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RESEARCH ARTICLE

Comparison between Free and Encapsulated Form of Epicatechin in Liposomes and In Polymeric Nanoparticles Against the Paraquat-Induced Toxicity of NRK-52E Cells

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ABSTRACT

Both liposomes and polymeric nanoparticles have lately been utilized as carriers of conventionally prescribed medications with the aim of improving their activity in various ways. Epicatechin is a flavonoid with a limited bioavailability that can be found in natural sources. It is somewhat water soluble. However, because of its poor absorption and quick metabolism, it cannot function as it is expected. This work sought to enhance the pharmacokinetic features of epicatechin by encapsulating it in polylactic acid nanoparticles and liposomes. Liposomes were formed by the hydration of a lipid film to prepare large multilamellar vesicles, followed by membrane extrusion to formulate smaller unilamellar vesicles. On the other hand, polymeric nanoparticles were produced by double emulsification solvent diffusion method using polylactic acid as the polymer. Both products were then characterized for their particle size, zeta potential, drug loading, antioxidant activity, toxicity on cell lines (NRK-52E cells) and protection against paraquat oxidation. The mean particle size of liposomes was 183.8 ± 80.1 nm and for polylactic acid nanoparticles it was 350.9 ± 87.4 nm. Their surface zeta-potentials were -11.3 ± 3.93 and -32.9 ± 7.54 mV; respectively. The encapsulation of epicatechin in liposomes was 10.23 ± 1.54% and in polylactic acid nanoparticles 5.35 ± 3.35% (%encapsulation efficiency = 18.09 ± 1.95%). Microscopic images presented both sorts of nanoparticles to be sphere-shaped. Encapsulation of epicatechin into both liposomes and polylactic acid nanoparticles enhanced the %internalisation remarkably from 4.18 ± 0.03% to 27.05 ± 1.07% and 36.29 ± 0.09%; respectively. The toxicity test found all three forms not to be harmful to the NRK-52E cells within the concentration ranges tested. Examining the in vitro activity results showed that the same concentration of epicatechin in the liposomal form showed more protection against paraquat than epicatechin in its free form. Moreover, a lower epicatechin concentration was used in the polylactic acid nanoparticle form and still found to be more protective. From these results it can be concluded that epicatechin-loaded liposomes and polylactic nanoparticles offer protection to NRK-52E cells against paraquat induced toxicity.

Keywords: epicatechin; liposomes; PLA nanoparticles; antioxidant; NRK-52E cells; tyrosine nitration test; ABTS assay; Paraquat.

1. Introduction

The main limitation to the use of epicatechin to treat oxidant-induced damage is its pharmacodynamic and pharmacokinetic properties, such as low lipid solubility, low bioavailability and fast excretion[1]. The use of nanoparticles to deliver antioxidants is an extensively researched area. Several attempts have been made to improve its physiochemical properties such as [1,2,3,4] with varying degree of success. The use of nanoparticle delivery is attempted in these studies, in order to improve its antioxidant activity by increasing intracellular intake and prolonging their availability inside cells.

Liposomes are vesicles within the nano to micro range. They contain of a liquid core fenced by one or more bilayers of phospholipids[5]. Due to their phospholipid composition, they have the benefit of being pharmacologically inactive and are fairly safe[6]. Polymeric nanoparticles, however, are nano-sized solid colloidal units consisting primarily of macromolecules. They have the capability to surge the solubility of the carried drugs and/or reduce their side effects by applying targeted delivery. Furthermore, they may be able to increase the stability of their cargo and therefore, increase their shelf-life and/or duration of action[7].

The purpose of this study was to enhance the bioavailability of epicatechin by encapsulating it into either liposomes or polymeric nanoparticles. The aim was to produce a preparation to increase its delivery into cells to ensure activity. Liposomes were created by first hydrating a lipid film to create big multilamellar vesicles, then extruding a membrane to create smaller unilamellar vesicles. Polymeric nanoparticles were produced by double emulsification solvent diffusion (DESD) method using polylactic acid (PLA) as the polymer. PLA nanoparticles and liposomes were tested for their particle size, zeta potential, morphology, loading efficiency and drug delivery. Liposomes were also studied for their *in vitro* antioxidant action using inhibition of tyrosine nitration by peroxynitrite and ABTS assay. The liposomes and PLA nanoparticles formed were furthermore, tested for their cellular toxicity and effectiveness for antioxidant activity against paraquat (PQ) oxidation using the NRK-52E cell line.

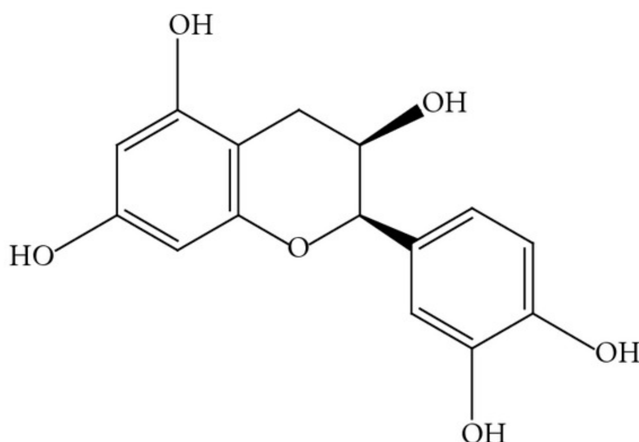


Figure 1: Chemical structure of epicatechin

2. Materials and methods

2.1. MATERIALS

Epicatechin (>98%), trolox, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulphate, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), PLA, Dimethylsulfoxide (DMSO) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and other chemicals and solvent were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK. The NRK-52E cell line was purchased ECACC (Porton Down, Wiltshire, UK). All cell culture supplements and consumables such as Dulbecco's Modified Eagle's Medium (DMEM), Trypsin/ethylenediaminetetracaetic acid (EDTA), foetal bovine serum (FBS), non-essential amino acids (NEAA) and penicillin-streptomycin (PenStrep) were purchased from Fisher Scientific Ltd. All other chemicals and solvents were of laboratory and HPLC grade.

2.2. ENCAPSULATION OF EPICATECHIN IN LIPOSOMES

Liposomes were prepared by the hydration of a lipid film to prepare large multilamellar vesicles, followed by membrane extrusion to form smaller unilamellar vesicles[8]. The method was modified as required.

An aliquot of ethanolic DPPC solution (5ml at 8 mg/mL concentration) was transferred into a round-

bottomed flask and evaporated to dryness using a rotatory evaporator at 40°C, 100 rpm under100 mbar (IKA Rotary evaporator RV 3 V-C) resulting in the formation of a lipid film. 4 mL Epicatechin solution in water (1.25 mg/mL) at 45°C was added to this lipid film followed by vigorous shaking and sonication in an ultrasonic bath for a total duration of 20 min to ensure complete detachment of film from flask wall.

