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Cancer therapeutics strategy using nano-carrier mediated natural drugs

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ABSTRACT

Purpose: Nucleolin is a multifactorial protein, having a significant role in chromatin remodelling, mRNA stability, ribosome biogenesis, stemness, angiogenesis, etc., thus, it is potential therapeutic target in cancer. The purpose of this paper is to study porous silicon (pSi) nanocarrier-based natural drug delivery system targeting dysregulated nucleolin expression for cancer therapeutics.

Design/methodology/approach: Quercetin was loaded in pre-synthesized and characterized pSi nanoparticles, and release kinetics was studied. The study compared the inhibitory concentration (IC50) of quercetin, synthetic drug doxorubicin, and quercetin-loaded pSi nanoparticles. Further, mRNA expression of a target gene, nucleolin, was tested with a quercetin treated breast cancer cell line (MCF-7).

Findings: Quercetin-loaded pSi nanoparticles followed first-order release kinetics. IC50 was determined at concentrations of 312 nM, 160 μM, and 50 μM against doxorubicin, quercetin, and quercetin-loaded pSi nanoparticles, respectively. The results further indicated 16-fold downregulation of nucleolin mRNA expression after 48h of quercetin treatment of exponentially growing MCF-7 cells.

Research limitations/implications: Whether pSi nanoparticle loaded quercetin can significantly downregulate nucleolin protein expression and its impact on apoptosis, cell proliferation, and angiogenic pathways need further investigation.

Practical implications: The practical application of the proposed nanocarrier-based drug delivery system potentially lays out a path for developing targeted therapy against nucleolin-dysregulated cancer using natural products to minimize the side effects of conventional chemotherapeutic drugs.

Originality/value: Inhibition of nucleolin and nucleolin regulated pathways using natural compounds and its targeted delivery with nanocarrier is not yet done.

Keywords: Breast cancer, Porous silicon nanocarrier, Quercetin; Nucleolin; Targeted therapy

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BIOMEDICAL AND DENTAL ENGINEERING AND MATERIALS

1. Introduction

The cancer burden is among the major public health burden globally. As per World Health Organization (WHO) 2016, cancer imposes the largest global burden accounting for more than 244.6 million Disability Adjusted Life Years (DALYs) affecting both men and women [1]. While breast cancer accounts for more than 25% of cancer occurrences and is one of the most common malignancies in women. Breast cancer has heterogenetic pathological characteristics, as some depict slower growth and proliferation with excellent prognosis while others display rapid proliferation and metastasis [2]. Although with technological advances in medical science, earlier diagnosis of breast cancer and introduction of the treatment regime has evolved, yet the most common treatment includes conventional therapies, including radiotherapy, chemotherapy, and surgery. Yet, the major drawbacks of these therapies involve the uneven or non-specific distribution of antitumor agents and the low scope of monitoring [3,4]. Thus, effective strategies to overcome drug resistance are crucial for cancer treatment. Towards this goal, the identification of therapeutic targets and their regulation by natural products is warranted.

Nucleolin (Ncl) is a multifunctional, shuttle nucleolar protein present ubiquitously in the cell surface, cytoplasm as well as a nucleus in a variety of cancer [5,6], having an essential role in various intracellular pathways, including cell survival, differentiation, apoptosis, angiogenesis, etc. Dysregulation of Ncl was implicated in cancer development and maintenance and hence could be a potential target in anti-cancerous therapy [5]. It also regulates microRNAs (miRNA) like miR-21, miR-222, miR-103, etc., whose overexpression was found in breast cancer progression, metastasis, and therapy resistance [6]. Although it is an important therapeutic target, inhibitors targeting Ncl are still elusive [7]. Furthermore, various studies have reported its overexpression in cancer, including breast, leukaemia, lung, colorectal, and gastric [5,6,8,9]. Hence, Ncl can be a potential target in developing anticancer therapy.

