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ORIGINAL RESEARCH

Potential of Methylglyoxal-Conjugated Chitosan Nanoparticles in Treatment of Fluconazole-Resistant *Candida albicans* Infection in a Murine Model

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Background: Fungal infections are becoming more prevalent and threatening because of the continuous emergence of azole-resistant fungal infections. The present study was aimed to assess the activity of free Methylglyoxal (MG) or MG-conjugated chitosan nanoparticles (MGCN) against fluconazole-resistant *Candida albicans*.

Materials and Methods: A novel formulation of MGCN was prepared and characterized to determine their size, shape and polydispersity index. Moreover, the efficacy of fluconazole or MG or MGCN was determined against intracellular *C. albicans* in macrophages and the systematic candidiasis in a murine model. The safety of MG or MGCN was tested in mice by analyzing the levels of hepatic and renal toxicity parameters.

Results: *Candida albicans* did not respond to fluconazole, even at the highest dose of 20 mg/kg, whereas MG and MGCN effectively eliminated *C. albicans* from the macrophages and infected mice. Mice in the group treated with MGCN at a dose of 10 mg/kg exhibited a 90% survival rate and showed the lowest fungal load in the kidney, whereas the mice treated with free MG at the same dose exhibited 50% survival rate. Moreover, the administration of MG or MGCN did not induce any liver and kidney toxicity in the treated mice.

Conclusion: The findings of the present work suggest that MGCN may be proved a promising therapeutic formulation to treat azole-resistant *C. albicans* infections.

Keywords: chitosan nanoparticles, fluconazole-resistant, systemic Candidiasis, methylglyoxal

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Introduction

Candida albicans is a normal commensal, but it can act as a pathogen in immunocompromised persons, ie patients receiving chemotherapy for organ transplantation and cancer treatment, or persons on prolonged antibiotic or steroid therapy.[1–3] *Candida albicans* causes infections that range from the mucosal to the systemic candidiasis in immunocompromised subjects. Azoles (fluconazole, itraconazole) are commonly used antibiotics to treat fungal infections in the human population, but their widespread use has resulted in the emergence of azole-resistant fungal pathogens.[4–7] Amphotericin B, a polyene antibiotic, is a gold standard and the most effective antifungal drug used in the treatment of deep-seated fungal infections, but the acute hepatic and renal toxicity limits its use in human beings.[8,9]

The status of the immune system plays a critical part in the outcome of active *C. albicans* infection. Since neutrophils and macrophages are the front line cells that start an onslaught against *C. albicans*, mice deficient in leukocytes and macrophages

showed severe susceptibility to *C. albicans*.[10,12] Moreover, human immunodeficiency virus (HIV)-infected individuals showed greater susceptibility to orogastric candidiasis owing to the impairment of macrophages.[13] The CD4$^+$ T helper cells and CD8$^+$ cytotoxic T cells play an important role in mounting anti-fungal immunity to candidiasis.[14]

Methylglyoxal (MG) is a reactive dicarbonyl compound that is produced as a natural by-product of glucose and fatty acid metabolic pathways. It has been shown to kill the cancerous cells through the inhibition of glycolysis and mitochondrial respiration.[15] MG, an active component in Manuka honey, has shown efficacy against the biofilm formation in *Pseudomonas aeruginosa* and *Staphylococcus aureus*.[16] MG has been shown to act as an antibiotic adjuvant and increases the activity of linezolid against *Staphylococcus aureus*.[17] A combination of honey and phage has been shown to possess a synergistic effect against the biofilm formation by *Escherichia coli*.[18] Antibacterial activity of MG is suggested to be mediated through the structural changes of bacterial fimbriae and flagella. Furthermore, MG has demonstrated antimalarial activities as well.[19]

Chitosan is a natural and biodegradable polysaccharide with a broad-spectrum antimicrobial activity that is correlated to its deacetylation as a higher degree of deacetylation increases the antimicrobial activity of chitosan.[20–22] Besides, the antimicrobial activity of chitosan is also pH-dependent. It shows increased activity at lower pH because of its cationic nature.[21] Cationic chitosan binds to the negatively charged cell membrane that causes an interference in the energy metabolism of pathogens.[23] Chitosan exerts its activity against *Candida albicans* through the inhibition of SAGA complex and was supported by the fact that chitosan inhibited ADA2 and ada2–regulated cell wall-related genes. Moreover, it reduced the levels of chitin and β-glucan that alters the structure of cell wall and plasma membrane via the inhibition of SAGA complex component expression.[24]

In recent years, various drug delivery systems including liposomes and nanoparticles (NPs) have been developed as the carriers of therapeutic agents. We have earlier shown that the encapsulation of antifungal drugs in liposomes resulted in enhanced activity against *C. albicans, Aspergillus fumigatus* and *Cryptococcus neoformans*.[25–27] In the present study, we determined the activity of MG or MGCN against *C. albicans* both in vitro or in vivo studies as well. The results of the present study demonstrated that MGCN, a formulation of methylglyoxal-conjugated chitosan nanoparticles, effectively eliminated *C. albicans* infection from a murine model.

Materials and Methods
Materials
Methylglyoxal and fluconazole were procured from Santa Cruz Biotechnology (Dallas, TX, USA). RPMI, glutaraldehyde and low-molecular-weight chitosan were purchased from Sigma-Aldrich (St. Louis, MO, USA). ALT, AST, BUN and creatinine kits were purchased from the Quimica Clinica Aplicada (Amposta, Tarragona, Spain). Sodium sulfate, sodium metabisulfite, Tween 80, percholic acid and 1,2-diaminobenzene (DAB) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Methods
Preparation of MG Chitosan Nanoparticles (MGCN)
A formulation of MGCN was prepared by dissolving 200 mg of chitosan in 1% glacial acetic acid aqueous solution for overnight on a magnetic stirrer as described earlier.[23] To this solution, 1 mL of MG 40% aqueous solution was added in a drop-wise manner to chitosan solution, followed by the addition of 1 mL of Tween-80. Subsequently, 500 μL of 20% sodium sulfate solution, 50 μL of 50% glutaraldehyde solution and 1 mL of 10% sodium metabisulfite were added to the above solution. After overnight incubation, the final reaction mixture was concentrated by Amicon filters. All components used in the preparation of nanoparticles were nonpyrogenic. Sham nanoparticles were prepared using the same procedure without MG and sodium sulfate.

Characterization of MGCN
Determination of Size and Polydispersity Index
Size of MGCN was measured by Malvern Nano zeta sizer (Malvern instruments, Southborough, Massachusetts), using dynamic light scattering technique.

