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Research Article

Preparation and *In-Vitro* Evaluation of Ketoconazole-Loaded Niosome (Ketosome) for Drug Delivery to Cutaneous Candidiasis

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Abstract

Background: Recently, niosomes are becoming popular in drug delivery. The current work aimed to investigate the characteristics, cellular safety, and antifungal activity of ketoconazole-loaded niosome (ketosome).

Methods: Ultrasonic approach was employed to prepare ketosome including cholesterol, nonionic surfactant and ketoconazole. The size characteristics and morphological features of ketosome and physicochemical properties of ketoconazole in ketosomes were evaluated using dynamic light scattering (DLS), differential scanning calorimetry (DSC), powder x-ray diffractometer (PXRD), scanning electron microscopy (SEM), and attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy. Also, the dissolution rate, cellular safety test and antimycotic properties of ketosome were examined.

Results: According to the results, the particle size of the ketosome decreased from 491.400±10.622 to 121.300±7.274 nm by the increment of cholesterol. According to further research, changes in the cholesterol:surfactants ratio can modulate the zeta potential from -27.866±1.069 to -12.500±1.153 mV. The highest entrapment of ketoconazole was about 87% when the cholesterol concentration in the ketosome was high. Ketosome with the maximum cholesterol:surfactants ratio showed the fastest drug release. Furthermore, the cell viability assay revealed that the ketosome had lower cytotoxicity in comparison with pure drug. The cell viability of the ketosome was estimated to be about 90% (HGF cell line). The ketosome had a lower MIC than the pure drug when tested against *Candida albicans*.

Conclusion: The results of this study revealed that the optimized ketoconazole-loaded niosome could be used as a possible nanovesicle for ketoconazole drug delivery, potentially opening up new ways for the management of cutaneous candidiasis complaints.

Introduction

In recent years, the prevalence of invasive fungal infections has risen. *C. albicans* are common human opportunistic fungal pathogen causing diseases ranging from mucosal to systemic infections for a variety of immunocompromised patients. The main risk factors for fungal infections are immunosuppression and the deterioration of anatomical defenses such as the skin.[1] Cutaneous candidiasis is a widespread skin infection that affects people of all ages, accounting for about 1% of all outpatient and 7% of all inpatient visits to dermatological clinics. *Candida* may cause skin disorder on its own or as a result of other skin conditions such as atopic dermatitis, psoriasis, or existing diaper dermatitis. In the last fifty years, several therapies such as imidazoles for cutaneous candidiasis have been studied.[2]

There are some limitations to antifungal agents. Many antifungals, such as imidazole derivatives, have poor solubility, which presents pharmaceutical formulation challenges in achieving effective therapeutic dosage forms.[3]

However, by modification of formulations, therapy will be improved while playing the main role in

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future antifungal approaches.[1] Ketoconazole (Ket) is an antimycotic with a wide range of activity against candidiasis. Because of its efficacy in treating seborrheic dermatitis as well as other imidazoles, Ket has emerged as the top candidate among treatment choices.[4] Ket prevents the formation of ergosterol, the fungal cell membrane's main sterol derivative. Ergosterol depletion causes changes in membrane permeability, which are incompatible with fungal survival and fertility.[5] Ket is provided as parenteral and oral dosage forms causing severe adverse effects such as hepatotoxicity and adrenal insufficiency.[6] Ket with a molecular weight of 531.4 Da and logP 4.3 has poor solubility in water.[7,8] Poor solubility of Ket has made it difficult to form an effective drug delivery system.[9] The dissolution rate of poorly water-soluble drugs regularly becomes a rate-restraining phase in their absorption from the GI tract.[10] There are several solubilizing approaches for increasing the dissolution features and drug solubilities, such as using a surfactant, polymeric conjugates, solid dispersion, and colloidal nanoparticle carriers.[11]

Colloidal drug carriers based on nonionic surfactants, including niosomes, have been proposed as an effective product for transporting higher amounts of medicine substance through the membrane at sustained release for systemic absorption.[12] Niosomes are generally nontoxic, have minimum manufacturing costs, and are stable for a longer period of time.[13] Ket-loaded niosomes would be used as a vehicle for extended and sustained drug targeting, potentially improving the drug's antifungal properties.[14] Few studies on the antifungal activity of Ket-loaded niosomes against *Aspergillus niger* are accessible.[15] In general, niosomes are prepared by conventional thin-film methods, which often require the induction of organic solvents at one or more stages of the process. With these processes, residual solvents pose health risks to humans. Moreover, the multistep process with evaporation steps is expensive and time-consuming. To overcome these complications associated with thin-film hydration methods, a facile, ecofriendly, green, and cost-efficient preparation approach (ultrasonication method) is required for the development of niosomes without the use of organic solvents.[16]

To the best of our knowledge, there has been no research into using the green ultrasonication method to manufacture ketosome, thereby preventing the use of unsafe additives. In order to achieve ketosomes with the highest degree of penetration, the effect of the cholesterol:surfactants (Chol:sur) ratio (w/w) was also investigated. The *in vitro* dissolution profile of the improved ketosome was evaluated, as well as the efficacy of Ket, using an *in vitro* mycotic infection pattern and cellular safety test.

Materials and Methods

Materials

Ketoconazole (Ket) was gifted from Darou pakhsh Co. (Tehran, Iran). Sorbitan monolaurate (Samchun Pure Chemical Co., Ltd. Pyeongtaek-si, Korea) was also used. Poloxamer 188 and Chol were purchased from Sigma (Hamburg, Germany). A Human Power 2 device was used to purify distilled water (human Co., Seoul, Korea). Roswell Park Memorial Institute (RPMI 1640 medium) was purchased from Merck Co (Frankfurt, Germany). Dulbecco's Modified Eagle Medium (DMEM medium) was obtained from Gibco (New York, USA).

