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ORIGINAL RESEARCH ARTICLE

Curcumin Encapsulated PEGylated Nanoliposomes: A Potential Anti-Infective Therapeutic Agent

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Abstract Exploration of novel bioactive molecules or potentiation of the existing bioactive molecules is necessary to reduce the burden of the infectious diseases for the better human health. Curcumin is a promising molecule with huge therapeutic potential. Despite high bioactivity, its therapeutic suitability is shadowed by poor bioavailability, limited aqueous solubility, and short shelf life. Nanotechnology has generated new avenues to overcome these challenges. In the current study polymer assisted nanoliposomes, PEGylated Curcumin nanoliposomes with good loading efficiency were prepared. These particles have shown 1000 fold enhanced curcumin hydrophilicity and tenfold higher stability. In vitro release kinetic indicates two fold higher curcumin release in the simulated gastric and intestinal environment. Various bioactivity assays have confirmed enhanced bioactivity of nanocurcmin in comparison of the native curcumin. PEGylated Curcumin nanoliposomes could be employed for treating various diseases.

Keywords Curcumin · Nanoliposomes · Antibacterial · Antifungal · Antioxidant · Quorum sensing inhibitors · Bioactive

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Introduction

Turmeric is a well known natural non-toxic food additive, as well as a potential remedy for the treatment of various diseases without any side effect [1]. Curcumin, is a hydrophobic polyphenolic compound of the turmeric [2]. Bioactive properties of curcumin like antioxidant, antimicrobial, antifungal etc. make it a potential therapeutic candidate for treating of various diseases and a potential anticancer agent [1, 2].

Despite of these properties, its use as a therapeutic agent is limited by its properties such as hydrophobicity, very low aqueous solubility, slow release, low stability and poor bioavailability [2]. A number of efforts were made to overcome these problems that include making the curcumin conjugates with biodegradable polymers, liposomes, metals and inorganic oxides [3–10]. Wang et al. reported the inorganic metal oxide based curcumin nanocomposites have enhanced water affinity and pH dependent controlled curcumin release [3]. However, the majority of inorganic metal oxides show toxic behavior [4]. However, inorganic metal oxides and polymers have low toxicity, high stability and ability to act as a carrier for the drug [5]. Polymeric nanoparticles-encapsulated curcumin synthesized by adopting the free radical copolymerization method was used in the human cancer therapy [5]. Among different polymers used for enhancement of the bioavailability of the compounds, Polyethylene glycol (PEG) was found to be safe and less toxic [6, 7]. PEGylation has gained attention due to its hydrophilic nature, biocompatibility, availability and low biofouling nature [7]. PEGylation is also known to improve the diffusion of nanoparticles through mucus which in turn enhances its bioactivity. Enhancement in the penetration power of drug in the microbes, helps in improvement of the antimicrobial activity [8]. Curcumin-

PEG nanoconjugates were found to have high water solubility and bioavailability as evident by their antiadipogenic activity, cellular uptake and cellular retention in 3T3-L1 cell line [8, 9]. The effect of the ratio of poly (lactide)-poly (ethylene glycol) addition in curcumin was found to increase curcumin solubility and its encapsulation efficacy [10]. However, PEG nanoconjugates have shown poor curcumin loading efficiency [6]. On the other hand, liposomes are small vesicles mad of natural non-toxic phospholipids and cholesterol which impart hydrophobic and hydrophilic characteristics, making them a promising system for drug delivery [11]. Combined approach of using of polymer assisted nanoliposomes enhance bioavailability as well as stability of drugs, providing an efficient drug delivery system [12]. In this study, we have utilized biocompatibility associated properties of the polymer and enhanced drug loading properties of liposomes to improve the therapeutic potential of the curcumin.

Materials and Methods

Materials

All reagents used in the experiments were of high purity analytical grade.

