Research Article

Poly(lactic-co-glycolic acid) Nanoparticles Loaded with *Callistemon citrinus* Phenolics Exhibited Anticancer Properties against Three Breast Cancer Cell Lines

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Fruit and vegetable diets rich in phenolic compounds reduce the risk of various cancers and offer multiple other health benefits due to their bioactivity and powerful antioxidant properties. However, the human health benefits of most phenolic compounds are restricted due to their limited aqueous solubility, low absorption, restricted passive cellular efflux, and poor gastrointestinal stability. Nanotechnology has been used to deliver various therapeutic drugs to specific targets overcoming many of the limitations of direct treatments. This study was designed to develop poly(lactic-co-glycolic acid) (PLGA) nanoencapsulated phenolic-rich extracts from *Callistemon citrinus* and berberine and to evaluate their effectiveness against extremely invasive MDA-MB 231, moderately invasive MCF-10A, and minimally invasive MCF-7 breast cancers. We have achieved about 80% encapsulation of phenolics from *C. citrinus*. Most encapsulated nanoparticles were polygonal with particles sizes of 200 to 250 nm. Release of phenolics from encapsulation during storage was biphasic during the first week and then levelled off thereafter. Nanoencapsulated phenolics from *C. citrinus* extract, berberine, and combination of both enhanced their bioactivity against the three breast cancer cell lines by nearly 2-fold. Growth inhibition of cells was a linear curve relative to phenolic concentration, with a maximum inhibition of nearly 100% at 0.1 mg/ml compared to control.

1. Introduction

Voluminous *in vivo* and *in vitro* studies have confirmed that diets containing fruits and vegetables, which are rich in phenolic phytochemicals, reduce the risk of several types of cancers and provide multiple human health benefits [1–3]. Recent research and review articles have demonstrated that dietary phenolic compounds reduce the risk of UV-induced oxidative and free radical damage, prevent cancers of the

breast, stomach, prostate, and skin, and protect against inflammation, diabetes, and neurotoxicity [2, 4, 5]. It has been suggested that dietary phenolics deliver better preventive and therapeutic options, by improving phytonutrient bioavailability and enhancing drug activity, while exerting low toxicity, compared to conventional drug treatments [6].

The genus *Callistemon* consists of 34 species widely grown in several parts of the world. Leaves, inflorescence,

and oils collected from this genus have for centuries been used in tribal medicine to treat gastrointestinal disorders, various pains, and infectious diseases [7]. Scientific evidence collected over the last few decades indicates that several *Callistemon* species contain bioactive compounds with medicinal properties against cardiovascular diseases and inflammation and anticancer and antidiabetic activities [7]. The medicinal properties of many of these species have been attributed to their rich content of polyphenolic antioxidants, flavonoids including flavanols, flavanones, terpenoids, and tannins, and several nonphenolic bioactive compounds including alkaloids, glycosides, and saponins [7, 8]. Significant differences in polyphenolic concentrations and bioactivity were observed among different species within the *Callistemon* genus and between different plant parts of the same species with leaves of *C. viminalis* containing as high as 44% per dry weight polyphenols [9, 10].

Several studies have examined the antibacterial and antifungal properties and bioactivity of oil extracts from several *Callistemon* species including *viminalis*, *comboyensis*, *lanceolatus*, *citrinus*, *rigidus*, and *linearis* [11, 12]. For example, nitisinone extracted from *C. rigidus* was reported to lower tyrosinaemia type 1, which can lead to buildup of tyrosine and its byproducts causing serious illnesses [7], while extracts from *C. lanceolatus* were reported to possess anticholinesterase activity [13]. However, limited research has been done on the anticancer properties of the nonoil bioactive compounds (primarily phenolics) found in *Callistemon* species. *C. citrinus* (Curtis) Skeels, commonly known as crimson or lemon bottlebrush, is very rich in phenolics and other bioactive compounds; however, there are very limited data on the potential of this species to reduce cancer [14, 15].