Quality by design (QbD) - niosome batch experimental design

The design of experiments provided in Table 1 was generated using a QbD approach. The two categories shown are the niosome formulation. Critical Material Attributes and the Critical Processing Parameters considered in this study. The process parameters and formulation element along with the manufacturing process for the niosomal dispersion, are shown in Table 1.

Table 1. Design of experiments by a Quality by Design (QbD) for niosome formulation.

	Critical Material Attributes				Critical Processing Parameters						Results
Formulation	Ket (mg)	Sorbitan monolaurate (mg)	Poloxamer 188 (mg)	Chol (mg)	T (°C)	Chol:sur ratio	Rate of mixing (RPM)	Mixing time (min)	Sonication power (Amplitude)	Sonication time (min)	Organoleptic characteristics
Ketosome 1	50	150	150	30	70	1:10	600	30	20	3	White-milky stable
Ketosome 2	50	150	150	60	70	1:5	600	30	20	3	White-milky stable
Ketosome 3	50	150	150	90	70	3:10	600	30	20	3	White-milky stable
Ketosome 4	50	150	150	120	70	2:5	600	30	20	3	White-milky stable
Ketosome 5	50	150	150	150	70	1:2	600	30	20	3	White-milky stable
Ketosome 6	50	150	150	200	70	2:3	600	30	20	3	White-milky stable
Ketosome 6	50	150	150	300	70	1:1	600	30	20	3	White-milky stable

Niosome production by Ultrasonic method

An ultrasonic processing technique was used to make the ketosome. A hotplate stirrer was used to mix the Chol, sorbitan monolaurate, Ket and poloxamer 188 at 70 °C. The water was made warm at an equal temperature to the oily phase. A magnetic hot plate was used to combine the two phases and produce a pre-emulsion at 70°C. After that, a probe sonicator (Bandelin; 3100; Germany, with a 20% amplitude for 3 minutes continuously) was used to sonicate the mixture, which was then immediately cooled via submersing in an ice bath to achieve Ket-loaded niosomes.

Recognizing physicochemical characteristics

The zeta potential, average particle size, and polydispersity index (PDI) of ketosomes were evaluated using a Zetasizer Nano ZS system (Malvern Instruments, Worcestershire, UK) and a dynamic light scattering (DLS) technique at a 90° angle at 25 °C.

Organoleptic Properties

Niosomal dispersions were characterized for visual appearance, color, and odor to confirm their stability.

Drug Entrapment Examinations

For Ket entrapment analysis, niosomal formulations were ultracentrifuged at 18000 rpm for 30 minutes at 4°C using an ultracentrifuge (Sigma 3-30KS, Germany) to separate the Ket-loaded from the dispersion.[17] The absorption of the acquired final solution was measured with a UV–vis spectrophotometer set to 244 nm (UV-Vis JASCO V-630, UK). The quantity of free pharmaceutical substance in the filtered solution was calculated utilizing a spectrophotometer. Eq. 1 was also used to calculate the entrapment efficiency percentage (EE %).

$$EE\,\% = \frac{W_{initial} - W_{free}}{W_{initial}} \qquad \text{Eq. (1)}$$

W_{free} is the amount of Ket in the supernatant, and $W_{initial}$ is the amount of drug primarily added.

Scanning electron microscopy (SEM) Analysis

SEM was used to examine the morphology of the ketosome. A few amounts of the specimen (one drop) were placed on a copper grid that had been carbon-coated. After that, the specimen was dried in the room air, and a gold sputter coating was added to it to make it conductive. The images were captured using a scanning electron microscope (HITACHI S-4160) with a 20 kV accelerating voltage.

Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy

A diamond was used as an ATR on a Cary 630 FTIR spectrophotometer (Agilent Technologies Inc., CA: USA) to investigate Ket-excipient interactions. ATR-FTIR analysis was performed on Ket, Chol, sorbitan monolaurate, poloxamer 188, physical mixture and ketosome powder. To obtain ketosome powder, Ket-loaded niosomes were separated from suspension through centrifuging (3–30 ks Sigma centrifuge). An alpha 1–2 LDplus freeze dryer (Marin Christ, Osterode, Germany) was used to dry the separated niosomes under decreased pressure. A small amount of samples was scanned at 4000-650 $cm^{-1,}$ and the spectra were recorded with a resolution of 2 cm^{-1}.

Differential scanning calorimetry (DSC) examination

A Pyris 6 (PerkinElmer, USA) was used to perform a DSC examination of the ketosome powder and other pure components. The 5 mg freeze-dried ketosome powder, physical mixture, sorbitan monolaurate, Chol, and Ket specimen were weighed and closely packed in aluminum pans. For 30 minutes, the closed pans were placed in an isothermal state at 20°C. The samples were then calorimetrically scanned in an inert N_2 atmosphere. The thermograms were taken at the rate of 20 °C/minute and between 20 and 250 °C. It should be mentioned that indium was used to calibrate the DSC before the analysis of the specimens.

Powder x-ray diffractometer (PXRD) analysis

X-ray diffractometer (X' Pert MPD Philips, Netherlands) was used to obtain the diffraction patterns using CuK radiation. The XRD instrument was equipped with a nickel-filtered graphite monochromator with a wavelength of 1.5406 and operated at 40 kV voltage and 30 mA current with an X'celerator detector. The scans were performed in the 5-80° (2°) range. Diagnostic peaks of pure Ket, Chol, physical mixture, sorbitan monolaurate and ketosome powder in the samples were used to confirm the solid state of the substances used in the ketosome. To obtain ketosome powder, niosomes were separated from niosome suspension through centrifuging (3–30 ks Sigma centrifuge). Then, an alpha 1–2 LDplus freeze dryer (Marin Christ, Osterode, Germany) was used to dry the separated niosomes under decreased pressure.