Liposomes were extruded (21 times) using an Avanti mini-extruder with 200 nm pore size polycarbonate membrane and glass air-tight syringes resulting in their size-reduction. The temperature was set at 41°C, which is slightly above the melting transition temperature for DPPC. Due to the instability of the liposomes in a freeze-thaw cycle, all liposome samples were freshly prepared and tested within two days. During this short period samples were stored at 2-8°C. Blank liposomes containing only the lipid were prepared in an identical manner and used as controls. All liposome samples were prepared in triplicate.

2.3. ENCAPSULATION OF EPICATECHIN IN POLYMERIC NANOPARTICLES

The technique used in the preparation of PLA nanoparticles in this work was based on the method developed by Buhecha *et al*, 2019 using DESD[9], which was adopted and modified as required. Initially, 2% (w/v) PVA stock solution was made by dissolving 2 g of PVA in 100 mL purified water. A magnetic stirrer (EW-04801-01 - Stuart Equipment) was used to aid dissolving, while heating to 70°C. 5 mg Epicatechin was added to this aqueous solution. In a separate beaker 200 mg PLA was dissolved in 10 mL DCM and 10 mL of the PVA solution was added. An emulsion was prepared by using a high-speed homogeniser (IKA® T25 digital Ultra-Turrax®) at 15000 rpm for 15 min. 10 mL acetone was added and further homogenised for a further 15 min at the same speed. This combination was then transported into an extra volume of water (100 mL) while continuing homogenisation for 15 min at 15000 rpm. Any organic solvent was then removed by using a rotatory evaporator, rotating at 130 rpm, heating at 40°C and vacuum pressure 70 mbar and left overnight to guarantee whole removal of organic solvents. Examination using microscope was made to confirm development of nanoparticles. The later solution was then centrifuged (using a Sorvall RC-6 Plus Ultracentrifuge) at 15000 rpm and 4°C for 15 min. We kept the supernatant for further investigation. Pellets (presumably, containing the nanoparticles) were frozen at -80°C for a minimum of 1 hour after which were freeze dried (Benchtop, 8L, -50°C, PTFE-Coated Collector, Labconco). Samples were then stored between 2-8°C for further investigation. Blank PLA nanoparticles were made in the same manner for comparison. All samples were produced in triplicates.

2.4. CHARACTERISATION OF NANOPARTICLE MORPHOLOGY, PARTICLE SIZE AND ZETA POTENTIAL

Particle size distribution and surface morphology of the liposomes were investigated by light microscope using a Motic™ BA210E Trinocular Compound Microscope. PLA nanoparticles were inspected for their shape using scanning electron microscopy (SEM). Samples for SEM were prepared by mounting the powders on 12 mm aluminium pin stubs after sticking double-sided conductive Leit-C-carbon self-adhesive mounting pads on them. Any additional powder was detached by gentle tapping. Samples were additionally coated with a platinum coat of 4-5 nm thickness using a sputtering coater (Quorum technologies Q150 ES sputter coater) and inserted into a ZEISS Sigma Field Emission Gun SEM with an Everhart Thornley-Secondary Electron detector for imaging (Zeiss Evo LS-15 SEM). The accelerating voltage used ranged between 5-10 kV with a working distance around 8.5 mm.

The particle size and charge of the nanoparticles (liposomes and PLA nanoparticles) were determined also tested by photon correlation spectroscopy (PCS) (Malvern Zetasizer Nanoseries, Nano-ZS690 Malvern Instruments, UK). A precise amount of the prepared nanoparticles sample was diluted in 10 ml of 18.2 MΩ deionized water and mixed using a vortex mixer for 30 seconds. For particle size testing, the samples were then moved to a plastic disposable cuvette and placed in the Zetasizer. On the other hand, for zeta potential testing, samples were transferred to folded capillary cuvettes, with gold plated electrodes. Tests were carried out at 25°C and each test was carried out in triplicate.

2.5. DETERMINATION OF DRUG LOADING EFFICIENCY IN LIPOSOMES AND POLYMERIC NANOPARTICLES

Nanoparticles were first passed through NAP-25 columns (pre-packed with Sephadex™ G-25 resin) for the purpose of purification and removal of any unencapsulated drug. Epicatechin was then extracted from the nanoparticles into a suitable solvent and then analysed by high performance liquid chromatography (HPLC) to determine the loading efficiency (%LE) of the antioxidant in the encapsulated liposomes and PLA nanoparticles. Agilent 1220 series, equipped with Clarity-Chromatography SW, DataApex software, Agilent 1220 pump system with degasser, manual injector

with a 20 µL injection loop and an Agilent 1220 UV-visible detector. A 5 µm Fortis c18, 250 * 4.6 mm column was used with 0.1% OPA: acetonitrile (The gradient method was: at time 0.01 min 11% B; at 20 min 25% B; and 21 to 30 min 11% B) as mobile phase, at a flow rate of 1.5 ml/min at 25°C with a detector wavelength of 280 nm. Standard concentrations of all the drug over the range of 0.1–20 µg/ml were prepared, and calibration graphs were plotted. Loading efficiency was calculated as described by Li et al.[10].

2.6. EVALUATION OF ANTIOXIDANT ACTIVITY USING *IN VITRO* METHODS

2.6.1. ABTS decolorization assay

The assay was carried out by the interaction free antioxidant and antioxidant encapsulated in liposomes with the ABTS radical cation, prepared as described by Re et al.[11]. A solution of 2.45mM potassium persulphate was added to a stock solution od ABTS (7mM-final concentration) and allowed to react overnight, generating ABTS radical. This was diluted in deionised water to obtain an absorbance of 0.7 ± 0.02 at 734 nm prior testing the samples.

Stock solutions of standard epicatechin and epicatechin encapsulated into liposomes were made such that, after the addition of 30 µl of dilution to 3 ml $ABTS^{\cdot+}$ solution, an inhibition of 20%-80% in the absorbance, compared to blank absorbance at 734 nm. Absorbance was measured after exactly 6 min of initial mixing, using a PerkinElmer UV-visible spectrophotometer with single cell holder. All determinations were carried out in triplicates. Trolox solutions with a concentration between 0-15 µl was used as a standard[11]. Appropriate solvent blanks were tested in each assay. The %inhibition in the absorbance was calculated and plotted against concentration of epicatechin.