Significant advances have been reported in recent years in nanoparticle systems, especially polymeric nano/microparticles used in cancer therapy [16]. Nanoparticles have been developed from various biocompatible and biodegradable materials that may be natural and/or synthetic and therefore display putative ability as carriers for treating various kinds of diseases, especially cancer [17]. In addition, polymeric nanoparticles are nontoxic and display a prolonged circulation potential and a wide payload spectrum for therapeutic agents. Synthetic polymers/copolymers such as PLGA, PLA, and PVA are preferred materials for the development of polymeric nanoparticles, as these systems can deliver drugs for days or even weeks compared to natural polymers, such as chitosan and sodium alginate nanoparticles, which have a shorter period of drug delivery and often require potentially toxic organic solvents [18]. Polymeric nanoparticles can be degraded enzymatically or nonenzymatically in vivo and thus produce biocompatible and safe byproducts that can be easily cleared from the body. Drugs encapsulated in polymeric nanoparticles are either dispersed inside the polymer matrix or attached/conjugated to polymer molecules allowing their release from nanoparticles upon their degradation [18].

Even though there is conclusive evidence to support the positive effects of phenolics on human health, their low solubility and bioavailability may have limited their health

benefits in many of the previous studies [19]. Nanoparticle delivery systems have successfully been used to encapsulate bioactive compounds and deliver them to intended targets in order to enhance their absorption and/or bioavailability [20, 21]. The aim of this study was to utilize advances in nanotechnology to encapsulate phenolic-rich extracts from *C. citrinus*, examine stability and effectiveness of nano-encapsulated *C. citrinus* nanoparticles, and evaluate their effects on growth and proliferation of three types of breast cancer cell lines.

2. Materials and Methods

2.1. Materials. Biopolymer poly(lactic-co-glycolic acid) (PLGA) 50:50, berberine chloride hydrate (mol. wt. 371.9 Da), gallic acid, sodium carbonate, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and polyvinyl alcohol (PVA) (99.3–100% hydrolyzed, average mol. wt. 85,000–124,000 Da) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetone and methanol were purchased from Fisher Scientific Laboratory (NJ, USA), and potassium phosphate buffer was purchased from EMD Chemicals, Inc. (Gibbstown, NJ, USA). Human breast carcinoma (MCF-7, MCF-10A, and MD-MB 231) cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle medium, fetal bovine serum (FBS), and penicillin-streptomycin/EDTA solution were purchased from Gibco (Carlsbad, CA, USA).

2.2. Tissue Sample Preparation. Bottlebrush *Callistemon citrinus* leaves and stems were collected from mature plants grown in Chakwal District of Punjab Province, Pakistan. Fresh tissue was frozen at -70°C , freeze-dried, and ground into fine powder. A subsample of 20 g freeze-dried tissue was mixed with 200 ml methanol and homogenized, and the homogenate was placed on an orbital shaker (VWR, Hampton, NH, USA) for 12 hours. The mixture was filtered through a Whatman (#4) filter paper (Marlborough, MA, USA), and the filtrate was concentrated in a rotary evaporator (Buchi Co., New Castle, DE, USA). Fresh extracts were prepared for each assay.

2.3. Preparation and Loading of PLGA Nanoparticles. Nanoparticles were prepared by a nanoprecipitation technique [22] with slight modifications as described by Pereira et al. [23]. An organic phase was formed when a fraction of 5.0 mL of the previously prepared methanolic tissue extracts was combined with 50 mg PLGA prepared in acetone solution and stirred at 150 rpm for 45 minutes. An aqueous phase of 1% polyvinyl alcohol (PVA) solution was prepared by dissolving 1.0 g of PVA in 100 ml of ultrapure H_2O and then heated and stirred to dissolve PVA. The organic fraction of the PLGA tissue extract was added to 20 mL PVA solution in a dropwise manner.

Similarly, a standard of 1.0 mg of berberine chloride was dissolved in 1.0 ml of methanol and 7 mg of PLGA prepared in 1.0 ml acetone. Berberine chloride solution was added to