Drug release

Immersion cells with acetate cellulose membrane (MWCO 12 kDa) were used for the *in vitro* release study. The specimens were located in the cells, and the acetate cellulose membrane was employed on top of the cells, followed by a cap closing. The Immersion cells were placed in the USP dissolution apparatus no. 2. The dissolution vessels were changed with 250 mL beakers, and the dissolution medium was 70 mL ethanol:buffer phosphate pH 6.8 (50:50) at 37 °C. 5 ml of the dissolution medium were discarded at various times (2, 4, 6, 8, and 24 h). Finally, to keep the volume of the dissolution medium constant, the same volume of fresh dissolution medium was added to the receiver phase. The amount of drug has been determined using UV-Vis spectrophotometry (UV-Vis JASCO V-630, UK) at 244 nm.[18]

Stability Studies and shelf-life determination

The stability of the optimum ketosome was evaluated at 4 and 25 °C for up to three months according to ICH guidelines. Physical stability was monitored, and the effects of temperature and time on the size, zeta potential, EE%, and PDI, as well as the changes in the color of the formulation, the creation of precipitate aggregation or lipid ingredients and shelf-life, were determined.

Non-specific cell toxicity assay

The cell toxicity of the formulations was investigated *in vitro* using HGF cells (Human Gingival Fibroblast) which were supplied by the National Cell Bank (Pasteur Institute of Iran; Tehran, Iran). The cells were then seeded in the 96-well microplates for 24 hours at a density of 10^5 cells/well with various concentrations of ketosome, plain niosome without Ket, and Ket (15, 10, 5, 2.5, 1, and 0.5 µg/mL). They were then rinsed with PBS before removing the material, and cell survival was determined using MTT-formazan colorimetry. MTT (0.5 mg/mL) was used, and the cells were incubated for 4 hours at 37 °C. Furthermore, the supernatant was decanted, and the sediment was dissolved in 100 µL of dimethyl sulfoxide (DMSO) containing the formazan crystals. After shaking the microplates for 20 minutes, the optical density was determined at 560 nm with a multi-walled spectrophotometer (Eq. 2). The viability of the cells was calculated by testing different concentrations three times with six additional controls (the cells in medium).

$$\text{Cell viability}(\%) = \left(\frac{\text{optical density (sample)}}{\text{optical density (control)}}\right) \times 100 \qquad \textbf{Eq. (2)}$$

Antifungal susceptibility testing (AFST)

Isolates

To test the effectiveness of ketosome as a drug-binding site protector, we exposed it to nine Ket-susceptible *Candida albicans* strains as well as standard *Candida albicans* strains (ATCC 14053).

The strains were collected from Iranian patients subjected to skin and subcutaneous candidiasis. A PCR-RFLP system using a MspI restriction enzyme was used to classify them to species level.[19] Stock cultures were grown for 48 hours on yeast extract peptone dextrose agar (YEPD; 1% yeast extract, 2% Bacto Peptone, 2% dextrose) at 35 °C to achieve new viable yeast cells. To test antifungal sensitivity, the isolates were inoculated onto Sabouraud Dextrose Agar (SDA; Merck, Germany) and incubated for 24 hours at 35 °C.

Strains and Antifungal agents

Antifungal susceptibility research was done using Ket and ketosome. Ket was dissolved in sterile DMSO, and a two-fold dilution was prepared in RPMI 1640 medium (with L-glutamine but no bicarbonate) with a 0.165 M solution of MOPS buffered pH 7.0 (3-N Morpholinepropanesulfonic acid, which is a perfect buffer for several biological systems at near-neutral pH).

Antifungal susceptibility testing

The Clinical and Laboratory Standards Institute (CLSI) M27-A3 (2018) documents were used to measure the minimum inhibitory concentrations (MIC) for Ket and ketosome. Ket and ketosome were placed into 96-well microdilution plates at a required concentration of 0.031 to 16 µg/mL and 0.002% to 2%, respectively. Isolates that were cultured for 24 hours were used to make *Candida* blastospore suspensions. Yeast cultures were submerged in sterile saline solution and spectrophotometrically measured at 530 nm to an optical density (OD) of 0.09 to 0.13. The final sizes of the stock inoculums ranged from 10^6 to 5×10^6 CFU/ml.[20] Two times test inoculums (10^3 to 5×10^3 CFU/ml) were obtained after a 1:10 dilution of the stock suspension and a 1:100 dilution of the working suspension with RPMI medium.

Statistical analysis

The data were reported as mean±SD, as previously stated. The data was then analysed using SPSS 22.0 (IBM Co., USA). The identified variables were statistically analysed using analysis of variance (ANOVA) and the Tukey test LSD's post-hoc test. Statistical significance was described as a P-value of less than 0.05.

Results and Discussions

Characteristics of prepared ketosome

The ultrasonication method was used to prepare Ket-loaded niosomes. Various Chol to nonionic surfactant (a binary mixture of poloxamer 188 and sorbitan monolaurate) ratios (Chol:sur), were used in this process (1:10, 1:5, 3:10, 2:5, 1:2, 2:3, 1:1 w/w).

The properties of the ketosome compositions are described in Table 2. The particle size of the ketosome significantly declined from 491.4±10.622 to 121.3±7.274 nm ($P< 0.05$) as the Chol:sur ratio increased from 1:10 (ketosome 1) to 1:1 (ketosome 7). The presence of Chol has been shown that improve the cohesion and rigidity of ketosome bilayers.[21] The size of niosomes was reduced as a result of this. According to Chaw *et al.*[18] and Akbari *et al.*[22] studies, niosomes with more Chol can have a smaller radius. Also, The possible reason for this behavior was the increased energy output of the probe leading to excessive heat generation, which caused the decreasing particle size. Moreover, by using the binary mixtures of surfactants (sorbitan monolaurate and poloxamer 188), which regulates the HLB value, the properties of the Ket vesicles can be modified to achieve an optimum value in terms of size.[23]

Each ketosome encapsulation efficiency (EE percent) is also shown in Table 2. The percentage of Ket entrapped in ketosome ranged from 17.989±3.960 to 86.36 ±0.413 percent, as seen in Table 2. The EE percent enhanced as the Chol:sur ratio was increased to 5:5, owning to increment of

Table 2. The investigated ketosome component and physicochemical properties (w/w %). The mean and standard deviation of three determinations were represented in the results (n=3).

