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Separation of Curcumin from *Curcuma longa* L. and its Conjugation with Silica Nanoparticles for Anti-cancer Activities

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ABSTRACT

Curcumin is the natural bio-active component of turmeric (*Curcuma longa* L.) with known therapeutic properties; nevertheless, its biological applications are limited due to its poor bioavailability. To overcome this limitation, curcumin was conjugated with silica nanoparticles. Curcumin was separated from turmeric by microwave-assisted extraction and silica nanoparticles were developed from rice husk. Conjugation of curcumin with silica nanoparticles was performed through ultrasound-assisted wet impregnation. XRD, FTIR and UV-visible analyses confirmed the successful synthesis of the conjugate; the drug loading in the nanoparticle was 39% as determined by HPLC analysis. TEM and AFM analyses indicated the spherical morphology of the conjugate with average particle size of 85.9 nm. The cell killing activity of the conjugate was tested against HeLa, MCF-7 and Saos-2 cancer cell lines and normal fibroblast cell line using MTT assay. The silica:curcumin conjugate was effective for destruction of cancerous cells, especially HeLa cells, with minimum side effects on healthy fibroblasts.

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1. INTRODUCTION

Curcumin is a polyphenolic compound derived from the rhizome of turmeric (*Curcuma longa* L.) [1, 2]. This bio-active compound has shown numerous amazing therapeutic properties as anti-oxidant, anti-inflammatory, anti-proliferative, anti-angiogenic, wound healing, anti-microbial, anti-tumor, anti-HIV, anti-viral, anti-diabetic, hepatoprotective, anti-atherosclerotic, antithrombotic, anti-arthritic and cholesterol lowering agent. Despite these highly encouraging health benefits, curcumin has very low aqueous solubility, rapid hydrolytic degradation, poor membrane permeation, low physico-chemical stability and poor bioavailability [3]. The poor aqueous solubility of curcumin is presumably the major reason for its low bioavailability and might be considered as the main challenge for its clinical development.

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In selection of suitable delivery systems which could incorporate the bio-active compound, meanwhile modify its dispersion status and improve its physico-chemical stability, several considerations have to be taken into account among which are biocompatibility, cost-effectiveness and biodegradability of the developed biomaterial [4, 5]. Silica derived from natural resources has found numerous potential applications in biomedical fields and current toxicological applications. Among the available agricultural waste residues, rice husk is considered as a valuable non-metallic and cost-effective bio-precursor for biogenic silica synthesis that can be used as a versatile vehicle for biomedical applications [4, 5]. Silica-based nanoparticles have widely been used for biomedical applications because of their low toxicity and high biocompatibility [6]. Alshatwi et al. [5] demonstrated that biogenic silica nanoparticles are biocompatible with human lung fibroblast cells.

Silica nanoparticles were reported to have interesting properties such as high specific surface area, biocompatibility and low toxicity, capacity for encapsulation, hydrophilic surface favoring protracted

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circulation, low density and versatile silane chemistry for surface functionalization [7, 8]. They can be widely used in materials such as fillers, pharmaceuticals, catalysts and chromatography [9].

In previous studies, silica matrix was used for improving bioavailability and/or delivery of different drugs. Gangwar et al. [10] showed that the conjugation of curcumin to synthetic silica nanoparticles improved the bioavailability of curcumin. Furthermore, their synthesized nanoparticles had anti-cancer effect against HeLa cell lines. Singh et al. [11] loaded curcumin on silica nanoparticles and found that the phototoxic effect of curcumin in oral carcinoma cells improved.

In present work, curcumin was first isolated from turmeric by microwave-assisted extraction method. The extracted curcumin was then conjugated with rice husk-derived biogenic silica nanoparticles through a simple wet impregnation method. The developed curcumin conjugated silica nanoparticles were then characterized and their anti-cancer properties on HeLa, MCF-7, Saos-2 and normal fibroblast cell lines were investigated.