Analysis of Functional Groups by Fourier Transforms Infrared Spectroscopy
FTIR was performed to analyze and compare the changes due to the conjugation of polymer with drug in the formation of nanoparticles. FTIR spectra of MGCN were recorded on the FIR spectrophotometer using a sensitive pyroelectric detector. For this purpose, the lyophilized pressed pellets were prepared by grinding the pellets with KBr in a mortar in a 1: 100 ratio.[28]

Determination of Diameter and Shape

The size and shape of MGCN were determined by the Transmission electron microscopy (TEM). Samples were prepared by drop-casting samples on a carbon-coated copper grid. This grid was placed on a drop of staining agent for more than 5 s followed by drying. The images were recorded on the high-resolution TEM operating on 200 kV.[28]

Surface Morphology

This was done with the Scanning electron microscopy (SEM). A small drop of the sample was placed on a clean coverslip and dried over for a day. The dried sample was coated with a gold sputter coater.[28]

Determination of the Quantity of Methylglyoxal Associated with Nanoparticles

The quantity of MG associated with nanoparticles was calculated by plotting the standard curve and then determining the amount of MG in an unknown sample using the standard plot.[29,30] Since MG does not have any aromatic ring for absorbance, we can use substrate for MG and subsequently plot standard curve for their product. About 200 μL of nanoparticle formulation was taken and treated with 0.5 M perchloric acid to release the entrapped MG. It was then treated with 1.8 mM 1,2-diaminobenzene (DAB) to form 2-methylquinoxaline, the spectra of which were recorded using a UV-Vis spectrophotometer between 200 and 400 nm after the addition of DAB. The maximum absorbance was recorded at 336 nm. The unknown concentration was calculated from the standard curve.

Test Strain

An isolate of *C. albicans* (KFH 121) was acquired from the Department of Microbiology, King Fahad Hospital, Buraydah, Saudi Arabia and was maintained on Sabouraud Dextrose Agar plates. The identification of *C. albicans* strain was confirmed in chromogenic media and by germ tube induction test in serum.

Determination of the Activity of MG and Fluconazole Against *C. albicans*

The antifungal activity of MG was determined by the agar well-diffusion method as described earlier.[31] Sabouraud dextrose agar (SDA) plates were prepared and spread with *C. albicans*. The wells (8 mm in diameter) were made and a 50 μL volume containing 12.5, 25 and 50 μg of MG was loaded in each well and incubated at 37ºC for 48 h. The anti-candida activity of MG was assessed by measuring the growth inhibition zone. The well containing equal volumes of saline was considered as a negative control.

Minimum inhibitory concentration (MIC) of MG and fluconazole was determined by the broth microdilution antifungal susceptibility method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI), Wayne (NCLLS).[32] The final concentration of fluconazole and MG was taken in the range of 0.125 to 128 μg/mL. Antifungal susceptibility testing was done in 96-well round-bottom microtitration plates. The inoculum of *C. albicans* was prepared in 5% dextrose and was diluted in RPMI buffered with MOPS (pH 7.0) to give a final concentration of 5×10^3 cells per mL. The corning 96-well cell culture plates (Sigma-Aldrich, St. Louis, USA) were incubated at 37°C for 48 h. The optical density was measured at 530 nm (BIO-TEK EL310 Microplate Auto-Reader, Biotek instruments). The MIC was the lowest drug concentration that caused 99% growth inhibition as compared to growth control without drug.

Effect of Free MG or MGCN on Hyphae Formation in *C. albicans*

Candida albicans were incubated with human serum for 8 h at 37°C in the presence or absence of 1 and 2 μg/mL of fluconazole or MG or MGCN as described earlier.[33] After 8 h, a very small aliquot of cell suspension was spread on a slide to visualize the formation of hyphae under a microscope with ×100 magnification.

Efficacy of MGCN Against Intracellular Replication of *C. albicans*

J774 macrophages (ATCC® TIB-67™) were plated in triplicates in 24-well Costar plates (Sigma-Aldrich, St. Louis, USA) with 1×10^6 cells/well in DMEM (Sigma-Aldrich, St. Louis, USA) supplemented with 10% fetal bovine serum for 24 h. Macrophages were infected with *C. albicans* at the multiplicity of infection of 2 (MOI=2). After 6 h of infection, cells were washed with 0.1 M Phosphate-buffered saline (pH 7.4) to remove unphagocytosed *C. albicans*. *Candida albicans* infected macrophages were treated at the doses of 10 and 20 μM of fluconazole or free MG or MGCN. After 48 h, macrophages were treated with 1% Triton X-100 (Sigma-Aldrich, St. Louis, USA) in order to release phagocytized

yeasts. Various dilutions of lysate were prepared in PBS and 100 μL of each aliquot was platted on SDA. CFUs were calculated by multiplying the numbers of colony-forming units (CFUs) by the dilution factor as described earlier.[34]

Mice

Female BALB/C mice of 12 weeks were obtained from the animal house facility of the college of Applied Medical Sciences, Qassim University, Buraydah, Saudi Arabia. All experiments in the mice were performed after approval from the animal ethics committee of the College of Applied Medical Sciences, Qassim University, Buraydah, Saudi Arabia. An anesthesia was given by injecting a combination of ketamine and xylazine mixture (90 mg/kg + 10 mg/kg) through the intraperitoneal route as described earlier.[35]

Infection Model

Each mouse was infected by injecting 7×10^5 CFUs of *C. albicans* through the lateral tail vein as described earlier.[36]

Fluconazole Therapy of *C. albicans* Infected Mice

Treatment was started by injecting various doses of fluconazole on days 1, 3 and 5 post 24 h of *C. albicans* infection. Mice were divided into the following groups and each group contained 10 mice:

(1) Normal saline
(2) Fluconazole −5 mg/kg
(3) Fluconazole −10 mg/kg
(4) Fluconazole −20 mg/kg

Determination of Antifungal Activity of MG or MGCN

MG or MGCN at the doses of 1, 5 and 10 mg/kg were tested to assess the efficacy against *C. albicans*. A 40% aqueous stock solution of MG was diluted with sterile normal saline and administered in *C. albicans* infected mice through an intraperitoneal route. Mice were divided into eight groups:

(1) Normal saline
(2) Sham chitosan nanoparticles (SCN)
(3) MG −1 mg/kg
(4) MGCN-1 mg/kg
(5) MG −5 mg/kg
(6) MGCN-5 mg/kg

(7) MG −10 mg/kg
(8) MGCN-10 mg/kg.

Evaluation of the Severity of *C. albicans* Infection

The efficacy of fluconazole or MG or MGCN against the systemic infection of *C. albicans* was evaluated by the survival rate and fungal burden in the kidney tissues.[36] Mice in various groups were observed for 40 days post *C. albicans* infection. In order to determine the severity of *C. albicans* infection, three mice from each group were sacrificed on day 4 postinfection and the kidney was taken out and homogenized. The tissue homogenate was cultured on SDA plates to determine the colony-forming units.

