Antifungal activity and antioxidant potential of Vitamin E in combination with ketoconazole

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ABSTRACT

Fungal infections encompass a spectrum from minor skin and mucosal issues to severe organ damage, posing a global health challenge. Concurrently, the emergence of Infections caused by fungi that are resistant to drugs and the restrictions imposed by currently available antifungal therapy options highlight the critical need for novel therapeutic techniques.

-In Vitro Agar Well-Diffusion Method This standard procedure is used to assess the antibacterial efficacy of both ketoconazole and Vitamin E at varying doses.

-In VIVO Mice were divided into seven groups and shaved back hair. Two injection sites were chosen for Candida albicans injection. The treatment involved ketoconazole and specific concentrations of vitamin E in DMSO and glycerine. The positive control group received glycerine. Group (A) was treated with a Solution-*Ketoconazole* (0.2) g only, and Group (B) was treated with a Solution of (0.2) g Vit. E with (0.2) g of *Ketoconazole*, Group(C) was treated with a Solution of (0.1) g Vit. E with (0.2) g of *Ketoconazole*, Group(D) was treated with a Solution of (0.05) g Vit. E with (0.2) g of *Ketoconazole*, Group(E) was treated with a Solution of (0.025) g Vit. E with (0.2) g of *Ketoconazole*, and finally Group (F) blank group untreated (negative control To compare the progress of the treated and untreated groups, the wound area was measured using the rule approach (length by width) and the rats' weights were recorded on days 0-3 and 6, 9-12, and 15 for each group.

The study investigated, anticandidal activity of different Vitamin E concentrations with Ketoconazole The highest inhibition zone, was observed in concentration., of 0.125/0.5ml with KTZ, which was directly proportional to the concentration of Vit. E. High concentrations of Vit. E

led to a moderate zone of inhibitions. In vitro efficacy of ketoconazole and Vitamin E, was highest at a concentration of Vit. E 0.125 mg /0.05 ml with KTZ 1 mg /0.05 ml. In vivo efficacy evaluation showed significant differences in wound healing between candida-inoculated groups of rats. The groups with different Vitamin E concentrations combined with Ketoconazole underwent an outstanding healing process, while the group treated with Ketoconazole only healing was marginal and experienced moderate weight loss.

Keywords: in vitro, in vivo, *Candida albicans*, vitamin E synergism, Antioxidant, Azole

Introduction

From simple skin and mucosal infections to serious organ damage, fungal infections can cause various diseases. Serious infections that reach the body are infrequent but can be fatal in those with compromised immune systems, such as those with HIV/AIDS, autoimmune diseases, or those receiving therapies like anti-cancer or organ transplantation. In contrast, despite innovations in medical care, immunocompromised people are now significantly more likely to have such invasive diseases. [1]; Pathogenic fungi die 1.5 million people around the world [1].

Candida species fungal infections are rapidly becoming a leading cause of illness and death and a significant financial burden on healthcare systems and hospitals worldwide. Invasive systemic candidiasis and septicemia are on the rise and are major causes of death, particularly in immunocompromised people [2].

Fluconazole or ketoconazole are used to treat systemic fungal infections. Modifications have caused resistance to most azole medicines. is one of the most significant challenges seen in clinical practice today [3]; Azole usage also has clinically observed negative effects [4].

In the past decade, Drug-resistant fungal infections have increased due tolarge part to the widespread use of antifungals for prevention, empirical therapy, and guided treatment of a wide variety of fungal infections. Some drug-resistant bacteria have emerged in environmental reservoirs due to using antifungal medicines with a medical history in agriculture.[5]

Combination therapy is a method used to treat various infections, including candidiasis and fungal infections. In animal models, combinations of agents have shown effectiveness in treating various conditions. Systemic candidiasis in mouse models, amphotericin B plus flucytosine showed synergy, but not as effective as monotherapy. A little more efficacy was seen when fluconazole and amphotericin B were used together., but not significantly better than monotherapy for aspergillosis. Combinations with terbinafine have been lacking in animal studies. [6] Antioxidants when given with antifungal drugs, they exhibit increased activity in vitro.