Synthesis of PEGylated Encapsulated Curcumin Nanoliposomes

Synthesis of PEGylated encapsulated curcumin nanoliposomes (PCNL) was performed by hydrating thin lipid film followed by sonication and extrusion [12, 13]. Phosphotidylcholine and cholesterol were used to synthesize the lipid matrix. Cholesterol and phosphatidylcholine (ratio of 1:7.5) were dissolved in 10 ml alcoholic solution of olive oil (2% (v/v%)) to produce a transparent yellow suspension. Thereafter, 10 mg of curcumin was added to the mixture followed by mixing by magnetic stirrer for 15 min at room temperature. Ethanol was evaporated by heating at 45 °C. A 10 ml aqueous mixture of Polyethylene glycol (PEG) 2000 (0.001% (w/v%)), Tween 80 (0.002% ((v/v%)) was added dropwise into the lipid matrix by continuous mixing by magnetic stirrer at 45 °C for 30 min. The resulting mixture was ultrasonicated with a constant power output of 650 W for 7 min with on/off cycle of 10 s. The resultant solution was stored at 4 °C for further characterization and downstream experiments. Native PEGylated nanoliposomes were prepared following the same strategy except curcumin was removed from the lipid matrix.

Characterization of PEGylated Encapsulated Curcumin Nanoliposomes

Morphology and size of PEGylated encapsulated curcumin nanoliposomes were analyzed using transmission electron microscope (TEM) (Hitachi (H-7500)) at Sophisticated Analytical Instrumentation Facility, Punjab University. Infrared (IR) spectrum of PCNL and PEGylated nanoliposomes were read with F.T.Infra-Red Spectrophotometer Model RZX (Perkin Elmer).

Curcumin Loading, Stability and In Vitro Release Kinetics

Encapsulation efficiency was calculated as described earlier [12]. Curcumin loading was estimated by suspending 200 µl of PCNL solution in 1 ml of methanol. Methanol fractions were centrifuged at 13,000 rev min^{-1} for 15 min and the supernatant was collected. Optical density of the supernatant was read at 425 nm with UV Visible spectrophotometer (PG Instruments, UK) to calculate curcumin encapsulation. Curcumin stability assay was performed for PCNL and native curcumin in an aqueous environment for 12 h. A 100 µl fraction was collected at various intervals (0 h, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h) and mixed with 900 µl ethanol before reading the absorbance at 425 nm.

Curcumin release from PEGylated curcumin nanoliposomes was studied in artificial gastric juice (PBS adjusted to pH 2.0 with HCl) and intestinal juice (PBS at pH 7.4). The amount of curcumin in aqueous solution was determined [5]. Results were expressed as mean + SD. Quantitative of the curcumin was estimated using the following equation:

$$\%\ \text{age release} = \frac{amount\ of\ curcumin\ in\ aq.solution}{amount\ of\ curcumin\ loaded} * 100$$

Determination of Aqueous Solubility

The solubility of CUM in the nanoconjugates was determined spectrophotometrically. In order to find out the aqueous solubility, 500 µl of the curcumin nanoconjugate samples was dispersed into a fixed volume of Ultrapure water (750 µl) and incubated at 37 °C with constant shaking at 200 rev min^{-1} for 24 h. After the incubation, the samples were filtered through a 0.22 µm Millipore membrane followed by centrifugation at 13,000 rev min^{-1} for 10 min. The aqueous layer was collected leaving insoluble and nonpolar fraction. Absorbance of the collected aqueous supernatant was recorded at 425 nm and solubility of the encapsulated/loaded curcumin was

calculated in various nanoconjugates. Results were expressed as mean ± SD.