Formulation	Formulation composition						Niosome's characteristics			
	Ket[a] (mg)	Chol[b] (mg)	sorbitan monolaurate (mg)	Poloxamer 188 (mg)	Chol-sur ratio[c]	Water up to (ml)	Particle size (nm)	PDI[d]	Zeta potential (mV)	EE [e] (%)
Ketosome 1	50	30	150	150	1:10	20	491.40±10.62	0.784±0.024	-27.86±1.06	17.98±3.96
Ketosome 2	50	60	150	150	1:5	20	340.93±22.65	0.632±0.018	-23.36±1.19	28.46±2.40
Ketosome 3	50	90	150	150	3:10	20	247.60±8.72	0.651±0.025	-21.76±1.65	42.01±1.24
Ketosome 4	50	120	150	150	2:5	20	218.13±20.11	0.511±0.011	-19.56±0.70	59.17±2.40
Ketosome 5	50	150	150	150	1:2	20	148.20±24.26	0.456±0.038	-18.66±0.83	58.06±3.76
Ketosome 6	50	200	150	150	2:3	20	140.23±23.27	0.415±0.013	-15.80±0.65	71.54±4.91
Ketosome 7	50	300	150	150	1:1	20	121.30±7.27	0.338±0.012	-12.50±1.15	86.36±0.41

[a]Ketoconazole
[b]Cholesterol
[c]Cholestrol:surfactant ratio
[d]Polydispersity index
[e]Entrapment efficiency

bilayer rigidity and reduction in drug leakage from niosome membrane. Chol has been shown to affect membrane permeation and EE percent, resulting in less permeable niosomes.[24] Surfactants with the high and low HLB values could also be distributed in the aqueous and oily phases, respectively, thus leading to the better physical stability of the surfactant films at the interface. Also, Chol associates with the surfactant molecules, which in turn changes their physical characteristics and vesicular structures via the regulation of bilayer strength and cohesion, which induces an increase in EE%.[24] The addition of Chol reduces the gel-liquid transition temperature of the vesicles, thus rendering them more stable and less permeable to drug leakage, which can increase the EE%.[25] Mokhtar *et al.*[26] investigated the impact of formulation variables such as Chol amount on flurbiprofen-loaded niosome entrapment. They found that as the Chol:sur ratio increased, the EE percent increased.[26] Abdelbary *et al.*[27] used a coacervation-phase method to load Ket into proniosomes using different concentrations of nonionic surfactant and lecithin. When comparing the EE percent of their sample to the current study, it was discovered that the EE percent achieved using sorbitan monolaurate alone was significantly lower (57%) than the EE percent obtained in the present work (86%).

The higher amounts of Chol reduced the surface charge of ketosomes, as seen in Table 2. For example, when the ratio of Chol:sur was enhanced to 1:1, the negative zeta potential was reduced (-12.5±1.153 mV), while when the concentration of Chol was smaller, the zeta potential was -27.866 ±1.069 mV. Due to electrostatic stabilization of the niosome, ketosome 1 with a maximum zeta potential of -27.866 ±1.069 mV may be preferable because it offers high stability to the ketosome during storage and when combined with water.[28] It was also discovered that the percentage of Ket entrapment decreased when the zeta potential of particles increased, which may be attributed to the distribution of free Ket in the water state or the diffusion layer potential. These findings are in accordance with previous research.[18,29] Also, it was interesting to note that the nonionic surfactants used produced a negative zeta potential surrounding the colloidal particles. This may be attributed to the dipole nature of the ethoxy groups of nonionic surfactants.[30]

The PDI value typically ranged from 0 to 1, with values near zero indicating a uniform and homogenous dispersal.[31] The PDI value described in Table 2 varied from 0.784±0.024 to 0.338±0.012. PDI values higher than 0.7 typically indicate a broad distribution of particle size.[32] It is noticeable from Table 2 that formulations with 300 mg Chol exhibited the minimum PDI value (0.338±0.012). In contrast, the ketosome with the lower amount of Chol (ketosome 1) indicated the maximum PDI value (0.784±0.024). Reduction in PDI value is not only limited to Chol concentration but also relies on some parameters, including the type of surfactant, HLB value of surfactant, sonication power, and particle charge.[16,33] Also, on a regular basis, the Chol reduces the chain order of gel state bilayers and the radius and number of the bilayer.[34] At this end, according to the thermodynamic scaling effect for soft self-assembling particles, the reduction relative to radius or number of bilayer caused the normalized distribution becomes narrower.[35]

The present method for preparing niosome has an advantage over other published methods because it does not use an organic solvent. The physical background of conventional (such as thin-film hydration) and Ultrasonic techniques is quite different when considering the formation of niosomes. The ultrasonic processor technique is the high energy technique in which high energy input to the system during a short period produces niosomal structures. The ultrasonic processing technique induces high cavitation forces, resulting in efficient mixing of the system and yielding smaller sized niosomes in comparison to the conventional method. On the other hand, thin-film hydration is a multistep process, where changes are taken place much slower. In general, niosomes are prepared by conventional thin-film methods, which often require the induction of organic solvents at one or more stages of the process. With these processes, residual solvents pose health risks to humans.[16]

