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Research Article

SYNTHESIS AND CHARACTERIZATION OF QUERCETIN-MEDIATED ZINC OXIDE NANOPARTICLES: *IN-VITRO* DRUG RELEASE KINETICS AND ANTICANCER EFFICACY AGAINST HELA AND MDA-MB-231 CANCER CELL LINES

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ABSTRACT: Quercetin (QC), a naturally occurring flavonoid having antioxidant properties and found in common diet ingredients. The therapeutic use of QC is hampered by its low oral bioavailability. To overcome this the present study was designed to synthesize and characterize the quercetin mediated zinc oxide nanoparticles (QZnO NPs) and their evaluation for anticancer activity against HeLa cervical cancer and MDA-MB 231 breast cancer cell lines. The size of the synthesized zinc oxide nanoparticles (ZnO NPs) and QZnO NPs were approximately 75 nm and 168 nm with a zetapotential of -14.5 mV and -36.7 mV, respectively. The polydispersity index of ZnO NPs and QZnO NPs were 0.226 and 0.277, respectively suggests that they were uniformly dispersed nanoparticles. The *in-vitro* drug release profile of QZnO nanoparticles showed a significant increase in sustained release at pH 4.8 (54.76% at 24 hours) compared to pH 7.4 (21.07% at 24 hours). This indicates that drug release is more sustained and pronounced in the acidic tumor microenvironment. *In-vitro* cytotoxicity assay was carried out in HeLa cervical cancer cells and MDA-MB 231 breast cancer cell lines for free quercetin, ZnO NPs and QZnO NPs at various concentrations in triplicate. Cells were incubated with all the three compounds for 48 h. Significant differences in IC_{50} values were observed for quercetin, ZnO NPs, and QZnO NPs in both cell lines ($p<0.01$), attributed to the uptake of nanoparticles by the cancer cells and the slow release of the drug within the cells. The results indicated that QZnO NPs were effectively inhibited the *in-vitro* cancer cell progression. Therefore, further studies are warranted to fully characterize the toxicity and the mechanisms involved with the anticancer activity of QZnO NPs and therefore there exists a vast scope to exploit QZnO NPs after appropriate clinical modeling.

Keywords: Quercetin; Zinc oxide nanoparticles; Breast cancer; Cervical cancer; Drug release kinetics.

INTRODUCTION

Cancer is the leading cause of death world-wide with nearly 10 million deaths per year accounting for one out of six deaths occurring [1]. According to WHO, 22.6% of the reported cases in 2020 are breast cancer cases and the leading type of cancer reported throughout the world. Cervical cancer also remains a significant cause of cancer-related mortality among women in low- and middle-income countries. Mortality is less in breast cancer compared to other types of cancers with mortality-to-incidence ratio of 15% [2]. Mortality can further be decreased by early detection and treatment of the disease. The disease is mainly associated with social and life style factors. Majority of breast cancer cases can be attributed to hormonal therapy, pregnancy associated factors, life style factors like obesity, inactivity, alcohol intake, low fiber diet and smoking [3]. Despite advances in screening and diagnosis, effective treatment remains a challenge due to therapeutic resistance, severe side

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effects from conventional chemotherapeutic agents, and high recurrence rates. The current treatment strategies employed for cancer include chemotherapy, radiation, hormone therapy, and surgery. However, these strategies pose undesirable effects such as systemic toxicity and immune suppression, which leads to reduction in quality of life. Additionally, tumor heterogeneity and drug resistance further limit the long-term effectiveness of chemotherapy. Consequently, there is a growing emphasis on the development of alternative therapeutic strategies that are both effective and safe, particularly using naturally derived bioactive compounds. Consequently, there is a growing emphasis on the development of alternative therapeutic strategies that are both effective and safe, particularly using naturally derived bioactive compounds.

Numerous studies have showed that phytochemicals are effective in treating or preventing various diseases. Quercetin (QC), also known as 3, 3′, 4′, 5, 7-pentahydroxyflavone is a poly phenolic flavanol, a member of flavonoid compounds [4] found in wide range of plants, particularly concentrated in fruits, vegetables, leaves, seeds etc. Quercetin is yellow in colour, sparingly soluble in hot water and quite soluble in alcohol and lipids [5]. It is usually found conjugated to sugars as QC glycosides. Quercetin is known for its multifaceted biological activities including anti-inflammatory, antioxidant [6], anticancer [7], antiviral [8] and antibacterial [9] activities.

The anticancer activity of QC can be attributed to its radical scavenging activity [10], inhibiting cell cycle progression and inducing apoptosis [11] or by altering PI3K/Akt/mTOR signaling pathway [12]. In addition, QC exhibits chemo-sensitizing effects, where it can enhance the efficacy of chemotherapeutic drugs like cisplatin [13] and tiazofurin [14] by decreasing the chemotherapeutic drug dose and counteracting the drug resistance making it a highly attractive candidate for integrative cancer therapy.