Curcumin Bioactivity Assays

The antibacterial activity of the PCNL, native Curcumin and native liposomes was analyzed in triplicate against native and antibiotic resistant microorganisms like *Escherichia coli DH10B*, *Klebsiella pneumoniae* (MTCC No. 109), *Bacillus subtilis* (MTCC No. 2057), *Mycobacterium smegmatics* (MTCC No. 992) and *Chromobacterium violaceum* (MTCC No. 2656) 89 [14, 15]. The antifungal activity of the PCNL was analyzed against *Aspergillus niger* (MTCC No. 514); *Candida albicans* (MTCC No. 514) and *Fusarium oxysporum* (MTCC No. 7392) with well diffusion method by measuring the zone of inhibition [16]. Quorum sensing inhibition activity of nanoliposomes was checked by qualitative analysis of inhibition violacein production by *C. violaceum* [17]. Antioxidant property of PCNL, native Curcumin and native liposomes was assessed using varying amount of test material (0.1 μg/ml^{-1}, 0.2 μg ml^{-1}, 0.4 μg ml^{-1}, 0.6 μg ml^{-1}, 0.8 μg ml^{-1}, 1.0 μg ml^{-1}, 2.0 μg ml^{-1}) with DPPH radical scavenging assay, ABTS radical scavenging assay and Nitric oxide scavenging assay [18].

Statistical Analysis

All experiments were performed in triplicate and results are expressed as mean ± standard deviation (SD, n = 3). p value was calculated using a parametric T test and a significant difference was set at $p < 0.05$.

Result and Discussion

Nanotechnology has been made significant advancement in the last decade and developed a number of novel materials [19], biomaterials [20], nanomedicines [14, 21], nanosensors [22]. Nanosized materials/biomaterials have shown significant improvement in their physical, as well as chemical properties. Nanomaterials have known to enhance stability, solubility, cellular infusibility [23, 24]. Curcumin has huge therapeutic potential, however, its poor stability and limited bioavailability make it difficult for pharmaceutical applications [1, 3]. As nanomaterials have proven to overcome these limitations, hereby in the present study an effort has been made to use nonmaterial as curcumin loading agents.

Preparation and Characterization of the PEGylated Encapsulated Curcumin Nanoliposomes

The microscopic examination of the resulting mixture has confirmed the synthesis of the liposomes (Fig. 1a). Morphological analysis of the PEGylated encapsulated curcumin nanoliposomes with transmission electron microscopy has confirmed the presence of uniformly sized (∼ 52 nm) spherical symmetry vesicles, along with the presence of very few large size aggregates (Fig. 1b). Despite size differences, the similar morphological observation was recorded for polymer assisted nanoliposomes [5]. Small particle size of the nanoconjugates was responsible for efficient uptake of the drug and PCNL with smaller particles exhibits a better possibility of its uptake and transport through the gut-associated cells [3, 5]. Infrared (IR) spectrum of PEGylated curcumin nanoliposomes showed the presence of absorption peaks at 3734 cm^{-1}, 2856 cm^{-1}, 2340 cm^{-1}, 1811 cm^{-1}, 1428 cm^{-1}, 1262 cm^{-1}, 1084 cm^{-1} (Fig. 1c). The presence of these peaks may be attributed to the strong and sharp stretching vibration of the hydroxyl group, C–H medium stretching of the alkane, CO_2, carbonyl group of the curcumin, bending of C–H, ether group of curcumin and C–O stretching in the 1° alcohol respectively. Similar signature peaks for liposomes and encapsulated curcumin were also observed in other studies [5, 23]. These characterization studies indicate the successful formation of PEGylated encapsulated curcumin nanoliposomes.

Physiological Characterization of Nanoconjugated Curcumin

The PEGylated curcumin nanoliposomes (PCNL) showed significant improvements in characteristics like loading, solubility, stability and release kinetics. PCNL have shown a good curcumin loading (60.65 ± 1.68 μg/μl) and a good encapsulation efficiency of 76.3% which was higher than previous polymer or liposome based studies [25]. The large surface area of nanoliposomes and hydrophobic internal environment might allow better diffusion of the curcumin within nanoliposomes that might have significantly improved curcumin loading. A similar observation of higher drug loading has been observed in previous studies using PEGylated nanoliposomes as a drug delivery system [12]. Enhanced drug loading or encapsulation is an inherent property associated with PEGylated nanoliposomes [24].

