RESEARCH-ANIMAL

Detection of *Candida spp.* from mastitis cattle milk and study of the antifungal activity of ketoconazole-loaded solid lipid nanoparticles and nanostructured lipid carriers against Candida isolates

Isa Swadi Touhali[1], Khairi Jameel Al Ruaby[2], Alwan Al Qushawi[3]

Abstract

Objective: To isolate candida species from cattle milk isolates and investigate the advances of ketoconazole-loaded solid lipid nanoparticles and ketoconazole-nanostructured lipid carters, and to analyse their properties and compare their antifungal performance compared to free ketoconazole.

Method: The cross-sectional study was conducted from October 2021 to April 2022 in Waist Governorate of Iraq, and comprised milk samples obtained from cows infected with mastitis. The study was initiated after obtaining approval from the IRB of the veterinary hospital in the Governorate of Iraq. The samples were cultured on 4% savoured dextrose agar and incubated independently at 37°C before they were subjected to a range of tests. Mastitis was checked with a commercially available cultural and morphological identity card system and through candida chromogenic agar. The data was analyzed by using the programmed SPSS IBM version 20. The chi-square test was used to check the inhibition zones due to the anti-fungal activity of KTZ-LPN and free KTZ. The significance level was kept at $p<0.05$.

Results: Of the 225 samples, 116(51.5%) were obtained from cows infected with mastitis on the basis of a commercially available cultural and morphological identity card system. Among the 61(52.6%) candida albicans isolated, resistance to amphotericin B was shown by 60(98%) and to nystatin by 58(95%), while those sensitive to ketoconazole were 32(52%) and to fluconazole 31(51%).The ketoconazole nanostructured lipid nanoparticle (KTZ-NLC) preparations showed higher antifungal activity than other tested preparations at 0.25µg/ml with a minimum inhibition zone of 15±2.4 mm followed by ketoconazole solid lipid nanoparticle (KTZ-SLN) preparations with minimum inhibition zone of 15±1.3 mm. Free ketoconazole showed the lowest inhibition zone values at the same concentration (0.25µg/ml), which was 11±1.3 mm. Both these lipid nanoparticles formulations, especially ketoconazole nanostructured lipid carriers (KTZ-NLC), could represent a promising formulation for antifungal application and use.

Conclusions: Ketoconazole-nanostructured lipid carter formulations were found to be effective for drug delivery.

Keywords: Nanoparticles, Fluconazole, Amphotericin, Ketoconazole, Nystatin, Drug, Milk, *Candida*, Mastitis.

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Introduction

Certain agents have been reported to be behind mastitis which causes economic losses to the dairy industry by reducing milk production and raising the expense of antibiotic treatment.[1] Fungus mastitis is frequently caused by *candida species (spp.)*. Long-term antibiotic treatment was adopted which led to resistance against many microorganisms. It was consequently expected that mastitis associated with fungi would pose an important problem in the future.[2] Not all antifungal agents have been successful in treating *candida spp*.[3] Through the past decade, significant attention has been paid to developing new transport systems, like loading antifungals on nanostructured lipid carriers (NLCs), in order to provide lasting drug release and amplifying the healing process. Solid lipid nanoparticles (SLNs) and NLCs have been attractive systems for the purpose due to their solid matrix that may prevent the burst release obtained in a conservatively preventable formulation. They are stable at room and body temperatures, and have frequent benefits, such as drug security in harsh environmental situations and the ease of a large-scale process. While NLC cores have been fully characterised to improve drug bioavailability, their newer cousins have determined some of the shortcomings associated with the former. These lipid nanoparticles (NLC) differ from all other lipid nanoparticles as the core composition release is prolonged due to the matrix being composed of solid and liquid lipids.

