release rate constant were predicted for each kinetic model. In general, the value (r^2) closer to 1, the better the fit or relationship between the two factors. From the analysis of kinetic models, it was observed that, the zero- and first-order kinetics regression coefficient (r^2) values are very closer to 1, hence these kinetic models were considered to be a good fit. Additionally, based on the Korsmeyer-Peppas model, it was found that the r^2 value was higher than 0.96 in all of the studies that were examined. Fickian diffusion was the desired result of these quercetin release kinetics. The quercetin release from quercetin-loaded EPS NPs, which was primarily regulated by the diffusion mechanism, may exhibit Fickian behaviour. This kinetics model indicated that the best-fitting model for the mechanism of quercetin release from exopolysaccharides nanoparticles was first-order kinetics. As a result, the kinetic model mechanism also illustrates the nanoparticles uniform disintegration and controlled release.

Antioxidant potential of the Nano formulation

DPPH radical scavenging assay

Figure 7 (a) shows the free radical scavenging effect of varied concentrations of quercetin-loaded EPS NPs on DPPH* radical. The observed results indicate that various concentrations of 6.25, 12.5, 25, 50, and 100 $\mu\text{g mL}^{-1}$ quercetin-loaded EPS NPs displayed $22.046 \pm 1.540\%$, $32.773 \pm 1.498\%$, $43.133 \pm 0.203\%$, $66.56 \pm 0.869\%$, and $84.737 \pm 0.566\%$ respectively, in a concentration-based manner. Further, 6.25 $\mu\text{g mL}^{-1}$ of ascorbic acid showed $31.963 \pm 0.436\%$ scavenging of DPPH* radicals.

ABTS radical scavenging assay

The standard drug has been used to compare the relative antioxidant effects to scavenge the ABTS radical. Using potassium persulphate, the stable form of the ABTS* radical cation was generated. After producing stable absorbance, the reaction medium is supplemented with an antioxidant quercetin-loaded exopolysaccharides nanoparticles, and the antioxidant power is measured by observing decolorization. The varied concentrations of 6.25, 12.5, 25, 50, and 100 $\mu\text{g mL}^{-1}$ of quercetin-loaded EPS NPS scavenged ABTS radical in a concentration-dependent mode as shown in Figure 7(b). At a maximum concentration (100 $\mu\text{g mL}^{-1}$) of ethyl acetate of quercetin-loaded EPS NPs exhibited highest ($75.806 \pm 1.452\%$) decolourization of ABTS* radicals.

FRAP assay

When quercetin-loaded EPS NPs react with a ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex to produce a coloured ferrous tripyridyltriazine (Fe^{2+} -TPTZ), the FRAP assay determines the reducing potential of the ferric ions. The free radical chain breaking takes place by donating an electron. Varied concentrations of quercetin-loaded EPS NPs were screened by FRAP assay and standard ascorbic acid. In the results obtained, the better-reducing potential was exhibited $164.06 \pm 6.159 \mu\text{g mol}^{-1}$ (Fe(II)) g^{-1} at a concentration

of 100 $\mu\text{g mL}^{-1}$ of quercetin-loaded EPS NPs. In the observed results, quercetin-loaded EPS NPs showed higher activity than reference standard ascorbic acid as shown in Fig. 7(c).

Anticancer activity of nanoformulation

Cytotoxicity assay

The cytotoxic efficacy of quercetin-loaded EPS NPs was studied against MCF-7 cells through MTT assay. From the results, it was found that $72.453 \pm 0.878\%$ of the viability of cells was observed for the $6.125 \mu\text{g mL}^{-1}$ concentration of the quercetin-loaded EPS NPs. The control drug, Doxorubicin $0.25 \mu\text{M}$ showed $42.83 \pm 2.424\%$ viability of the cells. The IC_{50} value of the quercetin-loaded EPS NPs was calculated as $14.313 \mu\text{g mL}^{-1}$. Figure 8(a) illustrates the % viability of cells at various concentrations ($\mu\text{g mL}^{-1}$). And Figure 8(b) and 8(c) shows the control cells and changes in the morphology of the MCF-7 cells after treatment with the quercetin-loaded exopolysaccharides nanoparticles, respectively. The IC_{50} concentration ($14.313 \mu\text{g mL}^{-1}$) was then selected in order to further examination of anticancer activities of the quercetin-loaded EPS NPs.

AO/EtBr staining for analysis of apoptosis

The abnormalities in the cells were observed when the cells were treated with the quercetin-loaded exopolysaccharides nanoparticles (IC_{50} concentration: $14.313 \mu\text{g mL}^{-1}$) and then stained with AO/EtBr. By using fluorescent microscopy, the morphology of MCF-7 cells during apoptosis, living cells, and necrotic cells was noted. The cells treated with quercetin-loaded EPS NPs showed early apoptotic cells in orange and late apoptotic cells with abnormal shapes, chromatin in the nucleus, and distorted membranes in red (Figure 9(b)). The control cells have not undergone any notable alteration (Figure 9(a)).

Apoptosis analysis by flow cytometry

The results of flow cytometry analysis of apoptosis induction by quercetin-loaded EPS NPs were represented in Figure 10. In comparison to control cells (early apoptosis 1.63%, dead cells 0.020%), MCF-7 cells treated with $14.313 \mu\text{g mL}^{-1}$ (IC_{50} conc.) of quercetin-loaded exopolysaccharides nanoparticles induced early apoptosis at a rate of 0.23% and dead cells were 6.87%. When compared to control cells (Late apoptosis: 0.44%), the quercetin-loaded EPS NPs was found to have significantly increased the proportion of late apoptosis (10.8%). The percentage proportion of living, early-stage, late-stage, and dead cells is shown in Table 2.

Analysis of mitochondrial membrane potential ($\Delta\Psi_m$)

The dye Rhodamine-123 was used to detect changes in mitochondrial membrane in the MCF-7 cells after 24 h of exposure with quercetin-loaded exopolysaccharides nanoparticles (IC_{50} conc.). The mitochondrial membrane potential in MCF-7 cells was noticeably lower after 24 h of incubation with quercetin-loaded EPS NPs treatment. The disruption and depolarization of the mitochondrial transmembrane was

observed, which was indicative of the earliest intercellular event of apoptosis (Figure 11). The penetration of the quercetin-loaded EPS NPs into mitochondrial membrane results in loss of the membrane and cell death (MCF-7). The event releases cytochrome C from the mitochondria, activating the mitochondrial permeability transition pore and triggering the apoptosis (Wani et al., 2013).

DAPI

DAPI fluorescent labelling, made it possible to determine the type of the nucleus and the overall morphological alterations that quercetin-loaded exopolysaccharides nanoparticles caused in MCF-7 cells. The results are shown in Figure 12. The nuclear DNA's adenine and thymine sections attach to the DAPI dye. The induction of apoptosis in the MCF-7 cells after 48 hours of treatment with quercetin-loaded exopolysaccharides nanoparticles is evident. The treated cells saw cell shrinkage, increased chromatin condensation, and damage to the nuclear DNA. The blue fluorescence was also less intense in the quercetin-loaded EPS NPs-treated MCF-7 cells than in the control group. All these processes increase the number of cells that undergo apoptosis.