Aqueous solubility studies indicate higher curcumin availability in such systems. The amount of soluble curcumin in PCNL (20.4 ± 2.47 mg/ml) was 1000 times more than the reported value for the native curcumin (20 μg/ml) [26]. Small particle size could be responsible for the high curcumin solubility. Similarly, an enhanced solubility of

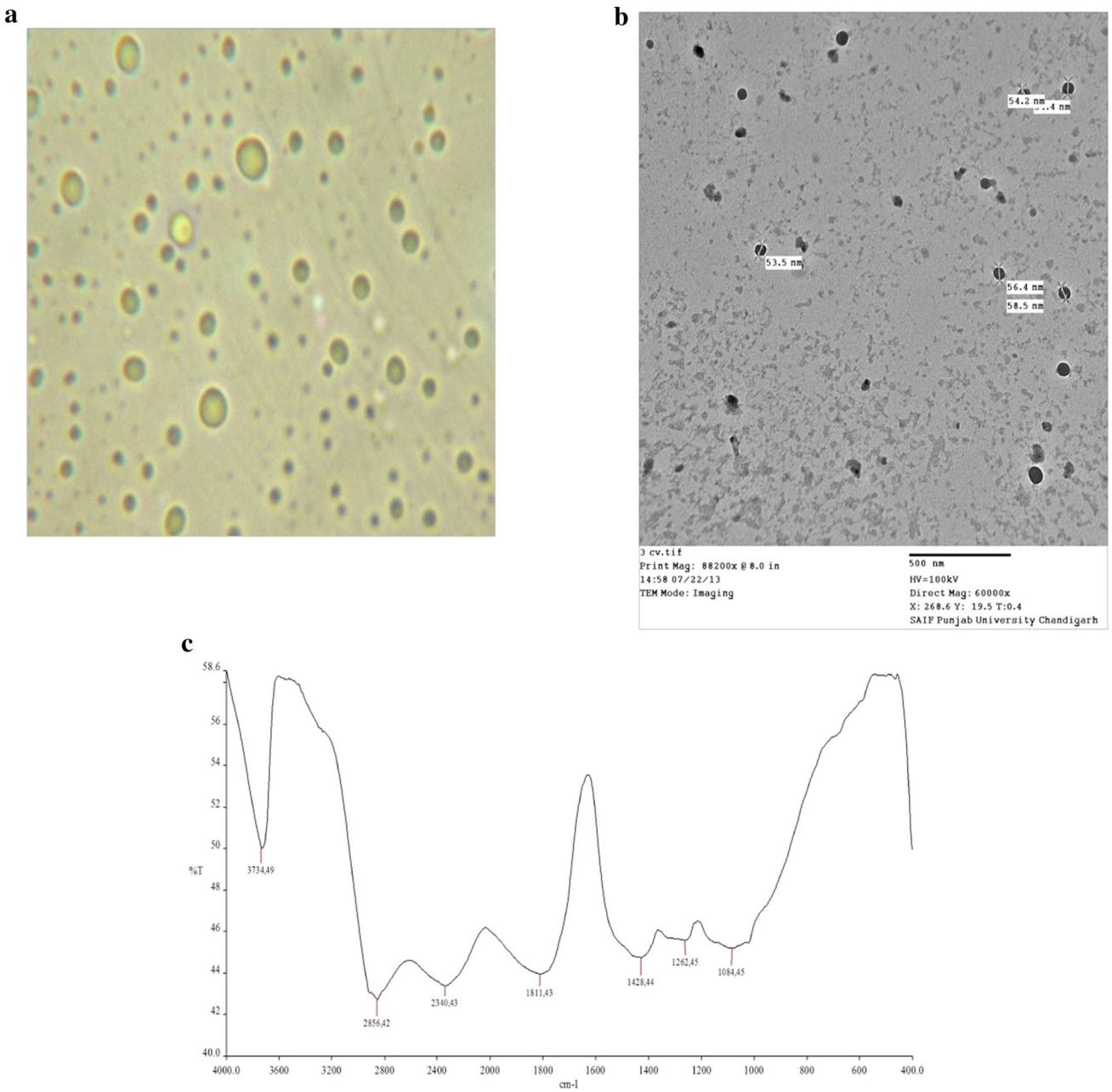

Fig. 1 Characterization of PEGylated curcumin nanoliposomes. PEGylated curcumin nanoliposomes prior to the ultrasonication (**a**) and after the sonication (**b**). FTIR spectra of PEGylated curcumin nanoliposomes (**c**)

ceramide C6 was reported after its incorporation within liposomes, as well as no trace of insoluble precipitates were identified [27]. Similarly, usage of glucosides has improved the aqueous solubility of the curcumin [28]. The higher dissolution rate of PCNL than the native curcumin was attributed to modification in the surface properties due to the addition of water soluble polymer [28]. PEGylation of nanoparticles also help in improving the aqueous solubility of the particles [7, 8]. The better curcumin solubility may also be ascribed to the increased hydrophobic interactions between the hydrophobic core of polymer and the drug [7, 8].

It is well known that the native curcumin is less stable and degrade easily in water which was the reason behind the poor pharmacological efficiency of native curcumin [2, 7]. Thus it became important to analyze the stability of conjugated curcumin in aqueous environments. Stability studies with liposome encapsulated curcumin have indicated that conjugated curcumin is highly stable and only $18 \pm 0.6\%$ curcumin degradation was observed after 12 h of incubation in an aqueous

environment (Fig. 2). Comparatively more than 90% of the native curcumin degrades after 12 h of incubation in aqueous environment. Additionally, almost tenfold higher degradation was observed for native curcumin in comparison to the PCNL. It is shown that the curcumin is more stable in PCNL than the native curcumin. This enhanced stability could be due to its limited exposure to the aqueous environment. The nanoliposomes membrane, as well as polyethylene glycol might have extended protection to the curcumin. Similar pattern of enhanced drug stability was observed when PEGylated nanoliposome as a drug delivery system [29].