In the last few decades, several investigations have focused on the biological actions of plant-derived phytochemicals, yet only one report showed the effect of curcumol against Ncl [10]. Quercetin is another potential natural compound that belongs to a class of polyphenolic flavonoids that physically mimics the structure of endogenous estrogen 17beta-estradiol [11]. It exhibits various biological properties, including anti-cancer, antioxidants, antiviral, cell-cycle modulation, apoptosis-induction, and angiogenesis inhibition in several cancer types including breast cancer [12-16]. Despite the promising cytotoxicity and role in molecular mechanisms promoting cancer progression, quercetin has shown the problem of poor solubility and low bioavailability, making it a challenging compound for therapeutic application [17].

In the past few decades, nanotechnology-based therapeutics have gained immense attention due to manufacturing materials at molecular and atomic scales. In the past few decades, nanomaterials have gained immense popularity due to its plethora of success in pre-clinical studies in drug delivery [18,19]. Besides, encapsulating the therapeutics molecules, including chemotherapeutic agents, RNAi, small molecule inhibitors, and so on, can increase the solubility and bioavailability of the molecules and alter their bio-distribution, thus facilitating their entry to the target cells [18]. However, the nanocarrier used to date in clinical studies has gained approval only via passive targeting. The studies conducted in past years have shown minimal nanocarrier deposition, even in high-EPR xenograft tumours [18]. The major psychological barrier faced by both active and passive targeting of the nanocarrier-mediated delivery includes the endothelial barrier, cellular barrier causing endosomal escapes, and alteration in the mononuclear phagocytic system. Although T7-modified PLGA nano-particle system consisting of combinational drugs, i.e., curcumin and paclitaxel, showed synergistic effects as well as increased blood-brain barrier permeability [20]. This result is encouraging in the field of nanotechnology as it can be potentially exploited to maximise its therapeutic use.

Interestingly, developing a controlled drug release system is important for maintaining drug concentration in blood or targeted site for longer. The release kinetics of the drug can be classified into a statistical method, model-dependent method, and model-independent method among which the model-dependent method incorporates various mathematical functions to analyse dissolution profiles of the drugs based on various set parameters [21]. This approach has different models, including the zero-order, first-order, Higuchi, Maker-Lonsdale, Weibull, regression, and linear or first-order regression models [21,22]. For instance, in the zero-order model, the drug has a very slow-release rate. It is suitable for drug application in the transdermal system or low soluble drug provided with an outer coating or drug release based on an osmotic environment. While the first-order model is known for either absorption or elimination of some drugs initially followed by slow release of drugs within the system that gets exhausted with time. Such model is suitable for developing water-soluble drugs loaded inside matrix system [21,22]. Similarly, another model has its distinguished application depending on the drug's nature, matrix system uses, and applicability. Hence, the kinetics study of drug release is an important aspect for determining qualitative and quantitative changes as well as formulation optimization.

Several types of nanocarriers have gained attention and are approved as safe for therapeutic use, including organic, hybrid, and inorganic compounds. Organic nanocarriers include solid lipid nanoparticles (SLNs), polymeric nanoparticles (PNPs), liposomes, etc. Some of the prominent inorganic nanoparticles explored in cancer therapeutics include gold (Au), silver (Ag), porous silicon nanoparticles (pSi), etc. [6,11]. The previous study has demonstrated that oral administration of spherical Ag nanoparticles (22, 42, 71 and 323nm size) at dose of 1 mg/kg concentration had no histopathological changes in bodily organs, including the liver, lungs, testicles and kidney, while administration of 20 nm Ag nanoparticles at a concentration of 50, 150, and 300 mg/kg showed relative accumulation and cellular infiltration [23]. While intravenous administration of colloidal Au-nanoparticles (25 nm) showed an increased alanine aminotransferase, aspartate aminotransferase and blood glucose level, indicating interference in pancreatic function [23]. Due to the presence of relative toxicity in the case of both Ag and Au nanoparticles in the body, inorganic biodegradable nanoparticle pSi was chosen for the current study. pSi possesses high biocompatibility, increases the dissolution rate of poorly water-soluble drug or compounds like quercetin, tunable surface for tailoring desirable biological activities, and high drug loading capacity [19, 24, 25, 26]. pSi for biomedical use is generally functionalized via targeting ligands, polyethylene glycol (PEG), and stimuli-responsive moieties for improving its bioavailability at the specific target site. The functionalization of pSi enhances its behaviour by improving its chemical stability and facilitating controlled drug release [26]. The biocompatibility of both silica-based nanocarriers as well as PEG is reported in various studies. FDA also approves it, thus making it suitable substance for drug delivery [27,28]. Thus, nanocarrier-mediated drug delivery can be the potential therapeutic approach in cancer therapeutics.