In spite of the diverse therapeutic potential, QC has certain disadvantages like poor solubility, short biological half-life, hydrophobic nature, low permeability and instability in physiological medium thereby limiting its utilization as therapeutic agent. Quercetin also exhibits extensive first pass metabolism in liver before reaching the systemic circulation resulting in poor bioavailability [15].

In light of these challenges, it is desirable to have an alternate drug delivery system for QC. Nanotechnology, to some extent, may offer a better platform to address the disadvantages associated with the use of QC as therapeutic agent. The use of nanoparticles as drug carriers can improve drug stability, solubility, extends half-live, and enhances drug concentrations at the disease site [16, 17, 18]. Several studies have attempted to load or encapsulate quercetin into nanocarriers such as liposomes, polymeric nanoparticles, and micelles. However, many of these methods involve complex synthesis procedures, use of toxic organic solvents, or result in poor drug loading and stability. To address these limitations, we explored a green synthesis approach to fabricate quercetin-mediated zinc oxide nanoparticles (QZnO NPs), wherein quercetin itself acts as both the reducing and stabilizing agent. Hence, the current study was therefore designed to synthesize, characterize and to evaluate *in-vitro* release kinetics of quercetin mediated zinc oxide nanoparticles, and to compare the *invitro* anti-cancerous activity of quercetin, and quercetin mediated zinc oxide nanoparticles in MDA-MB (triple-negative breast cancer) and HeLa (cervical cancer) cancerous cell lines. The novelty of our approach lies in the dual role of quercetin in nanoparticle formation and therapy, as well as in establishing a clear relationship between physicochemical properties, drug release behaviour, and cytotoxicity.

MATERIALS AND METHODS

Chemicals and reagents

Quercetin dihydrate (≥95% purity), zinc acetate dihydrate, dialysis membranes (12,000–14,000 Da MWCO), MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine, and non-essential amino acids were procured from Sigma-Aldrich (USA) unless otherwise stated. All other solvents and chemicals used were of analytical grade.

Synthesis of quercetin mediated zinc oxide nanoparticles (QZnO NPs)

The synthesis and characterization of QZnO NPs were carried out at Nanotechnology Laboratory, IFT, RARS, ANGRAU, Tirupati as per the method described by Prasad *et al*. [19]. A 2% QC solution was prepared by dissolving the appropriate quantity in distilled water and adjusting the volume to 100 mL. Subsequently, a 0.1 M ZnO acetate solution was prepared by dissolving 2.19 g of zinc acetate dihydrate in 100 mL of distilled water. 90 mL of zinc acetate solution was slowly added dropwise to 10 mL of 2% quercetin solution under

vigorous magnetic stirring at 65°C for 30 minutes. The resulting yellowish suspension was cooled to room temperature and allowed to incubate for 24 hours in a dark environment to ensure complete reaction and nanoparticle formation. The mixture was centrifuged at 10,000 rpm for 15 minutes to collect the nanoparticles, which were then washed thrice with distilled water and once with ethanol to remove unbound quercetin. The final pellet was lyophilized with freeze-dryer (Alpha 1-2 LD plus, MartinChrist®, Germany), and the resultant nanoparticles were stored for characterization at 4°C.

Characterisation of nanoparticles

Scanning electron microscopic (SEM) analysis

SEM analysis was done using Hitachi S-4500 SEM machine to determine the size, shape and distribution pattern of the synthesized NPs. Thin layers of the specimen were deposited onto a carbon-coated copper grid by gently dispensing a minute quantity of the sample onto the grid. Subsequently, the SEM grid with the film was dried by exposing it to a mercury lamp for 5 minutes.

Zeta sizer analysis

The dynamic light scattering (DLS) method employing the zeta sizer (Nanopartica®, HORIBA, Japan), following the protocol outlined by Yegireddy *et al.* [20], was utilized to evaluate the zeta potential, average hydrodynamic diameter, and polydispersity index of both ZnO NPs and QZnO NPs in cell culture media.

The equipment utilized a laser beam with either 10 mW or 100 mW power, operating at 532 nm wavelength. The surface charge of the NPs was determined through electrophoretic light scattering using folded capillary cuvette. The zeta potential measurements were carried out with an electrode voltage of 3.9 V at a temperature of 25°C

UV-Visible spectra analysis

The UV-VIS spectrophotometer (UV-2450, Shimadzu, Japan) was utilized to analyze the UV-Visible spectrum of ZnO NPs and QZnO NPs. A small quantity of the sample was diluted in distilled water, and absorption maxima were scanned over the wavelength range of 200-800 nm.