By influencing molecular structure, antioxidants may improve Azole's contact activity in various organisms.[7]

In the current study, the antifungal activity of ketoconazole was evaluated both alone and in combination with vitamin E.

Materials and Methods

Antifungal drugs, Azole antifungal drugs (Ketoconazole), and Vitamin E were pure sub-stances from Pioneer Co. – For Pharmaceutical Industries. Distal water and normal saline both from Pioneer Co. – For Pharmaceutical Industries\Iraq. Sabouraud Dextrose Agar (SDA) and glycerine from HiMedia (India).

(Sabouraud Dextrose Broth=SDB)

Forty grams of dextrose and ten grams of peptone were dissolved in one thousand millilitres of distilled water, and the resulting solution was poured into sterile test tubes. McGinnis (1980) says.

To prepare inoculum

Sub-culturing all tested isolates on Sabouraud Dextrose Agar (SDA) from sterile plates ensures purity and viability. Keep the temperature at 37°C during incubation. After 24 hours of Candida spp. cultures, 5 ml of sterile normal saline was utilized to suspend certain colonies for inoculum work. CLSI M38-A2, 2002

Stir the mixture for 15 seconds and adjust the cell density with a spectrophotometer by adding enough sterile saline to reach the transmittance of a (0.5) McFarland standard. This approach yields a yeast stock suspension of 1–5 x 106 cells/ml.

The method of agar well diffusion

The agar well-diffusion method is one of the most common approaches taken when investigating the antibacterial properties of plant or microbial extracts [32,33]. The procedure for inoculating the surface of the agar plate is quite similar to the one that is utilized in the disk-diffusion method. The inoculation process involves spreading a volume of the microbial inoculum throughout the whole surface of the agar plate. Following this, an aseptic hole measuring between 6 and 8 millimetres in diameter is punched into the well using a sterile cork borer or a tip. Following this, a volume of antimicrobial agent or extract solution containing the necessary concentration is then put into the well. After that, the agar plates are incubated at conditions that are appropriate for the bacterium that is being tested. In the agar medium, the antimicrobial agent is able to diffuse, and as a result, it is able to limit the growth of the microbial strain that was tested.

The Diffusion Method

The antifungal efficacy of Vitamin E and antifungal drugs against different species of *Candida,* including *C. albicans*. was investigated using a well-diffusion technique [8]. Briefly, 25 ml of sterile SDA medium was added to sterilized petri plates and set aside to cool at room

temperature. The fungal strain was swabbed on the surface of plates using swabs dipped in the inoculum suspensions when the medium was solidified. After drying the infected plates, a six mm diameter well was formed on the agar surface using a sterilized sterile pipette and Sabouraud dextrose agar plates had a hole drilled into them that was six millimetres in diameter, then 50 µl of different concentrations of Vitamin E (500, 375, 250, 125) µg/50µl, represent solution (A, B, C, D, E, F), 1000 µg/50 ml of Ketoconazole cleaned and sterile before being added to the well. The effectiveness of the antifungal was determined after 24 hours of incubation at 37°C by measuring the size of the well's surrounding inhibitory zone in millimetre's,. As a placebo, DMSO was utilized. (9,10)

Laboratory Animals and Strains

The University of Basrah College of Science and Biology's diagnostic microbiology facilities provided the clinical isolate of Candida albicans, strain OK631832.

The animal tests for this study took place between January 2022 and April 2023 in the animal house at the University of Basra's College of Pharmacy. The animal shelter is in the Basra City, college of Veterinary Medicine provided 42 adult male Swiss rats weighing between 177 and 215 g. Seven groups of rats (n = 6) were housed in individual plastic cages for a week at 30% humidity, 22 4°C, and a 12-hour dark/ 12-hour light cycle in the animal house. In this experiment, rats had free reign over a diet of regular provender and a water supply. All animal handling protocols described herein were sanctioned by the Basrah University, animal ethics committee (No. 32/2013).