It is made due to the amalgamation of solid and liquid fats, so as to augment the load of the drug and avoid its exclusion. In addition, the nanostructured lipid matrix offers more elasticity in modulating drug release.[4,5]

The current study was planned to isolate and recognise *candida spp*. in cattle milk isolates, to investigate the

[1]Department of Medical Microbiology, Wasit University, Iraq.
[2]Department of Biology, Wasit University, Iraq.
[3]College of Dentistry, Wasit University, Iraq.
Corrospondience: Khairi Jameel Al Ruaby
email: khairi.iraq2009@gmail.com

advances of ketoconazole (KTZ)-loaded SLNs and KTZ-NLCs, to analyse their properties and to compare their antifungal performance compared to free KTZ.

Materials and Methods

The cross-sectional study was conducted from October 2021 to April 2022 in Waist Governorate of Iraq, and comprised random milk samples obtained from cows infected with mastitis in 10 farming villages after acquiring approval from veterinary hospital in Governorate. The cows were subjected to California Mastitis Test in line with literature.[6] For milk sample sets, the nipple tips were cleaned with swabs saturated with 70% alcohol, and were left to dry. After the initial few milk jets, 2-5ml of milk samples were put into the disinfected glass vials. They were then brought to the microbiology laboratory under cold chain conditions.

The sample size calculation was done by applying the formula:[7]

$$\frac{Z_{1-\alpha/2}^{2} p^{(1-p)}}{d^{2}}$$

For the isolation and detection of *candida spp*. the samples were cultured on 4% savoured dextrose agar, and incubated independently at 37°C. The increment was checked on a daily basis, and after 72h candida colonies were categorised as creamy, mucous and soft at 37°C. They subsequently turned into furrowed, creamy white colonies after 1 week of incubation with yeast scent. All creamy white colonies were subculture on chromogenic candida agar that was used according to the manufacturer's instructions. Green colonies were identified as candida (C.) albicans. The blue colonies were recognised as C. tropicalis. Other candida colonies, including C. krusei and C. parasitosis, were pink and white, respectively. Finally, pure candida isolates were established by (VITEK 2 compact system), (BIOMERIEUX, France) according to the manufacturer's instructions.

Antifungal susceptibility tests were performed on *candida spp*. using Clinical and Laboratory Standards Institute (CLSI), M-44 antifungal disc diffusion sensitivity test method.[8] The antifungal tablets used in the tests included KTZ 10mcg, fluconazole 10 mcg, miconazole 10mcg, amphotericin B 20mcg, nystatin 100IU] and flucytosine 1mcg.

For nano-pharmacological analysis, KTZ-SLN and KTZ-NLC were prepared using stearic acid (Gattefosse, France), stretch 80%, %, polysorbate 80 (tween 80), sodium acetate, acetic acid, methanol, Sapporo dextrose agar (SDA), and Roswell Park Memorial Institute (RPMI) medium were used as orientation models. All chemicals were used with analytic reagent status.[9]

SLN and NLC were prepared using high-pressure, hot-pressure homogenisation procedures.[10] The lipid phase was melted at 90°C, disseminated in a hot aqueous surfactant solution and heated at the similar temperature, using ultra-torrox (T25, Germany) at 8000rpm) for 1m. The acquired pre-emulsion was homogenised at 90°C) using micron lab 40 (APV, Germany), a pressure of 500 bar, and 3 homogenising cycles. To prepare KTZ-SLNs, 5% (m/m) of the lipid matrix was mixed with the drugs. KTZ was dissolved in the molten lipid phase before dispersal in surfactant solution.

In vitro drug release from KTZ-LNPs was investigated by using the dialysis bag diffusion method.[11] A beaker containing 40mL potential of hydrogen (pH) 7.4 phosphate buffer saline (PBS) with 1% tween 80, as dissolution media was put in a dialysis bag loaded with LNP formulations. The process was carried out at 200rpm. Following 0, 1h, 2h, 4h, 6h, 8h, 10h, 12h, 14h, 16h, 18h, 20h, 22h, 24h, 48h, 72h, 96h, and 120h, the samples (2mL) were drawn. Using an ultraviolet (UV) spectrophotometer, the sample absorbance was determined and the quantity of drug release was assessed. In the UV-visible spectrophotometer, the samples were examined for drug content by measuring the absorbance at 255nm. Triplicate data was graphically investigated, and the ratio of drug release time graph was plotted.[12]