ROS

The imbalance between the amounts of reactive oxygen species and antioxidants has a significant role in the initiation and progression of cancer. The outcomes of analysing the generation of ROS by quercetin-loaded exopolysaccharides nanoparticles in MCF-7 cells using the H₂DCF-DA staining technique. In comparison to the control, cells treated with 14.313 µg mL⁻¹ of quercetin-loaded exopolysaccharides nanoparticles showed an increased green fluorescence (Figure 13(a) and 13(b)), confirming the initiation of apoptotic nature in MCF-7 cells. ROS generation measured as relative fluorescence intensity using fluorescence microscope. Results expressed as mean ± standard deviation of triplicate measurements (p < 0.05) Figure 13(c).

Discussion

Endophytic bacterial EPS was isolated and characterized, further it was converted into nanoparticles encapsulated with quercetin. The drug loading and drug release kinetics were studied followed by the characterization of nanoparticles. The endophytic bacteria were isolated from *T. terrestris* L. and was identified as *Pseudomonas otitidis*. Endophytic bacteria are associated with plants and are highly beneficial for the plant growth. The metabolites of endophytic bacteria exhibited for various applications including therapy. Here, the exopolysaccharides of *P. otitidis* is used to deliver quercetin to control proliferation of cancer cells. The crude exopolysaccharides and nano-formulations with quercetin was characterized by FTIR and NMR. Structural analysis of exopolysaccharides and its derivatives was identified by FTIR which unveiled the bonding between the elementary links and their rotational isomerism (Mitić et al. 2009). Followed by drug loading and release was performed *in vitro*. Significantly, the encapsulation efficiency and loading capacity of quercetin was observed as of 81.4066 ± 4.758% and 8.03 ± 0.018%, respectively with exopolysaccharides nanoparticles. Besides, the drug release kinetics

showcase that $48.323 \pm 0.798\%$ of quercetin was released at 15 h (pH 5) and maximum release ($82.523 \pm 0.854\%$) was observed at 25 h (pH 5). Raveendran *et al.* reported the synthesis of sulfated exopolysaccharide from halophilic bacterium, *Halomonas maura*. The exopolysaccharides-chitosan were formulated with the size range of 30 to 200 nm for the delivery of 5-fluorouracil. The encapsulation efficiency was reported as 60% (w/w) and drug release was observed at three different pH 4.5, 6.9, 7.4 for 12 days. At the pH 7.4, 40% of drug release was observed at 6th day of incubation (Raveendran et al. 2013a). Since, the nano-formulation is designed for anti-cancer activity, the anti-oxidant activity of drug loaded nanomaterials was analyzed in vitro by DPPH, ABTS, and FRAP assay with ascorbic acid as standard. Quercetin revealed anti-oxidant activity in concentration dependent manner, however the maximum activity was observed at $100 \mu\text{g mL}^{-1}$. Monumental research papers provide strong evidence for the anti-oxidant effect of quercetin (Sharma et al. 2018; Nguyen et al. 2017; Cho et al. 2003). Quercetin-loaded nanoparticles were studied for their cytotoxic effect against breast cancer cells in vitro. The half maximal inhibitory concentration of Quercetin-loaded nanoparticles was found to be $14.313 \mu\text{g mL}^{-1}$. Quercetin arrests the growth of cancer cells in time and dose dependent manner. Choe *et al.* reported that $92.4 \mu\text{M}$ of quercetin inhibited the growth of human breast cancer cells at 48 h (Chou et al. 2010). Indeed, in another reports the IC_{50} value of quercetin was reported as $37 \mu\text{M}$ against MCF-7 cells but not for MDA-MB-231 cells (Ranganathan et al. 2015). Delivery of quercetin with exopolysaccharide nanoparticles provide better results than previous reports since it is $14.313 \mu\text{g mL}^{-1}$ in 24 hours. The induction of apoptosis by quercetin loaded nanoparticles was analyzed with live/dead cell assay. Apoptotic cells were observed in quercetin-nanoparticles treated cells due to the DNA damage. The appearance of yellowish orange indicates DNA damage which is hall mark of dual staining method. Whereas the control cells are observed in green color. Differentiating the apoptotic- and normal cells by live/dead cell assay was validated by previous reports (Ranganathan et al. 2015). The cells treated with quercetin-loaded exopolysaccharides nanoparticles and control cells were analyzed with flow-cytometry with annexin-V to confirm the formation apoptotic bodies. Compared to control, cells treated with quercetin revealed the presence of early apoptotic cells (14%), and cells treated with quercetin exopolysaccharide nanoparticles exhibited late apoptotic bodies (38.9%). The anti-proliferative effect of Graviola fruit extract was analyzed against MCF-7 cells and its apoptosis mechanism was proved with flow-cytometry. The annexin-V/FITC assay proved the formation of apoptotic bodies in Graviola fruit extract treated cells after 72 h (Daddiouaissa et al. 2019). Our results corroborate with the results of Gao et al., (2012), where increase in annexin V positive cells were observed concentration dependent manner in quercetin treated group compared to control (Gao et al. 2012). Further, the mitochondrial membrane potential was studied with the Rhodamine-123 dye with the incubation period of 24 h. In comparison to control, the cells treated with quercetin-loaded exopolysaccharides nanoparticles displayed significant reduction in mitochondrial membrane potential. As a part of apoptosis, disrupted and depolarized mitochondrial transmembrane was observed. The mitochondrial membrane potential of polymer micelle-encapsulated quercetin was evaluated on ovarian cancer cells. Following quercetin treatment, a decrease in Rh123 accumulation in ovarian cancer cells (A2780S) was observed, which indicated the collapse of mitochondrial membrane potential followed by the treatment of quercetin (Gao et al. 2012). DAPI

staining was performed followed by mitochondrial potential analysis. The DAPI staining results unveiled the increased chromatin condensation, and damage to the nuclear DNA. Further, the effect of quercetin in influencing the balance between ROS and antioxidants was studied. Generation of ROS in quercetin treated cells was observed with H₂DCF-DA staining technique. Our results corroborated with the work reported by Jeon *et al.* 2019 (Jeon et al. 2019). The antiproliferative effect of quercetin was experimented in the Hepatocellular Carcinoma Cell Line HepG2. Quercetin is reported for the upregulation of intracellular ROS levels, activation of p53 a tumor suppressor protein, and for inducing the secretion of antioxidants such as glutathione. Increase in intracellular ROS levels were proved in HepG2 cells by H₂DCFDA assay (Jeon et al. 2019).

Conclusion

In this presented study, isolated endophytic bacterial EPS was successfully used for drug delivery applications. Initially, the EPS-producing endophytic bacteria was screened from the roots of *Tribulus terrestris* L. and it was confirmed as *Pseudomonas otitidis*. The producing EPS from the bacterial culture was isolated through the ice-cold ethanol precipitation method, and partially characterized and conformed via FTIR, NMR and their reducing sugar and protein contents were also estimated. Further, the characterized EPS was applied for drug delivery applications. In this regard, quercetin-loaded EPS NPs was fabricated through the precipitation method. The fabricated quercetin-loaded NPs characterized by FTIR, XRD, DLS, and SEM for their functional integrity, physical properties, zeta potential, size distribution and morphological characteristics and confirmed. Further, the drug release studies of the fabricated quercetin-loaded EPS NPs was performed via varied physiological buffer medium (acetate buffer (pH 3.5 and 5.0) and PBS (pH 7.2)). The 82.523±0.854% quercetin was released from quercetin-loaded EPS NPs at 25 h in an acetic medium (pH 5.0). The result of drug release data was that quercetin-loaded EPS NPs fit the first-order kinetic model. In addition, the quercetin-loaded EPS NPs showed significant antioxidant and anticancer properties. The IC₅₀ value of quercetin-loaded EPS NPs found to be 14.313 µg mL⁻¹ against MCF-7 cells. The studies further demonstrated that quercetin-loaded EPS NPs triggered apoptosis in MCF-7 cells by excessive production of ROS, damaging nuclear material, and depleting mitochondrial transmembrane potential. Thus, quercetin-loaded EPS NPs serves as the significant antioxidant and anticancer agent through *in vitro* methods. However, structural investigation of the ESP required for further validation and applied for other biological applications.