***In-vitro* release kinetic studies**

The *in vitro* drug release kinetics were investigated using the dialysis membrane technique [20]. To perform this, 3mg of QZnO NPs were dispersed in 1mL of the release medium and placed in a pre-soaked dialysis bag (M/s. Sigma Aldrich, USA) having 20.5 cm^2 surface area (12,000–14,000 MWCO). The bag was then submerged in a beaker containing 50mL of test media maintained at 37±0.5˚C in a temperature controlled shaking water bath set at 50 rpm for 24 hours. The release media included phosphate buffer solution (PBS) with a pH of 7.4 and acetate buffer with a pH of 4.8.

To maintain the sink conditions, 1 mL of aliquot was collected at predetermined time intervals *viz*., 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 10.0, 12.0, and 24.0 hours and replaced with an equal volume of fresh media. A spectrophotometer (UV-2450, SHIMADZU, Japan) calibrated at 380 nm was used to measure the QC concentration in the release medium [21]. Each sample underwent triplicate analytical runs. The absorption intensity data were plotted over time to generate the desorption profile of QZnO nanoparticles.

The cumulative % release was calculated for each time point. The obtained data were analyzed using the KineticDS3 software [22] to determine the best-fitted *in-vitro* release kinetic model, assessing the rate of quercetin release from QZnO NPs. Various mathematical models, including Zero-order, First-order, Korsmeyer-Peppas, Higuchi, Hill, and Hickson-Crowell kinetic models, were applied to characterize QC release kinetics.

***In-vitro* anticancer activity assessment**

Cell lines

The MDA-MB 231 human breast cancer cell lines and HeLa, human cervical cancer cell lines used in this study were obtained from NCCS, Pune, India. Both cell lines were cultured in Dulbecco's modified eagle's medium (DMEM) containing 0.1 mM sodium pyruvate, 0.5 mM L-glutamine, 1 mM non-essential amino acids (M/s. Sigma-Aldrich, USA) with 10% fetal bovine serum (M/s. Gibco, USA) at 37°C in a CO_2 incubator. All the experiments were performed when cells were at 80-90% confluency.

MTT assay

The cells were plated at a density of $1x10^6$ cells per well in 96-well plates. Subsequently, both cell types were exposed to test compounds at escalating concentrations for a duration of 24 hours. After 24h, 20 µL of 5 $mg.mL^{-1}$ MTT (3-(4, 5-dimethylthiozol-2-yl)-2,5-diphenyltetrazoliumbromide) was added and incubated for another 4h. At the designated time point, cell lysis was induced by adding 200 µL of dimethyl sulfoxide (DMSO) to all wells to dissolve the MTT formazan crystals formed. In control experiments, cells were cultured in the same medium without test

compounds. Absorbance readings were taken at 570 nm immediately after the appearance of a purple hue. The formazan produced in control cells was regarded as indicative of 100% viability. Relative cell viability was determined based on the amount of MTT converted into insoluble formazan salt. All experiments were conducted in triplicate, and mean ± standard error of the mean (S.E.M.) was calculated and reported as (%) cell viability versus concentration.

Statistical analysis

All experiments were performed in triplicate and results were expressed as mean ± SEM. IC_{50} values were determined using linear regression analysis. One-way ANOVA followed by Tukey's post hoc test was performed using GraphPad Prism 8.4.3, with significance set at $p < 0.05$.

RESULTS AND DISCUSSION

Characterisation of nanoparticles

Scanning electron microscopic (SEM) analysis

The SEM image analysis of ZnO NPs was depicted in Fig 1a. The synthesized particles were irregular and tubular in shape with uniform distribution. Nevertheless, on numerous occasions, particle agglomeration has been observed primarily due to the lack of protective ligands on the surface. The SEM micrograph clearly indicates that the average size of each particle measures 75 nm. Wang *et al*. [23] and Kumar *et al*. [24] reported the similar size and shape of ZnO NPs, whereas these results are in disagreement with some authors who reported the spherical shape and higher size range of 60–120 and 80–130 nm [25, 26].

The QZnO nanoparticles (NPs) primarily exhibited a tetragonal morphology with some instances of agglomeration. These QZnO NPs were polydispersed, with sizes ranging from approximately 200 to 250 nm (Fig. 1b). The higher size of the prepared NPs is due coating of the flavonoid around them. Sadhukhan *et al*. [27] reported similar size and shape of amino functionalized QZnO NPs, whereas, Jeyaleela *et al*. [28] reported small size of the QZnO NPs.

