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Role of Fluconazole Nanoemulsion in Inhibiting Liver Candidiasis in Female Mice and their Embryos

Sanaa H. Mohammed[1] , Ahmed H. Saleh[1] , Khaled H. Abu-Elteen[2] ,Batol I. Dheeb [3,*]and Safa M. Abdulateef[4]

[1]Biology Department, College of Science, University of Kirkuk, Kirkuk, Iraq; [2] Department of Biology and Biotechnology, Faculty of Science, The Hashemite University, Zarqa, Jordan; [3] Department of Pathological Analysis, College of Applied Science, Samaraa University, Iraq; [4]College of Science, Al Nahrain University, Jadriya, Iraq.

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Abstract

The current study aims to reveal the role of fluconazole nanoemulsion (FLX-NE) in inhibiting the candidiasis infection in female mice and their embryos. In the current investigation, thirty-two normal female Mus musculus mice, eight pregnant mice per group, were utilized. The mice were between the ages of twelve and sixteen weeks and weighed an average of twenty to twenty-five grams. At random, the animals were divided into four groups as follows: Group control, *Candida albicans* infected group, FLX-NE treated group, and the treated (*C. albicans* was administrated with a suspension and treated with FLX-nanoemulsion) group. The findings demonstrated (according to the HPLC) that the NE system preparation used glycerin oil as the oil phase, ethanol as the co-surfactant, tween 80 as the surfactant to prepare the optimum FLX-NE. Infecting the tested animals with *C. albicans* led to some histological changes in the liver, including degeneration and necrosis of hepatocytes and infiltration of inflammatory cells. Furthermore, some morphological abnormalities were found in the embryos which included a decrease in their number, short front limbs, and red spots on the skin. Nevertheless, after FLX-NE treatment, the liver tissues and embryos became semi-normal compared with the control group, where the central veins and hepatocytes appeared normal and decreased the levels of degenerative changes. The levels of CD163 in infected mice displayed significantly ($P \leq 0.05$) higher levels than untreated group. On the other hand, there was an improvement in CD163 levels in the treated group; however, a significant ($P \leq 0.05$) change was still observed when compared to the untreated group. In conclusion, the study showed that fluconazole nanoemulsion has a very strong effect against *C. albicans* infection.

Keywords: *Candida albicans;* nanoemulsion; Fluconazole; UTI; CD163.

1. Introduction

High morbidity and mortality rates are a hallmark of mycoses (fungal infections), which annually impact a sizable portion of the global population (Matthaiou *et al.,* 2015; Schmiedel and Zimmerli 2016; Bongomin *et al.,* 2017; Atiencia-Carrera *et al.,* 2022; Mohammed *et al.,* 2024). Humans have consistently been at risk from fungus infections (Vallabhaneni *et al.,* 2016). Immunocompromised individuals who are hospitalized are particularly the main victims of the growing number of fungal illnesses (Garnacho-Montero *et al.,* 2024; Leelambigai *et al.,* 2024). A fungus called Candida can cause candidiasis which affects more than 4 billion individuals, annually (McDermott 2022). One of these species is common opportunistic *Candida albicans* which drew a lot of attention in both basic and clinical biology (McManus and Coleman 2014). Given that approximately 75% of women experience human papillomavirus (HPV) infection at least once during their lifetime, and around 70% of adults harbor it as a benign commensal in their genitourinary and digestive tracts (Sarvtin *et al.,* 2014), it follows that these individuals are susceptible to one of the most prevalent reasons for gynecological consultations at basic healthcare facilities—vaginal candidiasis (Gonzalez *et al.,* 2011). Vulvovaginitis occurs when the infection affects the female genital areas and can result in intense itching and burning sensations, as well as potential white discharge (CDCP 2012). Materials utilizing nanoemulsion formulations hold great potential in this world. As practical dispersions of deformable nanoscale droplets with a range of flow characteristics and optical qualities, from opaque to almost transparent, nanoemulsions show considerable promise. These linkages and the formula's intriguing physical characteristics set them apart from common micro-emulsions (Lovelyn and Attama 2012). The nanoemulsion's droplet size ranges from 50 to 500 nm. The active components in citronella essential oil are anticipated to work more efficiently and effectively thanks to several benefits of this composition (Azmi *et al.,* 2019; Hussain *et al.,* 2024). Nanomaterials are typically described as environmentally friendly materials that have been used in a wide range of applications (Katva *et al.,* 2015). Previous research demonstrated the antibacterial properties of a nanomaterial in several ways. The