Antifungal activity was performed using the paper disc diffusion method.[13] The fungal strain was maintained after storage at 40°C on potato dextrose agar plates. A sterile Whatman No. 1 filter paper disc of approximately 5mm each, impregnated with 20µl LNP formulations, was located on a petri dish. The drug-free solution used as a positive control was examined. At 28°C, all plates were inoculated and kept for 1 week. Areas of inhibition across the discs were assessed.[14]

The isolates were tested for sensitivity to KTZ, fluconazole, nystatin and amphotericin. The results were interpreted by measuring the areas of embarrassment around the disc and comparing them with the refractive points interpretation as per the manufacturer's instructions; zone diameter 18mm indicated susceptibility (S), zone diameters 14-17mm indicated dose-dependent sensitivity (DDS), and 14mm zone diameter indicated confrontation.[15]

Statistical analysis: The data was analysed by using the programme SPSS IBM version 20.

Chi-square test was used to check the inhibition zones due to the anti-fungal activity of KTZ-LPN and free KTZ. The significance level was kept at $p<0.05$.

Results

Of the 225 samples, 116(51.5%) were found to have been infected with mastitis on the basis of the card system. Of them, 93(80.1%) were also found through candida chromogenic agar. C. albicans was the most common isolate 61(52.6%) (Table 1).

Among the C. albicans isolates, resistance to amphotericin B was shown by 60(98%) and to nystatin by 58(95%), while those sensitive to KTZ were 32(52%) and to fluconazole 31(51%) (Table 2).

In terms of physicochemical characterisations of LNP formulations, KTZ-NLC showed the lowest values 124.5±4.3nm. Polydispersity index (PDI) values ranged between 0.15 and 0.3 for submicron particles, suggesting size homogeneity. PDI values were low, but KTZ-NLC values 0.11±0.1 were significantly less than the other formulations (p<0.05).

All formulations had negative zeta potential (ZP_values after the addition of stearic acid (0.3% w/v), ranging from 25.52.1mV for KTZ-SLN to 32.41.3mV for KTZ-NLC (Table 3). Morphologically, structural equation modelling (SEM) images confirmed that the size of KTZ-SLN (Figure 1) and KTZ-NLC (Figure 2) particles was within the nano-scale. Maximum Entrapment Efficiency (EE) (values with KTZ-NLC were 93.41.5 (p=0.05).

Within 20h at pH 7.4, the free drug reached 100% of drug release, but other formulations provided a biphasic release model followed by continuous release with an initial burst release (Figure 3). KTZ-SLN confirmed faster initial burst release, KTZ-(15%) at pH 7.4 within the first 6 hours followed by a steady continuous release for 60 hours, while KTZ-NLC formulation obtained the lowest initial burst release of all measured formulations at pH 7.4, which was 10% within the first 6 hours followed by a constant

Table-1: Comparative results of candida (C.) species isolated from cattle's milk with mastitis between Vitek 2 system and chromogenic agar,

NO	Isolates species	No isolates	Vitek 2 system n (%)	Candida chromogenic agar n (%)
1	C. albicans	61	61 (52.5)	54 (58)
2	C. krusei	16	16 (13.7)	14 (15)
3	C. famata	11	11 (9.4)	8 (8.6)
4	C. parapsilosis	8	8 (6.8)	6 (6.4)
5	C. spherica	7	7 (6)	6 (6.4)
6	C. glabrata	7	7 (6)	3 (3.2)
70	C. kefyr	4	4 (3.4)	- (0)
8	C. tropicals	2	2 (1.7)	2 (2.1)
	Total	116	116 (100)	93 (80.1)

Values presented as means ± standard deviation (SD); n = 6 mice/group; capital letters denote the differences between groups; p<0.05 and 0.001 vs. control; small letters indicate the differences within group compared to baseline; p<0.05 and 0.001.