In this paper, we aim to develop a quercetin-loaded pSi nanoparticle system targeting breast cancer cells *in-vitro*. We hypothesize that the development of a quercetin-loaded pSi nanocarrier can resolve the problem of insolubility and increase its bioavailability.

2. Materials and methods

2.1. Materials

Cell line: MCF-7 cell line purchased from NCCS Pune; Quercetin (Sigma); Dulbecco's Modified Eagle Medium (DMEM) (Lonza); Fetal Bovine Serum (FBS); Streptomycin-Penicillin antibiotic; Phosphate Buffer saline (PBS); Trypsin-EDTA; Dimethyl sulfoxide (DMSO); functionalized porous silicon nanocarriers obtained from Dr. Tushar Kumeria's laboratory (UNSW, Australia), Ethanol, TRIzol, Chloroform, Isopropanol.

2.2. Cell culture

MCF-7 was grown in DMEM medium containing 10% FBS and 1% antibiotic. The cells were incubated and maintained in the presence of 5% CO_2 at 37°C followed by self-monitoring and medium change from time to time.

2.3. Synthesis and functionalization of pSi nanoparticles

Pre-synthesized, characterized, and PEGylated pSi was courteously obtained from Dr. Tushar Kumeria's laboratory, School of Materials Science and Engineering, University of New South Wales, Sydney, Australia. The pSi nanocarriers were well-characterized using ATR-FTIR, BET, TEM, DLS, and SEM methods [29-32].

2.4. Drug loading in pSi nanocarrier

0.33 mg of quercetin was dissolved in 500 µl of ethanol and loaded in 1mg pSi with 25% weight of the drug. The leading weight was calculated using the formula Loading wt.% = [(Wt. of drug) / (wt. of drug + wt. of particles)] *100. The required amount of the drug was dissolved in ethanol and then added to pSi particles and left overnight for shaking, followed by a vacuum centrifuge.

Further, MTT assay was performed with 20% loaded drug onto pSi nanocarrier. Here, 0.22 mg quercetin was loaded in 1 mg pSi.

2.5. Study of drug release kinetics

The study of the release kinetics of quercetin from pSi nanoparticles was done by plotting a standard curve by measuring optical density at 374 nm for different concentrations of quercetin. Ethanol was used as the solvent to dissolve quercetin for the standard curve.

Next, the drug release kinetics was done in DMEM complete media. The quercetin-loaded pSi nanoparticles were soaked in 10 ml of media under continuous shaking. OD was measured at regular time intervals up to 26 h.

2.6. Estimation of IC50 and resistance index

MCF-7 cells were harvested from the culture flask at the logarithmic growth phase via trypsinization for the

determination of IC_{50}values of quercetin (alone), pSi (alone), quercetin-loaded pSi, and doxorubicin. The effect of the respective drug was tested by seeding the cells in 96-well plates at a concentration of 5×10^3/well supplemented with DMEM medium with 10% FBS. Quercetin and Doxorubicin were prepared in DMSO that was managed at concentrations less than 0.1% in cells due to prevent relative toxicity. pSi and quercetin-loaded pSi were dissolved in DMEM medium. Varying molar concentrations of doxorubicin, quercetin, pSi, and quercetin-loaded pSi were added to the cells in 96-well plates and incubated in CO_2 incubator at 37°C. Thereafter, media was removed, followed by adding 100 µl of medium containing 5 mg/ml MTT in each well and incubated for 4 h. Further, the plate was swirled, followed by adding 100 µl DMSO per well and incubated at 37°C for 15-20 min with slight agitation. This allowed the dissolution of the blue-purple precipitate (Formazan) obtained from the MTT.

The experiments were repeated at least three times, and data are presented as the mean ± standard deviation (SD).