2.6.2. Inhibition of tyrosine nitration by peroxynitrite.

Peroxynitrite preparation and the tyrosine nitration test were carried out as described by Pannala *et al.*[12]. For the current assay, only freshly prepared peroxynitrite was used. 50 µl of peroxynitrite (500 µM final concentration) was added to a solution of tyrosine (100 µM final concentration) in the presence of increasing concentrations of liposomes encapsulated with antioxidant in 0.2 M phosphate buffer, pH7 to give a final volume of 1 ml. Appropriate blanks were carried out each time, to estimate concentration of 3-nitrotyrosine (3-NT) formed. Liposomes were also tested in the absence of peroxynitrite to exclude any effect from liposomes on the test accuracy. Samples were then analysed by HPLC using Fortis C18 column (25 cm x 4.6 mm) on an Agilent 1260 system with a Quaternary pump, an autoinjector, an auto sampler and a diode array detector. The system was equipped with Chem Station software. The mobile phase was 50 mM phosphate buffer, pH7: acetonitrile at a ratio of 95:5 v/v, at 1ml/min flow rate. Calibration plots were constructed using standard 3-NT (1-100 µM), monitored at 350 nm. The %inhibition in 3-NT was then plotted against theoretical concentration of antioxidant in liposomes. Different concentrations of peroxynitrite solutions (0.05-1 mM) diluted in 0.1 M NaOH were added to tyrosine solution (100 µM final concentration) in 0.2 mM phosphate buffer and the concentration of 3-NT formed was determined using the same HPLC method.

2.7. NANOPARTICLE CHARACTERIZATION UTILIZING RAT TUBULAR EPITHELIAL (NRK-52E) CELLS.

2.7.1. NRK-52E cell culture

NRK-52E cells (passage number 15 – 27) were regularly cultured in a cell culture medium made from DMEM supplemented with 5% (v/v) FBS, PenStrep (100 units/ml) solution and NEAA (CCM I). Cells were kept at 37°C in a humidified area of 95% air and 5% CO_2.

2.7.2. Effect of epicatechin loaded nanoparticles on cell viability

The cytotoxicity of free epicatechin and epicatechin encapsulated in both types of nanoparticles was determined by conducting MTT and LDH assays[13,14]. Cells were seeded at a cell density of 4000 cells/well in 24 well plates and cultured in CCM I for 24 h. Nanoparticle solutions diluted in CCM I, without FBS (CCM II) were added separately to the wells at a concentration range of 0.5 and 5%v/v (500 µL/well). Solutions of epicatechin (0-10 mM) were prepared in cell culture medium II. MTT solution was made at a final concentration of 5 mg/ml in the same media. After incubating the cells for 24 hours with the nanoparticles or drug solutions, 500µL of MTT solution was added to each well and the cells were incubated for a further 1 h. MTT solution was then carefully aspirated from the cells and DMSO (125 µL) was added to each well. The solutions were then transferred in duplicates into a 96 well plate and the absorbance of each well was measured at 540nm (Multiskan Ascent, v123). For LDH assay, 50µl LDH substrate was added to 50µl of cell culture supernatant. The reaction was carried out in duplicates in 96 well plates and the absorbance was measured at 540 nm (Multiskan Ascent, v123).

2.7.3. Effect of epicatechin loaded liposomes and polymeric nanoparticles on paraquat toxicity

Paraquat (PQ) was used to induce oxidative stress to NRK-52E cells in vitro (ref: https://doi.org/10.1016/S0300-483X(02)00382-7). NRK-52E cells were grown on 24-well plates to 80-90% confluence. The CCM I was removed and 500 µL of different concentrations of PQ (0-1 mM and 0-10 mM) prepared in CCM II was added. The cells were then incubated at 37°C in a humidified 5% CO^2 atmosphere for 24 hours. The cells were then inspected under light microscope and tested using MTT and LDH assay. Tests were repeated in replicates of six.

To compare the effectiveness of free epicatechin and their novel delivery systems under investigation (PLA nanoparticles and liposomes), they were tested for their ability to protect cells from PQ induced oxidative stress. All three preparations were prepared and diluted in CCM II to make 6.25 & 62.5 µg/mL theoretical drug concentration, regardless of their %LE and %internalisation. This was to ensure all other variables such as PLA, PVA and lipid concentrations were kept constant. The calculations, however, were made according to actual concentration of drug inside each preparation.

NRK-52E cells were grown on 24-well plates, to 80-90% confluence. The CCM I was removed and 500 µL of the two different concentrations of the antioxidant preparations prepared were added to the cells, separately. After 24 hours of incubation at 37°C in a humidified 5% CO2 atmosphere, the medium was removed and 500 µL of PQ was added at increasing concentrations (0, 0.2, 0.4, 0.6, 0.8, 1 mM). Cells were then tested after a further 24-hour incubation in the same conditions using the MTT and LDH assays. All tests were repeated in replicates of six.

3. Results

3.1. PREPARATION AND CHARACTERIZATION OF LIPOSOMES AND PLA NANOPARTICLES FOR THEIR MORPHOLOGY

Epicatechin-loaded DPPC liposomes were successfully prepared in suspended in an aqueous medium. Each passage of the liposomal suspension through the extruder produced a clearer solution and inspected under light microscope for the outer morphology of liposomes, which showed small spherical shapes (less than 400 nm). Blank liposomes that were produced had identical morphology.

PLA nanoparticles were prepared and stored in dry form after lyophilization resulting in the formation of a white fluffy powder. Morphological assessment of blank and epicatechin-loaded PLA nanoparticles was carried out using SEM which showed round spherical shaped particles with a wide size distribution. There was no evidence of free drug crystals shown [Figure 2].

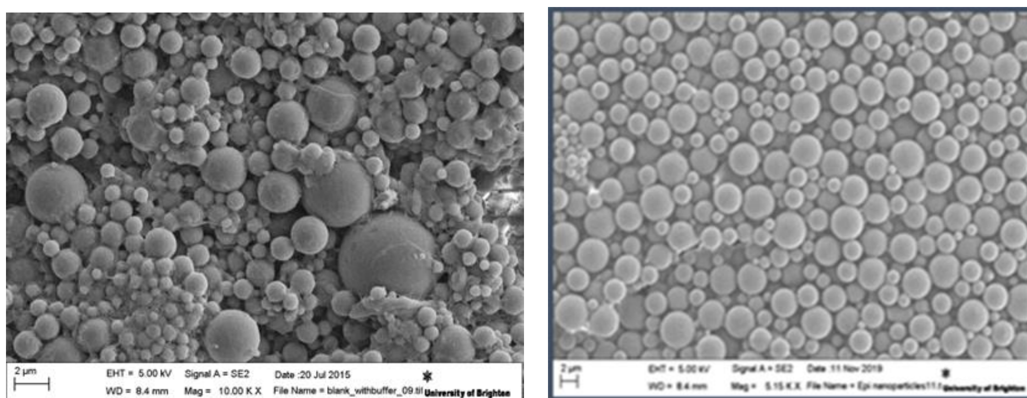


Figure 2: SEM images of PLA nanoparticles: Blank (left) and epicatechin-loaded PLA nanoparticles (right). The accelerating voltage used was 5 kV with a working distance from 8.5 mm. Nanoparticle were spherical in shape with smooth surface and variable sizes.