Table-3: Physicochemical characterizations of KTZ-LNP formulations (KTZ SLN and KTZ-NLC) and control formulations (free drug).

Formulation	ZP (mV)	Size (nm)	PDI	Entrapment Efficiency (EE%)
KTZ-SLN	−25.5±2.1	169.4±3.4	0.16.08	82.1±1.8
KTZ-NLC	−32.4±1.3	124.5± 4.3	0.11±0.1	93.4±1.5
SLN (free drug)	−30.2±1.2	166.6±2.3	0.17±0.2	-
NLC (free drug)	−24.5±1.2	126.4±6.3	0.12±0.1	-

KTZ-SLN: Ketoconazole-loaded solid lipid nanoparticles, KTZ-NLC: Ketoconazole-nanostructured lipid carter, ZP: Zeta potential, PDI: Polydispersity index.

Table-2: Antifungal susceptibility and resistance profile for candida (C.) isolates. (96 samples)

Antifungal	C. albicans No=61		C. krusei No=16		C. famata No=11		C. parapsilosis No=8	
	S	R	S	R	S	R	S	R
Ketoconazole	32(52%)	29(48%)	9(56%)	7(46%)	6(54%)	5(46%)	5(63%)	3(37%)
Fluconazole	31(51%)	30(49%)	6(38%)	10(62%)	4(36%)	7(64%)	3(38%)	5(62%)
Amphotericin B	1(2 %)	60(98%)	3(19%)	13(81%)	0(0%)	11(100%)	2(25%)	6(75%)
Nystatine	3(5%)	58(95%)	1(6%)	15(94%)	0 (0%)	11(100%)	0(0%)	8(100%)

R: Resistant, S: Sensitive.

Table-4: Comparison candida (C.) albicans inhibition zones towards the antifungal activity of serial concentrations for KTZ-LPN formulations and free KTZ.

Concentration µg/mL 0.125–16	0.125	0.25	0.5	1	2	4	8	16	32
KTZ-SLN	11±1.2	15±1.3	16±.1.6	19±1.4	23±1.9	26±2.3	28±2.1	29±1.4	30±2.3
KTZ-NLC	13±2.2	15±2.4	16±1.4	22±1.4	23±1.2	26±1.4	30±2.7	34±.1.5	36±3.5
NLC (free drug)	-	-	-	-	-	-	-	-	-
SLN (free drug)	-	-	-	-	-	-	-	-	-
KTZ	0	11±1.3	13±1.3	13±1.2	15±1.3	16±1.6	17±1.4	18±2.2	19±1.4

Data presented as mean ± Standard deviation, (n=3). p<0.05. KTZ-SLN: Ketoconazole-loaded solid lipid nanoparticles, KTZ-NLC: Ketoconazole-nanostructured lipid carter

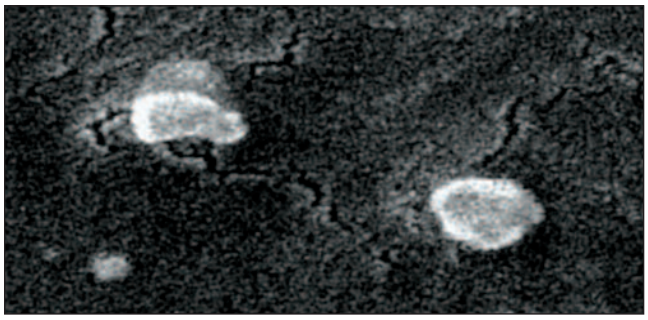

Figure 1: Structural equation modelling (SEM) graph of ketoconazole-loaded solid lipid nanoparticles (KTZ-SLN) formulation.