2. MATERIALS AND METHODS

Turmeric rhizome was purchased from a local market (Amol, Iran) and ground to an average size of 0.21 mm. Rice husk was obtained from a local rice mill (Amol, Iran); it was washed to remove dust and then dried in oven. Sodium hydroxide, acetone, methanol, hydrochloric acid, n-hexane, standard curcumin for HPLC analysis and ethanol were purchased from Merck, Germany. Dulbecco's Modified Eagle medium (DMEM), Roswell Park Memorial Institute medium (RPMI), fetal bovine serum (FBS), penicillin and streptomycin were purchased from ATOCEL, Austria. 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was provided from Alfa Aesar, USA.

Separation of curcumin from turmeric powder was carried by microwave-assisted extraction following our previous experiments [12]. In a typical experiment, 6 g of turmeric powder was mixed with 100 ml acetone and irradiated under microwave in an intermittent way, i.e. irradiation-cooling-irradiation; the input power was set at 100 W with fixed irradiation and cooling time of 2 min. After microwave extraction, the solid residue was filtered out and the solvent was evaporated in oven at 50 °C. For reducing the oil content of so-obtained oleoresin, the dried oleoresin was treated with n-hexane and then filtered and dried. Next, 0.13 g of defatted oleoresin was dissolved in 20 ml methanol, then 30 ml distilled water was added as anti-solvent. The resulting yellow solution was chilled in refrigerator at 4℃ upon which curcumin crystals were appeared. The crystals were then separated and dissolved in methanol to determine the curcumin content by HPLC analysis.

The method for synthesis of silica nanoparticle from rice husk was adopted from the work of Athinarayanan et al. [4] with some minor modifications. For this purpose, 40 g of rice husk was mixed with 200 ml of 1 N HCl and autoclaved at 121°C for 45 min under pressurized (15 psi) condition. The acid pretreated sample was then filtered and washed repeatedly with distilled water to remove the residues of hydrochloric acid; this was assured by measurement of pH in the filtrate. The rice husk was dried in oven at 45°C and then calcined in a muffle furnace at 700 °C for 1 h to obtain a white powder.

Ultrasound-assisted wet impregnation method was used for the synthesis of silica:curcumin conjugate which was very simple and fast. One mg curcumin and 10 mg of synthesized silica were mixed in a reaction flask containing 10 ml ethanol. The suspension was then sonicated in a sonicating water bath (Elmasonic, S10 H, Germany) for 5 min at ambient temperature. After sonication, the solvent in the reaction flask was evaporated in an oven at 50℃. The synthesized silica:curcumin conjugate was then subjected to several structural analyses.

X-ray fluorescence (XRF) analysis was used to detect and quantify the chemical composition of rice husk ash using an X-ray fluorescence spectrometer (XRF, ARL PERFOMIR'X). The surface functional groups of the developed nanoparticle were analyzed by Fourier transform infrared spectroscopy (FTIR, WQF-510). To investigate the crystallinity of the samples, X-ray diffraction analysis was performed using an X-ray diffractometer (XRD, D8-Advance Bruker Cu Kα1 (λ= 0.15406 nm)).The samples were analyzed for presence of curcumin by UV-visible spectrophotometer (Analytik Jena AG, Germany). Atomic-force microscopy (AFM, Easyscan2 Flex) and transmission electron microscopy (TEM, Zeiss – EM10C – 80 KV) were carried out to investigate the surface morphology and size of the particles.

The purity of extracted curcumin and loading percentage of curcumin in nanoparticles was determined using high-performance liquid chromatography analysis (HPLC, Knauer, Smartline, Germany) [13].