* Corresponding author. e-mail: batol.omran35@uosamarra.edu.iq ,batoolomran@yahoo.com .

formation of reactive oxygen species (ROS) and the interaction of proteins or DNA as intracellular inducing effects, for example, affect the bacterial division of cells (Makabenta *et al.,* 2021). Additionally, these substances may disrupt the bacterial film that is acting on intracellular components, leading to cellular dysfunction (Tacconelli *et al.,* 2018; Makabenta *et al.,* 2021). Fluconazole is a triazole antifungal drug that belongs to the triazole family (Zhou *et al.,* 2022). It is an FDA-approved medication for the treatment of different candidiasis infections. Additionally, it has been demonstrated that prophylaxis lowers the risk of candidiasis in bone marrow transplant recipients who are also undergoing radiation therapy or cytotoxic chemotherapy (Galgiani *et al*., 2016). Therefore, the current study aims to reveal the role of fluconazole nanoemulsion against the toxicity of *Candida albicans* on female mice and their embryos.

2. Materials and Methods

2.1. Collection of urine samples

Between February and May 2023, 50 urine samples were taken from women who visited Azadi Teaching Hospital (Iraq) and Maternity and Gynecological Hospital with signs and symptoms of infections of the urinary tract. The laboratory received the specimens and examined the microscopic E. coli bacteria.

2.2. Solubility studies

Solubility assessments were conducted for fluconazole in glycerin oil, olive oil, co-surfactants, and surfactants (Reddy *et al.,* 2012, Dheeb *et al.,*2022). In a glass tube with a screw cover, 2 ml of the excipient and fluconazole were combined. An isothermal shaker (Xiaoyang, China) was used to shake the mixture for 48 hours at 25º C in order to reach equilibrium. Before the HPLC analysis, each tube was spun at 5000 rpm for 15 minutes to determine the drug concentration. After that, the supernatant was diluted with methanol.

2.3. Construction of pseudo-ternary phase diagrams

To construct components for pseudo-ternary phase diagrams, a water titration technique was used to create oil, a mixture of surfactant and co-surfactant, and water (Schalbart *et al.,* 2010, Mohammed *et al.,* 2024, Hatem and Dheeb,2024). Co-surfactant and surfactant were combined in a variety of ratios (1:1, 1:2, 1:3, 1:4, and 2:1). Oil and surfactant: co-surfactant (Smix) were mixed in a variety of weight ratios up until the maximum ratio of Smix and oil was attained, following each phase diagram's instructions. The aqueous phase was then gradually added to each of these mixes, and each mixture was titrated using a moderate magnetic stirrer. By measuring the water concentration at which transparent-to-turbid transitions occurred, the titration's endpoint was identified (Ghareeb *et al.,* 2017).

2.4. Heating-cooling cycle (H/C cycle)

The stability of FLX-NE is dependent on temperature change, which was investigated by using a heating-cooling cycle. Six cycles, between 5 °C and 50 °C, were applied to the NE formulations, and they were kept at each temperature for at least 48 hours (Sadoon and Mowafaq, 2020).

2.5. Fourier transform infrared spectroscopy (FTIR)

The assessment of drug-formulation compatibility was conducted by employing FTIR with the acquisition of spectral data. It is utilized to pinpoint the functional groups' attachment methods as well as the molecule's unique fingerprint. The samples can be prepared using a reliable technique, such as Nujol mulls, and then scanned in FTIR at a slow scanning speed (4000 to 400 cm^{-1}) (Khalaf *et al*., 2018; Manyarara et al., 2018).

2.6. Field emission scanning electron microscope (FESEM)

Moreover, fluconazole nanoemulsions underwent a field emission scanning electron microscopy (FESEM) study. It was utilized to describe the size and shape of the droplets included within the generated fluconazole nanoemulsion. Furthermore, it is a device used to analyze the surface and dimensions of nanoemulsion (*Abu Bakar et al.,* 2011; Saleh and Abbood, 2020).