Evaluation of Toxicity of Free MG or MGCN in Mice

MG or MGCN was injected in mice at the doses of 5, 10 and 20 mg/kg for 5 days. On day 6, the blood was taken from three mice of each group to estimate the liver and kidney toxicity parameters, including Aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine as described earlier.[37]

Statistical Analyses

Survival data are shown by the Kaplan–Meier curve and analyzed by the Log-rank Chi-square test. The fungal burden data were analyzed by one-way ANOVA followed by a Bonferroni post-test using GraphPad Prism software, version 6.0 (La Jolla, CA, USA).
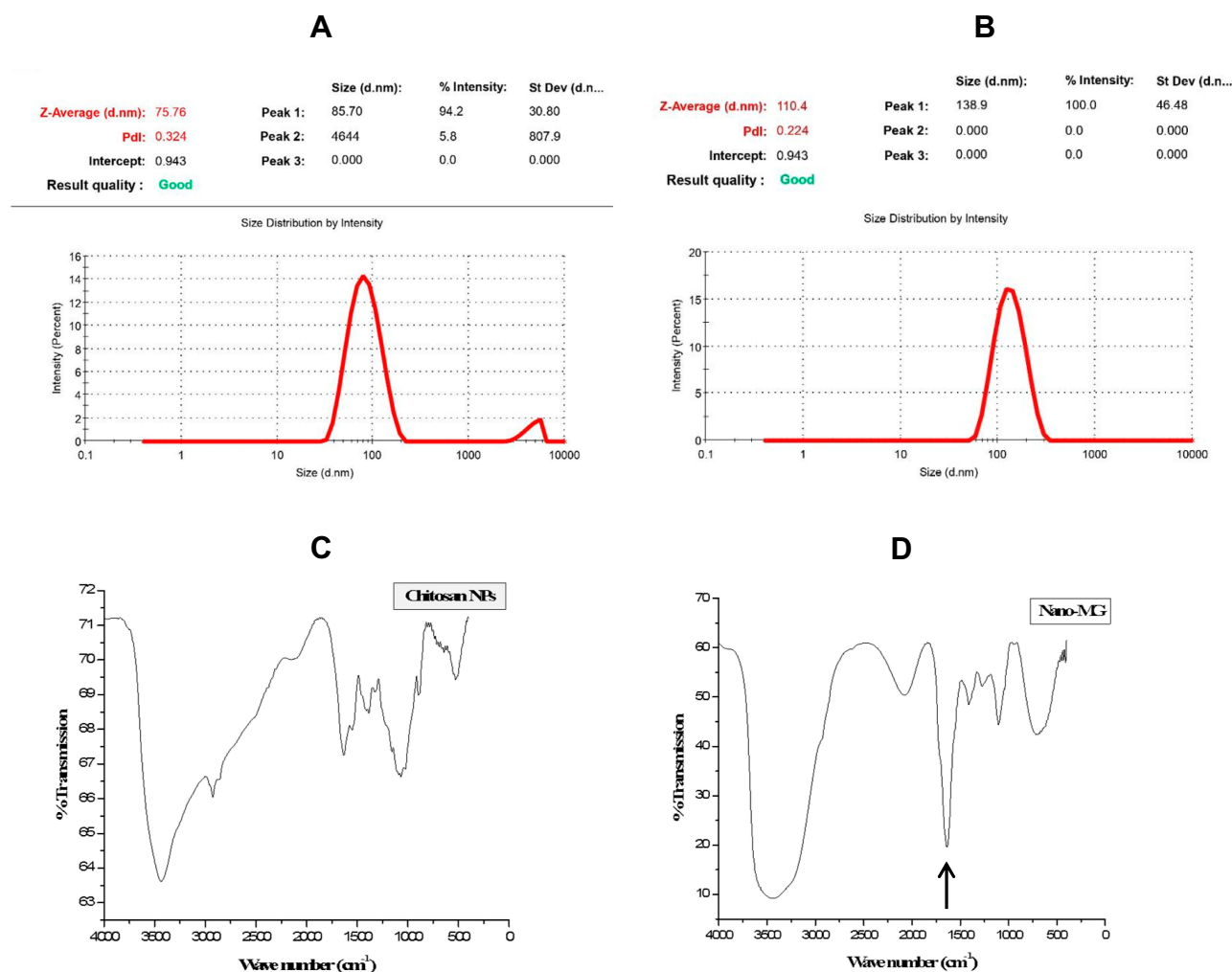
Results

Physical Characterization of Nanoparticles

Size, Polydispersity Index and Analysis of the Functional Group

The diameter of MGCN was measured by the dynamic light scattering (DLS) technique. This technique not only reveals the diameter, but also polydispersity index (PDI) of nanoparticles. It indicates the homogeneity of particles with the value between 0 and 1. Lower values indicate high homogeneity or more uniformity. The stability of the system is related to its PDI value, not by the zeta potential. The particles with higher PDI value have lower stability, whereas those with lower PDI value has high stability. The size of nanoparticles was in the range of 50–100 nm and PDI values were 0.2 to 0.4 as measured by DLS (Figure 1A and B).

A

	Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 75.76	Peak 1: 85.70	94.2	30.80
PdI: 0.324	Peak 2: 4644	5.8	807.9
Intercept: 0.943	Peak 3: 0.000	0.0	0.000
Result quality : Good			

Size Distribution by Intensity

B

	Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 110.4	Peak 1: 138.9	100.0	46.48
PdI: 0.224	Peak 2: 0.000	0.0	0.000
Intercept: 0.943	Peak 3: 0.000	0.0	0.000
Result quality : Good			

Size Distribution by Intensity

C

D

Figure 1 Physical characterization of chitosan nanoparticles. Zeta potential of (**A**) Sham chitosan nanoparticles and (**B**) Methylglyoxal-conjugated chitosan nanoparticles (MGCN). Fourier Transform Infrared Spectra of (**C**) Sham chitosan nanoparticles and (**D**) Arrow indicates the formation of imine bond in MGCN (Nano-MG).

Fourier Transform Infrared Spectroscopy (FTIR) is performed to examine the formation and breaking of the functional groups. Chitosan has a free primary NH_2 group present on its ring, whereas MG is a carbonyl compound. When chitosan interacts with MG, the formation of Schiff base takes place between primary -NH_2 group of chitosan and >C=O group of MG. This Schiff's base is an imine compound containing carbon nitrogen double bond. An imine bond is formed in the formation of MGCN, which gives FTIR peak in their characteristic C=N stretching range 1690–1640 cm^{-1} as indicated by an arrow (Figure 1C and D).