3.2. PARTICLE SIZE ANALYSIS AND SURFACE CHARGE

The mean particle size analysis of epicatechin-loaded liposomes and PLA nanoparticles revealed a size range of 183.8 ± 80.1 & 350.9 ± 87.4 nm; respectively and a polydispersity index (PdI) of 0.235 & 1; respectively. The blank liposomes and PLA nanoparticles on the other hand, showed a size range of 194.9 ± 114.0 and 345.0 ± 98.7 nm; respectively and a PdI of 0.256 & 1; respectively [Table 1]. This showed that incorporating the drug into the PLA nanoparticles preparation did not affect the size of the nanoparticles, significantly. Furthermore, the PdI shows a wide size distribution

for both the preparations which is more apparent in the PLA nanoparticles.

Epicatechin-loaded liposomes and PLA nanoparticles showed a zeta potential of -11.3 ± 3.93 & -32.9 ± 7.54 mV; respectively. There was no statistically significant difference in zeta potential when compared to their respective blank formulations: -10.9 ± 3.45 & -38.0 ± 3.85 mV; respectively [Table 1].

Table 1: Summary of the results obtained for particle size and zeta potential for different nanoparticles.

Drug in liposomes	Z-Average (D.nm)	PdI	Zeta potential (mV)
Blank liposomes	194.9 ± 114.0	0.256	-10.9 ± 3.45
Epicatechin-loaded liposomes	183.8 ± 80.1	0.235	-11.3 ± 3.93
Blank PLA nanoparticles	345.0 ± 98.7	1	-38.0 ± 3.85
Epicatechin -loaded PLA nanoparticles	350.9 ± 87.4	1	-32.9 ± 7.54

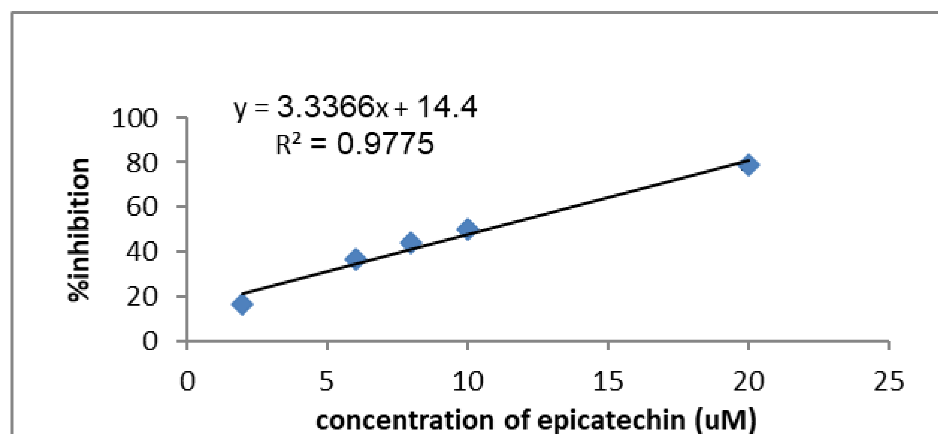
3.3. DETERMINATION OF DRUG LOADING EFFICIENCY AND CELL INTERNALISATION

The same HPLC method was used to measure and calculate both the amount of epicatechin loaded into both nanoparticle preparations and the amount delivered to the cells via these two preparations. The %LE of epicatechin in liposomes (10.23 ± 1.54%) was higher compared to PLA nanoparticles (5.35 ± 3.35%). Furthermore, the %internalisation of epicatechin using liposomes (27.05 ± 1.07%) was over 6 times higher than free epicatechin (4.18 ± 0.03%) and eight times higher using PLA nanoparticles (36.29 ± 0.09%).

3.4. *IN-VITRO* DETERMINATION OF ANTIOXIDANT ACTIVITY

3.4.1. ABTS decolorization assay

The extent in the decolorization of the $ABTS^{•+}$ by epicatechin and the prepared epicatechin-loaded liposomes was calculated as the %inhibition in the absorbance and plotted as a function of the concentration of epicatechin [Figure 3]. Trolox equivalent antioxidant activity (TEAC) was calculated by dividing the gradient of each plot against the gradient of the plot produced by using Trolox as standard. The TEAC values for free epicatechin and epicatechin-loaded liposomal preparation were 0.89 ± 0.01 & 0.56 ± 0.01; respectively. Blank liposomes were found to have negligible TEAC value - 0.034. This data demonstrates that encapsulated epicatechin is released from the liposomes in a suitable medium to exhibit its antioxidant activity.



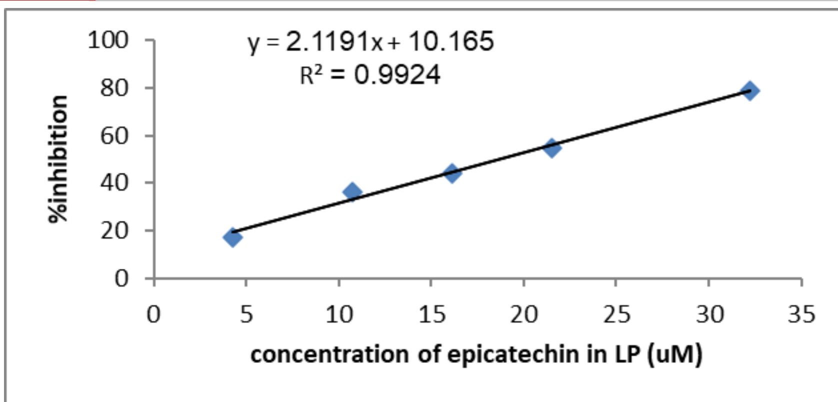


Figure 3: ABTS activity test. The %decolourisation to the ABTS solution by adding increased concentration of epicatechin solution (upper image) and epicatechin-loaded liposomes (lower image.

3.4.2. Tyrosine nitration assay

The addition of peroxynitrite to the tyrosine solution, resulted in the formation of 3-NT, which was confirmed by chromatograph comparison using HPLC. In this experiment, a final concentration of 500μM for peroxynitrite was used throughout the assay.

The ability of epicatechin-loaded in liposomes to inhibit the tyrosine nitration was determined. The liposomes were co-incubated with the tyrosine solution followed by the addition of peroxynitrite and quantifying the extent of 3-NT produced. A standard solution of 3-NT (0-100μM) was used to obtain a calibration plot (R^2=1). The %inhibition in the concentration of 3-NT formed was plotted against the theoretical concentration of epicatechin encapsulated in the liposomes [figure 4].

Results showed that the epicatechin-liposomes showed peroxynitrite scavenging properties by its ability to prevent tyrosine nitration. This inhibition increased with increased concentration, reaching 100% at high concentrations of epicatechin. A neglectable response was observed with blank liposomes, which did not exceed 13%, even at high concentrations. These observations confirm that encapsulated epicatechin demonstrates peroxynitrite scavenging ability.