2.7. Measurement of nucleolin mRNA expression

The expression of Ncl mRNA was done by reverse transcription-polymerase chain reaction (RT-PCR). The Ncl primer used 5'-GACCCAGGGGATCACCTAAT-3' and 5'-TCTACCACCACCTCGTCCTC-3'. Firstly, extraction and purified total RNA were done by TRIzol method, followed by the preparation of cDNA. The mRNA expression was determined by RT-PCR where the parameters set was 50°C for 2 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min.

3. Results

3.1. Microscopic observation

The control cells were viewed through an optical microscope at 20X magnification as shown in Figure 1a. Figure 1b shows the microscopic view of the cells after adding the drug loaded in pSi carrier. The black spots in the image in the background of the cells (Fig. 1b) indicate the pSi nanoparticles. A uniform distribution of the pSi particles can be observed.

3.2. Study of release kinetics

Figure 2a demonstrates the calibration curve of quercetin in ethanol. For studying the release kinetics of quercetin from pSi the calibration curve of quercetin in ethanol was

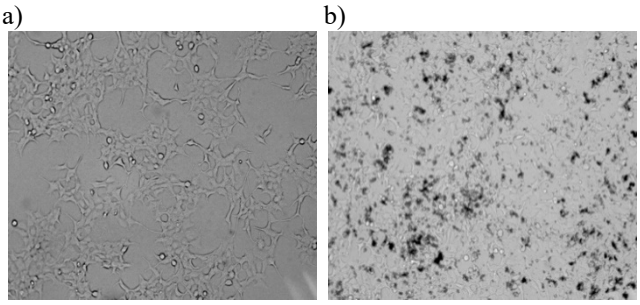

Fig. 1. Optical microscope images of MCF-7 cells (a) before and (b) after loading of Quercetin-loaded pSi nanoparticles

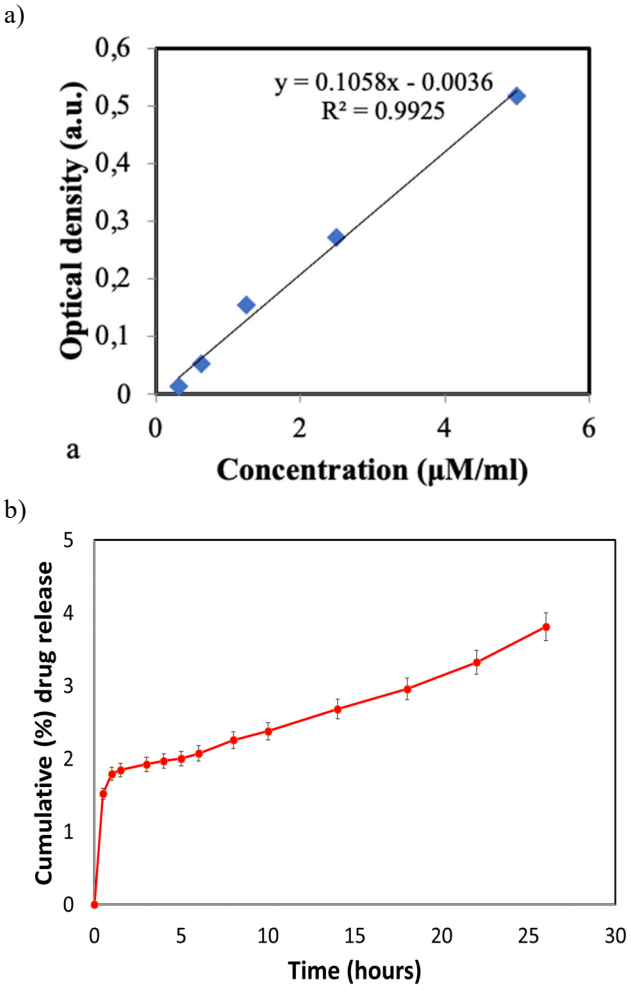

Fig. 2. a) Linear calibration curve of quercetin in ethanol, b) Release kinetics of quercetin from pSi nanoparticles at distinct time intervals