Morphological Characterization of Nanoparticles by TEM and SEM

The transmission electron microscopy (TEM) confirmed the size distribution of MGCN as obtained by DLS.

Although the size of MGCN was in a range of 50–100 nm, but the average size was approximately 70 nm (Figure 2A and B). TEM also confirmed the typical spherical shape of nanoparticles. The size distribution of MGCN through SEM reveals that the surface is smooth with an increase in size due to agglomeration and gold coating (Figure 2C and D).
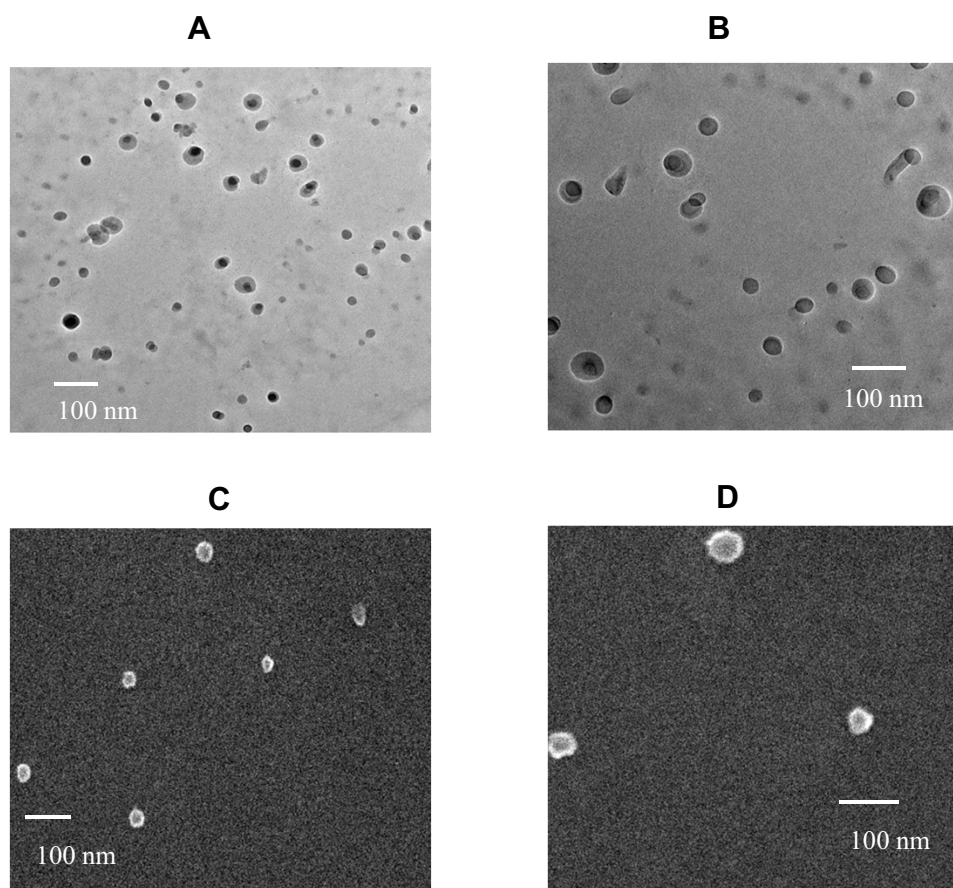
The Amount of MG Associated with Chitosan Nanoparticles

Chitosan associated MG (μM) = 2417.22 ± 61.3426 per mL of nanoparticle formulation. This is 2417.22 μM MG or 2.4 mM of MG in 1 mL.

Molarity = Weight/Molecular weight

Weight =Molarity × Molecular mass

=2.4 mM × 72 = 172 μg of MG per 1 mL

A

B

C

D

Figure 2 Transmission electron microscopy (TEM) images of (**A**) Sham chitosan nanoparticles and (**B**) Methylglyoxal-conjugated chitosan nanoparticles (MGCN). Scanning electron microscopy (SEM) images of (**C**) Sham Chitosan nanoparticles and (**D**) Methylglyoxal-conjugated chitosan nanoparticles (MGCN).

So, the final concentration was 172 µg of MG per 1 mL of formulation.

C. albicans Showed in vitro Susceptibility to Methylglyoxal

The agar well diffusion and the dilution methods were used to determine the susceptibility of *C. albicans* to MG. MG demonstrated remarkable activity against *C. albicans* by the agar well diffusion and dilution methods. A negative control well loaded with vehicle did not show any growth inhibition (Figure 3A), whereas MG at the concentrations of 12.5, 25, 50 µg/mL exhibited the inhibition zones of 19, 30 and 35 mm, respectively (Figure 3B-D).

The antifungal activity of fluconazole and MG was also determined by the dilution method as described earlier.[31] *Candida albicans* with MIC value >64 µg/mL of fluconazole has been categorized as a resistant strain according to the CLSI guidelines. The MIC of fluconazole for the present isolate of *C. albicans* (KFHR 098) was found to be >64 µg/mL, whereas the MIC value of MG was 32 µg/mL.

Methylglyoxal, Not Fluconazole, Inhibits the Hyphae Formation

The effect of lower doses of fluconazole or MG or MGCN was assessed on the transition of *C. albicans* yeast form into hyphae. *C. albicans* showed an extensive hyphae formation in fetal bovine serum (FBS) in the control group without any drug (Figure 4). Fluconazole at the concentrations of 1 and 2 µg/mL did not inhibit the hyphae formation (Figure 4). Free MG or MGCN considerably inhibited the hyphae formation in *C. albicans* at the same concentrations (Figure 4).

MGCN Effectively Inhibits the Intracellular Replication of *C. albicans*

Candida albicans has the ability to multiply intracellularly as shown by the proliferation of *C. albicans* in macrophages after 24 h of infection. The numbers of CFUs in *C. albicans*-infected macrophages were found to be 55,402 ± 5875 in the untreated macrophages (Figure 5). Treatment with fluconazole at the concentrations of 10 and 20 µM