Voluminous literature reports the significance of nanoparticles in peroral drug delivery systems with the advantage of slower but persistent release which reduces the amount of drug intake [24, 29]. A superior release of curcumin from the PEGylated curcumin nanoliposomes was observed in intestinal and gastric juice during in vitro release kinetics studies (Table 1). More than 70% release of the curcumin was obtained in intestinal juice and about 50.78% release was in gastric juice. The curcumin percentage release was found higher than the previous studies [5, 16]. The release kinetics of drug was dominated by the diffusion of the drug from the polymer pores but to a little extent also depend on the nature of media as obtained in the current report. Diffusivity of the drug depends on molecular weight and size of the nanoparticles formed. The decrease in the particle size of active ingredients in nano-range size has shown improvement in its efficacy, solubility, and bioavailability. Additionally, PEG associated nanoconjugates [9], nanoliposomes [11, 12], as well as admixture of both [24, 29] are known to improve drug release kinetics. A similar observation was recorded in the current study. Physiological characterization of PEGylated curcumin nanoliposomes indicates the current matrix has good curcumin encapsulation efficiency. Additionally this matrix enhanced curcumin stability and solubility, as well as significantly improved the curcumin release from this matrix in the artificial gastric and intestinal environment. These results clearly indicate that the current system has overcome the limitations of the native curcumin for its usage as a potential therapeutic agent. Though current system have overcome key physico-chemical challenges but the functionality of the encapsulated curcumin is still uncertain. Hereby curcumin bioactivity must be analyzed to assess the impact of current matrix on the curcumin bioactivity.