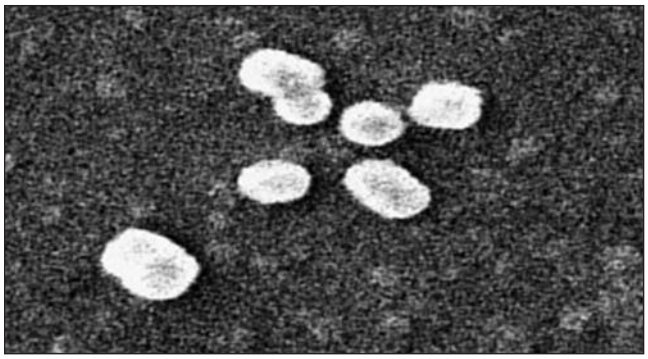

Figure 2: Structural equation modelling (SEM) graph of ketoconazole-nanostructured lipid carter (KTZ-NLC) formulation.

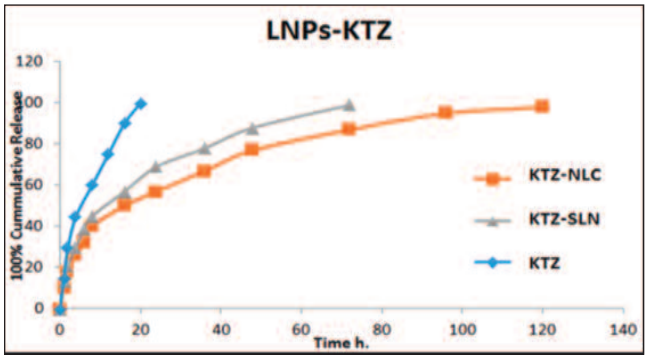

Figure 3: In vitro release profile of Ketoconazole (KTZ) from solid lipid nanoparticle (SLN) and -nanostructured lipid carter (NLC) formulations compared with free KTZ.

continuous release for 120 hours. Regarding free KTZ, it was completely released within 20 hours. Anti-fungal action in various formulations revealed that C. albicans was more susceptible to the prepared formulations of LNPs, KTZ-NLC demonstrated the most significant antifungal activity (p=0.05), with an inhibition zone of 0.25g/mL against the isolates, followed by KTZ-SLN, and free KTZ (Table 4)

Discussion

The current findings related to *candida spp*. were incompatible with an earlier study[16] which reported C. krusei (41.33%), C. lusitaniae (11.33%), C. famata (4.67%), C. albicans (1.33%), C. tropicalis (1.33%), C. glabrata (1.33%), and C. utilis (0.67%). Also, the current results are incompatible with a study in 2010[16] which reported the most frequently isolated species were C. krusei (34.8%), followed by C. albicans (10.1%), C. tropicals (9.2%), C. zeylanoides (5.8%), C. parapsilosis (4.3%), C. guilliermondii (3.4%), C. famata (1.9%) and C. glabrata (1.5%).

The high incidence of fungal mastitis is most likely due to a combination of unfavourable factors, including prolonged intraduodenal antibiotic administration, increased incidence of mastitis due to vitamin-mineral deficiencies, antioxidant deficiency accompanied by energy imbalance, and weather change. All of these are negative conditions, and reduce the resistance of the cow's udder to infections.[17,18] The antifungal profile of candida isolates from cow milk with mastitis revealed yeast resistance to antifungal agents, and their ability to produce slime and form biofilms has recently been investigated.[19] Treatment of mastitis on farms from where milk samples are often collected does not include the administration of antifungals. Even in cases of laboratory confirmation of candida mastitis, treatment is rarely applied.[20] The current results disagree with those of a study in Nigeria[21] that reported that C. albicans isolates from bovine mastitis were sensitive to nystatin and resistant to fluconazole. In Turkey, a study[22] isolated C. parasitosis from bovine mastitis proved that these isolates were sensitive to ketoconazole (80%), intermediately susceptible to amphotericin B and nystatin (100%) and miconazole (60%), and resistant to fluconazole (80%), which is inconsistent with the current study. The disagreement may be due to the differences in the types of local isolates from one area to another, the frequency of drug use patterns, and the professional skills of the researchers.