A   **B**

C   **D**
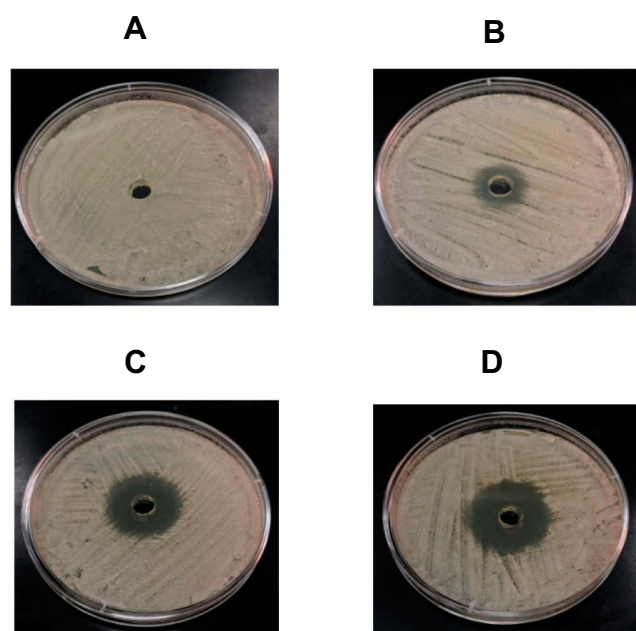
Figure 3 Anti-Candida activity of MG by agar well diffusion method. (**A**) Vehicle control, (**B**) 12.5 µg, (**C**) 25 µg and (**D**) 50 µg of MG.

was ineffective to reduce the intracellular multiplication of *C. albicans* in the macrophages (Figure 5). The CFUs were found to be 53,344 ± 3061 and 52,075 ± 3699 in *C. albicans* infected macrophages treated with fluconazole at the doses of 10 and 20 µM, respectively (Figure 5). Treatment with free MG at the doses of 10 and 20 µM reduced the fungal load to 13,453 ± 2933 and 7281 ± 2785 CFUs in macrophages and was significantly reduced as compared to fungal load in the untreated or fluconazole-treated macrophages (P<0.001). *Candida albicans* infected macrophages treated with MGCN at the doses of 10 and 20 µM exhibited the lowest CFUs of 5142 ± 1432 and 92 ± 76, which was significantly lowered as compared to CFUs in the untreated or fluconazole-treated macrophages (P<0.001). This shows that chitosan nanoparticles effectively delivered MG to intracellular locations in the macrophages.

Fluconazole Treatment Was Not Effective Against *C. albicans*

The antifungal activity of fluconazole at the doses of 5, 10 and 20 mg/kg was assessed against murine candidiasis. The results of the in vivo study correlated well to in vitro findings. Mice did not respond to fluconazole and all infected mice died before day 40 post-infection (Figure 6A). Median survival time of mice in saline-

treated group was found to be 4.5 days, whereas the median survival time of mice in the groups treated with fluconazole at the doses of 5, 10 and 20 mg/kg was found to be 5, 8 and 14 days, respectively (Figure 6A). The median survival time of mice in the group treated with fluconazole at a dose of 20 mg/kg was found to be significantly higher as compared to that of mice in saline-treated group (P =0.0019).

The severity of infection in kidney tissues revealed the highest fungal burden of 159,596 ± 19,869 CFUs per gram of kidney tissue in mice from saline-treated group (Figure 6B). Whereas, the mice in the groups treated with fluconazole at the doses of 5, 10 and 20 mg/kg exhibited the fungal burden of 158,591 ± 26,487, 147,103 ± 18,067 and 138,102 ±16,783 CFUs per gram of kidney, which were found to be insignificant as compared to CFUs in saline-treated mice (P>0.05).

MGCN Eliminates Fluconazole-Resistant Systemic Candidiasis

Free MG or MGCN at the doses of 1, 5 and 10 mg/kg were tested against *C. albicans* infection in mice. Free MG and MGCN showed antifungal activity against an isolate of *C. albicans* not responding to fluconazole therapy. Incorporation in chitosan nanoparticles increased the antifungal activity of MG. *Candida albicans* infected mice treated with MGCN at a dose of 10 mg/kg showed a 90% survival rate, whereas the mice treated with free MG at the same dose showed a 50% survival rate on day 40 (P = 0.0258) (Figure 7A). Mice treated at a dose of 5 mg/kg of MGCN showed a 60% survival rate, whereas the mice treated with free MG at the same dose showed a 20% survival rate (P = 0.0298) (Figure 7A). Treatment with SCN increased the median survival time of *C. albicans* infected mice to 15 days as compared to 5.5 days in saline-treated mice (P=0.0064).

The severity of infection was assessed by measuring the fungal load in the kidney tissues of mice untreated or treated with free MG or MGCN. Mice in the group treated at a dose of 10 mg/kg of MGCN exhibited the greatest reduction to 3175 ± 1281 CFUs as compared to 218,217 ± 38,919 CFUs in the saline-treated group (P<0.001) (Figure 7B). The fungal load of 15,578 ± 4145 CFUs in the kidney tissue of the mice treated at a dose of 5 mg/kg of MGCN was lower to 26,665 ± 4782 CFUs in the mice treated with free MG at a dose of 10 mg/kg (Figure 7B).

Control   **Flu-1 µg/ml**   **Flu-2 µg/ml**

MG-1 µg/ml   **MG-2 µg/ml**

MGCN-1 µg/ml   **MGCN-2 µg/ml**
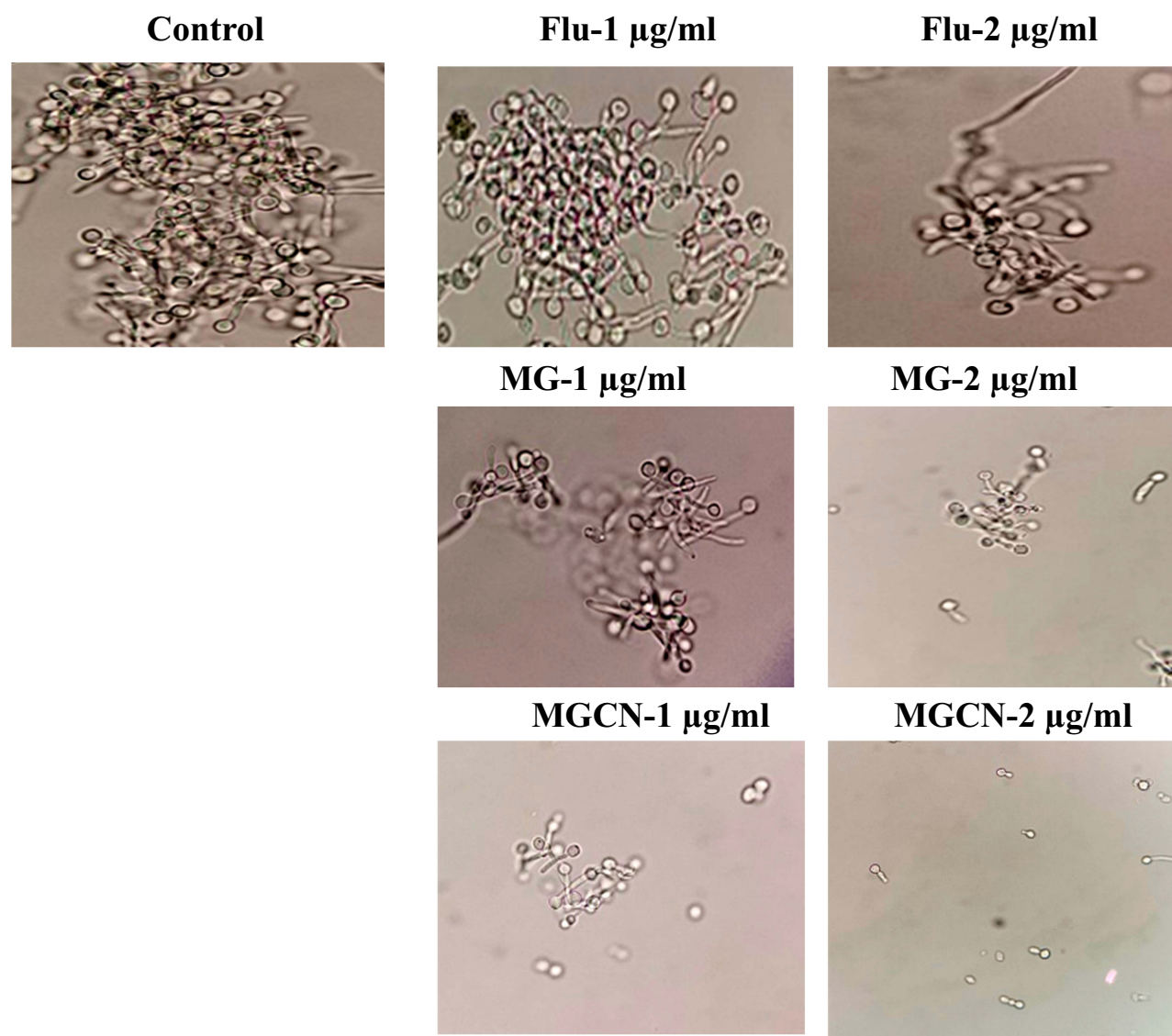
Figure 4 Effect of Fluconazole, MG and MGCN on the transition of *Candida albicans* yeast cells into hyphae form.