Declarations

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Author Contributions

S.K. supervision, project administration, funding acquisition, resources, writing review & editing; A.K.K., S.R.K., P.V. writing-original draft, formal analysis, investigation; T.P. S.J.K. M.S. A.C. conceptualization, writing, investigation and editing. All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this manuscript

Conflict of interest

The authors declare that they have no Conflict of interests.

Ethical approval

Ethical approval was not required for this research.

Consent to participate

Not applicable

Consent for publication

The authors give the consent for publication.

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Tables

Table 1: Drug release kinetics profile of the quercetin from quercetin-loaded exopolysaccharides nanoparticles.

Model	Parameter	Quercetin released from quercetin-loaded EPS NPs		
		pH 3.2	pH 5.0	pH 7.2
Zero order $F = K_0 \times t$	K_0	0.025	0.026	0.025
	r^2	0.9788	0.9722	0.9694
	AIC	282.657	295.884	295.309
First order $F = 100 \times [1 - \text{Exp}(-k_1 \times t)]$	K_1	0.000	0.000	0.000
	r^2	0.9408	0.9430	0.9451
	AIC	325.823	326.017	319.924
Higuchi model $F = K_H \times t^{1/2}$	K_H	0.838	0.868	0.813
	r^2	0.8231	0.8300	0.8229
	AIC	371.818	371.931	369.091
Korsmeyer–Peppas model $F = kKP \times t^n$	kKP	0.027	0.039	0.052
	r^2	0.9785	0.9717	0.9687
	n	1.022	0.777	0.691
	AIC	284.298	297.654	297.272
Hixon–Crowell model $F = 100 \times [1 - (1 - kHC \times t)^3]$	kHC	0.000	0.000	0.000
	r^2	0.9613	0.9623	0.9617
	AIC	307.969	308.685	304.841

AIC = Akaike information criterion, F = fraction of drug release in time t, K_0 = apparent rate constant of zero order release constant, K_1 = first order release constant, K_H = Higuchi constant, kKP = Korsmeyer–Peppas rate constant, kHC = Hixon–Crowell constant, n= diffusional exponent, and r^2 = Squared correlation coefficient.

Table 2: Flow cytometry analysis of annexin V/ PI apoptosis assay performed on MCF-7 cells treated with quercetin-loaded EPS NPs.

MCF-7 cells	Q4	Q3	Q2	Q1
	Live (%)	Early apoptosis (%)	Late apoptosis (%)	Dead (%)
Control	97.9	1.63	0.44	0.020
Quercetin-loaded exopolysaccharides nanoparticles	82.1	0.23	10.8	6.87

Figures

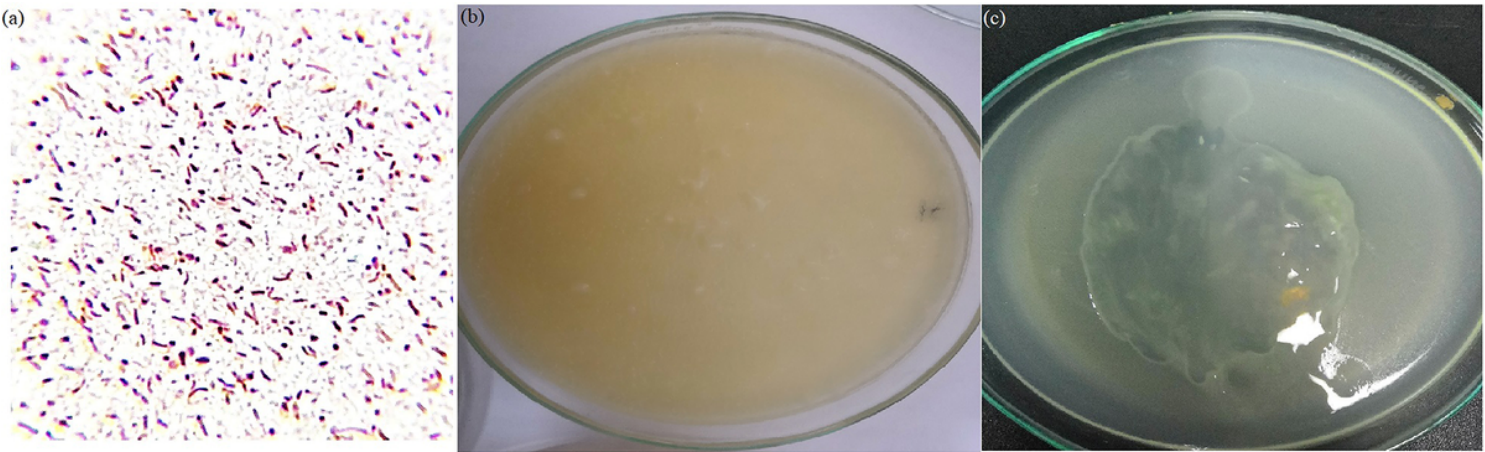


Figure 1

Gram’s staining of the endophytic bacteria (a); exopolysaccharides producing endophytic bacterial colony (b); and isolated exopolysaccharides from endophytic bacteria (c).