the PLGA solution and stirred for 45 minutes. The PLGA-berberine mixture was added to another 20 mL PVA solution in a dropwise manner and stirred for 10 minutes at room temperature. Mixing of the organic phase with the aqueous phase while stirring resulted in aggregation of PLGA nanoparticles, which was prevented by adding 2.0 mL purified H₂O. The solvents (acetone and methanol) were removed by rotary evaporation. Similarly, blank samples of PLGA nanoparticles were synthesized without the tissue extracts by adding only PLGA solution following the same steps outlined previously. The PLGA nanoparticles were centrifuged (Eppendorf centrifuge 5415C, Hamburg, Germany) at 14,000 rpm for 15 minutes and then collected and purified by ultrafiltration as previously described [24, 25]. PLGA nanoparticles were lyophilized by mixing synthesized nanoparticles with trehalose (EDM Chemicals, Philadelphia, PA, USA) at 1:1 ratio by pouring the PLGA nanoparticles into glass vials covered with a double layer of Parafilm. The PLGA nanoparticles were stored at -80°C for 24 hours and then freeze-dried (Labconco, Kansas City, MO, USA).

2.4. Scanning Electron Microscopy (SEM) of PLGA Nanoparticles. The prepared nanoparticles were evaluated under a scanning electron microscope (Hitachi S-4800 FE-SEM, Japan) for their size, morphology, and surface properties. Nanoparticles were washed three times with distilled water, freeze-dried, and coated with gold palladium to improve electrical conductivity before imaging under SEM at 15 kV.

2.5. Nanoparticle Properties. PLGA nanoparticle diameter (ϕ , size), surface property or zeta potential (ζ), and polydispersity index (PI), which determines nanoparticle penetration potential, aggregation capacity, and diffusion rate, were determined using Nano ZS90 Zetasizer (Malvern, UK) and Hitachi S-4800 scanning electron microscopy (SEM). PLGA nanoparticles were suspended in pure water in a cuvette (Malvern Panalytical, UK) and were sonicated to uniformly disperse the particles, in order to determine their size and any aggregate formation. Aggregation of PLGA nanoparticles was eliminated by filtering through a 0.2 μ m nylon filter (Acrodisc, Pall Corporation, Port Washington, NY, USA). The zeta potential of nanoparticles was determined by suspending PLGA nanoparticles in pure water and then transferring them into a zeta capillary cell (Malvern, Worcestershire, UK), which was loaded into a Malvern Zetasizer. The passage of the laser beam through PLGA nanoparticles and fluctuation in scattering intensity of nanoparticles produced the signal of an electrophoretic charge present on PLGA nanoparticles. All readings were recorded at 25°C and a scattering angle of 90° after dilution of samples with pure water to prevent multiscattering. Polystyrene was used as a standard to measure light scattering at an angle of 173°. Each sample was measured in triplicate.

2.6. Total Phenolic Entrapment Efficiency within Nanoparticles. UV spectroscopy was used to determine the

amount of polyphenol and polyphenol matrices of the *C. citrinus* extract, berberine, and *C. citrinus* extract-berberine mixture entrapped into PLGA nanoformulations. The amount of PVA applied as a stabilizer and surfactant were kept constant at 1% during formulation of PLGA nanoparticles. Entrapment efficiency ($\eta\%$) of total phenolics of the *C. citrinus* extract within nanoparticles was determined by measuring the UV absorbance of total phenolics entrapped within PLGA nanoparticles according to Pereira et al. [23] with a slight modification. The *C. citrinus* extract and berberine loaded PLGA nanoparticles were suspended in 95% methanol solution at a concentration of 1.0 mg/mL and placed in the dark for 30 minutes at 37°C. The mixture was filtered through a 0.2 μ m nylon filter. A 100 μ L fraction of the filtrate was mixed with 20 μ L Folin-Ciocalteu reagent, 830 μ L purified water, and 50 μ L sodium carbonate solution. Samples were placed in the dark at room temperature for 30 minutes, and absorbance was recorded at 725 nm using a UV160U spectrophotometer (Shimadzu Corp., Kyoto, Japan). Gallic acid was used as a standard to estimate the total phenolic content in the encapsulated PLGA nanoparticles [25, 26]:

$$\eta = \frac{\text{amount of total phenolics entrapped}}{\text{total phenolic quantity used for encapsulation}} \times 100. \quad (1)$$

2.7. In Vitro Release of Total Phenolics. The release of total phenolics from nanoparticles was estimated by combining lyophilized nanoparticles in a phosphate buffer saline (pH 7.2) at 1.0 mg/mL, which was suitable to create a sink condition. The mixtures were incubated at 37°C in a water bath for 24 hours and then filtered through a 0.2 μ m Acrodisc nylon filter [25]. Later, the filtrate was collected, and total phenolics released were measured as described in Section 2.5.