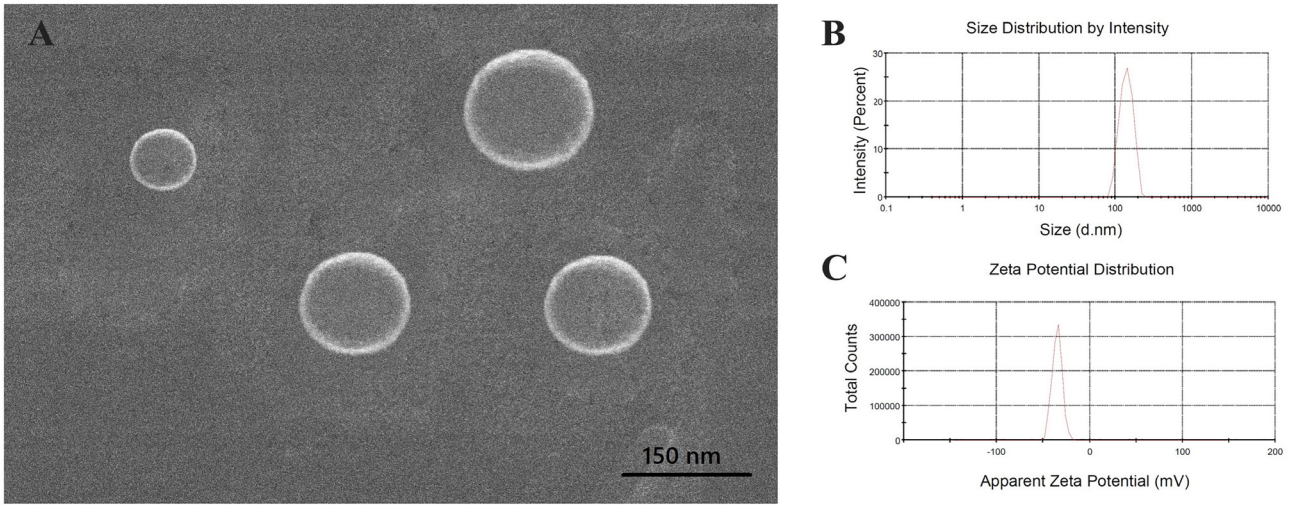

Figure 1. Characteristics of ketosome 7 as selected formulation. (A) SEM micrograph with a magnification 20,000× (B) Particle size distribution. (C) Zeta potential.

Furthermore, none of the studies examined the effect of the Chol:sur w/w percent ratio, which is needed to improve ketosome efficiency. The optimum ketosome 7 prepared by ultrasonication has a particle size, PDI, zeta potential, and drug entrapment percentage of 121.3 ±7.274 nm, 0.338± 0.012, -12.51.153 mV, and 86.36± 0.413 %, respectively.

Organoleptic Properties

Niosomal formulations were described as being milky white in color, odorless dispersions with a fluid-like consistency, and stable (Table 1).

SEM analysis

These niosomes were spherical and separate from each other, according to the SEM micrograph of ketosomes (Figure 1). The micrograph also revealed that the size of the ketosome is properly uniform, as expected given their morphological dimensions.

ATR-FTIR analysis

The potential chemical interaction of Ket with niosomal components was investigated using ATR-FTIR spectroscopy (Figure 2). The ATR-FTIR spectra of pure Ket, Chol, sorbitan monolaurate, physical mixture, and poloxamer 188 have been presented in Figure 2. The ATR-FTIR spectrum of Ket demonstrated characteristic peaks at 3118 cm^{-1} (aromatic C-H stretching), 2880 cm^{-1} (aliphatic C-H stretching), 1644 cm^{-1} (C=O stretching), and 1300-1000 cm^{-1} (C-O stretching). The ATR-FTIR spectrum of Chol exhibited peaks at 3400 cm^{-1} (O-H stretching), 3000-2850 cm^{-1} (C-H of CH_2 and CH_3 groups, asymmetric and symmetric stretching), 1462-1376 cm^{-1} (C-H bending), and 1055 cm^{-1} (C-O stretching). The ATR-FTIR spectrum of sorbitan monolaurate showed peaks at 3390 cm^{-1} (O-H stretching), 2924 cm^{-1} (-CH_2- asymmetric stretching), 2853 cm^{-1} (-CH_2- symmetric stretching), and 1738 cm^{-1} (C=O stretching). The ATR-FTIR spectrum of poloxamer 188 indicated peaks at 3409 cm^{-1} (O-H stretching), 2881 cm^{-1} (C-H stretching), and 1099 cm^{-1} (C-O stretching). The ATR-FTIR outputs showed that did not exist any chemical interaction between Ket and the respective excipients in the selected formulation.

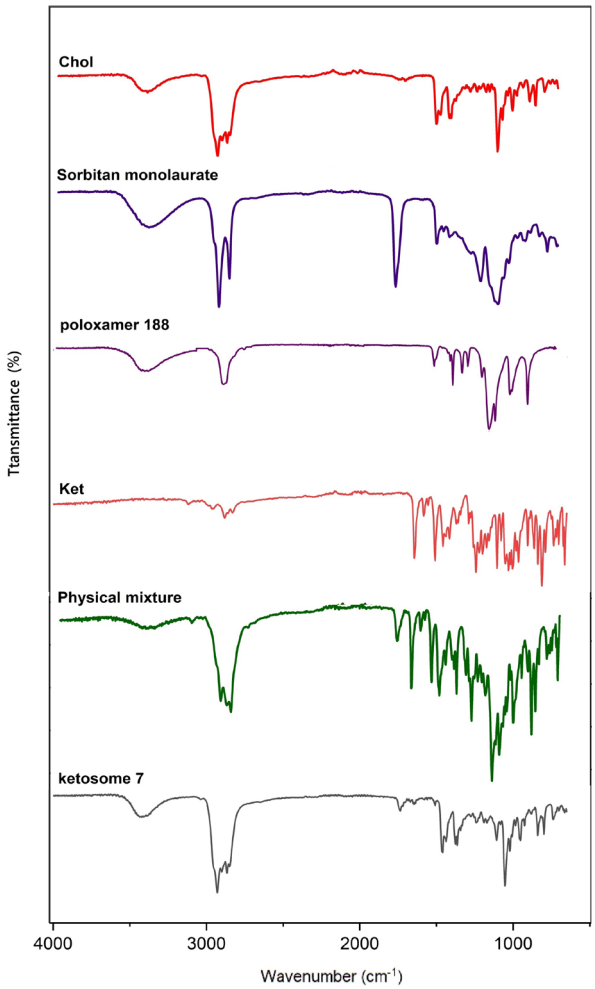

Figure 2. ATR-FTIR spectra of Chol, sorbitan monolaurate, poloxamer 188, Ket, physical mixture and ketosome 7.