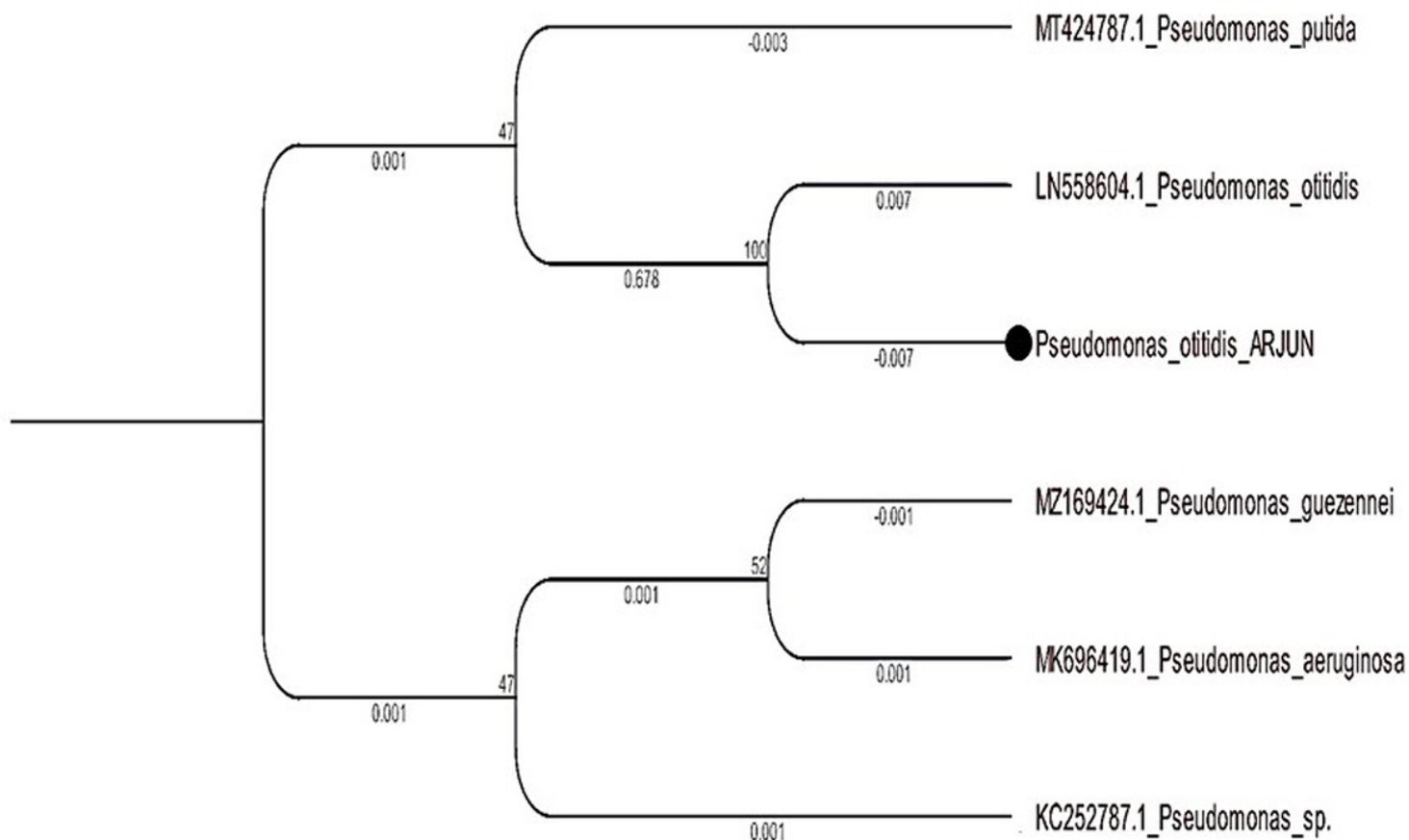


Figure 2

Phylogenetic tree showing relatedness of endophytic bacteria isolated from *Tribulus terrestris*L. and their relatives in the *Pseudomonas* by neighbor-joining grouping of the aligned sequences of the 16 S rRNA gene. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA 6.

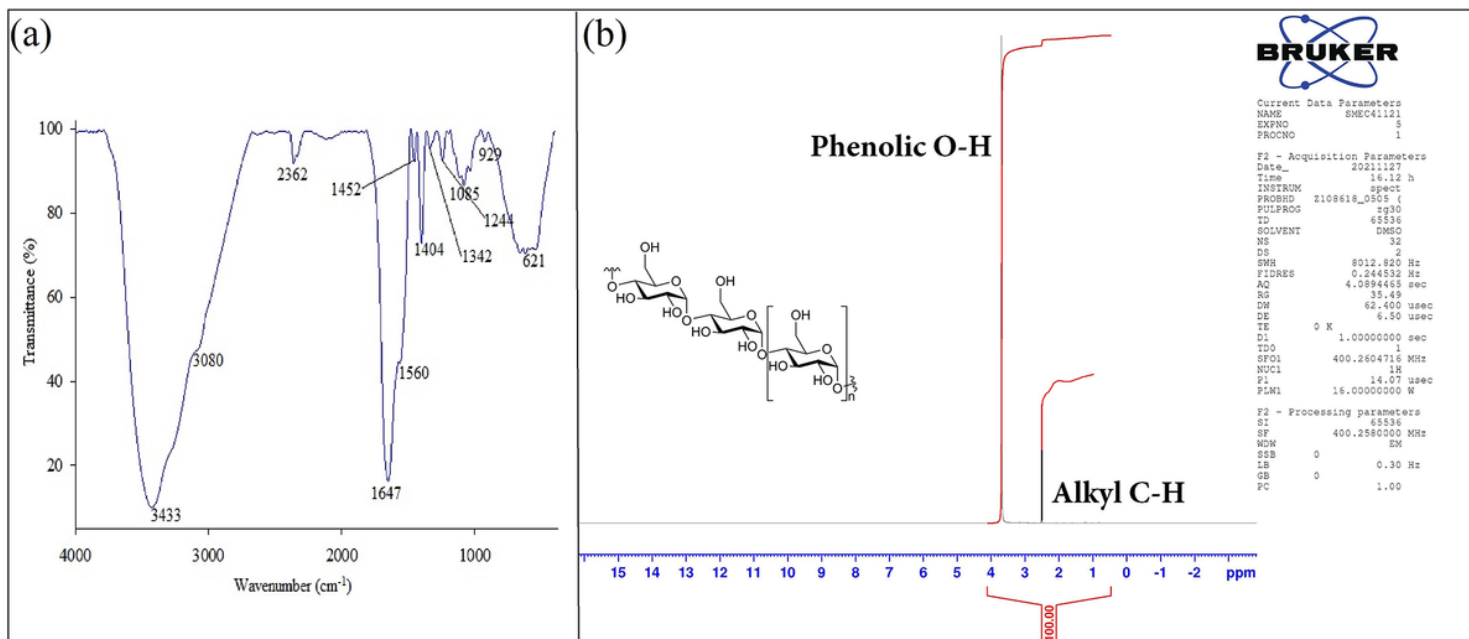


Figure 3

FTIR spectrum of the exopolysaccharides from endophytic bacteria (a); ¹H-NMR spectrum of the exopolysaccharides from endophytic bacteria (b).