2.7. HPLC technique

The examination and determination of potential interactions between oil, drugs, and other excipients employed the HPLC method. A water-based HPLC system with an SPA-20A detector was used. Breeze software was used to control the system. The mobile phase had an acetonitrile:water ratio of 65:35 v/v, was flowing at a rate of 1 ml/min, and the injection volume was 10 l. The 239 nm detective wavelength was chosen. Before use, the mobile phase was filtered via a millipore solvent filtration device (0.45 m) (Channabasavaraj *et al*., 2010., Hashim *et al.*,2023, Hussain *et al.,* 2024).

2.8. Study animals

In the current study, 32 healthy female Mus musculus mice, aged 12 to 16 weeks, with average weights of 20 to 25 grams, and 8 pregnant mice per group, were employed. The animals were randomly divided into four groups as follows: Control group: administered with normal saline. infected mice: C. albicans (isolated from urinary tract infections) was administered (orally) at a concentration of 1.5 x 10^8 cfu/ml. FLX group: FLX-nanoemulsion, was administered with 0.1 ml. treated mice: C. albicans was administrated with a suspension and treated with FLX-nanoemulsion. The administration was made before pregnancy, after 10 days from fertilization, and continued until the 18th day of pregnancy, which represents the dissection day. Ethical approval was obtained from the Research Ethics Board at Kirkuk University (Ethical Approval number: KIEC\ 0544\0043 dated May 29, 2023).

2.9. Histological study

With a 4 mm ear-punch of 2% xylocaine as the anesthetic, liver samples were collected. The biopsies were routinely processed, fixed in 10% formalin, embedded in paraffin slices, stained with hematoxylin and eosin, and seen under a microscope (Abdul *et al*., 2023).

2.10. Investigation of the CD163 levels

Every study subject's serum contained CD163, which was identified. The standardized sandwich ELISA method was used to accomplish this. In summary, 50 µl of each specimen was added in each well that had previously been coated with sCD163 antibody. The wells were then incubated for 120 minutes at 37°C, washed with phosphate

buffer saline, and then 50 µl of streptavidin horseradish peroxidase was added. The wells were then again incubated for two hours at 37°C, and again were washed with phosphate buffer saline. Each well received 50 µL of substrate components A and B until color development. To terminate the reaction, 50 µL of a stop solution was added. Using an ELISA reader, the optical density at 450 nm was measured.

2.11. Statistical Analysis

Analysis of variance (ANOVA) was performed to examine experimental mean values; the one-way ANOVA test was employed to ascertain the degree of significance within a single experimental group, and the LSD (Fisher's least significant difference) test was used to determine the difference between different means, and $P < 0.05$ was considered statistically significant (Ahmed and Saleh 2021).

3. Results & Discussion

3.1. Diagnosis of Candida albicans isolates

After collecting urine samples from mice infected with *C. albicans* as well as from the control group, the urine samples were cultured on Sabouraud dextrose agar ((HiMedia, India) media. The group of infected mice showed positive growth of the fungus. *C. albicans* was identified based on cultural and microscopic characteristics and biochemical tests. *C. albicans* was also diagnosed on CHROM Agar Candida medium (CHROMagar Candida, France). To confirm infection with the fungus, the WBC count was examined among the infected mice before treatment, as well as in the control group.

The results showed that the mice injected with the fungal suspension had a significant (P>0.01) increase in the WBC count (16.28±2.83 $X10^3$ cell/mm^2) compared to the control group (5.72±1.64 $X10^3$ cell/mm^2). The significant increase in the number of WBC in mice injected with the fungal suspension than control mice indicates the extent of the intense immune response to the fungal infection, as white blood cells are considered the first line of defense for that response following various infections (Anderson 1980, Al Zaher *et al.*,2024, Abdulateef *et al.*,2024). In general, most parasitic and fungal infections are characterized by a noticeable increase in the number of white blood cells, which is one of the most prominent signs of the immune response to the infection. The rise is caused by histamine being released, particularly because it is a chemo-attractive agent, and a condition that leads to the destruction of mast cells, and thus the release of histamine from its granules helps to increase white blood cells, especially acidic ones (Archer 1980, Al-Sarraj *et al.,* 2024).

3.2. Fluconazole nanoemulsion properties

Some properties of Fluconazole nanoemulsion were studied, including Saturation solubility, FESEM, FTIR spectra and Validation of the HPLC method.

3.2.1. Saturation solubility of fluconazole

The NE system preparation used glycerin oil as the oil phase, tween 80 as the surfactant, and ethanol as a co-surfactant following the saturated solubility data obtained as shown in Table 1.