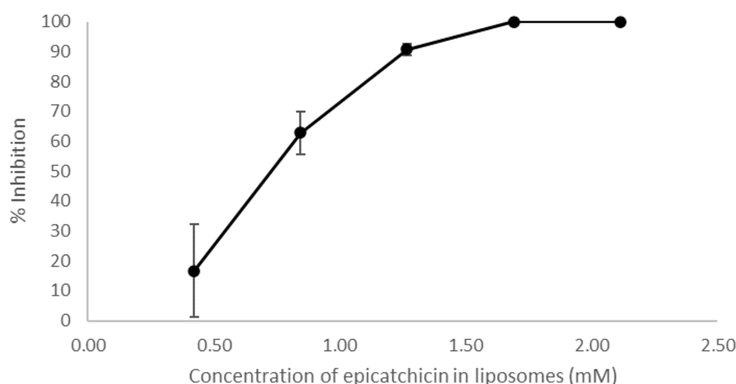


Figure 4: The effects of different concentrations of different epicatechin-loaded liposomes on the concentration of 3-NT formed in the tyrosine nitration assay (n=3).

3.5. EFFECT OF EPICATECHIN-LOADED LIPOSOMES AND POLYMERIC NANOPARTICLES ON THE VIABILITY OF NRK-52E CELLS

A range of equivalent concentrations of epicatechin solution and epicatechin-loaded liposomes and PLA nanoparticles were tested for their toxicity on NRK-52E cells [Figure 5]. This allowed a comparison to be made between the effects of the drug in solution and their effects when encapsulated in liposomes or PLA nanoparticles.

Epicatechin-loaded liposomes did not cause substantial effect on cells, where %viability ranged between 91.19 – 97.24% and %increase in LDH release ranged between 0.39 – 6.69%. Additionally, the PLA form of epicatechin caused a minor insignificant increase in %viability (99.32 – 106.76%) with minimal variation in %increase in LDH release (1.24 – 6.05%). Furthermore, epicatechin solution (free form) caused insignificant change in %viability (92.56 – 100.49%) and in %increase in LDH release (1.92 – 9.44%) [Figure

5]. To summarise, all three forms of epicatechin were found safe on the NRK-52E cells.

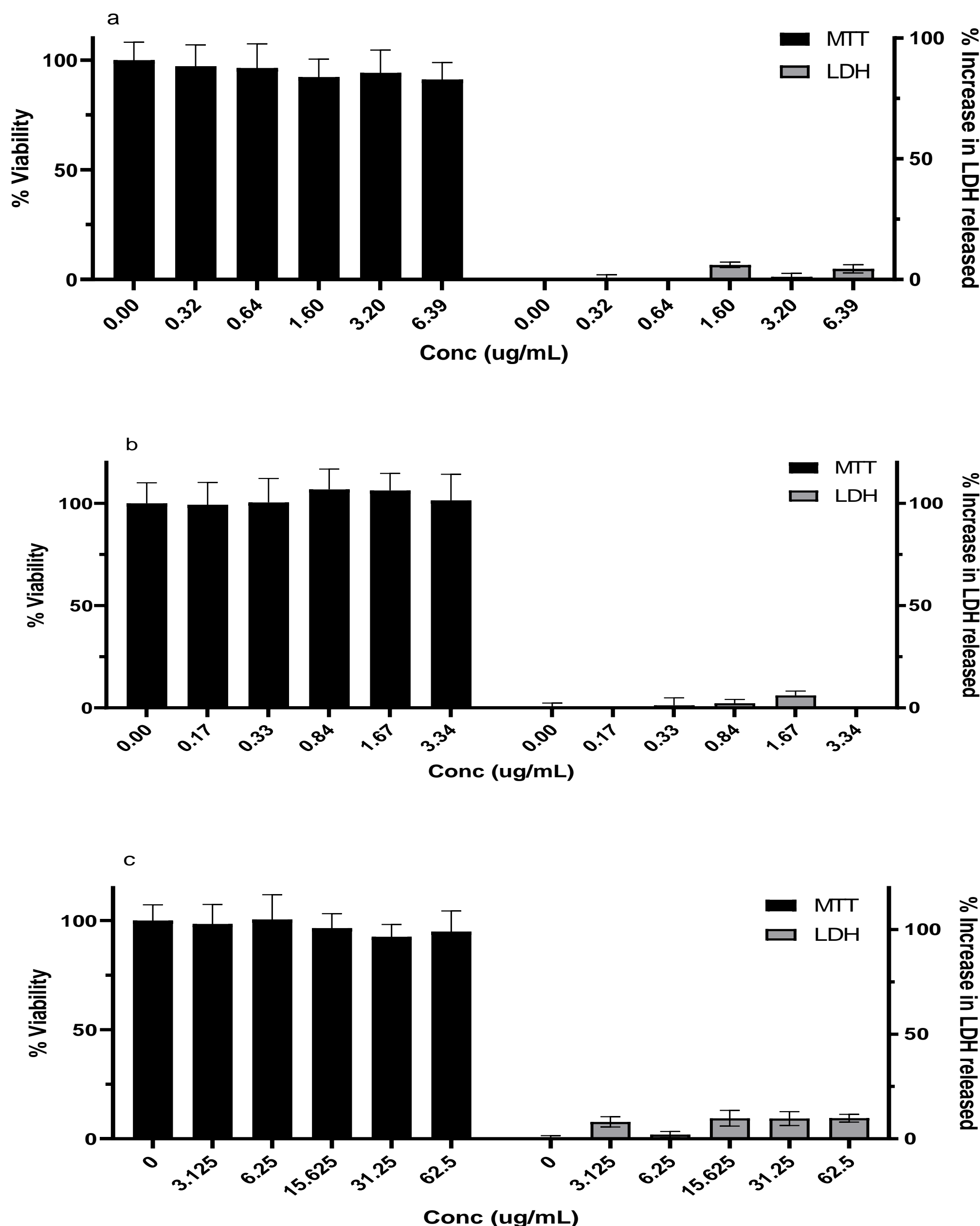


Figure 5: The effect of increased concentration of epicatechin in three different forms on NRK-52E cells measured using two different assays: MTT and LDH. a. epicatechin-loaded liposomes. b. epicatechin-loaded PLA nanoparticles. c. epicatechin solution. (mean ± SD, $N = 6$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs* blank [0 µg/mL]). Data analysed using ordinary two-way ANOVA, followed by Bonferroni's multiple comparisons test with no significant difference in the results.

3.6. EFFECT OF EPICATECHIN-LOADED LIPOSOMES AND POLYMERIC NANOPARTICLES ON PARAQUAT TOXICITY

PQ was used to induce oxidative damage in NRK-52E cells as described in previous work (15). Epicatechin was prepared as free (solution) and encapsulated forms in liposomes and PLA nanoparticles. Details of the calculated concentrations within each sample, when considering the %LE are shown in table 2.