plotted using UV-visible spectroscopy by measurement of OD at 374 nm. Quercetin showed a strong absorbance peak

at 374 nm; hence the OD measurements were done at this wavelength. The R^2 value of 0.97 was highly reliable in estimating the unknown released quantity of quercetin from the pSi nanocarriers. The release kinetics was then recorded and is shown in Figure 2b. A burst release is observed in the initial 1.5 h, followed by a slow release. The calculation shows 3.8% drug release at 26 h, indicating a slow drug release. The burst release is most likely due to some drug on the outer surface of the nanocarrier while the loading process. The graph can be considered first-order kinetics as there is rapid release initially followed by slow release of the sustained component.

3.3. Estimation of IC_{50} and resistance index

Figure 3 displays the inhibitory effect of the synthetic drug, i.e., Doxorubicin, at a lower concentration of 312 nM as only 53.9% of cells were viable. The IC_{50} was observed between 312 nM and 625 nM at 48 h intervals. This indicates the high cytotoxicity of doxorubicin against MCF-7, even at a lower concentration. Figure 4 demonstrates the inhibitory effect of Quercetin against MCF-7 cells in 48 h. The treatment was provided at concentrations of 0, 20, 40, 80, 120, 140, 160, 180, 220, 240, 320, and 640 µM/ml. The IC_{50} determined was at 160 µM where 50.3% cell viability was observed.

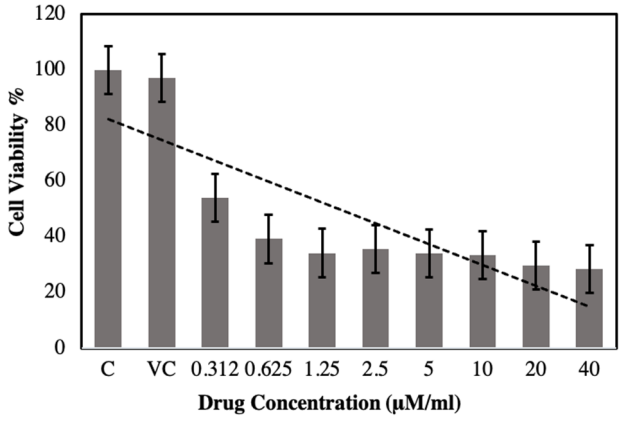

Fig. 3. Inhibitory effect of doxorubicin on MCF-7 proliferation

Figure 5 represents the comparative effect of pSi and quercetin-loaded pSi on MCF-7 cells. It can be observed that pSi alone does not have any inhibitory effect against MCF-7. While the effect of quercetin-loaded pSi at 50 µM/ml concentration showed reduction in cell viability to 60.3%.

Figure 6 Ncl expression was observed to be 16-fold decreased in quercetin-treated sample compared to the control sample where no drug was present. This might indicate that quercetin can potentially target Ncl mRNA level expression in MCF-7 cell line.