Zetasizer analysis

The hydrosol's hydrodynamic diameter (HDD) and *zeta* potential were measured using the DLS technique. The HDD of the ZnO NPs and QZnO NPs were measured using DLS technique and recorded as 68.6 nm (Fig. 2a) and 165.8 nm (Fig. 2b), respectively. These measurements align well with the findings from SEM analysis. The measured polydispersity index value of the ZnO NPs and QZnO NPs were 0.226 (Fig. 2c) and 0.277 (Fig. 2d), respectively indicates the monodispersity of the formed nanoparticles. The measured *zeta* potential value of the ZnO NPs and QZnO NPs were -14.5 mV (Fig. 2c) and -36.7 mV (Fig. 2d), respectively, indicates the higher stability synthesized NPs. The findings align closely with prior research of Ahamed *et al*. [29] reported ZnO nanorods suspended in cell culture media had an average HDD of 97 nm and a *zeta* potential of -29 mV. Similarly, Kumar *et al*. [30] observed the mean HDD of 185 nm with a *zeta* potential of -26 mV, while Yedurkar *et al*. [26] found 145.1 nm with a *zeta* potential of -49.19 mV for ZnO NPs. On the other hand, smaller ZnO loaded quercetin loaded nanocomposite was prepared by Satishkumar *et al*. [31] with a diameter of 21–39 nm. The increased hydrodynamic diameter and more negative zeta potential of QZnO NPs compared to bare ZnO NPs suggest enhanced colloidal stability and potential for improved cellular interaction.

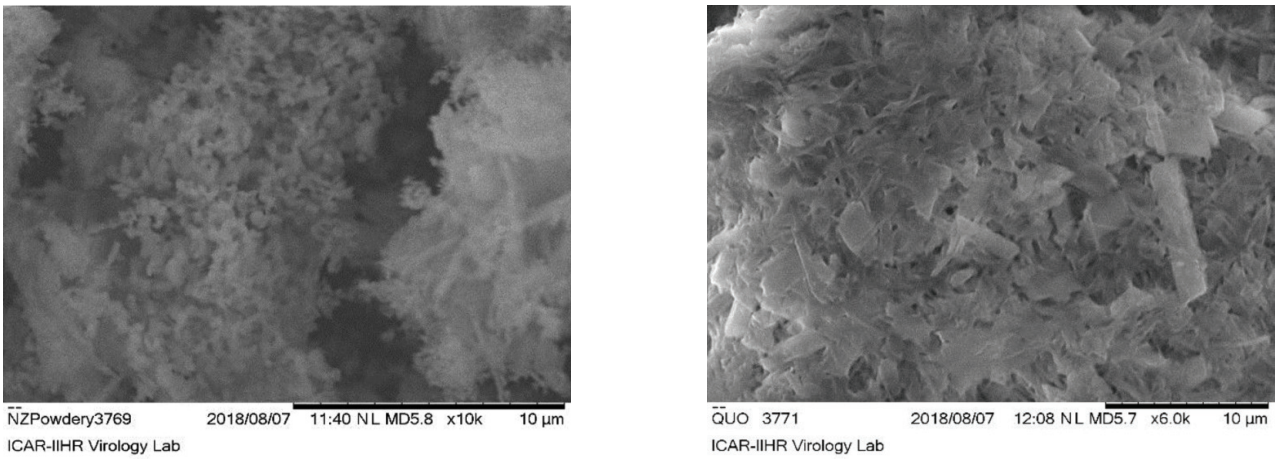
Table 1. Inhibitory concentration of quercetin and Quercetin mediated zinc oxide nanoparticles (QZnO NPs) against HeLa and MDA-MB 231 cancer cell lines.

Compound	HeLa Cell line		MDA-MB 231 Cell line	
	$IC_{50(\mu g/ml)}$	$IC_{99(\mu g/ml)}$	$IC_{50(\mu g/ml)}$	$IC_{99(\mu g/ml)}$
Quercetin	21.499 (7.61±60.75)	184029.307 (65132.37 ± 519968.57)	20.596 (10.56 ± 40.17)	5361.490 (2748.92 ±10457.03)
QZnO NPs	1.406 (0.816 ± 2.425)	81.305 (47.160 ± 140.171)	2.572 (1.488 ± 4.445)	221.926 (128.390±383.606)

*The values in the backets are lower and upper limits of respective inhibitory concentrations with 95% Fiducial CI.

UV-Visible spectra analysis

The localized surface plasmon resonance of the ZnO NPs and QZnO NPs was determined using UV spectral analysis. The scan was conducted over a



(a) **(b)**

Fig. 1. The representative Scanning electron microscopy (SEM) images of (a) Zinc oxide nanoparticles, (b) Quercetin mediated zinc oxide nanoparticles.

wavelength range of 200-400 nm, and the resulting spectrum is shown in Fig. 3. The spectral range of ZnO NPs was broad and it falls between 230 – 330 nm [32]. The maximum absorbance peak of ZnO NPs was found to be around 249 nm. These results are in good agreement with the previous reports [33, 34, 35]. The synthesized QZnO NPs exhibited an absorbance peak at approximately 360 nm, which is characteristic of QC. This confirms the presence of ZnO in the formulation and indicates that there is no chemical degradation of QC in the prepared compound.