Table 2: The concentration of epicatechin in each sample and its given code

Drug	Form	Theoretical conc (µg/mL)	%LE	Actual conc (µg/mL)	code
Epicatechin	liposomes	6.25	10.23%	0.64	EL-1
Epicatechin	liposomes	62.5	10.23%	6.39	EL-2
Epicatechin	PLA nanoparticles	6.25	5.35%	0.33	EP-1
Epicatechin	PLA nanoparticles	62.5	5.35%	3.34	EP-2
Epicatechin	solution	6.25	NA	6.25	ES-1
Epicatechin	solution	62.5	NA	62.5	ES-2

Remarkable, effect was observed from liposomes on the viability of the cells. For example, pre-incubation with EL-1 & EL-2 increased the viability of cells from 88.41 ± 4.7% when treating with 0.2 mM PQ to 111.12 ± 9.63% and 101.03 ± 10.23%; respectively. The protective effect was even observed even at the highest concentration of PQ, where the %viability increased from 6.10 ± 2.08% when treated with 1 mM PQ to 17.41 ± 9.99% and 18.8 ± 7.59% after pre-incubating with EL-1 & EL-2; respectively [Figure 7]. These results were confirmed using the LDH assay, for example, the %increase in the LDH release was reduced from 10.87 ± 1.51% after treating with 0.2 mM PQ to 2.28 ± 1.10% and -2.63 ± 3.16% when pre-incubating with EL-1 & EL-2; respectively. As with the MTT results, the protective effect was observed up to the highest concentration of PQ (1 mM), where the %increase in the LDH release was reduced from 218.04 ± 4.62% to 191.2 ± 3.22% & 173.11 ± 3.98% when pre-incubating with EL-1 & EL-2; respectively [Figure 8].

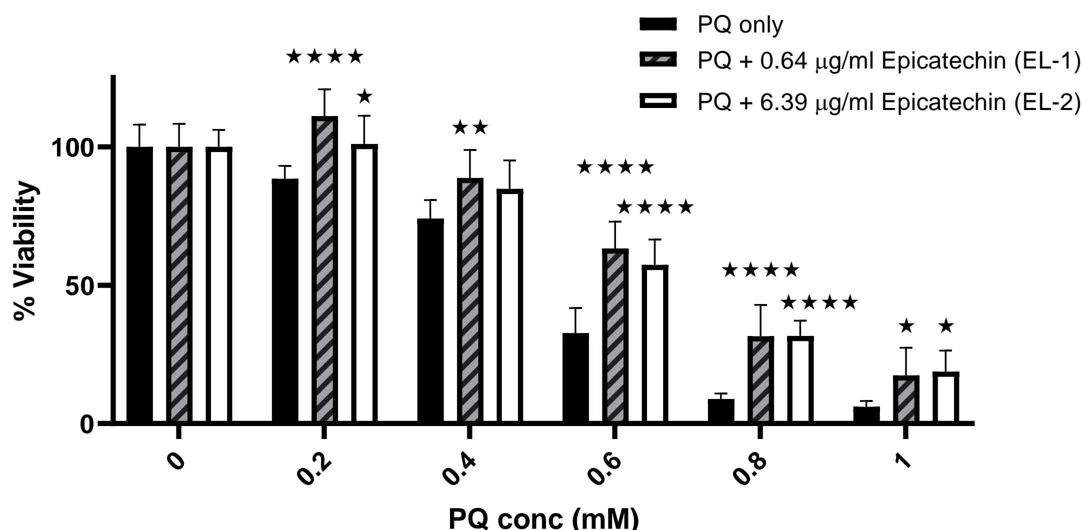


Figure 6: The effect of two different concentrations of epicatechin-loaded liposomes plus PQ compared to PQ only on %viability of cells using MTT assay. Mean ± SD, $N = 6$, $p < 0.0001$ (using two-way ANOVA), ** $p < 0.01$, **** $p < 0.0001$ compared to PQ only (using Bonferroni's *post hoc* test).

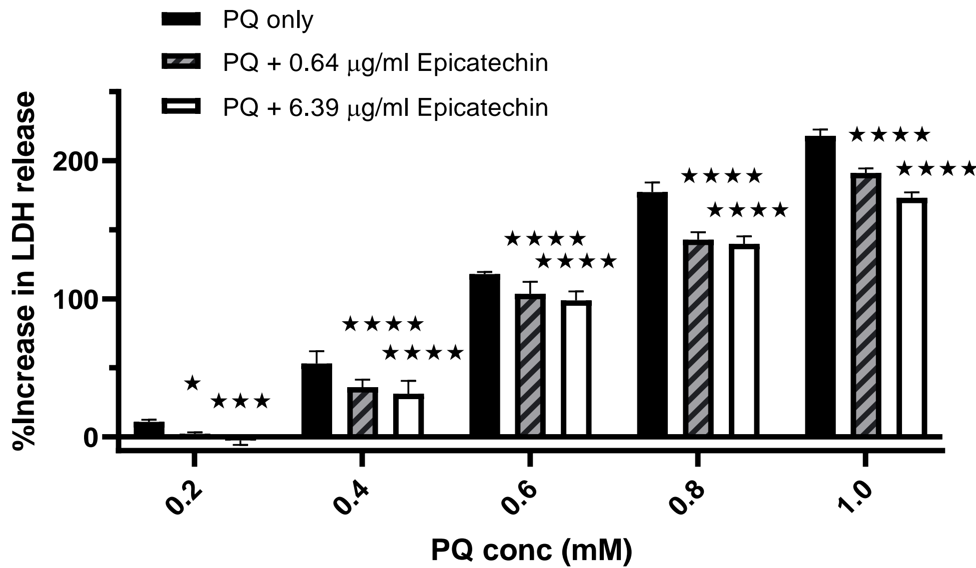


Figure 7: The effect of two different concentrations of epicatechin-loaded liposomes plus PQ compared to PQ only on the %increase in LDH release from cells. Mean ± SD, $N = 6$, $p < 0.0001$ (using two-way ANOVA), * $p < 0.05$, **** $p < 0.0001$ compared to PQ only (using Bonferroni's *post hoc* test).

In the same way as liposomes, epicatechin-loaded PLA nanoparticles were tested for their activity. Although not as effective, a good protection was still observed in the MTT and LDH results. The highest protection was observed at 0.4 mM and 0.6 mM PQ, where the %viability increased from 79.94 ± 6.82% & 32.85 ± 8.88%; respectively after treating with PQ only to 99.83 ± 7.36% & 53.1 ± 9.64; respectively when pre-incubating with EP-1 and to 93.17 ± 9.98% & 48.9 ± 10.46%; respectively when pre-incubating with EP-2 [Figure 9]. The protective effect in the LDH results was also observable at all PQ concentrations. For example, the %increase in the LDH release after treating with 0.2 mM PQ (17.87 ± 4.08%), almost returned to zero when pre-incubating with EP-1 (5.44 ± 1.66%) & EP-2 (3.49 ± 2.5%). Even at a high PQ concentration (1mM), the %increase in the LDH release was reduced from 200.54 ± 4.88% to 187.71 ± 9.97% & 191.17 ± 8.97% when pre-incubating with EP-1 & EP-2; respectively [Figure 10].