Constructing Models and Intervening in Groups

Mice were grouped into seven different sets of six, with their back hairs removed and their injection locations chosen at random. All of the (A, B, C, D, E, and F) groups were injected with a stock suspension of Candida albicans, OK631832, ranging in cell density from 1 x 106 to 5 x

106 cells/ml. For the positive control group, we prepare the therapy by dissolving (0.2 g of ketoconazole) in 2 ml of DMSO and then finishing it off with glycerine. In contrast, for the other groups we create the medication (2 ml of DMSO containing 0.2 g of ketoconazole and a variety of vitamin E doses)., then the solution with glycerine, and detailed procedures as follows:

Group (A) Vehicle(control), (B) KTZ 0.2 mg /0.05 ml. only (C) Vit.E 0.5 mg /0.05 ml with KTZ 1 mg /0.05 ml. (D) Vit.E 0.375 mg /0.05 ml with KTZ 1 mg /0.05 ml. (E) Vit.E 0.250 mg /0.05 ml with KTZ 1 mg /0.05 ml.(F) Vit.E 0.125 mg /0.05 ml with KTZ 1 mg /0.05 ml.

To compare the treated and untreated groups and to see how the wound surface changed over time, we weighed the rats on days 0, 3, 6, 9, 12, and 15 and measured the wound area using the rule technique (length by breadth).

For best results when inducing cutaneous candidiasis, it is recommended to use Candida albicans (the Candida albicans, OK631832 yeast stock suspension). Using a hair removal shaving machine, the rats' back hairs are shaved, and a 2 cm2 region is chosen for the application of the produced formulations. The Derma roller, a device rolled against the skin to develop micropores, is used the following day to create the pores. Transdermal application of medication has many advantages. Successful medicine delivery to the dermis was facilitated by penetrating the stratum corneum using this method. The ready-made mixtures were then swabbed onto the skin of the rats. The rats received a single treatment once a day for six days. The control group, which was positive, was given simply ketoconazole, while the untreated group received nothing. After six days, the groups' responses were compared to those of a control group.

Result and Discussion

Zone of Inhibition and Minimum Inhibitory Concentration

The reason for this investigation was to compare the efficacy of several anticandidal Vitamin E concentrations with Ketoconazole; among them, Vitamin E in a concentration of)125 µg/50µl

(Vit. E with KTZ has shown a high and stable zone of inhibition (21.56 mm) compared to all other concentrations tested (Figure.1)

The concentration was straightforwardly relative to the zone of inhibition. Of Vitamin E, whereas high concentrations of Vitamin E led to a moderate zone of inhibitions (Fig. 1, 2).