In vitro release kinetics

The cumulative *in vitro* release of QC from QZnO NPs over a 24-hour period at pH 4.8 and 7.4 was shown in Fig. 5. The release curves indicate that QC release from QZnO NPs was slower at pH 7.4 compared to pH 4.8. Within 24 hours, 54.76% of QC was released at pH 4.8, while only 21.07% was released at pH 7.4. These results demonstrate that QC release from QZnO NPs is faster in acidic conditions, highlighting the significant role of pH in the release mechanism. It is crucial to remember that, in comparison to blood and healthy tissues, cancer cells have a greater acidic nature at higher temperatures. For the purpose of treating cancer, the drug delivery system should rapidly release the targeted medication at a pH of 5, while the same should be retained at a pH of 7 [31]. The release of QC from QZnO NPs in acidic conditions may be due to break down of the nanomaterial at pH 4.8.

The cumulative release data of QC from QZnO NPs dispersion was analyzed using various kinetic models, including zero-order, first -order, Korsmeyer-Peppas, Higuchi, Hill and Hickson-Crowell to understand the QC release mechanism from the QZnO NPs and the results were presented in Fig. 5 (a-f). The QC release mechanism was chosen to be the kinetic model with the greatest estimated correlation value (R^2). Accordingly, Higuchi kinetic model illustrates the best fit with R^2 of 0.9668 at pH 4.8 compared to R^2 of 0.9075 at pH 7.4 (Fig. 5d). The release exponent 'n', derived from the Korsmeyer-Peppas equation, indicates that the drug diffusion from QZnO nanoparticles exhibited anomalous and non-Fickian behavior. Specifically, the exponent values were determined to be 0.52 at pH 4.8 and 0.7 at pH 7.4, respectively. The release kinetic studies revealed that the QC release rate was quicker in acidic conditions compared to physiological pH. This favours the accumulation and rapid release of QC in the acidic environment of the targeted cancer site. The slow release of QC at physiological pH suggests that the QZnO NPs will be retained in the body for prolonged time. The sustained release profile at pH 4.8 suggests that QZnO NPs preferentially release quercetin in acidic tumor environments. This pH-sensitive behavior promotes endosomal escape and facilitates intracellular accumulation of quercetin, thereby enhancing cytotoxicity [31, 36].

In-vitro anticancer activity

In vitro cytotoxicity assay was carried out in HeLa cervical cancer cells and MDA-MB 231 breast cancer cell lines for both free QC and QZnO NPs at various concentrations in triplicate. The cells were treated with free QC and QZnO NPs and kept for 48 hours of incubation. The IC_{50} values, representing 50% growth inhibition, were determined using the MTT assay. Significantly different IC_{50} and IC_{99} values were