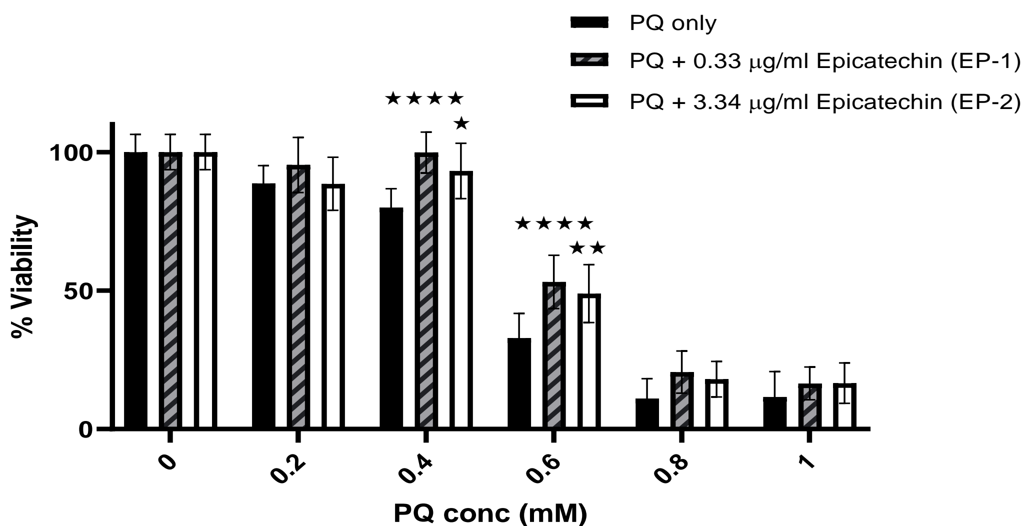


Figure 8: The effect of two different concentrations of epicatechin-loaded PLA nanoparticles plus PQ compared to PQ only on %viability of cells using MTT assay. Mean ± SD, $N = 6$, $p < 0.0001$ (using two-way ANOVA), * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ compared to PQ only (using Bonferroni's *post hoc* test).

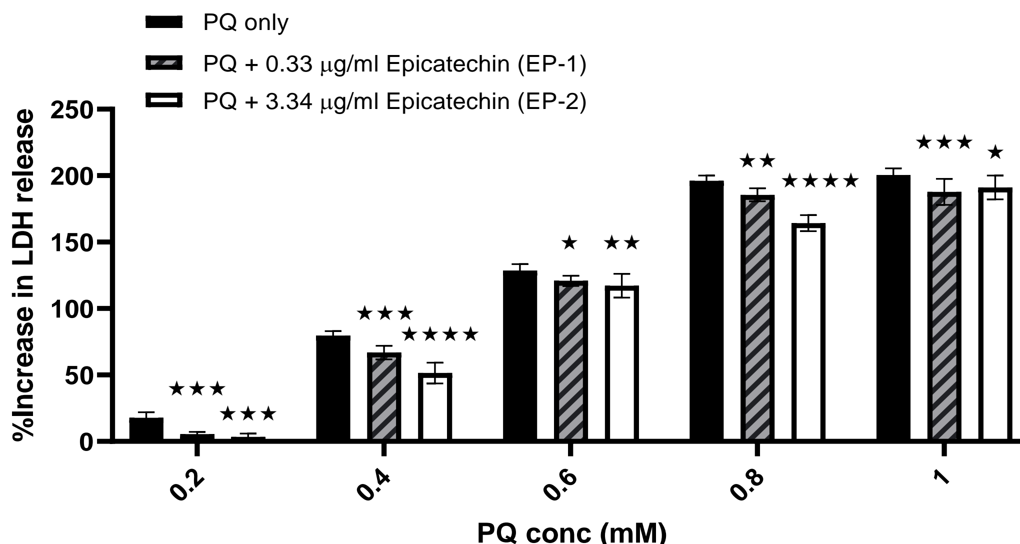


Figure 9: The effect of two different concentrations of epicatechin-loaded PLA nanoparticles plus PQ compared to PQ only on the %increase in LDH release from cells. Mean ± SD, $N = 6$, $p < 0.0001$ (using two-way ANOVA), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to PQ only (using Bonferroni's *post hoc* test).

Finally, to check whether the encapsulation of epicatechin into either liposomes or PLA nanoparticles had any influence on its activity, epicatechin solution (as free from) was tested in the same way as its encapsulated forms. The MTT results showed some protection from epicatechin against PQ. This was mostly evident at 0.4 mM PQ, were the %viability increased from 77.17 ± 9.45% to 96.20 ± 5.39% & 88.43 ± 4.59% after pre-incubating with ES-1 & ES-2; respectively [Figure 11]. Looking at the results from the LDH assay [Figure 12], the protection was only evedent using ES-2 at PQ concentartions ≥ 0.4 mM. However, there was a significant increases in the LDH release after pre-incubation with ES-2 at 0.2 mM PQ. No noticible effect from pre-incubating cells with ES-1 at all PQ concentrations tested as detected. The results indicate the benefits of encapsulating epicatechin into nanoparticles, mainly liposomes.

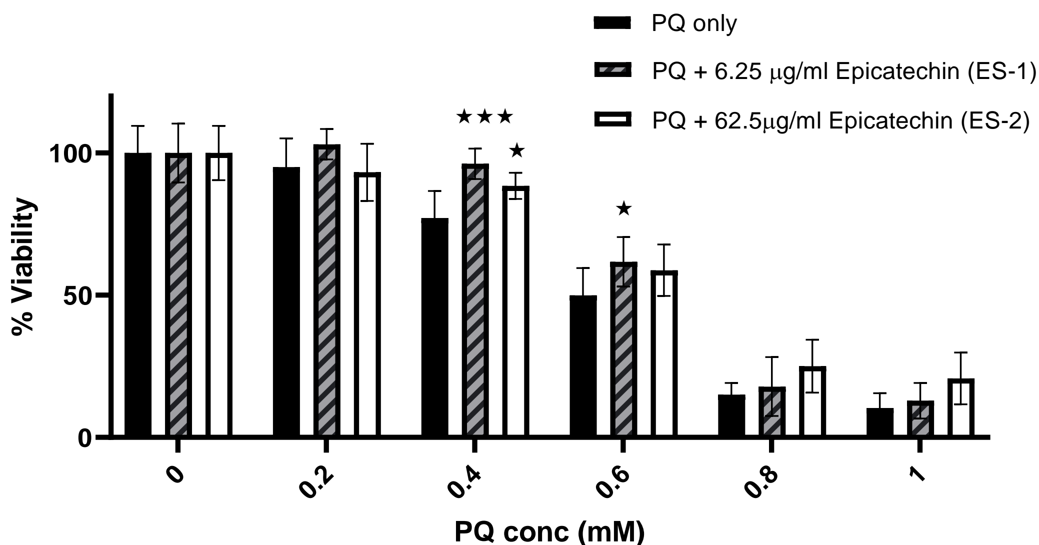


Figure 10: The effect of two different concentrations of epicatechin solution plus PQ compared to PQ only on %viability of cells using MTT assay. Mean ± SD, $N = 6$, $p < 0.0001$ (using two-way ANOVA), * $p < 0.05$, *** $p < 0.001$ compared to PQ only (using Bonferroni's *post hoc* test).