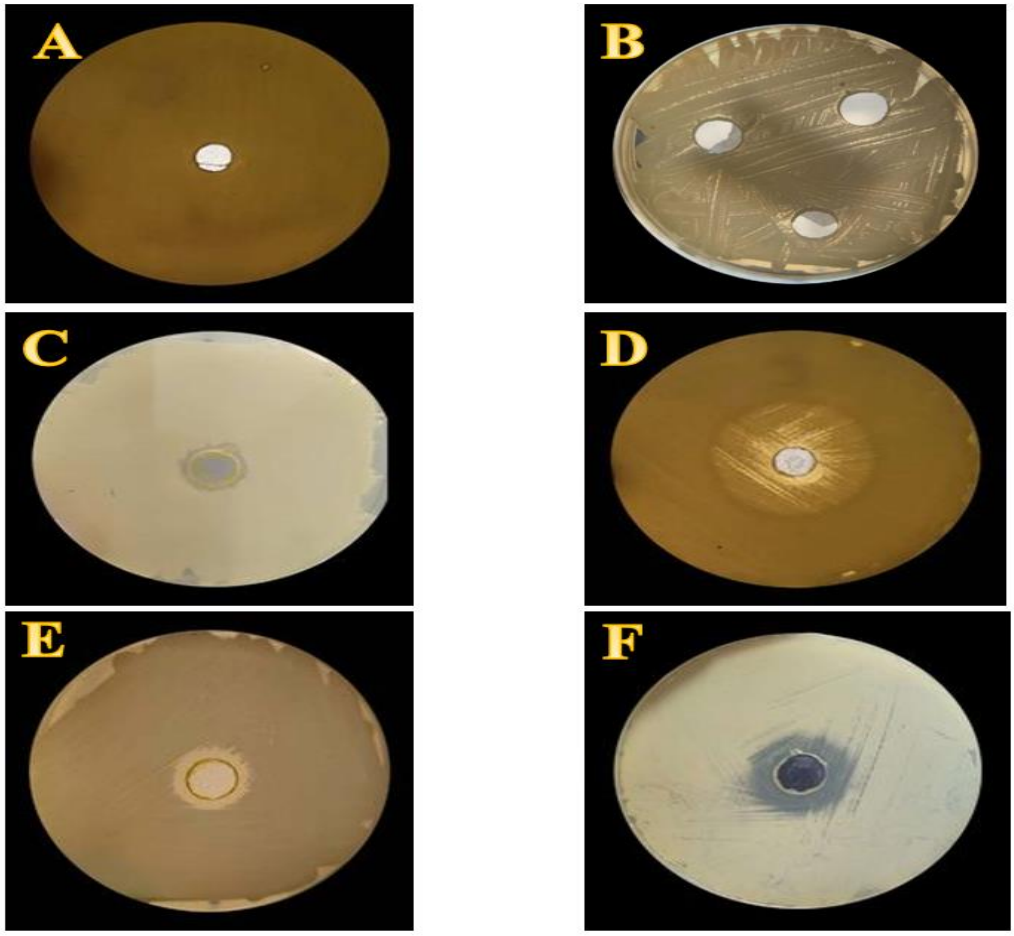

Figure [1] Activity of Ketoconazole or Ketoconazole in combination with Vitamin E In contradiction of *C. albicans* by the agar well diffusion method.

(A) Vehicle(control), (B) KTZ 1 mg /0.05 ml.(Positive control)

(C) Vit.E 0.5 mg /0.05 ml with KTZ 1 mg /0.05 ml.

(D) Vit.E 0.375 mg /0.05 ml with KTZ 1 mg /0.05 ml.

(E) Vit.E 0.250 mg /0.05 ml with KTZ 1 mg /0.05 ml.

(F) Vit.E 0.125 mg /0.05 ml with KTZ 1 mg /0.05 ml.