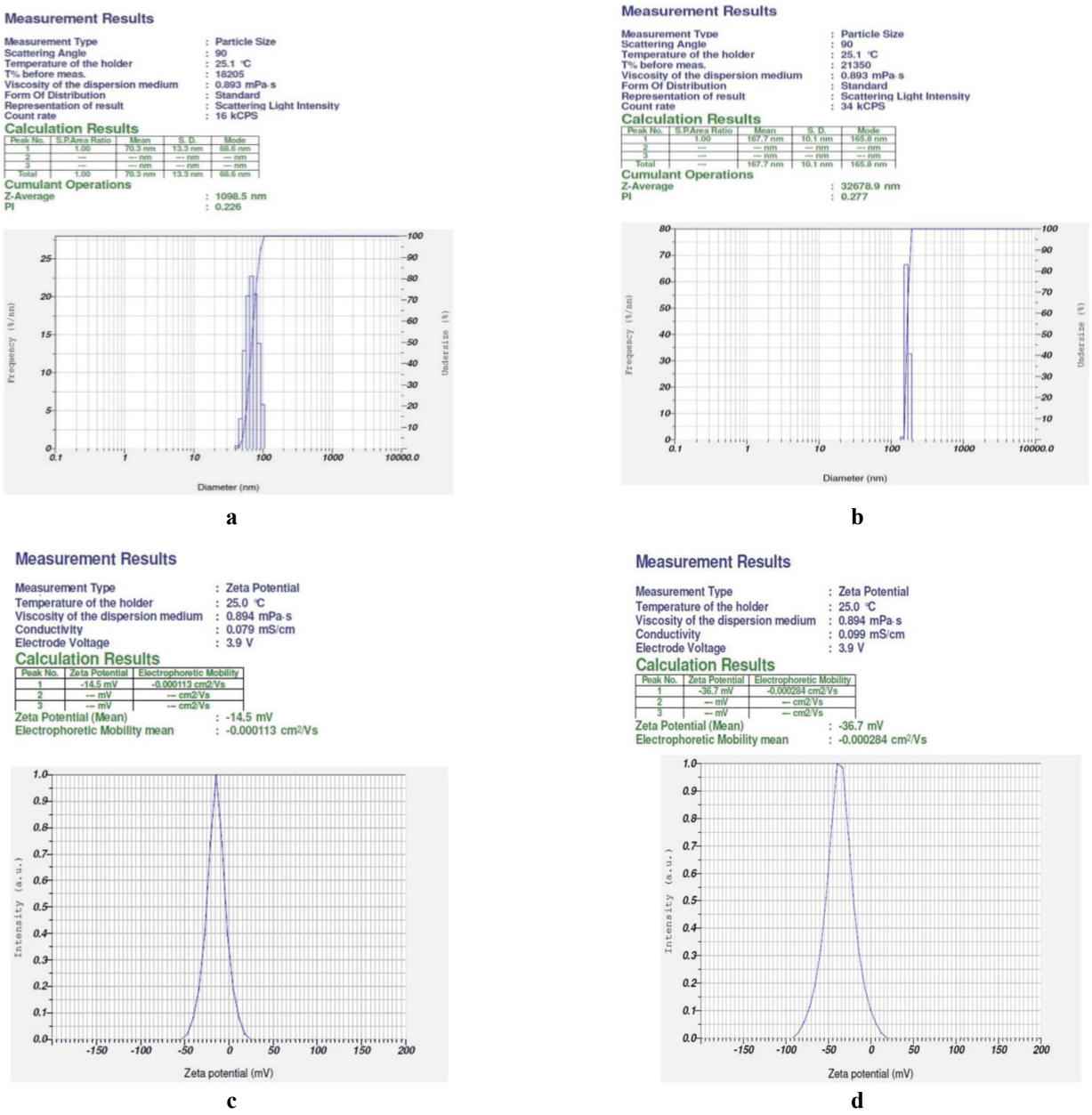


Fig. 2. The Hydrodynamic diameter and Zeta potential of synthesized nanoparticles. HDD of ZnO NPs (a) and QZnO NPs (b); zeta potential and PDI of ZnO nPs (c) and QZnO NPs (d).

*HDD - Hydrodynamic diameter; ZnO NPs - Zinc oxide nanoparticles; QZnO NPs Quercetin mediated zinc oxide nanoparticles.

observed between QC and QZnO NPs in both the cell lines (p<0.01). There is 15-fold, and 8-fold decrease in IC_{50} for QZnO NPs compared to QC in HeLa cervical cancer cells and MDA-MB 231 breast cancer cell lines, respectively (Fig. 6; Table. 1). This variance may be attributed to the uptake of nanoparticles by cancer cells and the gradual release of the quercetin at acidic pH environment within the cells. The smaller IC_{50} values for QZnO NPs imply that the nanoparticle formulation facilitates better cellular uptake, likely *via* endocytosis, and protects quercetin from premature degradation [37]. This prolonged intracellular retention and localized release may activate apoptotic pathways more effectively compared to free quercetin. These findings support the hypothesis that the physicochemical properties of QZnO NPs directly influence their cellular uptake and subsequent cytotoxic mechanisms. Free QC, on the other hand, is less hazardous to cells because it is more sensitive to the cells' efflux pump.

Numerous studies have documented QC's chemo

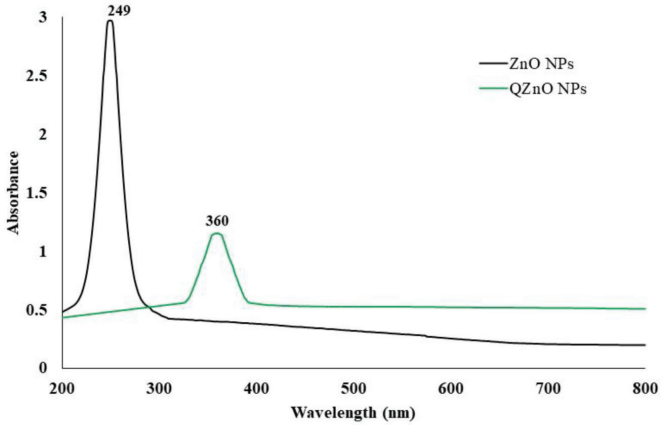


Fig. 3. UV spectrum of (a) Zinc oxide nanoparticles, (b) Quercetin mediated zinc oxide nanoparticles.