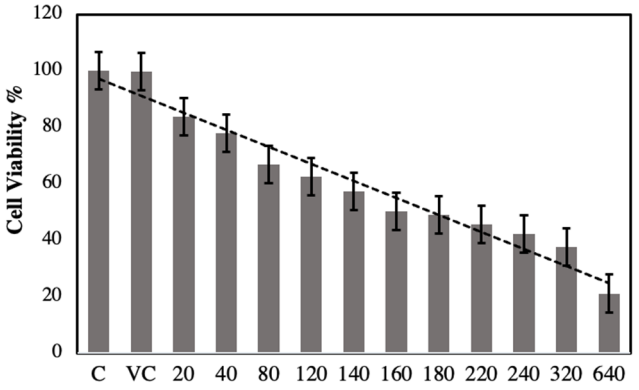

Fig. 4. Inhibitory effect of quercetin on the proliferation of MCF-7

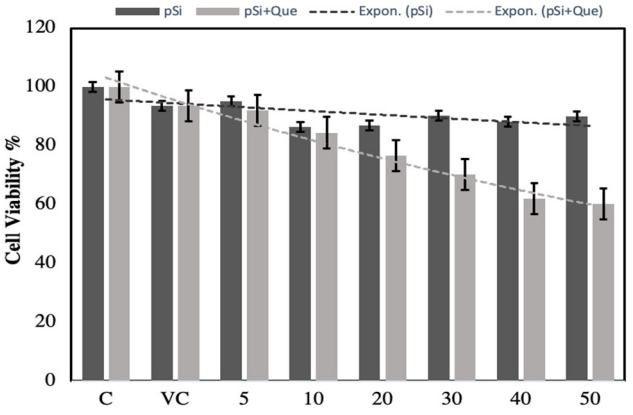

Fig. 5. Comparative representation of pSi alone and quercetin-loaded pSi on the proliferation of MCF-7

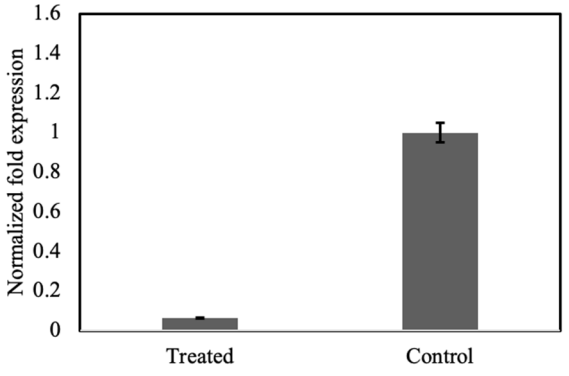

Fig. 6. mRNA expression of Nucleolin (Ncl) in MCF-7 cell line treated with quercetin

3.4. Measurement of nucleolin mRNA expression level

Ncl expression was observed to be 16-fold decreased in the quercetin-treated sample in comparison to the control sample where no drug was present (Fig. 6). This indicates that quercetin might potentially target Ncl and decrease Ncl mRNA level expression in MCF-7 cell line.

4. Discussion

Nucleolin is a multifaceted nonhistone phosphoprotein protein that is involved in the regulation of various pathways, including chromatin remodelling, maintaining of cancer stemness, dysregulation of the cell cycle, and promoting metastasis and angiogenesis [5]. The diagrammatic illustration of targeting nucleolin is presented in Figure 7.

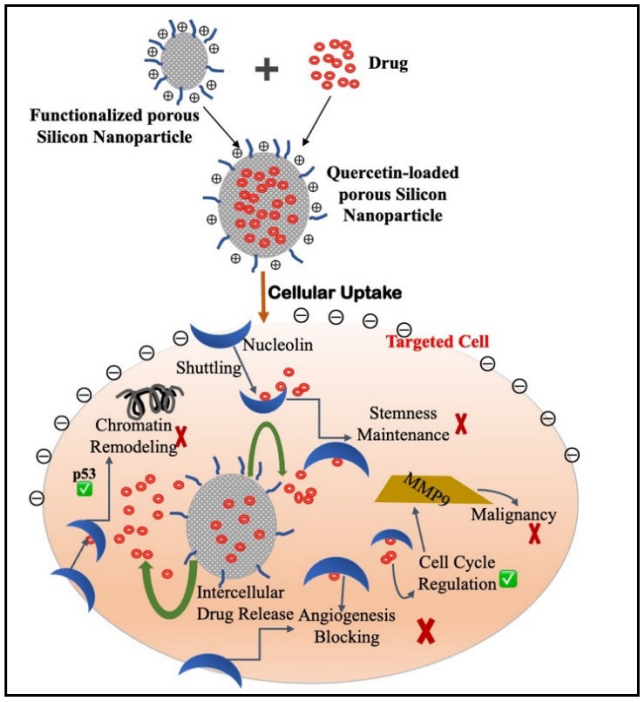

Fig. 7. Diagrammatic illustration of Nucleolin role in the regulation of various cellular pathways. Targeting Nucleolin receptors can potentially rectify various dysregulated cellular processes in cancer: 1) Chromatin Remodelling; 2) Stemness Maintenance; 3) Inhibition of Malignancy; 4) Blocking Angiogenesis, and 5) Promoting cell cycle regulation