DSC analysis

The thermal behavior of Ket, ketosome powder (freeze-dried formulation powder), poloxamer 188, sorbitan monolaurate, physical mixture and Chol was investigated using DSC (Figure 3). Chol, poloxamer 188, and Ket, totally had a single sharp endothermic peak in DSC traces, in accordance with their melting temperature of 150 °C,[36] 62.5 °C, and 160 °C,[37] respectively. When DSC traces of niosome formulations and physical mixture were compared to the different ingredients used to prepare ketosome, it was discovered that the endothermic peak for Chol existed, but the sign of a sharp endothermic peak for Ket wasn't found. This suggests that the Ket in the ketosome is in the amorphous state (Figure 3).

PXRD studies

Figure 4 shows the PXRD pattern of Chol, poloxamer 188, Ket, sorbitan monolaurate, physical mixture and ketosome 7. The PXRD of Ket presented characteristic peaks at 2θ of 17.3°, 19.75°, 23.5°, and 27.4°. Chol PXRD displayed peaks at 2θ of 5.30°, 10.60°,12.83°, 15.55°, 17.05°, 17.4°, 18.15°, 23.55°, 26.2°, 37.15°, and 42.40°.[30] Poloxamer 188 PXRD pattern demonstrated peaks at 2θ of 18.9° and 23.05°. Chol and Ket both had sharp peaks, indicating that they are highly crystalline materials. When PXRD of ketosome was compared to PXRD of absolute Ket and Chol, it was discovered that the key diagnostic peaks of Chol (2θ of 5.3°, 9.45°, 15.50°, and 26.45°) were apparent in ketosome, while the majority of the indicative peaks for Ket was absent. According to the data in Figure 4, Chol peaks in ketosome 7 did not shift, but their intensity decreased when compared to absolute lipid, which may be attributed to the existence of Chol and Ket between the sections of the niosome membrane, which altered Chol crystallinity. The small concentration of Chol in the niosome may also explain the lower crystallinity of ketosome compared to pure Chol. All the diagnostic peaks of all ingredients were presented in the physical mixture. The nonappearance of indicative peaks for Ket in the ketosome could mean that Ket is amorphous or molecularly scattered in the niosome.

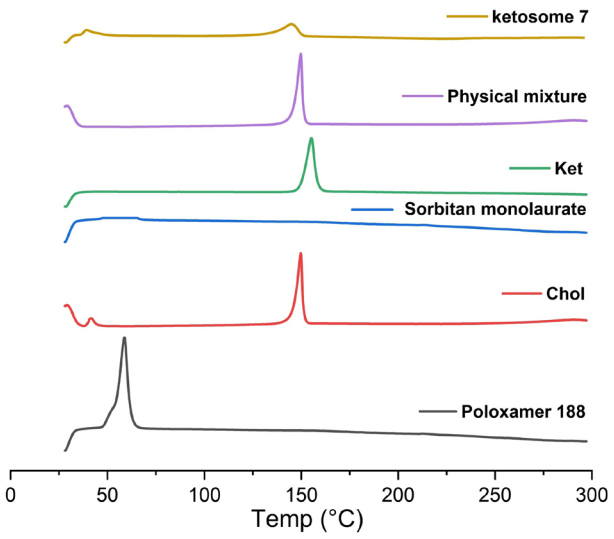

Figure 3. DSC thermogram of ketosome 7, physical mixture, Ket, sorbitan monolaurate, Chol, poloxamer 188

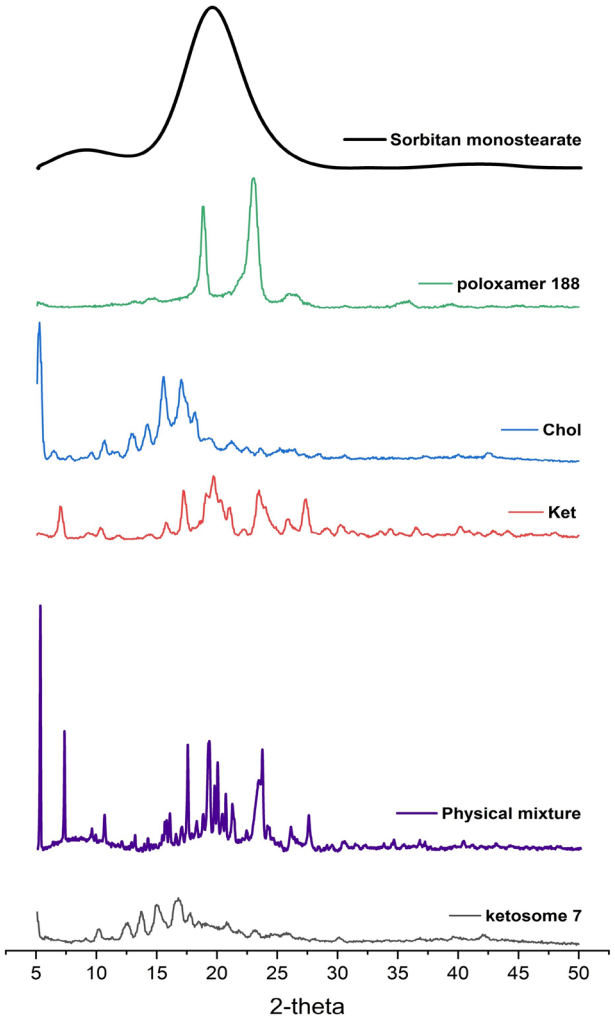

Figure 4. PXRD of sorbitan monolaurate, poloxamer 188, Chol, Ket, physical mixture and ketosome 7.