Free MG or MGCN Did Not Induce Any Toxicity in Mice

To evaluate whether the administration of MG or MGCN is associated with any toxicity, the hepatic and renal toxicity parameters were analyzed in the treated mice. The administration of MG or MGCN did not induce any hepatic toxicity as shown by the levels of AST and ALT (Figure 8A and B). Similarly, there was no significant change in the levels of BUN and creatinine in mice treated with MG or MGCN at the doses of 10 and 20 mg/kg (Figure 8C and D). These results suggest that MG or MGCN is completely safe at the doses used in the present study.

Discussion

Fluconazole is one of the most commonly used antifungal drug to treat fungal infections and its extensive use has resulted in the emergence of drug-resistant fungal infections, including *Candida* spp.[4,5] Multi-drug resistance genes like MDR1, CDR1 or CDR2 or ERG11 are over-expressed in fluconazole-resistant *Candida* isolates.[6,8,38] The findings of this study showed that fluconazole exhibited higher MIC value (>64 mg/L) against the present isolate of *C. albicans*. To examine whether in vitro findings correlate to in vivo results, the efficacy of fluconazole against *C. albicans* was assessed in a murine model. The findings of in vivo study were in accordance with in vitro
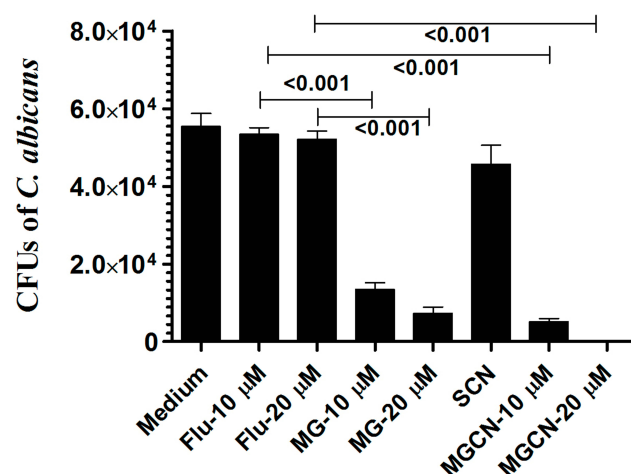
Figure 5 Macrophages (1 × 10⁶ cells/well) were infected with *C. albicans* at multiplicity of infection of 2 (MOI=2) followed by treatment with fluconazole or free MG or MGCN at the concentrations of 10 and 20 μM. After 48 h, the cells were lysed and intracellular yeasts were plated on Saboraud dextrose agar plates to determine the number of CFUs. Data are expressed as mean ± SE. A P value <0.05 was considered to be significant.

results. *Candida albicans* infected mice did not respond to fluconazole even at a dose of 20 mg/kg. The survival data were strengthened by the fungal load data in kidney tissues. *Candida albicans* infected mice treated with fluconazole showed substantially higher fungal load in their kidney.

Due to limited numbers of available current antifungal drugs, there is a critical need to find new antifungal agents to treat *C. albicans* infection. MG has been shown to possess the antimicrobial activity against *Pseudomonas aeruginosa, Staphylococcus aureus,*

Enterococcus faecalis and Salmonella typhi.[39,40] Moreover, MG and methylglyoxal-bis-guanylhydrazone have shown inhibitory effects against HIV and influenza virus.[41,42] None of the earlier studies have shown the effect of MG against fungal pathogens. In the present study, we determined the activity of MG and MGCN against *C. albicans* infection. Conjugation of MG to chitosan nanoparticles resulted in the increased activity of MG.[29,30]

Candida albicans shows an ability to grow as unicellular budding yeast cell and filamentous hyphae form. The hyphae form is considered a virulent factor as compared to the yeast form of *C. albicans*. The immune cells respond differently to the yeast and hyphae forms in relation to the secretion of cytokines. For example, yeast cells of *C. albicans* induce dendritic cells to secrete IL-12, whereas hyphae form induces the secretion of IL-4.[43] Moreover, hyphae did not induce IFN-γ secretion because hyphae show short of recognition by TLR4.[44] Thus, the hyphae form of *C. albicans* has the ability to evade the Th1 immune response. A therapeutic agent that is capable of inhibiting the transition of yeast *C. albicans* into the hyphae may thwart the immune evasion strategy of *C. albicans*. The findings of the current study demonstrated that MG or MGCN, not fluconazole, inhibited the transition of *C. albicans* yeast into hyphae at lower doses. Thus, MG formulations can effectively reduce the virulence of *C. albicans*. Interestingly, chitosan itself inhibits the growth of