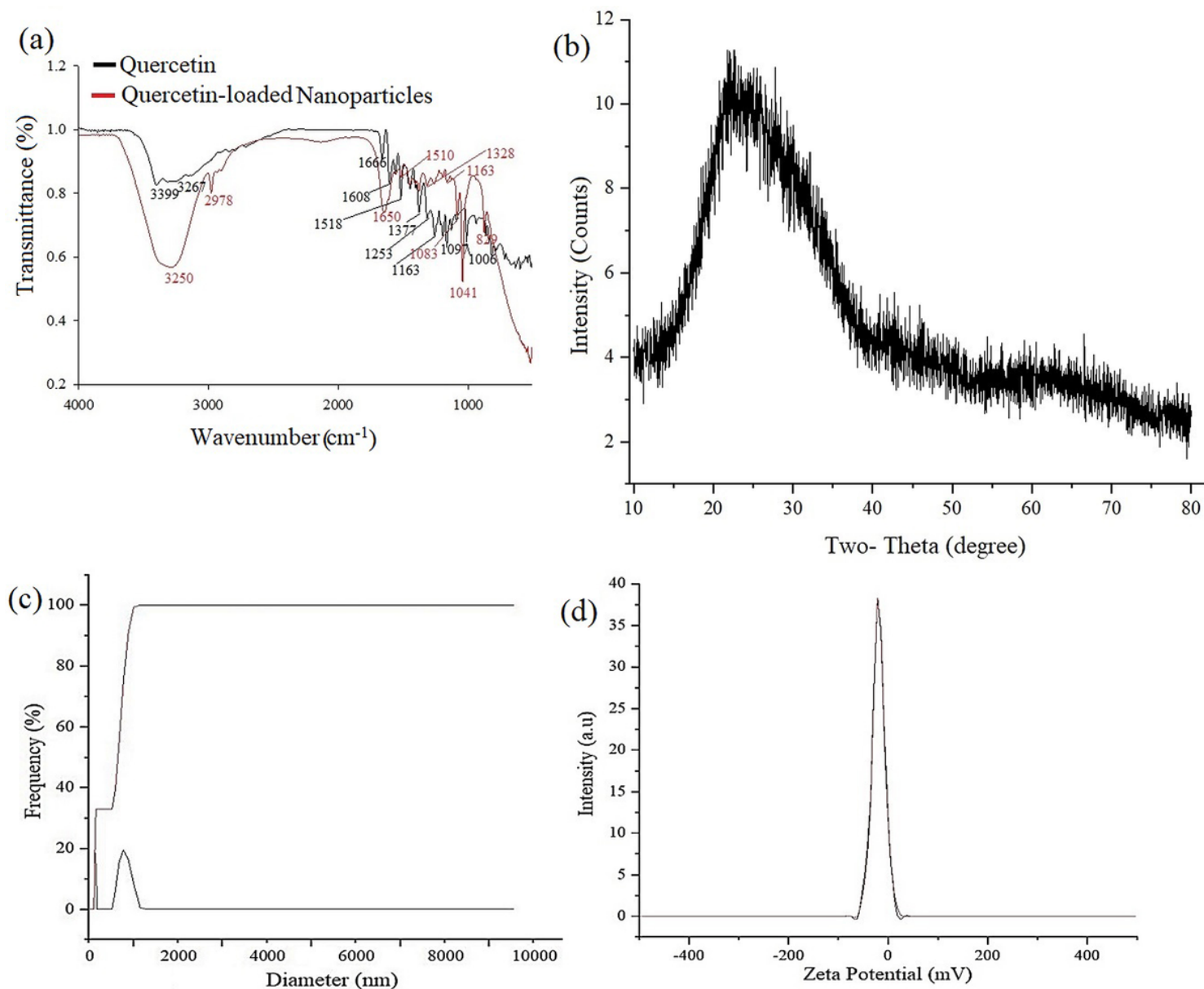


Figure 4

FTIR spectrum of quercetin-loaded EPS NPs (a); XRD spectrum of quercetin-loaded EPS NPs (b); average particle size (c), and zeta potential of the quercetin-loaded EPS NPs (d).