2.8. Stability of PLGA Nanoparticles. The stability of PLGA nanoparticles was evaluated by measuring the amount of total phenolics released after one week of storage at 4°C. In summary, 1.0 mg/mL subsamples of refrigerated PLGA nanoparticles were centrifuged at 3,000 rpm for 3 minutes. The supernatants were collected, and the amount of total phenolics released was measured as previously described. The results were compared to total phenolics released by freshly prepared PLGA nanoparticles.

2.9. Anticancer Activity Determination. The anticancer activity of *C. citrinus* and berberine extract loaded PLGA nanoparticles was determined by a sulforhodamine B (SRB) colorimetric assay (Invitrogen, Carlsbad, California) according to a protocol outlined by Vichai and Kirtikara [27]. The protocol was used for cytotoxic screening of PLGA nanoparticles based on cell density measurement by estimation of protein content. Three cancer cell lines (MCF-7, MCF-10A, and MDA-MB 231) were used to study the effect of PLGA nanoparticles and unencapsulated form of

C. citrinus and berberine. The human hormone-dependent breast cancer cell lines MCF-7 and MCF-10A were cultured in Dulbecco's modified Eagle medium (Gibco, Carlsbad, CA, USA), and MDA-MB 231 hormone-independent breast cancer cell lines were cultured in Leibovitz's L-15 medium (Sigma-Aldrich) in T75 cell culture flasks (Thermo Fisher Scientific), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin/EDTA solution (Gibco, Carlsbad, CA, USA). Cultured cells were immediately transferred to a 96-well plate (Thermo Fisher Scientific) and incubated overnight to a rate of about 1×10^4 cell density per well, and the percentage of growth inhibition of breast cancer cell lines was measured after a period of 48 hours.

Tested PLGA nanoparticles loaded with *C. citrinus* and berberine and free extracts from the same treatments ranged from 0.0008 to 0.1 mg/ml. Samples were placed in 96-well plates, incubated under 5% CO₂-enriched air and 100% relative humidity at 37°C for 48 hours, fixed by adding 50 μ l of cold 10% (w/v) TCA, and then stored at 4°C for 1 hour. Each sample well was washed four times with distilled water and then dried by a purified air stream at room temperature.

The staining step was performed by adding 100 μ L of 0.57% (w/v) sulforhodamine B (SRB) dye to each sample well. Treated samples were incubated at room temperature for 30 minutes and then washed four times with 100 μ l of 1% (v/v) acetic acid to remove the unbound dye. Finally, 100 μ l of 10 mM Tris base (pH 10.5) was added to each sample well, and the plates were placed on a shaker for 3 minutes. The absorbance of each sample was measured using a spectrophotometer set at 490–630 nm, and the growth inhibition percentage was determined using the following formula:

$$\text{growth inhibition (\%)} = \left[\frac{\text{mean OD of sample} - \text{mean OD}_b}{\text{mean OD of control} - \text{mean OD}_b} \right] \times 100, \quad (2)$$

where OD_b is the OD for a blank sample.

2.10. Statistical Analysis. Results are expressed as mean $\pm$ standard deviation (SD). Data were analyzed by one-way analysis of variance, and differences among the means of groups were analyzed by an unpaired, two-sided Student's *t*-test. Differences were significant at $P < 0.05$.

3. Results and Discussion

3.1. Preparation and Characterization of PLGA Nanoparticles. In our study, PLGA nanoparticles were synthesized by a nanoprecipitation technique, as outlined in Figure 1. Polyvinyl alcohol (PVA), a biodegradable and biocompatible synthetic biopolymer, was used as a stabilizer for PLGA nanoparticles. By using PVA as a stabilizer and PLGA as a carrier, we were able to obtain high yield of nanoparticles. PLGA has been widely used for drug delivery applications of hydrophobic drugs such as polyphenols. It is also biodegradable, biocompatible, and a highly stable

polymer. It has also been approved by the USA Food and Drug Administration.