A

B
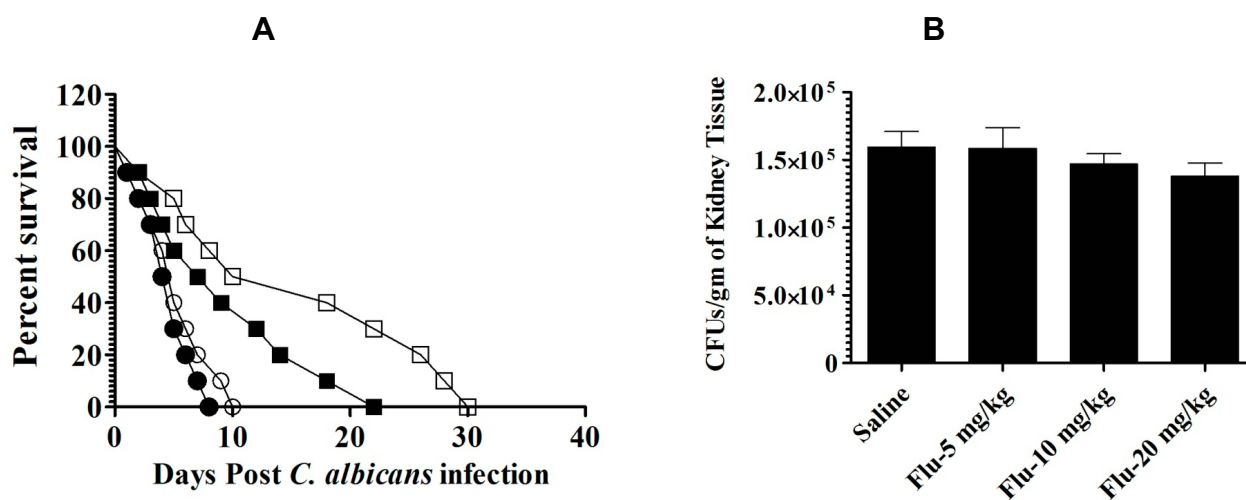
Figure 6 Fluconazole did not show antifungal activity against *C. albicans*. (**A**) Each mouse was infected with 7 × 10⁵ CFUs of *C. albicans*. Mice were treated with 5, 10, 20 mg/kg of fluconazole on days 1, 3 and 5 postinfection. Mice were observed for 40 days to monitor their survival. Saline (●), fluconazole (5 mg/kg) (○), fluconazole (10 mg/kg) (♦), fluconazole (20 mg/kg) (♦). (**B**) On day 4, three mice from each group were sacrificed and their kidney was taken to prepare tissue homogeninate. The kidney tissue homogenate was cultured in order to determine the fungal load. Data are expressed as mean ± SE. A P value <0.05 was considered to be significant.

A

B
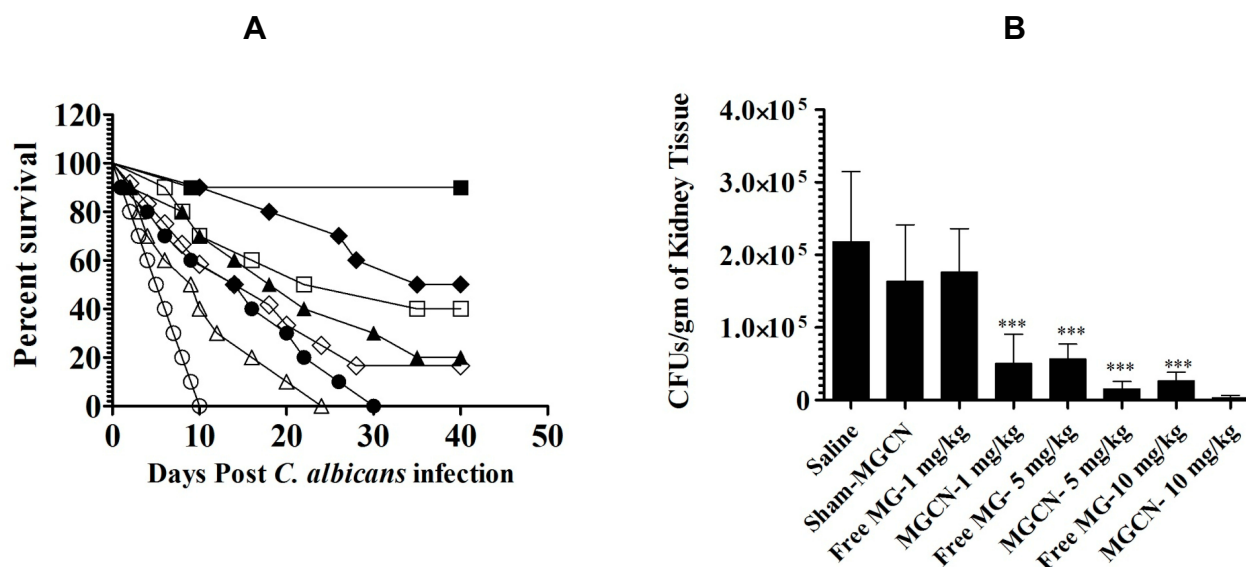
Figure 7 Treatment with MGCN showed the highest antifungal activity against C. *albicans*. (**A**) Mice infected with *C. albicans* (7×10^5 CFUs/mouse) were treated with 1, 5 and 10 mg/kg of MG or MGCN on days 1, 3 and 5 postinfection. Mice were observed for 40 days to monitor their survival. Saline (○), SCN (●), Free MG-1 mg/kg (Δ), MGCN-1 mg/kg (▲), Free MG-5 mg/kg (♦), MGCN-5 mg/kg (♦), Free MG-10 mg/kg (□), MGCN-10 mg/kg (■). Saline vs SCN (P=0.0064), Free MG-1 mg/kg vs MGCN-1 mg/kg (P=0.0284), Free MG-5 mg/kg vs MGCN-5 mg/kg (P=0.0298), Free MG-10 mg/kg vs MGCN-10 mg/kg (P=0.0258). (**B**) On day 4, three mice from each group were sacrificed and their kidney was taken to make tissue homogenate. The kidney tissue homogenate was cultured to determine the fungal load. A P value <0.05 was considered to be significant. *** (*P*<0.001).

important fungal pathogens, including *A. fumigatus*, *C. albicans* and *C. neoformans*.[45] Thus, the formulations of chitosan nanoparticles-loaded antifungal drugs may have additional effectiveness against fungal infections. This is also evident from the results that showed a greater survival rate of mice in the group treated with sham chitosan nanoparticles.