The amount of curcumin loaded on nanoparticles was determined by HPLC analysis. To this end, 1 mg of curcumin conjugated silica nanoparticle was dissolved in 10 ml methanol to disrupt the nanoparticles' structure and release the conjugated curcumin. The solution was then centrifuged at 6000 rpm for 5 min to separate the silica particles. The obtained clear supernatant was then analyzed by HPLC system for determination of curcumin content. Drug loading content and nanoparticles yield were calculated by Equations (1) and (2) [14, 15]:

$$\text{Drug loading content (\%)} = \frac{\text{Curcumin in nanoparticles}}{\text{Total curcumin input}} \times 100 \quad (1)$$

$$\text{Nanoparticles yield(\%)} = \frac{\text{Weight of nanoparticles}}{\text{Weightofsilica and drug fed initially}} \times 100 \quad (2)$$

HeLa, Saos-2, MCF-7 and normal fibroblast cell lines were obtained from Pasteur Institute of Iran (Tehran, Iran). The cancerous cell lines were maintained in RPMI supplemented with 1% penicillin, 1% streptomycin and 10% of fetal bovine serum at 37 °C in 5% CO_2 atmosphere. The fibroblast cell lines were cultured in DMEM medium (Dulbecco's Modified Eagle *Medium)* supplemented with 10% FBS, 1% penicillin and 1% streptomycin at 37 °C in 5% CO_2 atmosphere. All experiments were performed on the cells from fifth passage. To evaluate the cytotoxic effect of nanoparticles, MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay was used as described by Mosmann [16]. Briefly, cells from passage 5 were seeded at a density of 7×10^3 cells per well in 96-well plate. The plates contained described medium for cancerous and normal cell lines. Cells were treated with different concentrations of curcumin in its nanoformulation (1.25, 2.5, 5 and 10 µg/ml). Subsequently, cells were incubated at 37 °C for 24, 48 and 72 h in 5% CO_2 atmosphere. After incubation, 50 µl of MTT solution (5 mg/ml) was added to each well. The plates were incubated for 4 h at 37 °C in 5% CO_2 atmosphere. At this time, living cells converted MTT solution to insoluble formazan crystals. Subsequently, the purple formazan crystals were dissolved by the addition of 150 µl of acidified isopropanol solution into each well. The absorbance of the resulting solution was monitored at 570 nm using a 96-well plate reader (Rayto, Germany). The cell viability (%) was calculated by the following formula:

$$\text{Cell viability (\%)} = \frac{\text{Optical density of treated sample}}{\text{Optical density of untreated sample (control)}} \times 100 \quad (3)$$

The MTT assay results were represented as the mean cell viability ± SD obtained from triplicate cultures per condition.

3. RESULTS AND DISCUSSION

In this work, curcumin was separated from turmeric by microwave assisted extraction and then purified with methanol and water as anti-solvent. Quantitative analysis of the extracted curcumin was carried out using HPLC. Figure 1 shows HPLC chromatograms of (i) standard curcumin and (ii) extracted curcumin; both samples were prepared at a concentration of 5 µg/ml in methanol. From the HPLC analysis, the purity of isolated curcumin was found to be 70%.

As prior mentioned, biogenic silica has shown good biocompatibility and bioactivity for biomedical applications. In this study, rice husk as an abundant silica rich precursor was used to develop silica nanoparticles. To ensure the synthesis of pure silica nanoparticles, XRF analysis was carried out and the results are summarized in Table 1. As results show, 98.3% of the rice husk derived nanoparticles was composed of SiO_2 with minor amounts of metal oxides including CaO and Fe_2O_3 and traces of Al_2O_3 and MgO. The high purity of silica nanoparticles confirmed the effectiveness of the implemented synthesis method where the hydrochloric acid pretreatment hydrolyzed the lignocellulosic structure of rice husk and removed its inherent metal impurities; subsequent calcination step eliminated the organic substances and helped to develop amorphous silica nanoparticle. After successful synthesis of silica nanoparticles, the curcumin extracted from turmeric was conjugated with silica nanoparticles via a simple method and the obtained silica:curcumin conjugate was subjected to several characterization analyses which are discussed here.

The XRD patterns of the extracted curcumin, biogenic silica nanoparticles and synthesized silica:curcumin conjugate are shown in Figure 2(a).