3.2. Scanning Electron Microscopy. Sizes of PLGA nanoparticles loaded by berberine, *C. citrinus* extract, and mixtures of berberine and *C. citrinus* extract were 250.7 ± 0.06 , 278.8 ± 0.007 , and 274.8 ± 0.028 nm, respectively (Table 1). Nanoparticle shapes observed under SEM were polygonal (hexagonal) with diverse sizes, with unique morphological features (Figures 2(a) and 2(b)), as compared to a previously reported formulation [24]. Various studies have demonstrated that tailoring of the shape of nanoparticles could improve their effectiveness. For example, it was reported that polygonal nanoparticles have more antibacterial activity against *E. coli* than triangular and spherical nanoparticles [28]. In another study, it was observed that oblong-shaped nanoparticles improved targeted delivery of antibody nanoformulations [29]. Nanorods coated with trastuzumab exhibited a 66% increase in binding and uptake potential by BT-474 breast cancer cell lines compared to an equal dose of spherical nanoparticles, resulting in a 5-fold growth inhibition potential against BT-474 cancer cells [30]. While oblate nanoparticles exhibited a better degree of adherence to mammalian cells and a higher drug-carrying capacity compared to spherical particles [31], polygonal nanoparticles were reported in an earlier study to exhibit a prolonged stay in blood stream due to uptake failure of macrophages [32]. This mechanism may lead to enhanced growth inhibition due to higher penetration through cell membranes, subsequently releasing polyphenols into the cytoplasm, which contains various organelles that could be affected by the nanoformulations [25].

The enhanced growth inhibition feature of polygonal nanoparticles was observed during their cytotoxic effect on various types of cancer cell lines as these nanoformulations penetrate more efficiently into cells due to their morphological matrix [33, 34]. In addition to their morphology, nanoparticles' surface charges also determine their potency. The effectiveness of positively charged PLGA nanoparticles was higher than that of PLGA nanoparticles bearing negative charges [35]. Nanoparticles' surface charges also affect their aggregation. Aggregation of nanoparticles developed in this study was significantly reduced by filtration followed by addition of an excess solvent. Our PLGA nanoparticles were more efficiently separated when they had higher surface charges and tended to aggregate when they had lower surface charges.

3.3. Characterization of Nanoparticles. Nanoparticle size and polydispersity index (PDI) define efficient delivery of loaded materials. Both nanoparticle size and PDI determine drug loading and drug release profiles, stability, toxicity, and targeting potential, which are main requirements for an ideal cargo carrier system. The nanoparticle size obtained in the current study ranged from 200 to 250 nm (Table 1), which is considered an optimum size range for PLGA nanoparticles [36]. Zeta potential is another important characteristic that determines cytotoxicity

FIGURE 1: Flow chart for the synthesis and characterization of *C. citrinus* extract-loaded PLGA nanoparticles. (a) Mixing of *C. citrinus* extract solution with PLGA solution. (b) Preparation of 1% PVA solution. (c) PLGA solution containing *C. citrinus* extract was constantly stirred at 150 rpm for 30 minutes, leading to precipitation of PLGA NPs. (d) The solvent was evaporated by rotary evaporation. (e) Collection of PLGA nanoparticles by centrifugation at 14,000 rpm. (f) Collection of PLGA NPs was freeze-dried and stored at -4°C . (g) Characterization of PLGA-loaded nanoparticles by scanning electron microscopy, zetasizing, total content in extracts, loading of total phenolics (TP) into PLGA nanoparticles, and percentage TP release studies and stability profiles. (h) Assessment of growth inhibitory effect of PLGA NPs and their free counterparts against MDA-MB 231, MCF-10A, and MCF-7 breast cancer cells. (i) Mode of action of PLGA NPs on model cells.

TABLE 1: Estimates of zeta diameter, polydispersity index, and zeta potential of nanoformulations of BBR PLGA, CCE PLGA, and CCE BBR PLGA.