Breast cancer drugs available in the market work efficiently during the initial stage of cancer progression, but drug resistance is a major issue among more than 90% after relapse [3]. Besides, patients introduced to immunotherapy have also shown relapse in a short period, raising concerns related to the need for effective measures to limit drug resistance. The major cause of relapse due to drug resistance is due to the low therapeutic index of chemotherapeutic drugs; minor changes in the sensitivity of the cancer cells result in drug resistance [3]. Nanocarrier-mediated delivery of anti-cancerous natural compounds is a growing area in cancer therapeutics. To overcome the major limitations associated with chemotherapeutic drugs, researchers are constantly thriving to develop nanotechnology-based advanced drug delivery systems. Besides, nanocarrier-mediated delivery of the drug can be exploited for targeted therapy and thus reduce therapeutic relapse. Bioflavonoids from plant sources have displayed potential therapeutic properties, including anti-inflammatory, anti-cancer, antioxidant, antibacterial, etc. Furthermore, some previous studies have demonstrated the potential anti-cancerous property of quercetin, that is abundantly found in plant sources including onion, grapes, apples, berries, citrus fruits, etc. [29,30]. Quercetin has shown the ability to inhibit the proliferation of various cancerous cells, including colon, breast, ovarian, stomach, pancreatic, and gastric cancer, as well as induce apoptosis of the cells *in-vitro* [29,30].

In this study, the anti-cancerous activity inhibiting the proliferation of breast cancer cell MCF-7 was determined by MTT assays. It was observed that quercetin could reduce cell proliferation up to 50% (IC_{50}) at 160 µM in 48 h. Similar observations were reported in a previous study showing the inhibitory effect of quercetin against the MCF-7 cell line at concentrations of 40 mg/ml and cell apoptosis of 37.8% [33,34]. However, the cytotoxic effect of quercetin against breast cancer cell lines showed wide range of fluctuation between 50 µM and 200 µM, making it quite challenging to determine the exact dose that could be effective against breast cancer cells line [15,16]. This infers that quercetin might be an effective compound for inhibiting MCF-7 proliferation in a dose-dependent manner.

In the current study, we have tested MCF-7 cell line, which is triple positive, that is, estrogen-positive (ER+), progesterone-positive (PR+), and herceptin-positive (HER+), thus contributes to formation of hormone-positive (HR+) breast cancer. HR-positive breast cancer is generally slow growing compared to HR-negative breast cancer. Hence patient detected with HR-positive breast cancer has better prognosis in the short-term. However, the previous study has shown nucleolin to be one of the bcl-2 mRNA-binding protein [35] that is prominently expressed

among 40-80% expression among breast cancer patients [36]. Hence, targeting nucleolin can be important means of cancer therapeutics for both slow and fast-growing breast cancer.

The solubility of natural compounds, including quercetin is an issue inside the body due to its hydrophobic property. This is a major challenge in applying anti-cancerous natural compounds as the drug that can be overcome by using nano carrier-mediated delivery [37]. Besides, using carriers containing theranostic capabilities allows delivery of the drug at the targeted site efficiently, thus requiring its usage at lower concentrations [33,38]. Moreover, Quercetin is widely present in plant-derived food sources due to its toxicology study plays a crucial role in its therapeutic use. Several in-vitro studies have shown positive results regarding quercetin toxicity and mutagenic activity, including the Ames test at a lower concentration. However, in-vivo genotoxicity studies conducted via oral administration of quercetin in rats and mice showed no significant toxicity or mutational changes [39].

Furthermore, single-dose administration of quercetin dose up to 100-150 mg/kg body weight showed no toxic effect. Similarly, the administration of dietary quercetin doses at concentrations of 30, 300 and 3000 mg/kg body weight/day for 28 days showed no signs of chronic toxicity/carcinogenicity [40]. In the current study, the IC50 of quercetin is determined to be 160 µM, decreasing approximately 3 times when the drug is conjugated with pSi nanocarrier (µM/ml). Moreover, pSi alone showed no cytotoxic effect on MCF-7 cells. Hence, as quercetin in the form of a drug is used at very low concentrations, thus the possibility of its relative toxicity in the body system is quite low.