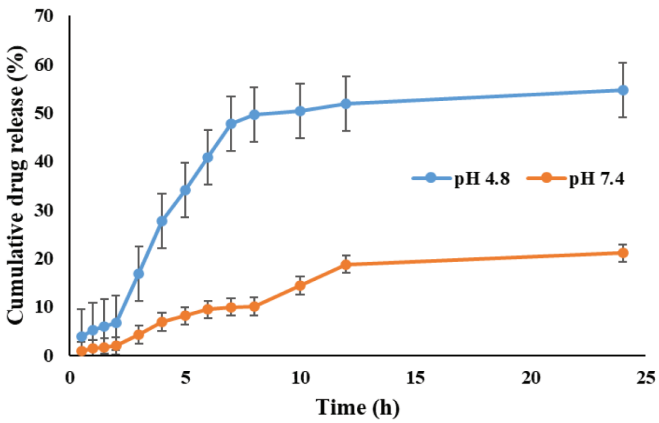


Fig. 4. Percent cumulative release of quercetin release from Quercetin Zinc oxide nanoparticles.

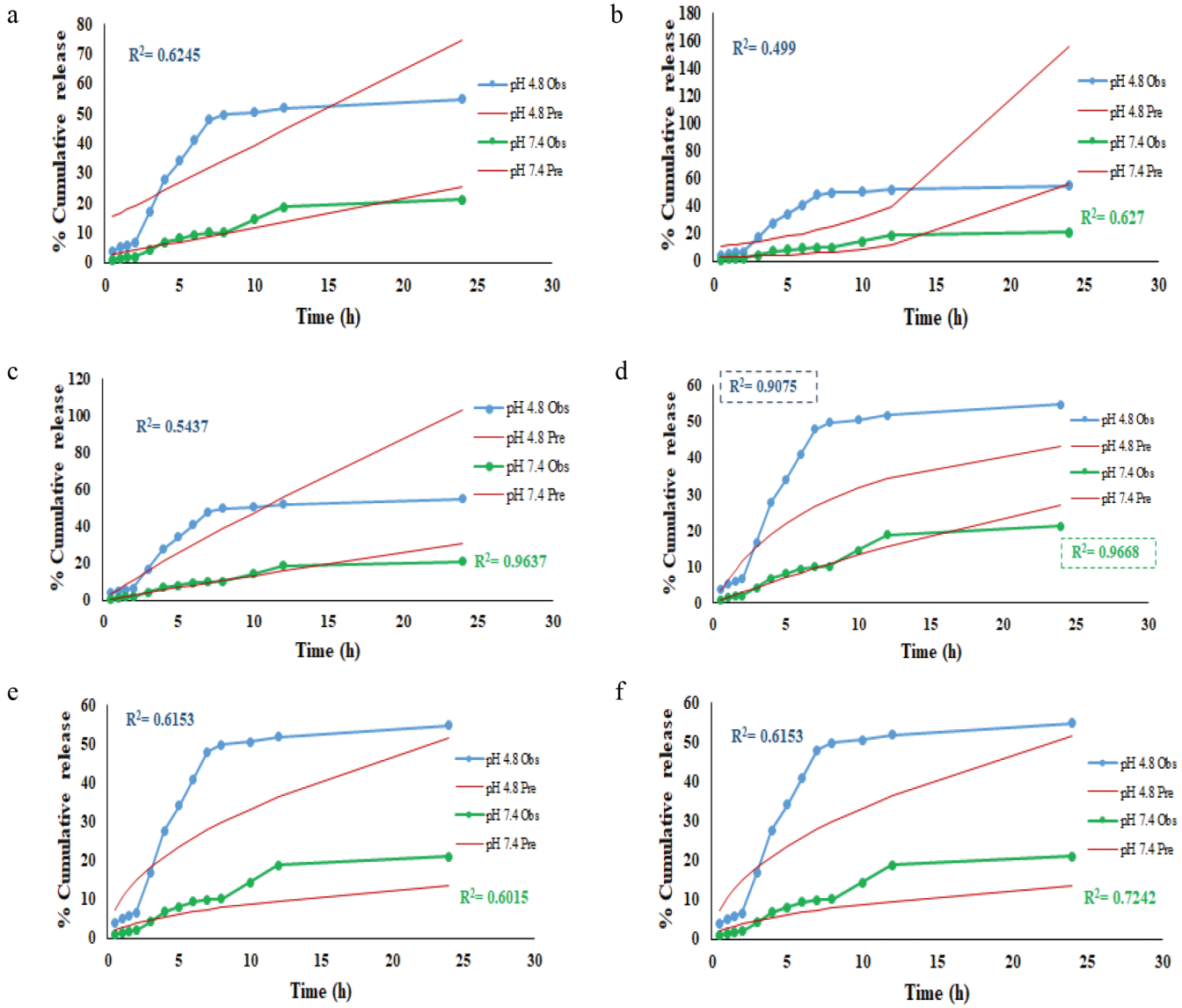


Fig. 5. Quercetin release kinetic models (a) Zero-order, (b) First-order, (c) Korsmeyer-Peppas, (d) Higuchi, (e) Hill and (f) Hickson-Crowell plots. (Obs- observed value; Pre- predicted value; R2- correlation coefficient).