NPs	Incubation temperature ($^{\circ}\text{C}$)	Zeta diameter (nm)	Aggregation (%)	Polydispersity index	Zeta potential (mV)
BBR PLGA	25	250.6 ± 0.08	0	0.361 ± 0.06	-8.50 ± 0.06
CCE PLGA	25	278.8 ± 0.00	0	0.545 ± 0.07	-20.06 ± 0.10
CCE BBR PLGA	25	274.8 ± 0.02	0	0.256 ± 0.04	-5.80 ± 0.09

Abbreviations: BBR, berberine; CCE, *Callistemon citrinus* extract; PLGA, poly(lactic-co-glycolic acid); NPs, nanoparticles. Data points represent mean $\pm$ SD ($n = 3$).

and clumping of loaded PLGA nanoparticles. The surface charge of PLGA nanoparticles loaded with berberine and a combination of berberine and *C. citrinus* was -8.50 ± 0.06 and -5.80 ± 0.09 , respectively, whereas the surface charge of PLGA nanoparticles loaded with *C. citrinus* alone was 2.4- to 3.5-fold lower than that loaded with berberine and mixture of berberine plus *C. citrinus*. Since nanoparticles' surface charge plays a significant role in inhibition of cancer growth [35], PLGA nanoparticles loaded with berberine plus *C. citrinus* could be more cytotoxic or more effective against cancer cells compared to PLGA nanoparticles loaded with *C. citrinus* alone. The PDI data in Table 1 and size distribution in Figures 2(c) and 2(d) show that the PLGA nanoparticles were of diverse sizes. Formation of diverse-sized PLGA nanoparticles is advantageous due to variation in their diffusion rates with smaller nanoparticles having higher diffusion than larger nanoparticles.

3.4. Total Phenolics and Their Entrapment in PLGA Nanoparticles. Berberine, an alkaloid antioxidant, reacted positively and linearly with the phenolic assay, but at a rate lower than that of *C. citrinus* phenolics. The total phenolic content of berberine, *C. citrinus*, and the mixture of *C. citrinus* and berberine was 1942.46 ± 0.01 , 609.35 ± 0.02 , and $1979.58 \pm 0.006 \mu\text{g/ml}$, respectively (Figure 3(a)). The data obtained suggest that phenolic concentration in the *C. citrinus* extract and *C. citrinus* extract plus berberine was 4-fold higher than that in berberine alone. Numerous articles have linked antioxidant properties of total phenolics to human health benefits but have very little information on the health benefits of their entrapment in nanoformulations.

Total phenolic entrapment of about 88% was observed in PLGA nanoparticles loaded with a blend of *C. citrinus* and berberine, followed by 82% entrapment from *C. citrinus* extract alone and only about 23% entrapment from berberine alone (Figure 3(b)). The solubility of the entrapped

FIGURE 2: Size distribution images of PLGA NPs obtained through Malvern Zetasizer and Hitachi S-4800 scanning electron microscopy synthesized by a nanoprecipitation technique. SEM images of (a) *C. citrinus*, (b) berberine, and (c) *C. citrinus* + berberine nanoparticles. Histograms of (d) *C. citrinus*, (e) berberine, and (f) *C. citrinus* + berberine nanoparticles.

material in water determines the percentage of entrapment into PLGA nanoparticles as extracts having more solubility will move out into the aqueous phase from the organic phase during synthesis of PLGA nanoparticles, resulting in a decrease in percentage entrapment efficiency as described by Pereira et al. [37]. The entrapment of berberine and *C. citrinus* extracts into PLGA is consistent with the encapsulation of the hydrophobic extract of guabiroba fruit

which was reported at between about 84 and 99% encapsulation into PLGA nanoparticles [38].

3.5. Percent Release of Total Phenolics from Loaded Nanoparticles. Release of total phenolics from PLGA nanoparticles was carried out at pH 7.4 and 37°C, resembling conditions of the gastric juices in the human body [39]. Data

FIGURE 3: (a) Total phenolic content of *C. citrinus* (CC), berberine (BB), and *C. citrinus* extract + berberine (CC + BB) encapsulated in PLGA nanoparticles. (b) Percent encapsulation efficiency ($\eta\%$) of PLGA nanoparticles loaded with *C. citrinus*, berberine, and *C. citrinus* + berberine. Data are represented as mean $\pm$ SD ($n = 3$).