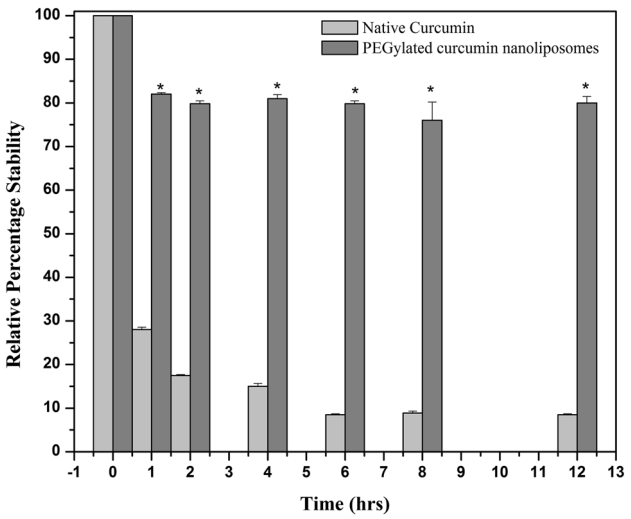

Fig. 2 Relative stability of the native and conjugated curcumin in aqueous solution. Here * indicates statistical significant ($p < 0.05$) improvement in conjugated curcumin stability over native curcumin. All experiments were preformed in triplicate

Bioactivity of the PEGylated Curcumin Nanoliposomes

Antimicrobial activities of the PCNL, native nanoliposomes and native curcumin were performed in triplicate against gram-positive i.e. *B. subtilis;* gram-negative i.e. *K. pneumoniae, C. violaceum,* native *E. coli DH10B* and acid-fast bacteria *M. smegmatis* using a disk diffusion assay (Table 2). PCNL have shown significant antibacterial activity against both all tested microbes including antibiotic resistant microbial strains. Additionally, the antimicrobial activity of the PEGylated curcumin nanoliposomes against all the tested strains was found to be enhanced as compared to the native nanoliposomes and native curcumin. Solubility assay has indicated that the aqueous solubility of nanocurcumin was more than the native curcumin. This is the basic requirement for its antibacterial application. The particle size is responsible for the better penetration and higher uptake by the cells which in turn was the rationale for the stronger activity of nanocurcumin [30]. PCNL showed a significant enhancement in the antimicrobial activity for acid-fast bacteria *M. smegmatis* and gram-negative *C. violaceum* as compared to other bacterial strains. The difference in inhibition zone for various bacteria is ascribed to differences in their constituents and structure of cell membrane. It is well known that the peptidoglycan layer in Gram-positive bacteria and phospholipid membrane in Gram-negative interact differently with the curcumin [30]. It is already known that particles with radius less than 100 nm directly target the cell membrane by the enhanced permeability and retention (EPR) effect [31]. As the size is below 100 nm so the curcumin nanocomposite may penetrate inside the cell by breaking the peptidoglycan layer and cause disruption of the original cell structure and kill the cell through lysis [16]. Enhanced antibacterial activity of the PCNL was the result of the combined effect of the improved curcumin

Table 1 Quantitative release of the curcumin in artificial intestinal and gastric juice

Sr. no.	Nature of curcumin nanoconjugate	Amount of released curcumin (mg/ml)	% of curcumin released in artificial juice
1.	PCNL in intestinal juice	21.4 ± 1.29	70.76
2.	PCNL in gastric juice	15.4 ± 1.9	50.78

Table 2 Antibacterial activity of PEGylated curcumin nanoliposomes, native nanoliposome, against various microbial strains