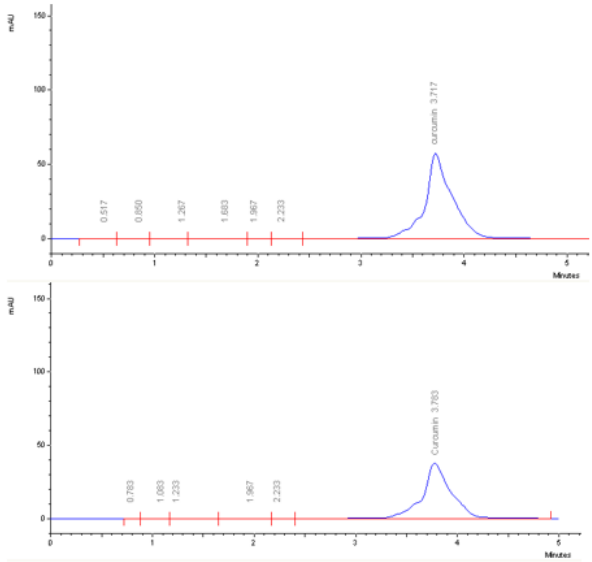


Figure 1. HPLC chromatograms of (i) standard curcumin and (ii) extracted curcumin at concentrations of 5 µg/ml in methanol

TABLE 1. XRF analysis of rice husk derived nanoparticles

Component	SiO_2	CaO	Al_2O_3	MgO	Fe_2O_3	LOI
Content (wt%)	98.3	0.5	0.07	0.04	0.13	0.96

The XRD pattern of extracted curcumin showed several characteristic diffraction peaks in 2θ range of 10º to 30° which correspond to the crystalline nature of hydrophobic curcumin [17]. The XRD spectrum of biogenic silica exhibited a broad peak at 2θ of 22°, characteristic of amorphous silica, without other impurities. The broadness of the XRD peak was an indication of nanoscale dimension of biogenic silica [4]. The XRD pattern of silica:curcumin conjugate showed typical amorphous pattern after conjugation, despite the crystalline nature of curcumin. This result confirms that after loading of curcumin onto the silica nanoparticle, the conjugate retained its amorphous or disordered phase; this is much desired for unimpeded and regular release of curcumin from the conjugate [17].

Figure 2(b) depicts the FTIR spectra of extracted curcumin, silica nanoparticles and silica:curcumin conjugate. The main peaks in the FTIR spectrum of extracted curcumin (spectrum (i)) were free O−H group appearing at 3504-3871 cm^{-1}, C=O and C=C (enol) groups located at 1450−1630 cm^{-1} [10, 18] and the peak at 1000−1300 cm^{-1} which was probably attributed to symmetric and asymmetric configurations of C−O−C chains [10]. The spectrum of silica nanoparticle (spectrum (ii)) displayed characteristic peaks corresponding to asymmetric vibration of Si-O (1100 cm^{-1}), symmetric vibration of Si-O (808 cm^{-1}) and Si-O bending vibration (469 cm^{-1}) [10, 19].The band corresponding to the stretching vibration of O-H appeared at 3200-3600 cm^{-1} and another one with lower intensity observed at 1573-1643 cm^{-1}; both were assigned to the water physically bound to silica [19].

The spectrum (iii) belongs to the curcumin loaded silica nanoparticles in which, signatures of both curcumin and silica can be observed. The intensity of the characteristic peaks of curcumin reduced in the spectrum of curcumin loaded nanoparticle which could be an indication of conjugation of curcumin with silica. It is most plausible that curcumin attaches to the silica via its enolic hydroxyl group (Figure 2 (d)) which resulted in shifts of Si−O stretching from 1100 to 1099 cm^{-1} and 469 to 460 cm^{-1} [10, 20].