in Figure 4(a) show linear curve responses with fast releases of berberine, *C. citrinus* extract, and mixture of *C. citrinus* and berberine from PLGA nanoparticles within the first 24 h, reaching their peaks at 7 days and then levelling off to negligible levels thereafter. The immediate fast release of berberine and *C. citrinus* extract could be attributed to rapid desorption and subsequent release from the outer boundary of PLGA nanoparticles as previously described during chlorambucil encapsulation [40]. All three treatments exhibited a similar pattern of release with encapsulated berberine being significantly higher than the other two treatments during the first 7 days, while combination of berberine and *C. citrinus* release was significantly lower than the other two treatments during the remainder of the study period (Figure 4(a)). The slow and sustained release of total phenolics was due to difference in their concentration within PLGA nanoparticles and to the phosphate saline buffer. The change in sink conditions significantly affected the release profile of total phenolics *in vivo* where it was enhanced due to rapid degradation of PLGA nanoparticles [39].

3.6. Stability of PLGA Nanoformulations during Storage.

Total phenolic release profiles in Figure 4(b) show that the prepared nanoformulations were very stable. Leakages from prepared PLGA nanoparticles containing berberine, mixture of berberine and *C. citrinus* phenolics, and *C. citrinus* phenolics alone showed less than 2% reduction over a 28-day storage period at 4°C (Figure 4(b)). The stability of these nanoformulations may be due to the presence of a PVA coating barrier, allowing for better formulation retention. The low percent leakage of phenolics over the 28-day storage suggests that the prepared PLGA nanoformulations are a very stable carrier making them suitable for drug delivery applications. The stability of the encapsulated liposomal

formulations reported in this study was very similar to that observed using curcumin under similar storage conditions [41], which indicates that storage of PLGA nanoparticles has no effect on their retention of polyphenols, and thus, they can be used as stable carrier systems for berberine and *C. citrinus* extract.

3.7. Cytotoxic Activity of Nanoformulations against Breast Cancer Cell Lines.

The nanoformulation technique presented in this study is a useful tool for loading of complex extracts into polymeric nanoparticles to evaluate their anticancer potential for *in vitro* trials. Recent studies have used nanoencapsulation of a single or multiple bioactive compounds to evaluate their anticancer effectiveness against several types of cancers *in vitro* [42, 43]. Sampath et al. [44] examined the bioactivity of *C. citrinus* extracts against skin carcinoma A431 and human keratinocyte HaCaT cell lines. They attributed the apoptotic effect of *C. citrinus* extracts to their content of the monoterpenoid 1,8-cineole, which they reported to enhance the expression of p53, a tumor-suppressing protein. Similarly, essential oils extracted from *C. citrinus* leaves and flowers exhibited highly significant growth inhibition of A549 cells and C-6 cancer cells (61% and 69%, respectively) but had no effect on growth of normal cells.

Data in Figures 5(a)–5(d) of nanoformulated and non-formulated treatments at different concentrations show different growth inhibition efficacies against the tested breast cancer cell lines. Figure 5(b) shows that, at 0.1 mg/ml, a mixture of nanoformulated berberine and *C. citrinus* induced the most potent inhibition (33%) of MDA-MB 231 cells. In contrast, the same concentration in nonformulated form induced only about 12% growth inhibitory, suggesting that encapsulation resulted in nearly a 3-fold increase in effectiveness of the

FIGURE 4: (a) Percentage release of TP of CC-, BB-, and CC + BB-loaded PLGA NPs over a period of 11 days under conditions resembling the human gastrointestinal tracts. (b) Percent release of TP from BB PLGA NPs (closed circle), CC PLGA NPs (closed square), and CC + BB PLGA NPs (closed triangle) over a period of 28 days in cold storage at 4°C. Data represent mean $\pm$ SD ($n = 3$).

FIGURE 5: Continued.

FIGURE 5: (a) Cytotoxic effects of BB, CC, and CC + BB extracts against MDA-MB 231 breast cancer cell lines. (b) Cytotoxic activity of BB PLGA NPs, CC PLGA NPs, and CC + BB PLGA NPs against MDA-MB 231 breast cancer cell lines. (c) Cytotoxic effects of BB, CC, and CC + BB extracts against MCF-10A breast cancer cells. (d) Extract-loaded PLGA nanoparticles against MCF-10A breast cancer cells. (e) Cytotoxic effects of BB, CC, and CC + BB extracts against MCF-7 cancer cells. (f) Cytotoxic effects of BB-, CC-, and CC + BB loaded PLGA NPs against MCF-7 breast cancer cells (mean $\pm$ SD, $n = 3$).