Table 1. Fluconazole solubility (mg/ml) in different oils, surfactants, and co-surfactants.

Compounds		Solubility mg/dl
Oil	Olive	8.5
	Glycerin	39.1
Surfactant	Tween 20	73.5
	Tween 80	96.2
Co-Surfactant	Ethanol	58.2
	Acetone	14.7

3.2.2. FESEM

Figure 1 demonstrates that the oil droplets produced by the improved formula are spherical in shape and accumulate smaller oil droplets (mentions the nano size of the droplets achieved), although the shape and size of the oil droplets did not significantly alter as they accumulated (Redzuan *et al.,* 2011; Bozdağ-Pehlivan *et al.,* 2011). This result appeared to be comparable with the findings of Yuan *et al. (*2008) on β-carotene nanoemulsion, where the oil droplet size remained unaltered after 1 month at 4°C storage.

3.2.3. FTIR Spectra

The typical peaks in the FTIR spectra of pure FLX powder are 3005.10 cm^{-1} due to vibrational (N-H) stretching, 2926.01-2854.65 cm^{-1} related to (=C-H) stretching, and 2673.34 cm^{-1} due to aliphatic (C-H) stretching. The stretching of ester (C=O) is responsible for 1710.86 cm^{-1}, aromatic C=C stretching is responsible for 1460.11 cm^{-1} and 1413.82 cm^{-1}, aliphatic CH bending is responsible for 1286.52 cm^{-1} and 1247.94 cm^{-1}, disubstituted orthobenzene stretching is responsible for 723.31 cm^{-1}, and CN stretching is responsible for 1089.78 cm1 and 937.40 cm^{-1}. Figure 2 shows the FTIR spectra of the FLX.

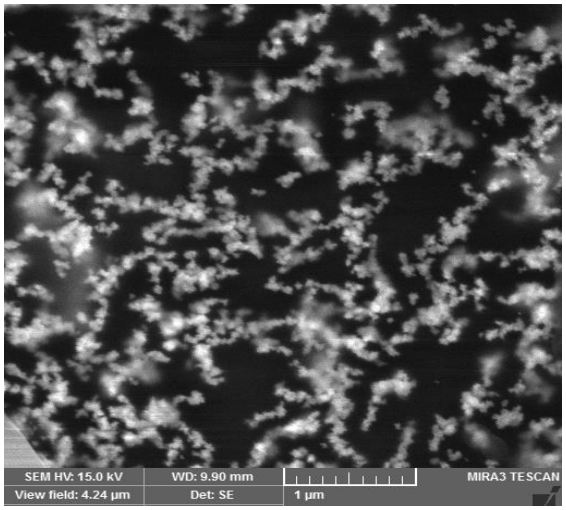

Figure 1. FESEM of optimized formula FLX-NE, the optimized formula produces spherical fluconazole oil droplets with a collection of smaller oil droplets.

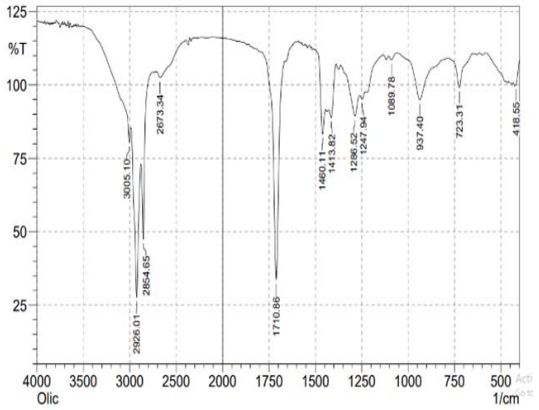

Figure 2. The FTIR spectra of FLX.3.2.4 Validation of the HPLC method

There was no difference in retention time between the chromatograms of Pure Drug FLX and FLX-NE, and no additional peaks were noted. Therefore, it was discovered that certain excipients were compatible with FLX. Chromatograms of FLX and FLX-NE were displayed in Figures 3 and 4, respectively.