Figure 2(c) exhbits the UV-vis spectra of the extracted curcumin, silica and silica:curcumin conjugate collected in the range of 350 to 800 nm using UV-vis spectrophotometer. The spectrum of standard curcumin showed an absorption peak at ~ 420 nm which is an indication of diarylheptanoid chromophore group of curcumin. The UV-vis spectrum of silica:curcumin conjugate showed the characteristic peak of curcumin at ~ 420 nm which indicates that the diarylheptanoid chromophore group of curcumin remained intact; nevertheless, the absorption band broadened due to conjugation of curcumin to silica. Previous researchers have expressed that the size of drug nanocarrier is important for effective cancer targeting [21].

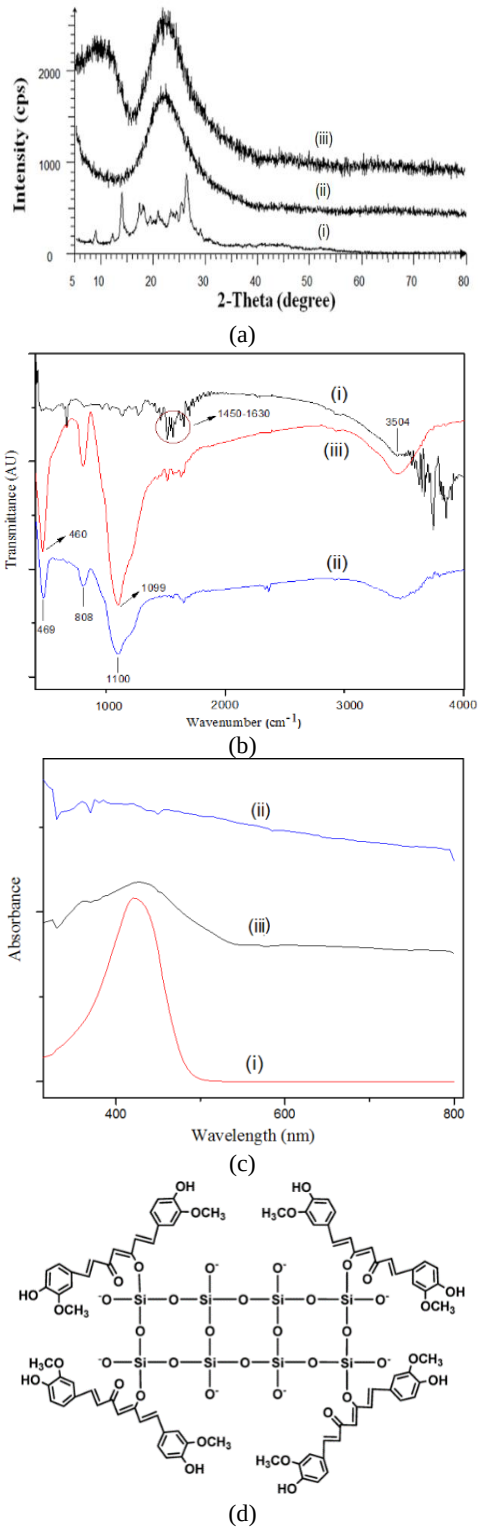
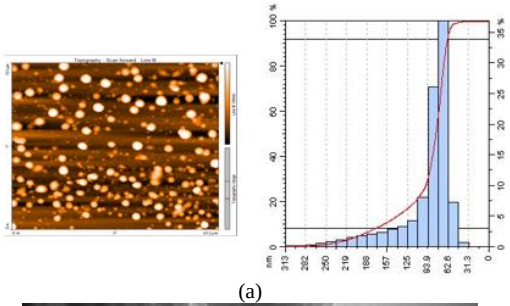


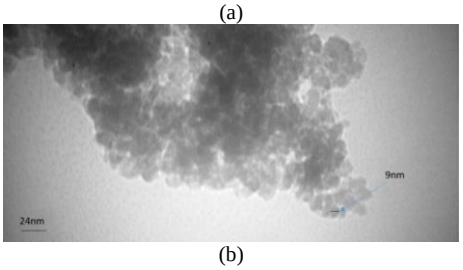
Figure 2. (a) XRD patterns of (i) extracted curcumin, (ii) silica nanoparticles and (iii) silica:curcumin conjugate, (b) FTIR spectra of (i) extracted curcumin, (ii) silica nanoparticles and (iii) silica:curcumin conjugate, (c) UV-vis spectra of (i) extracted curcumin, (ii) silica nanoparticle and (iii) silica:curcumin nanoparticle and (d) scheme of plausible conjugation of curcumin with silica nanoparticle