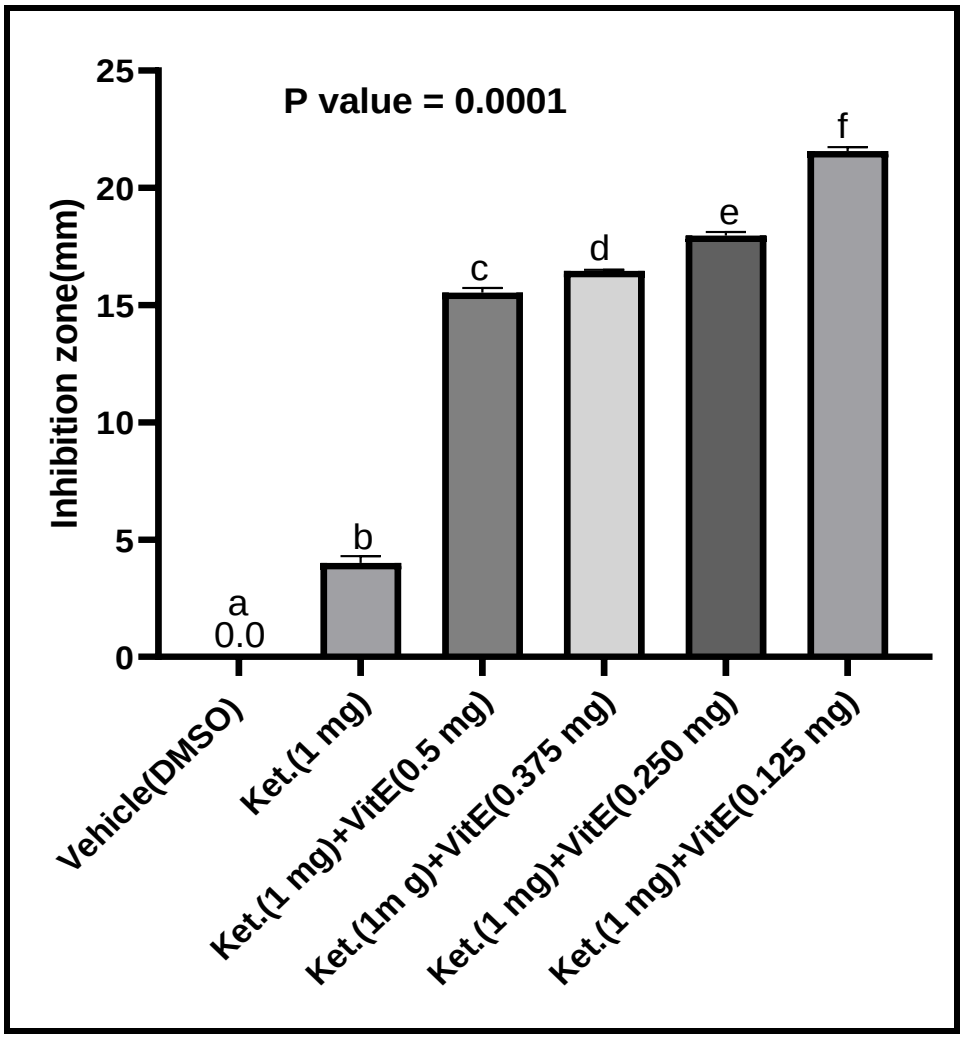

Figure [2]

The Ketoconazole Impact (KTZ) and different concentrations of vitamin E

On pathogenic *candida spp.*

In vitro efficacy of ketoconazole and Vitamin E, one of the highest levels of inhibition zone was recorded at (Vit**. E 0.125 mg /0.05 ml with KTZ 1 mg /0.05 ml)** in diameter of (21.56) mm against *C. albicans*, followed by (18) diameter at the concentration of) **Vit. E 0.250 mg /0.05 ml with KTZ 1 mg /0.05 ml,** (16.46) mm diameter at the **Vit. E 0.375 mg /0.05 ml with KTZ 1 mg /0.05 ml.**, and (15.53) in diameter at the concentration **VitE 0.5 mg /0.05 ml with KTZ 1 mg /0.05 ml.**, while the **ketoconazole** alone efficacy was low as (4) mm in diameter, as shown in Figure (1,2) and table 1.

Table 1. Represents the inhibition zone in (mm) of different concentrations of Vitamin E (mg/ml) against pathogenic Candida spp.:

Candida species filtration		Concentrion (mg\0.05ml)	Inhibition Zone (mm) For *C. albicans*			Mean
Vehicle (DMSO)	A	-	-	-	-	-
Ketoconazole only	B	1	4	4.5	3.5	4
(Vitamin E) at different Concentrations with Ketoconazole (1mg\0.05ml)	C	0.5	15.5	15.9	15.2	15.53
	D	0.375	16.5	16.5	16.4	16.46
	E	0.257	18	17.7	18.2	17.9
	F	0.125	21.5	21.9	21.3	21.56

For Vitamin E the results of statistical analysis showed a statistically significant difference between candida spp. And the zone of inhibition in mm at (P value =0.0001).