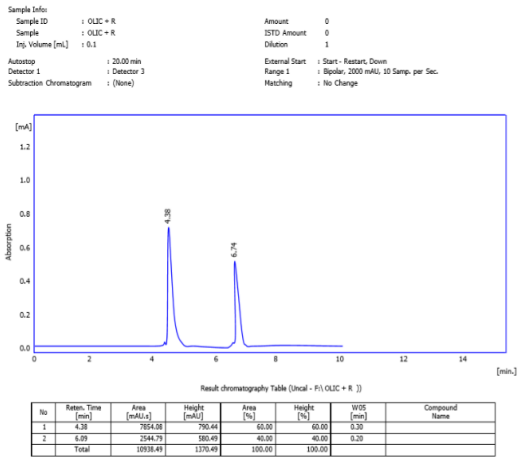

Figure 3. Chromatograms of FLX-NE

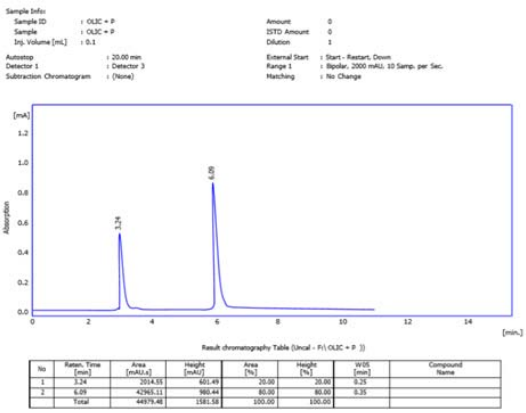

Figure 4. Chromatograms of Pure drug FLX**.**

3.3. In vivo study

The *in-vivo* study was conducted to reveal the role of FLX-NE against the toxicity of *C. albicans* which causes some histological changes in the liver. The section of liver of control group showed normal structure (Figure 5); after infection with *C. albicans*, the liver of pregnant mice underwent several histological changes, including hepatocyte degeneration and necrosis, thickening of blood vessel walls, fibrocyte presence, and infiltration of mononuclear inflammatory cells (Figure 6); also the liver of FLX-NE group showed normal structure and cells (Figure 7) as shown in table 2. Moreover, various morphological anomalies were discovered in the embryos, such as short front limbs, red spots on the skin, thickening of the skin in some places, and a decrease in the number of embryos table 3. After treatment with FLX-NE, the liver of treated group (Figure 8) was closer to the control group, and the morphological changes of the embryos were limited and were closer to the control group.

Table 2. Histological changes in the pregnant mice livers.

Groups Lesions	Control Mice	Infected Mice	FLX-NE Group	Treated Group
Degeneration	Trace	++	Trace	Trace
Necrosis	Trace	++	Trace	Trace
Congestion	Nil	+	Nil	Nil
Fibrocytes	Nil	Trace	Nil	Nil
Infiltration of lymphocytes	Nil	++	Nil	Trace
Thickening walls	Nil	+	Trace	Trace

Table 3. Morphological changes in the pregnant mice embryos.

Groups Changes	Control Mice	Infected Mice	FLX-NE Group	Treated Group
No. of embryos	10±2	5±3	9±2	9±3
Red spots	Nil	+	Nil	Trace
Thickening of the skin	Nil	+	Nil	Trace
Short front limbs	Nil	+	Nil	Nil

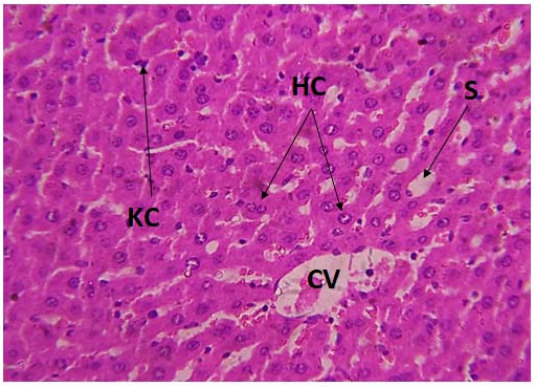

Figure 5. The liver of control group showing the normal shape of central vein (CV) and hepatocytes (HC) with normal sinusoids (S) and kupffer cells (KC) H&E X400.

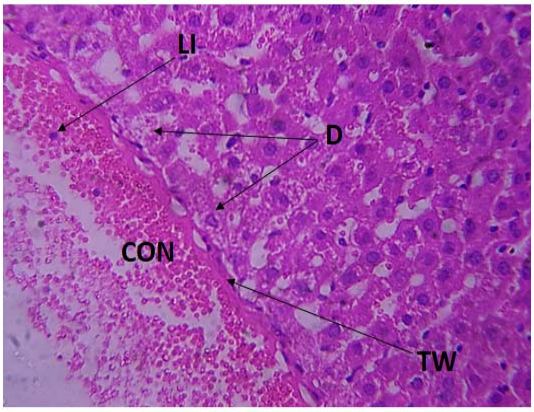

Figure 6. The liver of infected group showing thickening wall of central vein (TW) with congestion (CON), degeneration (D) of hepatocytes and lymphocytes infiltration (LI) H&E X400.