Experiments suggest that the size of nanoparticle therapeutics for cancer should ideally be in the range of 10–100 nm [22, 23], although some studies have pointed to the suitability of nanoparticles with diameters below 200 nm [24]. To determine the size and shape of the synthesized nanoparticles, AFM and TEM analyses were carried out. Figure 3(a) illustrates the AFM image and particle size distribution of silica: curcumin conjugate. The AFM results showed the spherical morphology of the conjugate and the average particle size was found to be 85.9 nm. The TEM image of the conjugate is depicted in Figure 3(b). The TEM result also confirmed the spherical morphology of the synthesized conjugate and that the size of the developed curcumin loaded silica nanoparticle is suitable for cancer therapy.

It was observed that silica:curcumin nanoparticles homogenously dispersed in water; whereas, intact curcumin could barely dissolve in water and curcumin particles aggregated on the water surface or deposited at the bottom of the bottle. Synthesis of water-soluble curcumin was a good achievement through which curcumin's cytotoxicity could be improved. The drug-loading and silica:curcumin nanoparticles yield were 39 and 89%, respectively, as calculated using Equations (1) and (2) through HPLC analysis. The results of FTIR and UV-vis indicated that the diarylheptanoid chromophore group of curcumin remained intact after conjugation which is much required for biomedical applications [10]. Due to such characters and considering the appropriate particle size of the developed soluble conjugate, it could be expected that the conjugate act as a potential anti-cancer agent.



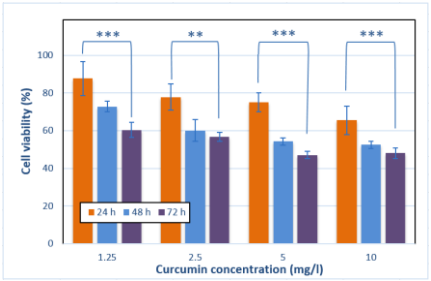
(a)



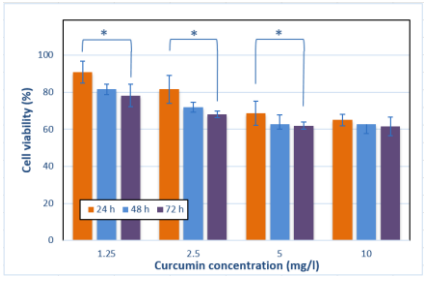
(b)

Figure 3. (a) AFM image and particle size distribution of the silica:curcumin conjugate and (b) TEM image of the synthesized silica:curcumin conjugate

To examine such permise, the cytotoxic activity of the silica:curcumin conjugate against some human cancer cell lines was evaluated. The cytotoxicity effect of the curcumin loaded nanoparticles was studied against some carcinoma cell types including HeLa (cervix), MCF-7 (breast), Saos-2 (bone) cell lines as well as normal fibroblast cell line using MTT assay. The cells were incubated with different concentrations (1.25, 2.5, 5 and 10 µg/ml) of curcumin; the cell viability was determined after 24, 48 and 72 h treatment. The results of this study are projected in Figure 4.