Using pSi as a nanocarrier is advantageous based on its biodegradability inside the human body. Yet, its biodegradability depends on various factors, including acidity of the surrounding environment, and intrinsic properties, including chemical composition, size, and porosity. Although it is established that the degraded product obtained from pSi is non-toxic in both *in-vitro* and *in-vivo* studies [33,38].

Besides, it is important to study the release pattern of the drug trapped inside the nanocarrier. This can be generally categorised under zero-order kinetics and first-order kinetics, where in zero-order, there is an almost restricted or slow release of the drug, while in first-order kinetics, there is rapid drug release at the initial stage followed by zero or slow release. In the above result, Figure 2b aligns with the first-order kinetics pattern as burst release is observed at the initial stage followed by slow drug release [21, 22]. Hence, the advantage of slow-release is that it enables controlled drug release that allows maintenance of the drug in the circulatory system for a longer duration.

In the current study, it can be observed that pSi alone incubated with MCF-7 cells for 48 h have a negligible effect on the cells, whereas quercetin-loaded pSi has shown significant cytotoxicity against MCF-7 cells introducing 35% cell death in 48 h at 50 µM concentration. Comparatively, it can be noted that quercetin alone has shown approximately 50% cell death at a higher 160 µM concentration [41]. This reflects that quercetin-loaded pSi can potentially show increased cytotoxicity against breast cancer cells. Thermally hydrocarbonized pSi nanoparticles conjugated with quercetin and its derivatives have been shown to increase the sensitisation of the cancer cells towards doxorubicin, thus reversing the condition of multidrug resistance (MDR). Moreover, the nanocarrier-mediated drug delivery has shown stimuli-responsive release of the drug, improved particle-cell interactions, and allowed monitoring of drug release [37].

Additionally, reports have highlighted the enhanced effect of quercetin-loaded pSi to show superoxide scavenging effects, indicating reducing the effect of reactive oxygen species in cancer development. Besides, a significant reduction in proinflammatory cytokines by macrophages, including interleukin one beta, tumour necrosis factor-alpha, and interleukin six production was reported on the use of quercetin-loaded silica nanoparticles in cancer cells [42]. Hence, nanocarrier-mediated quercetin delivery against cancer cells can potentially have multi-fold benefits, including increased sensitisation of the cancer cells for chemotherapeutic drugs, reduction in oxidative effect, inflammatory cytokines, and targeted therapy.

Nucleolin is a multifaceted protein of 76-kDa found extensively on the surface of highly proliferating cancer cells. It consists of a tripartite structure and acts as a shuttling protein between the cytoplasm and nucleus [37, 43]. Nucleolin involvement is reported in various pathways, including chromatin remodelling, blocking expression of p53, supporting matrix metalloproteinase (MMP)-9 mediated malignant transformation, mediates angiogenic activities via stabilising endostatin, etc. [16, 37]. This means that nucleolin not only serves as a biomarker in cancer detection but also is a potential target in cancer therapeutics as it has a role in promoting proliferation, angiogenesis, and metastasising of the cancer cells. In this study, we observed significant downregulation of Ncl downregulation in mRNA level at post-quercetin-treated MCF-7 cells. Further study is under process to investigate whether quercetin-loaded pSi could significantly downregulate Ncl mRNA as well as protein, followed by its impact on the rate of apoptosis, cell proliferation, and angiogenesis.

5. Conclusions

The current study examined the effect of free quercetin and quercetin-loaded pSi nanocarrier as an inhibitor of cell proliferation in MCF-7 breast cancer cells. The potential anti-cancerous effect of quercetin-loaded pSi may have a promising effect on breast cancer treatment. Furthermore, our result indicates that quercetin might be the potential agent that can increase the sensitisation of the breast cancer cells against cancer therapeutics by downregulating the level of Ncl where it is dysregulated. Nanocarrier-mediated delivery of quercetin can be a potential drug delivery system that can decrease nucleolin expression at a lower concentration. However, the action of quercetin-loaded pSi against nucleolin expression in MCF-7 must be further validated to confirm the efficacy of nanocarrier-loaded drugs, which is the future of this study.

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Additional information

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