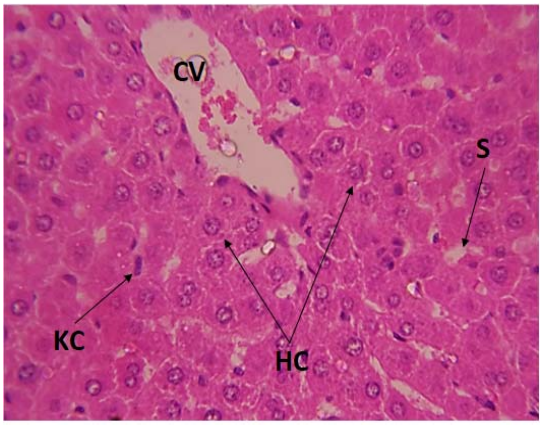

Figure 7. the liver of FLX-NE group showing the normal shape of central vein (CV) and hepatocytes (HC) with normal sinusoids (S) and kupffer cells (KC) H&E X400.

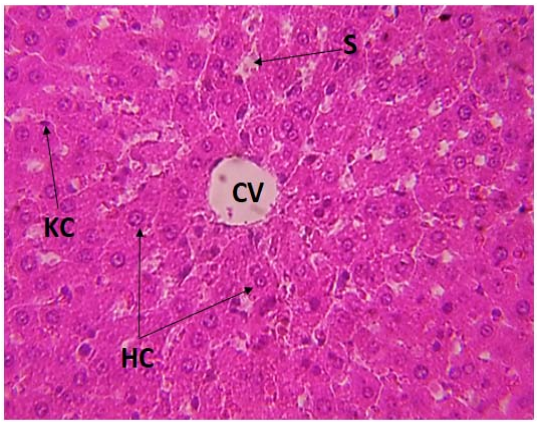

Figure 8. the liver of treated group showing the normal shape of central vein (CV) and hepatocytes (HC) with normal sinusoids (S) and kupffer cells (KC) H&E X400.

The results from the present study align with those from a previous investigation conducted by Mohamed *et al.* (2010). Their study similarly noted that the administration of *C. albicans* led to degenerative changes, irregular liver cell arrangement, and increased Kupffer cell presence, as well as the occurrence of congestion areas interspersed among the degenerative cells. Additional degenerative alterations encompassed the infiltration of mononucleated cells, the presence of necrotic foci surrounded by a high influx of inflammatory cells, and further degenerative changes. The results of our study align with those reported by AL-Naqeeb *et al*., (2019), illustrating histological abnormalities in the livers of mice infected with *C. albicans*, such as hepatocyte necrosis and degeneration. Treatment with both fluconazole and fluconazole nanoemulsion in our current study notably improved the external appearance of the embryos and liver tissues in pregnant mice.

Fluconazole exerts its influence by blocking the conversion of lanosterol to ergosterol (Graybill 2001, Awad *et al.,* 2020), thus inhibiting fungal sterol synthesis (Kołaczkowska and Kołaczkowski 2016) and impeding cell membrane formation. These effects collectively account for the observed outcomes. Notably, fluconazole has been efficacious in treating Candida infections (Watt *et al.,* 2015). Nevertheless, it is important to acknowledge the existence of Candida species that display resistance to azole medications (D'asheesh *et al.,* 2020, Abdulateef *et al.*,2024).

In our study, nanoemulsions played a pivotal role due to their non-toxicity at biocidal dosages to mucosal and gastrointestinal tissues (Sonneville *et al.,* 2004.,Salih *et al.,* 2022), underscoring their significance in the administration of fluconazole. Consequently, our study's killing kinetics demonstrated the enduring fungicidal effects of fluconazole nanoemulsion.

Nanoemulsions at biocidal concentrations are nontoxic to mucosal membranes and gastrointestinal tract tissues (Krishnamoorthy *et al*., 2021). This explains the absence of damage to liver tissue in the current study when using fluconazole nanoemulsion. On the other hand, the synergy between fluconazole and essential oil bioactive compounds, which produces strong antifungal activity against fungal infections, explains the current results (Al-Naqeeb *et al*., 2018., Abed *et al.,* 2022).