(a)



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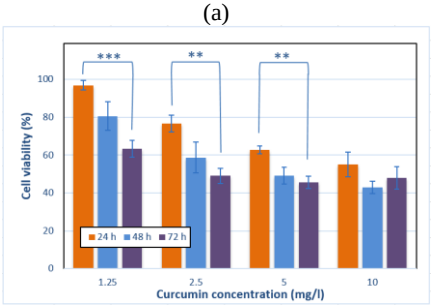
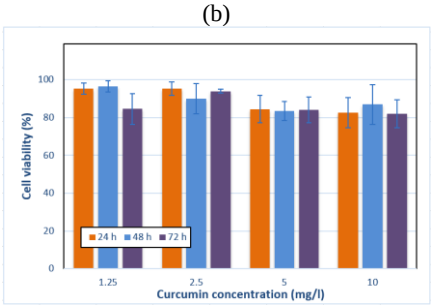


(c)



(d)

Figure 4. Cell viability percentage determined by MTT assay for (a) HeLa cell lines, (b) Saos-2 cell lines, (c) MCF-7 cell lines and (d) fibroblast cell lines after treatment with different concentrations of synthesized silica:curcumin conjugate. Significant differences are indicated by * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$)

The general trend was that with increase of curcumin concentration and incubation time the cytotoxic activity of silica:curcumin nanoparticles increased. Cytotoxicity results show that the cell viability of the HeLa, Saos-2, MCF-7 and fibroblast cells after 72 h of incubation with the nanoparticles containing 10 µg/ml of curcumin was 48.06, 61.60, 48.49 and 82.69%, respectively. According to the results, the curcumin conjugate was more toxic to HeLa cell lines compared to other cell lines.

4. CONCLUSION

In this work, curcumin was separated from turmeric using microwave-assisted extraction; the purity of extracted curcumin was 70% as determined by HPLC analysis. Attempts were made to overcome the low solubility of curcumin in aqueous environments through its conjugation with biogenic silica nanoparticles which were derived from rice husk. The conjugation was carried out through ultrasound-assisted wet impregnation which was simple and very fast. The successful synthesis of silica: curcumin nanoparticles was confirmed by XRD, FTIR and UV-visible analyses where curcumin loading in the conjugate was 39%. Moreover, the TEM and FTIR analysis showed the spherical morphology of the conjugate with average particle size of 85.9 nm which made it suitable to be used as anti-cancer agent. The cytotoxicity of the developed conjugate against some cancerous cell lines including HeLa, Saos-2 and MCF-7 cells as well as normal fibroblast cells was studied. Promising results were achieved where the conjugate could effectively kill the growing cancerous cells, while its impact on healthy cells was much less. Nevertheless, more complementary investigations are required before curcumin could be exploited as an injectable conjugate for cancer therapy.

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Anti-cancer Activity

چکیده

کورکومین ترکیب زیست‌فعال طبیعی موجود در زردچوبه (کورکوما لونگا) با اثرات درمانی شناخته شده است؛ با این حال، کاربرد بیولوژیکی آن با توجه به زیست‌فراهمی پائینی که دارد محدود است. به منظور غلبه بر این محدودیت، کورکومین با نانوذرات سیلیکا ترکیب شد. کورکومین با استخراج به کمک مایکروویو از زردچوبه جداسازی شد و نانوذرات سیلیکا از شلتوک برنج تولید شدند. ترکیب کورکومین با نانوذرات سیلیکا با روش خیساندن مرطوب با کمک امواج فراصوت انجام شد. آنالیزهای XRD، FTIR و امواج مرئی–فرابنفش سنتز موفق این ترکیب را تایید کردند؛ بارگذاری دارو روی نانوذره ۳۹٪ بود که با آنالیز HPLC تعیین گردید. آنالیزهای TEM و AFM نشان دادند که مورفولوژی نانوذرات کروی بوده و متوسط اندازه ذرات ۸۵/۹ نانومتر بود. خاصیت ضدسرطانی ترکیب سنتز شده روی سلول‌های HeLa، MCF-7 و Saos-2 و همچنین سلولهای فیبروبلاست نرمال با استفاده از تست MTT مورد بررسی قرار گرفت. نتایج نشان داد که ترکیب کورکومین:سیلیکا روی سلول‌های سرطانی، به خصوص سلول‌هایHeLa ، اثر مخرب داشت درحالی که کمترین اثر جانبی را روی سلول‌های فیبروبلاست سالم نشان داد.

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