Analysis of ketosome release profile

Figure 5 shows the results of the *in vitro* drug release analysis from ketosomes. In general, an increase in Chol concentration caused an enhancement in the dissolution rate of Ket from ketosome, as seen in the release profile. Ketosome with the maximum Chol:sur ratio released the drug quickly, while those with lower Chol concentrations released Ket slowly. When niosome formulations were compared to the control, it was discovered that incorporating Ket into niosome formulations would improve Ket dissolution (Figure 5).

Ket appears that affect lipid membrane structure and stability, especially in sorbitan monolaurate short lauryl chains (C_{12}), which are liquid at room temperature.[31] Ket is a lipophilic molecule found in hydrophobic core bilayers. The existence of a potential competition between Ket and Chol, which is incorporated into the niosomes, may explain

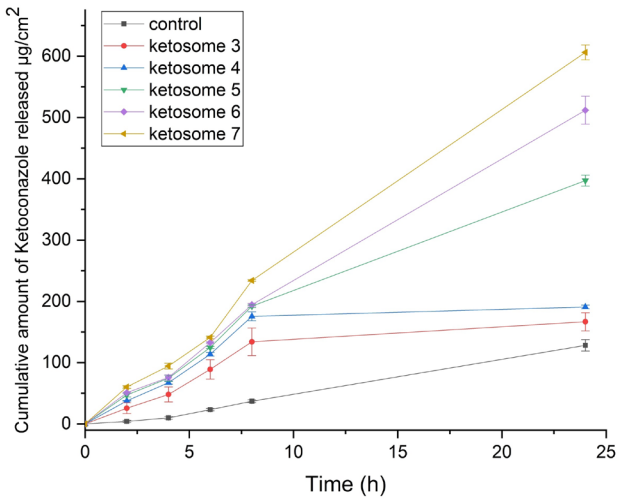

Figure 5. Dissolution profile of Ket solution and ketosome formulations.

this result.[38] This result was previously found in a niosomal design with Chol and carotenoids.[39] The observation of this behavior is justified because Chol serves two functions in the niosome membrane. The high levels of Chol in the formulation result in both slow and explosive release.[26] According to Cócera *et al.*[40] report, Chol has an optimal hydrophobicity, which reduces the formation of transient hydrophilic holes by decreasing membrane fluidity, which is responsible for medicine release through the liposomal membrane.[40] On the other hand, increasing the amount of Chol in ketosome may enhance the release of Ket. El-Samaligy *et al.*[41] showed enhancing Chol above a specified amount can dislocate the regular linear arrangement of the vesicular membrane and enhance medicine release. In addition, an enhancement in the quantity of Chol caused the release rate enhancement of curcumin from nanovesicle. Nanovesicle with the maximum Chol:sur ratio displayed the highest dissolution rate, while formulations with no or low Chol concentration showed the slowest dissolution.[18]

Stability Studies and shelf-life analyses

Stability was conducted based on zeta potential, entrapment efficiency, particle size, and PDI for three months at 4 and 25 °C (Table 3). The results indicated that the ketosome kept at both temperatures were within the same colloidal nanometer range. Ketosome kept at 25 °C showed an increment in the PDI (wider distribution due to particle growth) and a reduction in EE%. No pronounced alterations in size, PDI, and zeta potential were found for ketosome kept at 4 °C, confirming that 4 °C could be a suitable storage temperature for ketosome. Also, drug content in the formulation was about 95% at 4 and 25 °C after 3 months (Figure 6). The shelf-life prediction of the formulation was about 9 months.

MTT assay

MTT was used in this study to assess the cell survival of ketosomes. To that end, several concentrations of Ket solution, ketosome, and plain niosome were tested at 0.5–15 µg/mL. The cells were incubated for 24 h in the cell viability test using the HGF normal fibroblast cell line acquired from Pastor Institute (Tehran, Iran). The survival of the cells was significantly declined during 24 hours in the presence of ketosome and Ket ($P < 0.05$). According to Figure 7, the cell viability of the ketosome was greater than the cell survival of the Ket solution. After treatment, the cell viability of the ketosome was approximately 90%. According to previous research, niosomes can improve stability and solubility, and they are nontoxic, nonimmunogenic, and considered safe.[42]

It is well defined that drug nanoparticles can be harmful to body tissues, and small particles can be easily carried to cells and cause cell cytotoxicity. Additionally, the zeta

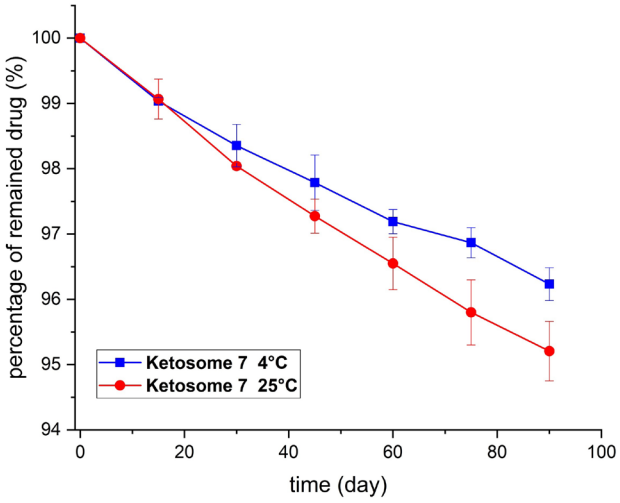

Figure 6. Percentage drug remaining versus time plot of ketosome 7 at 4 and 25 °C.

Table 3. Stability information of the ketosome 7 dispersion followed by storage for 3 months.