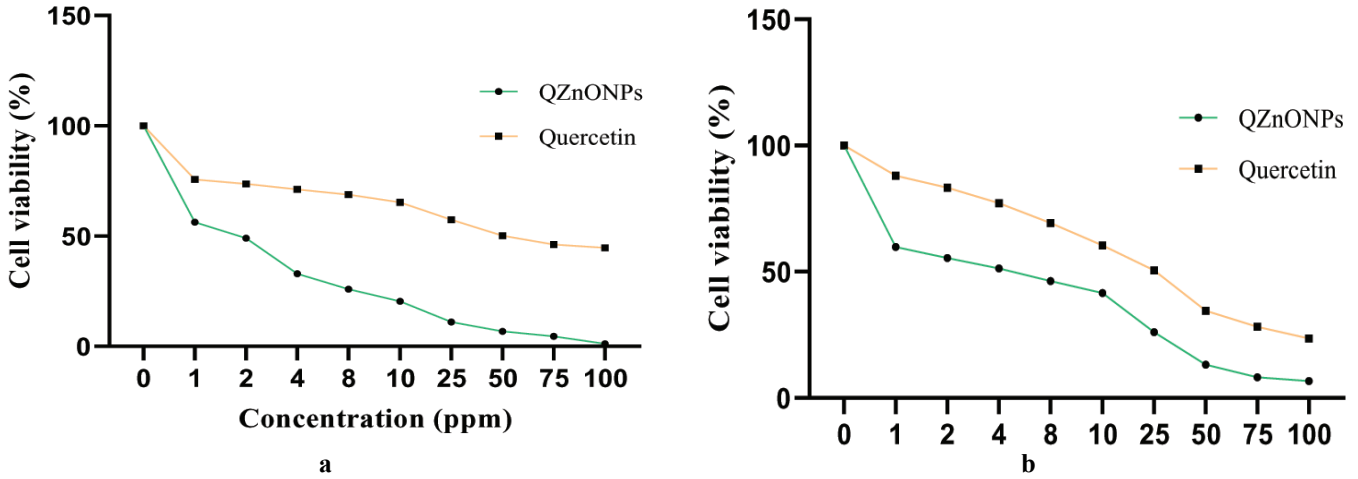


Fig. 6. Anticancer efficacy of quercetin and quercetin mediated zinc oxide nanoparticles against (a) HeLa cervical cancer and (b) MDA-MB 231breast cancer cell lines.

preventive impact *via* various anticancer pathways, including cell cycle arrest, apoptosis induction, enhancement of antioxidative defence enzyme activity, inhibition of cell proliferation, metastasis, and angiogenesis [37, 38, 39]. However, the bioavailability of QC at the tumor site is greatly limited by its low stability and insolubility in bodily fluids [40, 41].

In light of these challenges, this study focused on leveraging nanotechnology to target QC delivery to tumor sites as the nano carriers offer substantial potential in cancer therapy [42, 43]. The synthesized QZnO NPs hold promise for delivering the QC to tumor microvasculature, enhancing bioavailability due to their size, morphology, and ability to sustain quercetin release in acidic tumor environments. Consequently, the observed significant decrease in IC_{50} value of QZnO NPs may be attributed to sustained QC release in the acidic tumor environment, triggering apoptosis in both the cancer cell lines.

CONCLUSION

In conclusion, the use of QZnO NPs represents a novel and effective strategy to overcome the intrinsic limitations of quercetin delivery in cancer therapy. This approach not only improves the bioavailability and stability of quercetin but also leverages the advantages of nanotechnology to ensure targeted and sustained release within the tumor microenvironment. However, we acknowledge the limitations of the current study and the need for further investigations to enhance its translational significance. Future work should include evaluation across a broader pH spectrum and in-vivo relevance through detailed pharmacokinetic and pharmacodynamic studies, alongside mechanistic analyses such as Annexin V/PI staining, ROS quantification, and selectivity assessments using non-tumorigenic cell lines to better elucidate the therapeutic potential and safety profile of the synthesized nanoparticles. Collectively, these future directions are intended to bridge the gap between laboratory research and clinical application, ultimately facilitating the development of a safe, effective, and scalable nanomedicine platform utilizing quercetin and zinc oxide nanoparticles.

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