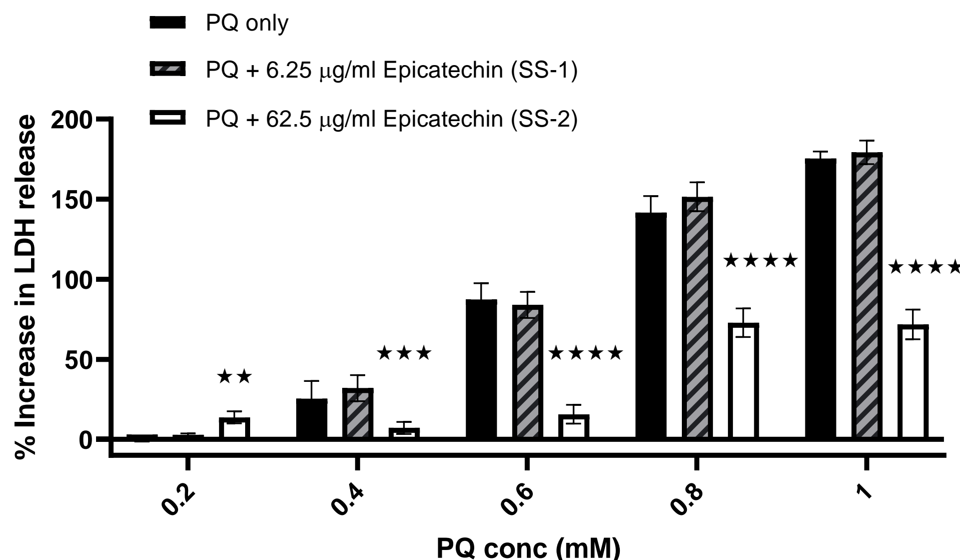


Figure 11: The effect of two different concentrations of epicatechin solution plus PQ compared to PQ only on the %increase in LDH release from cells. Mean ± SD, $r = 6$, $p < 0.0001$ (using two-way ANOVA), ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to PQ only (using Bonferroni's *post hoc* test)

4. Discussion

Epicatechin is a flavonoid that is available from natural sources, mainly green tea, with low bioavailability. Although, it has some water solubility, its activity is hindered due to low absorption and rapid metabolism. In this study it was encapsulated into liposomes and PLA nanoparticles to improve its pharmacokinetic properties. The mean particle size of liposomes was 183.8 ± 80.1 nm and for PLA nanoparticles it was 350.9 ± 87.4 nm. Their surface zeta-potentials were -11.3 ± 3.93 and -32.9 ± 7.54 mV; respectively. The encapsulation of epicatechin was found to be higher in liposomes (%LE = 10.23 ± 1.54%) than PLA nanoparticles (%LE = 5.35 ± 3.35%, %EE = 18.09 ± 1.95%). This was attributed to its hydrophilicity, as it has been reported that encapsulating hydrophilic compound is more difficult in PLA nanoparticles[9]. These results were comparable to the results obtained by Perez-Ruiz *et al*,[3]. They produced epicatechin-loaded lecithin–chitosan nanoparticles using molecular self-assembly, with %LE equal to 3.42 ± 0.85%. However, there are other studies that managed to produce nanoparticles with higher %LE for epicatechin such as Ghosh *et al*,[4]. Here, epicatechin and morin (another flavonoid) were encapsulated within albumin nanoparticles for the purpose of enhancing their solubility and anticancer activity. They used the desolvation method for preparing nanoparticles with %EE of around 88% for morin and 72% for epicatechin[4]. Additionally, epigallocatechin gallate (another polyphenol in green tea) was encapsulated in egg phosphatidylcholine liposomes (%LE = 63%) showing a 20-fold increase in drug deposition in basal cell carcinomas. This was contributed to the increased stability of the drug in the encapsulated form[1]. It should be noted however, that even though the method of preparation in the last study was similar to the current study, the lipid composition was quite different. They used egg phosphatidylcholine (4% w/v), cholesterol (1% w/v) and diacetylphosphate (0.25% w/v) for the lipid bilayer instead of DPPC. This might explain the higher %EE achieved.

Even with the slightly low %LE achieved, liposomes and PLA nanoparticles improved the %internalisation remarkably from 4.18 ± 0.03% to 27.05 ± 1.07% and 36.29 ± 0.09%; respectively. In a separate study, tea catechins also showed increased permeability across Caco-2 cells using tea catechins-loaded nanoparticles prepared from chitosan and an edible polypeptide (24%) as compared to their free form (less than 6%)[16], which was consistent with our study. Although, the toxicity test found all three forms to be unharmful to the NRK-52E cells within the concentration ranges tested. However, examining the in vitro activity results showed that the same concentration of epicatechin in the liposomal form showed more protection against PQ than epicatechin in its free form. Furthermore, a lower epicatechin concentration was used in the PLA nanoparticle form and still found to be more protective. This can be explained by the increased cellular uptake and was consistent with the results noted by Perez-Ruiz *et al*., 2018 who showed cytotoxic activity against breast cancer cell lines increased by four-fold using the

nanoencapsulated form of epicatechin[3]. Additionally, the increased antioxidant and cytotoxic activities of epicatechin was also improved using albumin nanoparticles which was attributed to the enhanced solubility which led to increased cellular uptake[4].

5. Conclusions

In summary, epicatechin was successfully encapsulated into liposomes and PLA nanoparticles. The liposomes characterized in this study possessed a narrow size distribution while the PLA nanoparticles were slightly larger and a wider size distribution. An acceptable zeta potential with good drug-loading efficiency for both liposomes and PLA nanoparticles was achieved making them suitable to be developed into a formulation for intravenous drug delivery. Cytotoxicity studies showed the formulations to be well-tolerated by kidney cells at clinically relevant concentrations. Additionally, the activity studies against the harmful effect of PQ showed the benefits of the encapsulation over free drug form. The fact that encapsulated epicatechin was internalised to a greater extent in the cells compared to epicatechin solution indicates that free epicatechin is likely to be easily metabolised and excreted, while encapsulation protects the drug and can improves its therapeutic activity.

Author contributions: Planning of the research work was equally shared between KIL, PKC, and ASP. Research work was carried out by KIL. All authors have contributed equally in the preparation of the manuscript and have given their consent to the final version of the manuscript.

Declaration of interest: Authors declare that there is no conflict of interest.

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