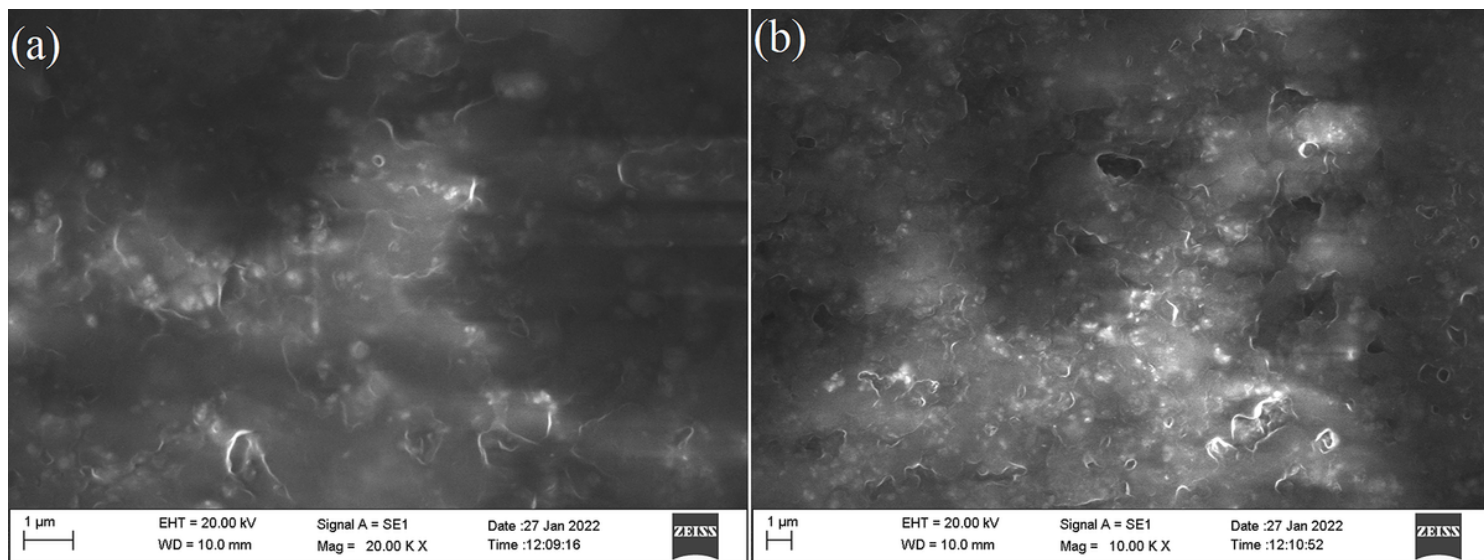


Figure 5

Scanning electron microscopy images of the quercetin-loaded EPS NPs

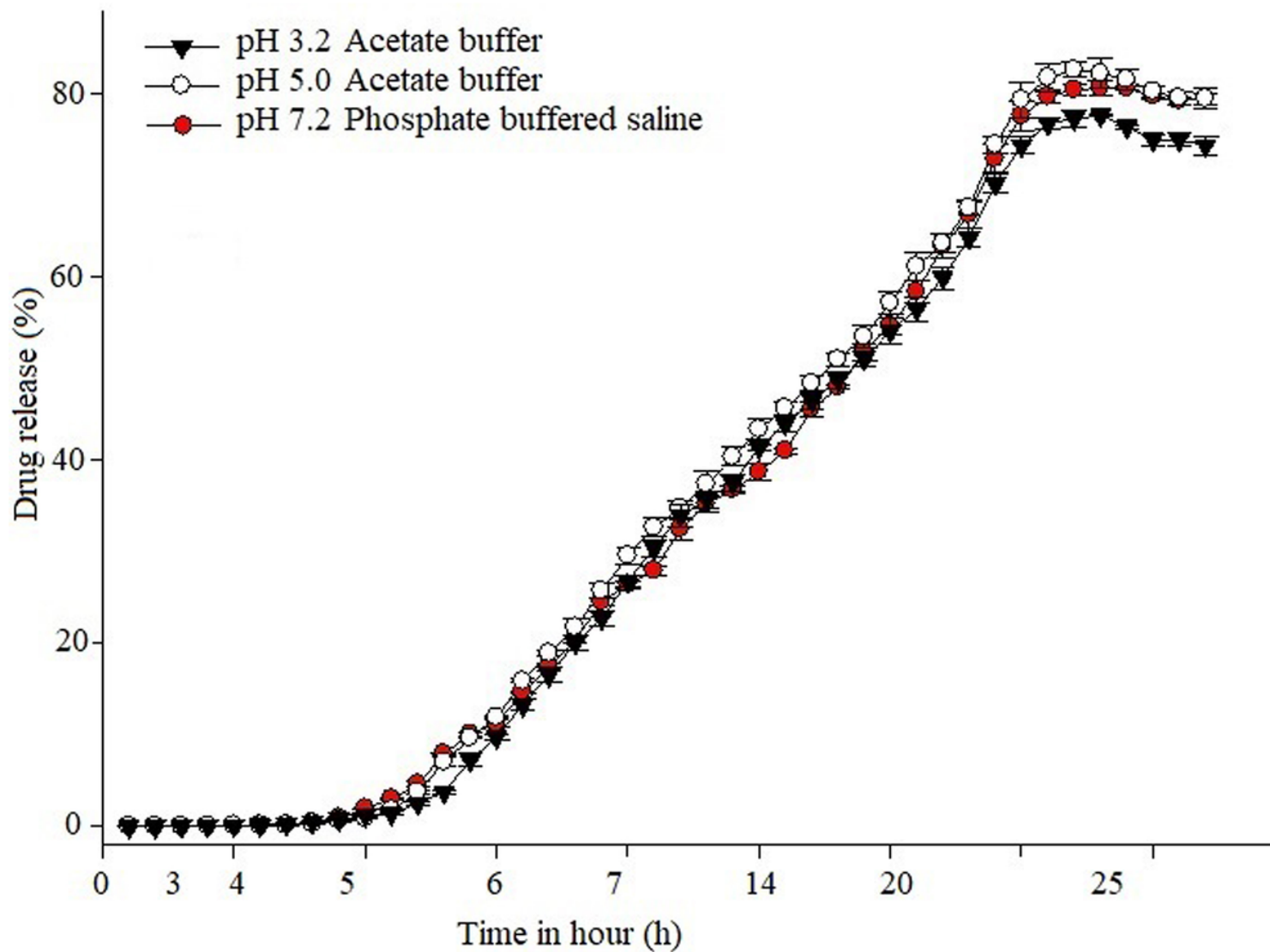


Figure 6

Drug release profile of quercetin-loaded EPS NPs

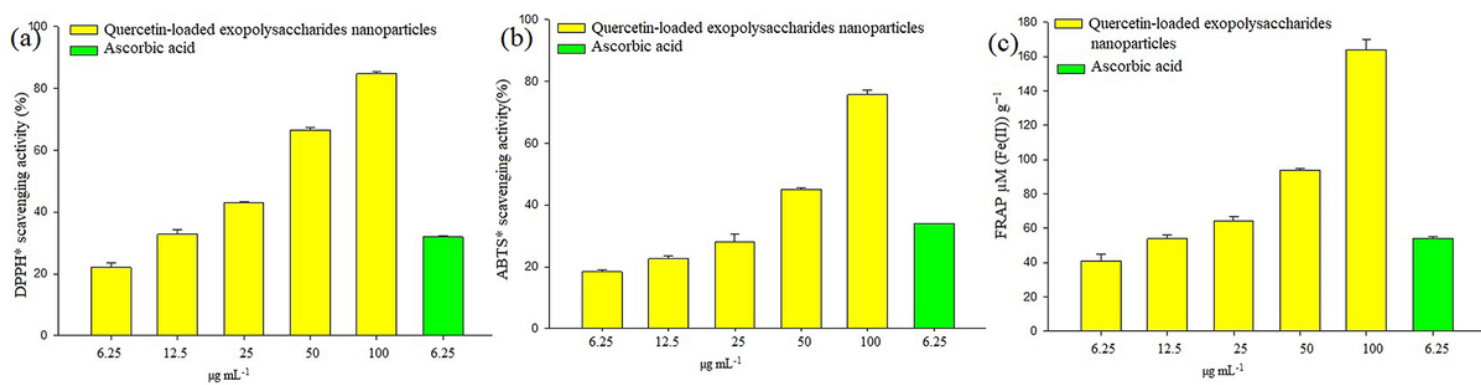


Figure 7

DPPH radical scavenging activities of various concentrations of quercetin-loaded EPS NPs (a); ABTS radical scavenging activities of various concentrations of quercetin-loaded EPS NPs (b); FRAP potential of various concentrations of quercetin-loaded EPS NPs

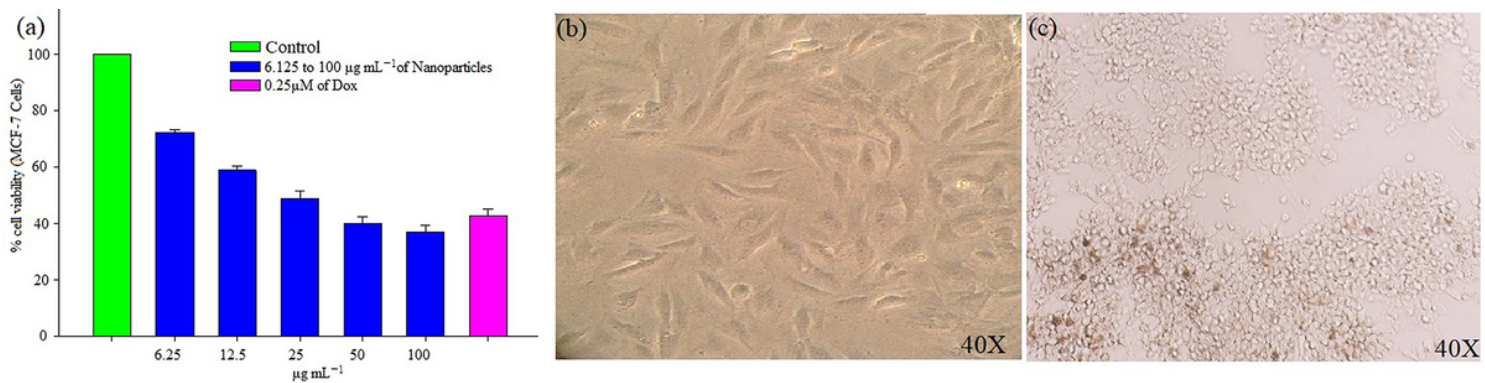


Figure 8

Cytotoxic activity of MCF-7 cells using different concentrations of quercetin-loaded EPS NPs after 24 h treatment. The percentage of apoptotic cells increased with the concentration proportionally: values are mean±standard deviation of triplicate measurements (p<0.05) (a); morphology of the control cells (b); morphology of treated cells (c).