The synergistic action between bioactive components of essential oils and nanodroplets is believed to contribute to the substantial antifungal activity against fungal infections, offering a plausible explanation for these findings (Ajaiyeoba 2000; Bala *et al.,* 2010; Abdullah *et al.*,2019; Hussein *et al.,* 2024).

3.4. Detection of CD16 in studied groups

Figure 9 showed significant ($P \leq 0.05$) changes in the levels of CD163 between studied groups, where the concentrations of CD163 in infected mice (29.51±6.03) showed significant ($P \leq 0.05$) elevated compared with control group (12.74±3.84); otherwise, there are non-significant ($P \leq 0.05$) differences in level of CD163 in FLX-nanoemulsion group (11.94±1.51) compared with control group. On the other hand, there was an improvement in CD163 levels in the treated group (20.42±2.36), but there was still a significant ($P \leq 0.05$) difference compared to the control group.

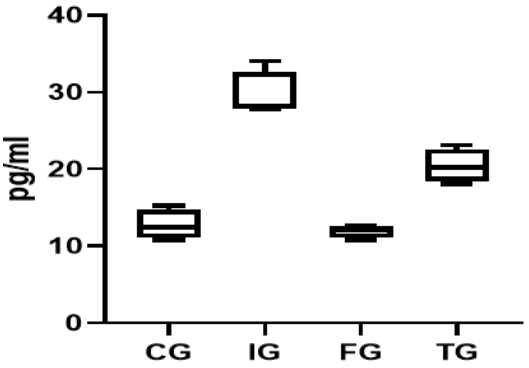

Figure 9. Levels of CD163 in studied groups.

CG: Control, IG: Infected mice, FG: FLX-nanoemulsion, TG: Treated group.

In the current investigation, it was discovered that pregnant female mice infected with candidiasis had elevated CD163 levels; following treatment, there was a minor improvement in comparison to the infection group. Melino *et al.* (2012) observed elevated circulating CD163 levels concurrent with elevated hepatic CD163 expression, which is consistent with our findings and suggests that changes in CD163 in inflammatory liver tissue can be observed in blood levels of CD163. These results provided credence to the idea that CD163 could serve as a non-invasive biomarker for the prognosis of hepatic inflammatory diseases. The findings of our investigation support the theory that some histological lesions in the liver are caused by candidiasis infection. The discovery was made by Kazankov *et al.* (2015), who found that patients with fatty liver disease had significantly higher levels of sCD163. This could be linked to early complications in the liver, activation of macrophages, and inflammatory changes that can happen before histological changes are noticed. Additionally, they asserted a correlation between progressive fibrosis and the amount of sCD163, indicating continuous activation of macrophages throughout the entire course of the disease. Additionally, sCD163 levels were higher in patients exhibiting acute liver failure. In the current study, FLX-nanoemulsion had a role in reducing CD163 levels after treatment. This may be because FLX-nanoemulsion treated the candidiasis infection that was the main cause of many histological changes in the liver, and thus CD163 levels improved in pregnant mice after treatment.

Since sCD163 levels are quite elevated in other acute inflammatory conditions such as liver disease including liver candidiasis (Coca *et al*., 2009, Dheeb *et al.,*2023), and some studies have showed the effective role of Fluconazole in treating Liver Candidiasis resulting from infection with C. albicans (Pfaller *et al*., 2004; Charlier-Woerther *et al*., 2006., Mahmood *et al.,*2019), which enhances the effective role of Fluconazole Nanoemulsion in reducing the inflammatory condition as a result of the antifungal activity and thus reduces the need for pro-inflammatory parameters including CD163.

4. Conclusions

Based on the results of the current study, it is evident that fluconazole nanoemulsion exhibits a potent effect against *C. albicans* infection. Furthermore, it effectively treats histological abnormalities and fetal lesions induced by *C. albicans* in both pregnant mice and their fetuses.

5. Author contributions

SHM and AHS contributed equally to the study conception, material preparation, data collection, and analysis and design. Supervision was carried out by BID. The initial write-up of the draft manuscript was done by SHM and BID. The review and corrections for the previous version of the manuscript were carried out by KHA. All authors read and approved the final manuscript.

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