Storage condition	Time (month)	Particle size (nm)	PDI	Zeta potential (mV)	EE%	Organoleptic
Initial	-	121.30±7.27	0.338±0.012	-12.50± 1.15	86.36±0.41	stable-milky
4°C	1	125.63±2.15	0.342±0.015	-12.10±1.05	85.07±0.29	stable-milky
	2	125.96±3.77	0.346±0.015	-12.03±0.95	84.44±0.70	stable-milky
	3	126.96±2.87	0.342±0.022	-12.50±1.15	83.07±0.24	stable-milky
25°C	1	129.23±1.89	0.343±0.009	-12.23±1.12	84.66±0.58	stable-milky
	2	132.05±0.90	0.348±0.010	-12.07±1.07	83.47±0.43	stable-milky
	3	133.13±1.21	0.347±0.011	-11.80±1.50	82.52±1.18	stable-milky

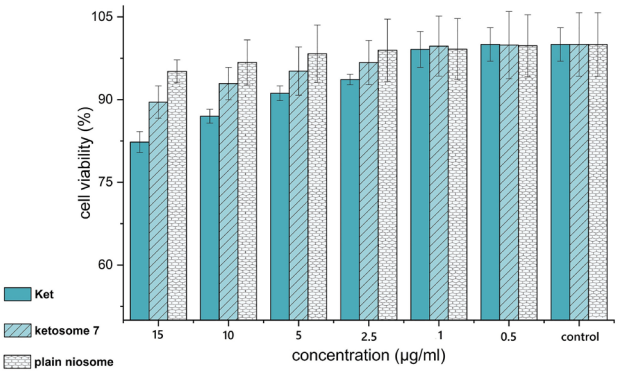

Figure 7. Cell survival of various concentrations of Ket, plain niosome and ketosome 7. Data were expressed as mean ± SD.

potential of noisome can enhance their reactivity with cells and proteins.[18]

Another theory is that extreme concentrations of the ketosome reduce cell survival; however, the ketosome improves cell viability to over 89 percent. This may be due to the nonionic-surfactant mixture in the nanovesicle such as niosome increasing the permeability, lysis, and solubilization of the lipid-protein-detergent interaction.[43] According to these findings, the cell toxicity of the medicine may be declined by encapsulating Ket on ketosome due to the amorphous form of Ket and absence of crystallinity or/ and the use of nonionic surfactants (sorbitan monolaurate) with considerable biocompatibility, resulting in a low cytotoxic effect of the vesicular formulations (plain niosome and ketosome)[44] because of the effect of the enzyme on the ester bond.[45] Furthermore, *in vitro* cell toxicity testing discovered no cytotoxicity of the optimum ketosome due to a higher percentage of cell survival of about 89%.

Antifungal Susceptibility Analysis

The growth of several strains was obviously evident after 24 h of incubation at 35 °C using the CLSI document M27-A3 susceptibility analysis technique for *C. albicans*. The findings, however, were defined after 48 hours of incubation at 35 °C, as suggested by CLSI.

The findings of the *in vitro* antimycotic susceptibility analysis of ketosome versus examined isolates are shown in Table 4. As shown in Table 4, using the green ketosome results in a significant decline in MIC values for each strain of *C. albicans* ($P < 0.05$). According to our findings, when ketosome was used, the MICs of Ket were significantly declined, implying that the clinical dosage and chance of drug side effects could be reduced. Furthermore, the amphiphile nature of the ketosome can allow the Ket to enter the yeast cells more effectively, easily, and quickly. To the best of our knowledge, there are just a few published studies on ketosome production. As a result, researching ketosome effect on a different range of *Candida albicans* is innovative. The nano scale ketosome in the niosomal formulation allows for direct contact with the targeted site and can improve drug penetration.[15] Furthermore, the size

Table 4. Effect of the Ket optimum formulation against *C. albicans* strains.

Isolates	Ket		Ketosome	
	MIC (µg/ml)	MIC50	MIC (µg/ml)	MIC50
C3	0.031	0.1875	0.0004	0.0004
C5	0.25		0.0004	
C11	0.50		0.0004	
C57	0.031		0.0004	
C69	0.031		0.0004	
C70	0.031		0.0004	
C75	0.125		0.0008	
ATCC	0.25		0.0004	

C: *C. albicans*

of the niosome may allow for sustained drug release.[18] Wagh *et al.*[46] studied the effect of itraconazole-loaded noisome on *C. albicans*. Results showed that the itraconazole-loaded in niosomes had a wider inhibition zone than the market production against *C. albicans*.

Conclusion

Ketosome have a great potential antifungal effect, with minimum dose administration and maximum safety profile. Ketosome could be converted successfully to a finished product to treat cutaneous candidiasis. Ket was successfully loaded into niosomes made of Chol and surfactant mixtures using an ultrasonic process (as a green method) in this study. On the report of solid-state studies, a Ket encapsulated into niosome was in an amorphous form with mean particle size, PDI, EE percent, and zeta potential of 121.300 ±7.274 nm, 0.338± 0.012, 86.360± 0.413 percent, and -12.500± 1.153 mV, respectively. According to a dissolution test, ketosomes offered higher drug release than pure drugs. Furthermore, an *in vitro* cytotoxicity test revealed that the improved formulation of niosomes containing Ket exhibited lower cytotoxicity in comparison pure drug. Cell viability (HGF cell line) for ketosome was approximately 90%. The results of this study revealed that when the optimum formulation was tested against *C. albicans*, the ketosome had a lower MIC than the pure drug. Thus, niosome offers a potential alternative to current delivery systems aimed at improving the biopharmaceutic efficiency of drugs with low aqueous solubility. The results of this study revealed that the optimized ketosome could be used as a possible nanovesicle for ketoconazole drug delivery to improve therapeutic effect, potentially opening up new ways to manage cutaneous candidiasis complaints.

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Authors contributions

KMS: Conceptualization, Visualisation. MS: Supervision, Formal analysis. JA: Supervision. MM: Formal analysis. AB: Writing - review and editing. RN: Investigation. MA: Investigation. ME: Investigation. SMHH: Writing - original

draft, Writing - review and editing.

Conflict of Interest

The authors report no conflicts of interest.

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