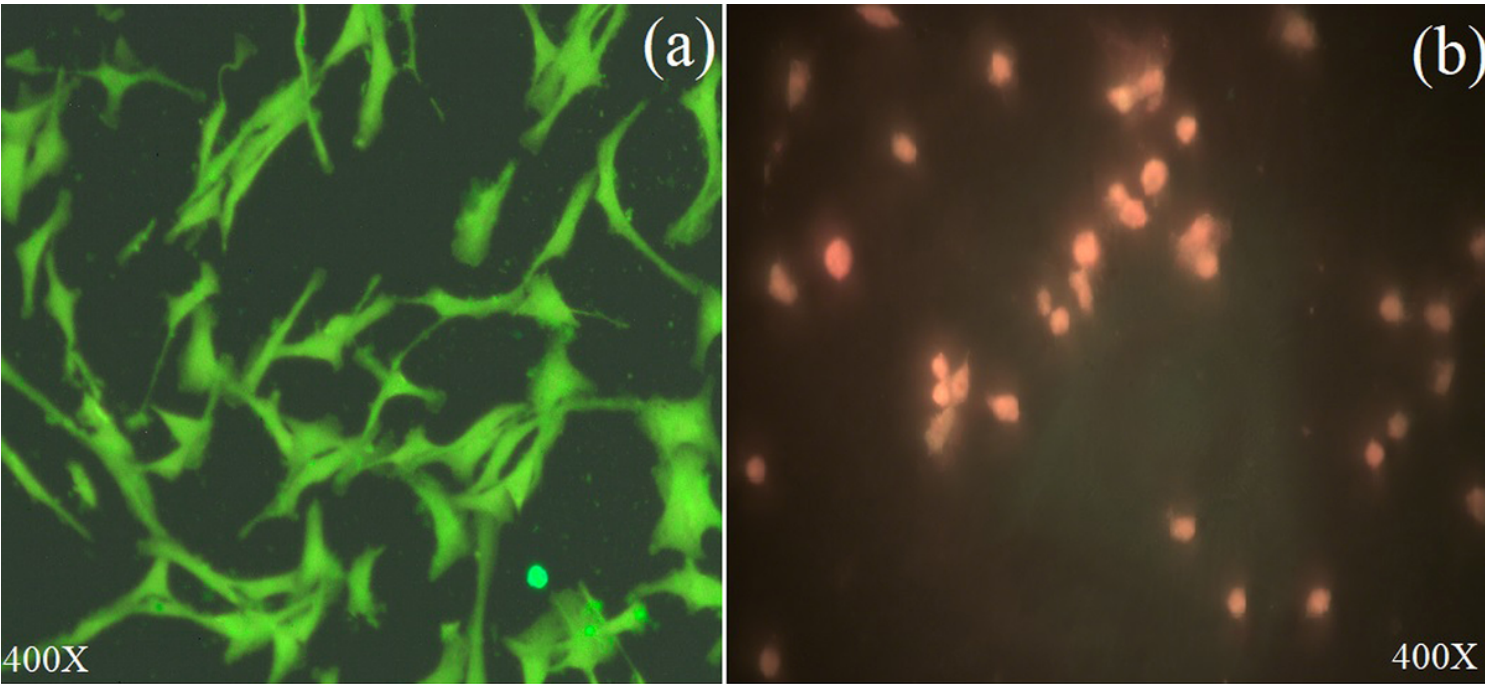


Figure 9

Apoptotic morphological variations of MCF-7 cells identified with AO/EtBr staining and observed using fluorescence microscope: control cells (a); 14.313 $\mu\text{g mL}^{-1}$ of quercetin-loaded EPS NPs at 24 h (b)

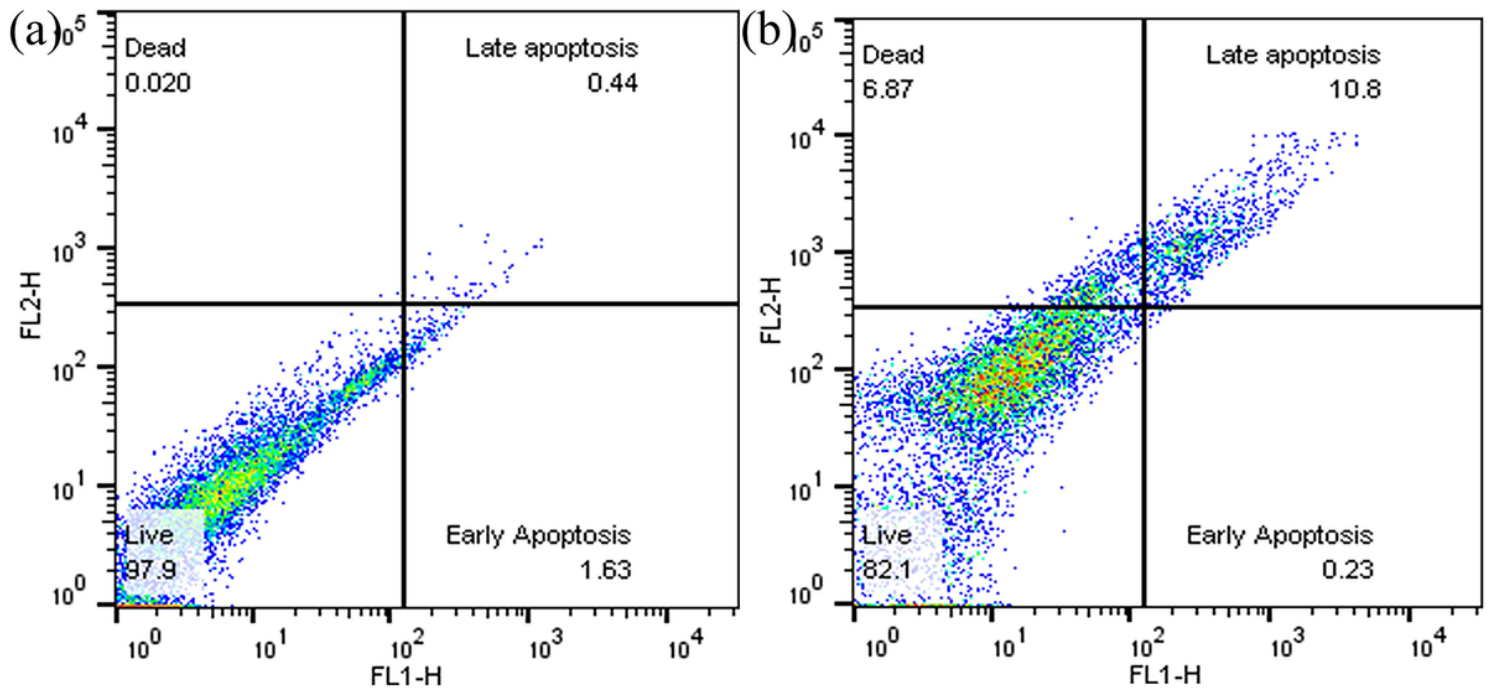


Figure 10

Apoptosis analysis by flowcytometry measurements: Before treating MCF-7 cells with quercetin-loaded EPS NPs (a); after treating MCF-7 cells with quercetin-loaded EPS NPs (b).