nanoformulated treatments (Figure 5(b)). The cytotoxicity of free and encapsulated *C. citrinus* forms reported in this study is in agreement with that reported in other studies [45], showing a marked difference in their anticancer potential. Contrary to an earlier study [46], we have observed a marked increase in effectiveness of the treatments against MDA-MB 231 breast cancer when their concentrations were increased (Figures 5(a) and 5(b)). The effect of the treatments against MCF-10A, a more invasive cancer cell line, is shown in Figures 5(c) and 5(d). The patterns of MCF-10A response to the treatments' concentrations were similar to those for MDA-MB 231; however, at 0.1 mg/ml nonformulated and nanoformulated

doses, the rate of inhibitions was about 2.5- and 5-fold higher, respectively, than those for MD-MB 231. The highest anti-cancer activities of the treatments were observed against MCF-7 cell lines (Figures 5(e) and 5(f)). More than 83% and nearly 100% cell growth inhibitions were observed in the non-formulated and nanoformulated treatments at 0.1 mg/ml, respectively (Figures 5(d) and 5(f)).

Treatment of cancer cells with *C. citrinus* extracts induced higher growth inhibition compared to berberine in both nonformulated and nanoformulated forms. However, cancer cell growth inhibition was significantly higher when a mixture of both materials was used in either nanoformulated

FIGURE 6: Values of IC_{50} of MDA-MB 231, MCF-7, and MCF-10A breast cancer cell lines treated with nanoformulated and non-formulated BB, CC, and CC + BB extracts (mean $\pm$ SD, $n = 3$).

or nonformulated forms. This combination was highly effective against MCF-7, intermediately effective against MCF-10A, and slightly effective against MDA-MB 231 cancer cell lines. Synergistic effects were observed between the tea polyphenols theaflavin-3-3'-digallate, ascorbic acid, and (-)-epigallocatechin-3-gallate [47]. In contrast, PLGA nanoparticles loaded with berberine alone were minimally effective. Results of this study support the previous study using MCF-7 breast cancer cell lines [24]. Thus, PLGA nanoformulations loaded with complex polyphenol matrices could provide effective treatment to reduce and/or inhibit the proliferation of less invasive breast cancer cells. These results highlight two important observations: (a) significant inhibition of cancer cell growth when extracts were encapsulated into PLGA nanoparticles and (b) higher breast cancer cell inhibition when cells were treated with a combination of berberine plus *C. citrinus*.

Another important observation was the correlation between the degree of invasiveness of a cancer cell line and the degree of its growth inhibition by the treatment. For example, *C. citrinus* and berberine PLGA loaded nanoparticle treatment had minimal effects against the growth of the extremely metastatic MDA-MB 231 cancer cell line (Figure 6), while very effective against the other two less invasive lines. Our data confirm earlier observations by Kim et al. [48] using berberine against the same cell lines. In another study [49], treatment of MDA-MB 231 cancer cell lines with magnolol, a natural compound, significantly inhibited cell growth by downregulating the matrix of metalloproteinase-9 (MMP-9), an enzyme required for invasion and tumor metastasis. In our study, nanoformulated *C. citrinus* extract plus berberine showed a more pronounced growth inhibition of MCF-7 cells, which suggests that the degree of invasiveness/proliferation is related to growth inhibition. A similar pattern of growth inhibition was demonstrated against MDA-MB 231 and T47D cells using metformin in nanoencapsulated and free forms [50].

4. Conclusion

Nanoencapsulation of *C. citrinus* into PLGA nanoparticles enhanced its bioactivity against MDA-MB 231, MCF-10A,

and MCF-7 breast cancer cell lines by about 2-fold. In addition, the combination of *C. citrinus* extracts with berberine further increased their cytotoxic potential in free and encapsulated forms. Data suggest that nanoencapsulation of anticancer compounds either alone or in combination with other bioactive compounds is a valuable tool for cancer chemotherapy.

Data Availability

Data used to support the findings of this study are included in the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest. Additionally, University of Illinois is an Affirmative Action/Equal Opportunity Employer dedicated to building a community of excellence, equity, and diversity.

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