There were many advantages of hot homogenisation, such as ease of conduct, no use of organic solvents, and lower particle size and PDI values. Ultrasonication, along with homogenisation, has also led to the generation of smaller sizes. However, the high-pressure homogenisation (HPH) technique is still the main procedure adopted for the production of lipid nanoparticles, which can be performed at either hot or cold temperatures.[10-14]

The current findings were similar to those of SLN and NLC research using stearic acid, a mixture of stearic acid and oleic acid, where the particle size depends on the amount of oleic acid in the formula.[9] PDI values range between 0.15 and 0.3 for sub-micron particles, indicating size homogeneity, while PDI >0.3 results in heterogeneity; a value closer to zero corresponds to an improvement in homogeneity in size. ZP is an essential technique for assessing the potential stability of the nanoparticle system and the surface charge. Since electrostatic repulsion

between particles of the same charge's limits aggregation, a high absolute or positive ZP value that is necessary for the stability of colloidal scattering is essential.[13] In maintaining the physical integrity of nano-formulations, the surface charge is a vital parameter. Typically, high absolute ZP prevents particles from aggregating among themselves because of electrostatic repulsion. In general, an absolute ZP -30mV suggests great stability.[23] In the morphological analysis, there was no perfect spherical shape in the LNP formulations seen, which could probably be correlated to the drying phase of the sample.[15] The spherical form of the KTZ-NLC particles was platelet-like. The KTZ-SLN particles have been stated to be normal with a smooth surface texture.[14]

Encapsulated drug estimation is very important because it specifies the pattern of release and therapeutic efficacy. The composition of NLC provided by both solids and lipids does not recrystallise and retains its amorphous structure, allowing room for many imperfections to encapsulate more KTZ. The NLC has been expanded to create molecules in which the oil is in a solid fat centre, which in turn improves the encapsulation of the compound by dissolving the compound in the oil and binding it to a solid fat.[14,25] An in vitro release study reported data from the formulation matrix on drug release behaviour, showing that drug trapping in LNPs impairs the release profile; the initial impulse release followed by the slow-release phase. This can be attributed to the fact that the expanded surface area is responsible for the small particle size, which runs along the diffusion path. Moreover, the trapping efficiency was lower, which affected drug release.[13] The rate of drug release from LNPs is determined by the partitioning of the drug in the dialysis bag between the lipid phase and the aqueous environment, then by the drug's diffusion through the membrane. The release profiles depend essentially on the formulation process, as it has been noted that the higher temperature and concentration of surfactants have increased the solubility of the drug.[14] a study stated that the pattern of drug release depends on its lipid matrix affinity because it might influence LNP matrix viscosity, which could play an essential role in regulating the pattern of release.[14] The considerably slower release pattern of NLCs may be due to its higher loading capacity. According to Fick's law of diffusion, the NLC release profile almost adopts the drug-core model because the lipid covers the drug as a membrane.[26]

The current results are in line with earlier studies.[6, 16] Compared to free drug, the improved in vitro antifungal efficacy of LNP formulations may be due to the increased solubility contributing to higher KTZ permeability across the fungal cell walls to prevent ergosterol synthesis.[27] This results in a significant reduction in KTZ-LNP formulations relative to KTZ, indicating that the clinical dosage could be decreased, thus reducing the risk of adverse drug effects. The process accountable for drug resistance recognised in pathogenic fungi is one potential reason for the effects of antifungal susceptibility.[28,29] The drug resistance process can occur in three distinct ways: by reducing the concentration of drugs that can occur with drug efflux, by modifying the drug target, and by genome sequence mutation.[29,30] Efflux is the most significant mechanism which leads to a decrease in drug concentration.[31,32]

Conclusion

KTZ-LNP formulations were successfully developed for effective drug delivery with sufficient particle size and a high Entrapment Efficiency (EE) . This novel drug delivery could face C. albicans strains that exhibit varying susceptibilities to the conservative formulation of KTZ. The novel drug formulations could increase KTZ bioavailability and dissolution rate to enhance the formulations' efficiency.

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