Test materials (→) Microbes tested (↓)	PEGylated curcumin nanoliposomes (mm)	Native nanoliposome (mm)	Native curcumin (mm)	Control disc (mm)
Escherichia coli DH10B	13.5 ± 1.5	Nil	10 ± 1	Nil
Bacillus subtilis	13.5 ± 0.5	Nil	11 ± 0.5	Nil
Mycobacterium smegmatis	16.5 ± 0.5	Nil	10	Nil
Klebsiella pneumoniae	11.1 ± 1.0	Nil	10 ± 1	Nil
Chromobacterium violaceum	16.5 ± 0.5	Nil	10 ± 2	Nil

loading, solubility, bioavailability and in vitro release of the curcumin in the human media [31]. Additionally, PCNL has significantly reduced the violacein production by the *C. violaceum* (MTCC No. 2656). It clearly indicates that the nanoconjugated curcumin act as quorum sensing inhibitors [17]. Quorum sensing inhibitors recently described as a novel strategy to counter the infections [32–38]. Quorum sensing inhibition property of PCNL made it a potent therapeutic agent [32–38].

Antifungal activities of PEGylated curcumin nanoliposomes, native nanoliposomes and native curcumin were performed in triplicate against *A. niger*, *C. albicans* and *F. oxysporum* using a disk diffusion assay (Table 3). PCNL also showed the enhanced (13.0 mm in *A. niger)* or similar activity in comparison to the native curcumin. Higher drug storing efficacy of polymer nanoparticles provides better pharmacokinetic profiles, bioavailability and evenly drug distribution [39]. Polymer encapsulated drug also showed improved adaptable behavior in human media such as gastric and intestinal juice [39]. In a previous study various cell cytotoxicity assay were performed and found that curcumin showed antifungal activity by affecting PM-ATPase, ergosterol biosynthesis, and proteinase secretion [40].

The antioxidative activity of the PCNL was analyzed with ABTS radical scavenging assay, DPPH radical scavenging assay and Nitric oxide scavenging assay in reference to native nanoconjugates. It was observed that the absorbance decreases with increase in the concentration of test material which indicates the enhancement in antioxidant activity with increase in concentration. In case of DPPH assay, the percentage inhibition was 49.5 ± 1.7 at 2 μg/ml concentration of nanoconjugate. In case of ABTS assay, %age inhibition was 23.5 ± 2.1 with nanoconjugate (2 μg/ml). Similarly, for nitric oxide assay, %age inhibition was 34.9 ± 1.5 using PCNL. Antioxidant activity assays indicate that the synthesized nanoconjugates showed superior antioxidant activity for DPPH and nitric oxide scavenging assay. It is well known that curcumin shows keto-enol equilibrium [41] and this equilibrium was responsible for physicochemical and antioxidant behavior. The antioxidant activities of curcumin nanoconjugates are ascribed to the removal of the H atom from the phenolic or methylene group [41]. In acidic medium keto form

Table 3 Antifungal activity of PEGylated curcumin nanoliposomes, native nanoliposome and native curcumin against various fungal strains

Test materials (→) Fungus tested (↓)	PEGylated curcumin nanoliposomes (in mm)	Native nanoliposome (mm)	Native curcumin (mm)	Control disc (mm)
Aspergillus niger	13.0	Nil	12	Nil
Candida albicans	11.5 ± 0.5	Nil	12	Nil
Fusarium oxysporum	10.5 ± 0.5	Nil	10	Nil

predominates and abstraction of H atom occurs from the C12 methylene group whereas in basic medium enol form predominates and removal of H atom occurs from phenolic groups [41]. The good antioxidant activity of the nanoconjugates also proved helpful for the improved antimicrobial activity of the nanoconjugates.

Bioactivity assays clearly indicate that nanoconjugated curcumin in PEGylated curcumin nanoliposomes not only maintains its inherent property like antibacterial, antifungal and antioxidant, as well showed better performance than the native curcumin. These results make PEGylated curcumin nanoliposomes as potential anti-infective and antioxidative molecule for therapeutic treatments.

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Compliance with Ethical Standards

Conflict of interest The author declares that they have no conflict of interest.

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