Macrophages are an important part of the innate immune response to *C. albicans* infection.[46] *Candida albicans* adopts various strategies to evade macrophage-mediated onslaught in order to replicate within macrophages.[47,48] The conversion of *C. albicans* from the yeast to hyphae is critical to sabotage the microbicidal action of the phagosome. In addition, *C. albicans* produces ammonia to alkalinize the acidic environment of phagosomes to facilitate the yeast to hyphae transition.[49] Because MG inhibits the transition of the yeast to hyphae, treatment with MG or MGCN can reduce the ability of *C. albicans* to avoid the onslaught of macrophages. Moreover, MG activates the macrophages to secrete TNF-α and IFN-γ that have a protective role against tumor and infectious diseases.[50] The results of the present study demonstrated the macrophage-activating property of MG because MG-treated macrophages exhibited the lowest intracellular fungal burden.

Because of the recent emergence of azole-resistant fungal isolates and toxic manifestations of antifungal drugs, it is important to find new antifungals that show increased efficacy with decreased toxicity. The finding of the present study showed that MGCN has strong antifungal efficacy. Interestingly, *C. albicans* infected mice treated with sham chitosan nanoparticles significantly increased the survival rate as compared to the saline-treated mice (P=0.0064). MGCN at a dose of 10 mg/kg was highly effective and mice in this group exhibited a 90% survival rate as compared to 50% survival rate of mice treated with free MG at the same dose (P=0.0258). Ironically, the most effective polyene antifungal drugs have shown considerable renal and hepatic toxicity in the treated subjects.[9,26] Importantly, administration of MG or MGCN was found to be safe, and treated mice did not reveal any substantial changes in liver inflammation markers such as ALT and AST and renal function parameters such as BUN and creatinine.

In conclusion, MGCN was prepared in this study through the conjugation of the aldehyde group of MG to free amino group of chitosan. Free MG or MGCN showed antifungal activity against an isolate of *C. albicans* that did not respond to fluconazole therapy. Chitosan also shows an antifungal activity against *C. albicans*. MGCN demonstrated a synergistic antifungal effect of MG and chitosan against *C. albicans* both in vitro and in a mouse model. Moreover, a combination therapy of MGCN with

A

B

C

D
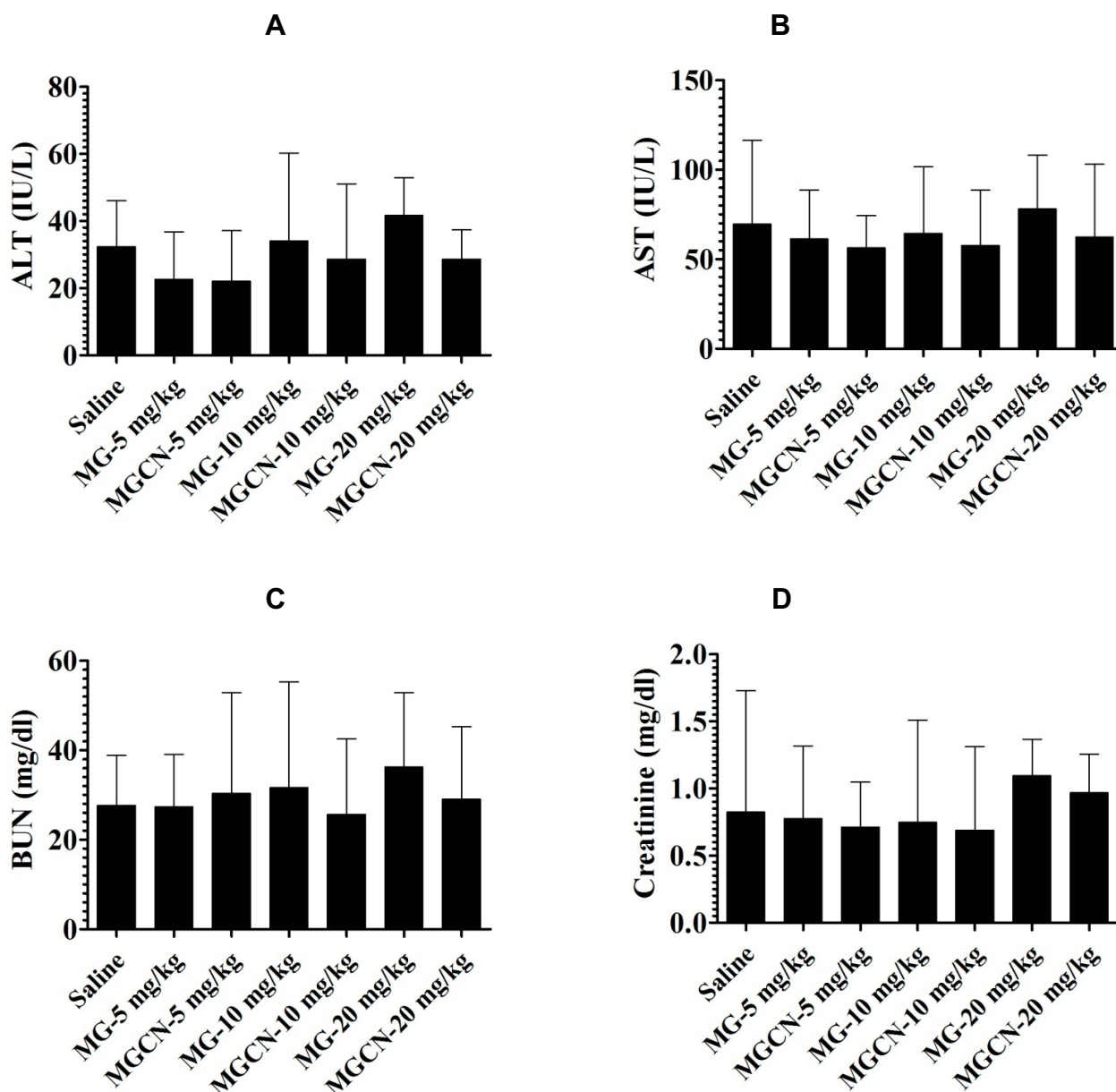
Figure 8 Administration of free MG or MGCN did not induce any toxicity. Mice were injected with MG or MGCN at the doses of 5, 10 and 20 mg/kg for consecutive 5 days. On day 6 postinjection, the blood was taken from three mice of each group to estimate the levels of (**A**) ALT, (**B**) AST, (**C**) BUN and (**D**) creatinine. Data are expressed as mean ± SE.

antifungal drugs such as fluconazole, itraconazole and amphotericin B may be considered to treat drug-resistant fungal pathogens. The results of the current study revealed that MGCN showed superior anti-*Candida* activity as compared to free MG at the respective doses. Owing to its ability to inhibit yeast to hyphae transition, intracellular killing, the enhanced in vivo efficacy and reduced toxicity,

MGCN may be proved a very potential chemotherapeutic agent to treat *C. albicans* infection.

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Disclosure

The authors report no conflicts of interest in this work.

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