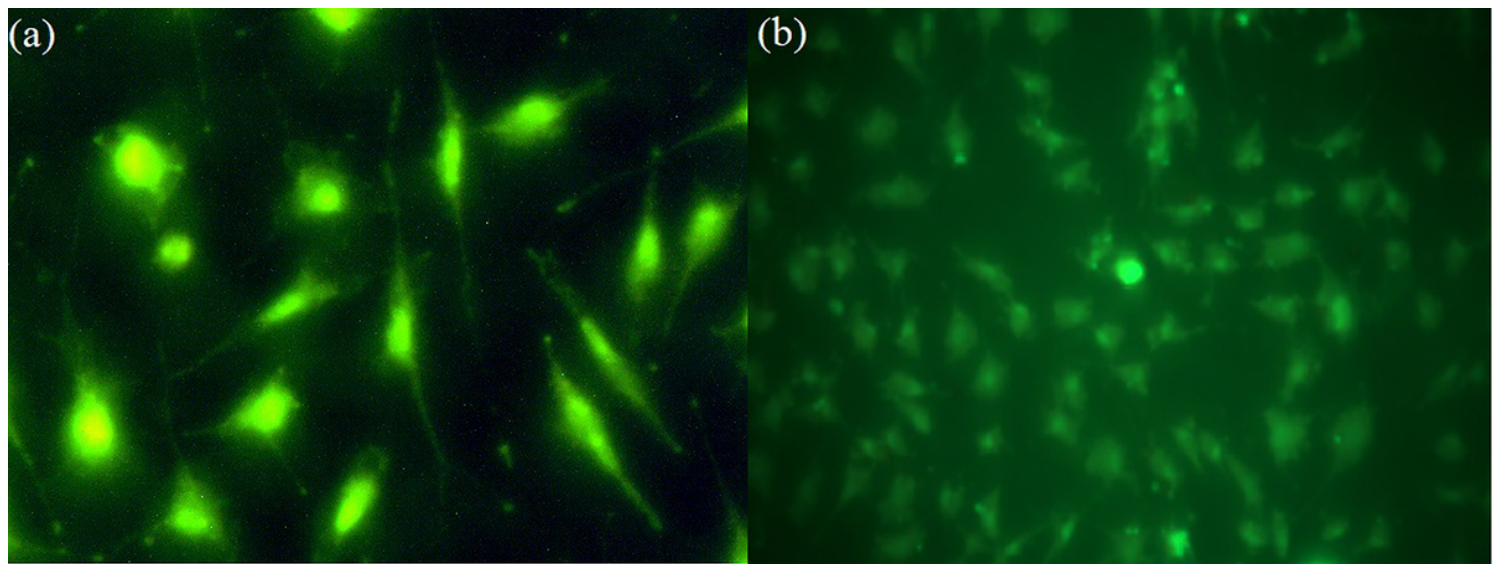


Figure 11

Effects of quercetin-loaded EPS NPs on mitochondrial transmembrane potential in MCF-7 cancer cells: control cells (a); notable loss of mitochondrial transmembrane potential in treated cells (b).

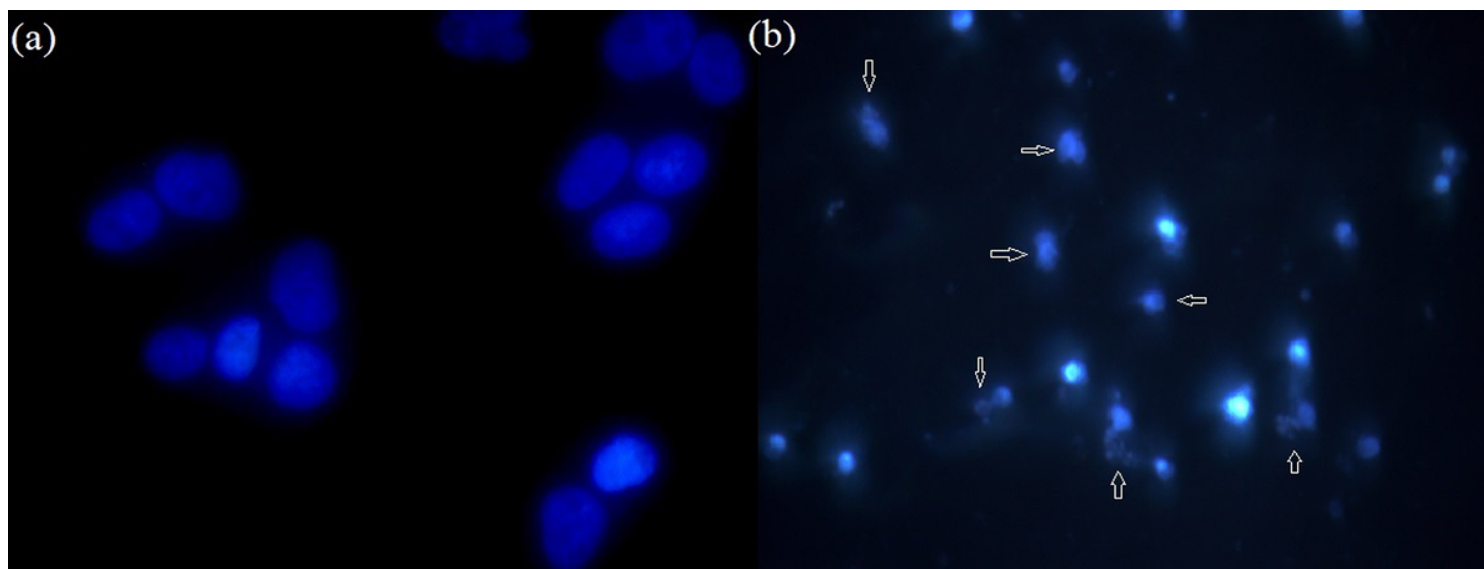


Figure 12

DAPI-stained image of MCF-7 cancer cells: control cell treatment with quercetin-loaded EPS NPs (a); chromatin fragmentation is shown with an arrow (b)

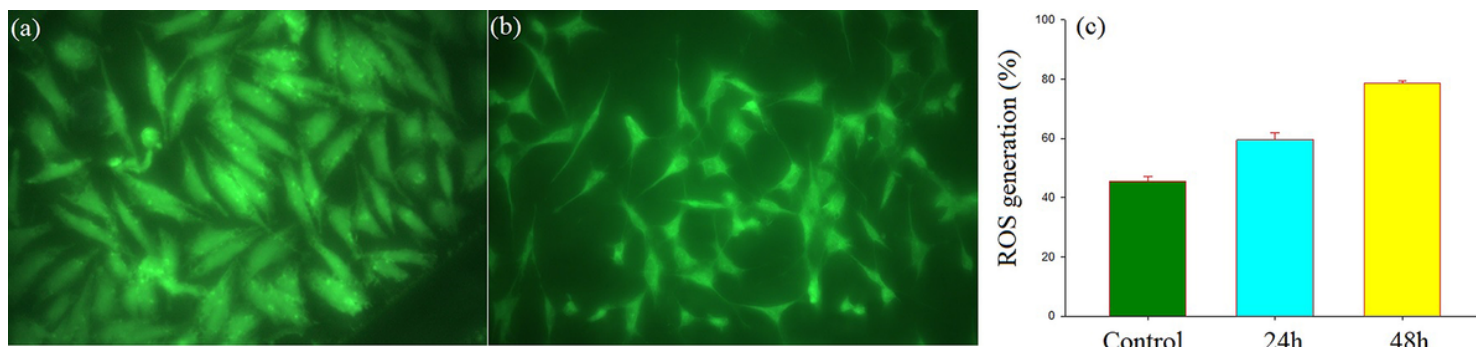


Figure 13

Effects of $14.313 \mu\text{g mL}^{-1}$ of quercetin-loaded EPS NPs on ROS generation (percentage of control) in MCF-7 cancer cells. ROS generation measured as relative fluorescence intensity using fluorescence microscope. Image of untreated control cells (a); increased ROS in *A. flavus* extract-treated cells (b). Results expressed as mean $\pm$ standard deviation of triplicate measurements ($p < 0.05$) (c).

Supplementary Files

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