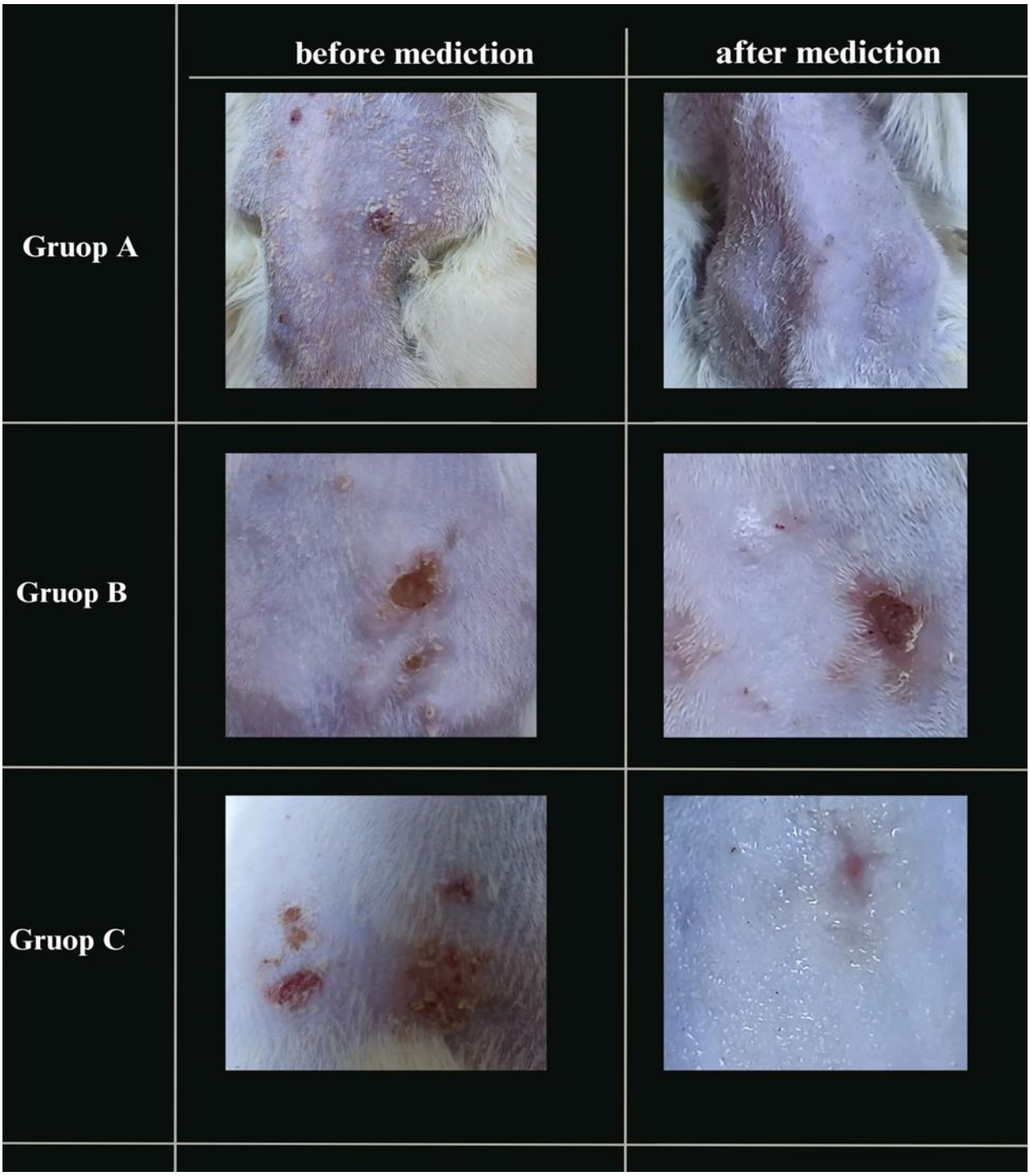

Figure (3)

Rat skin before and after treatment fungal infection with Candida albicans.

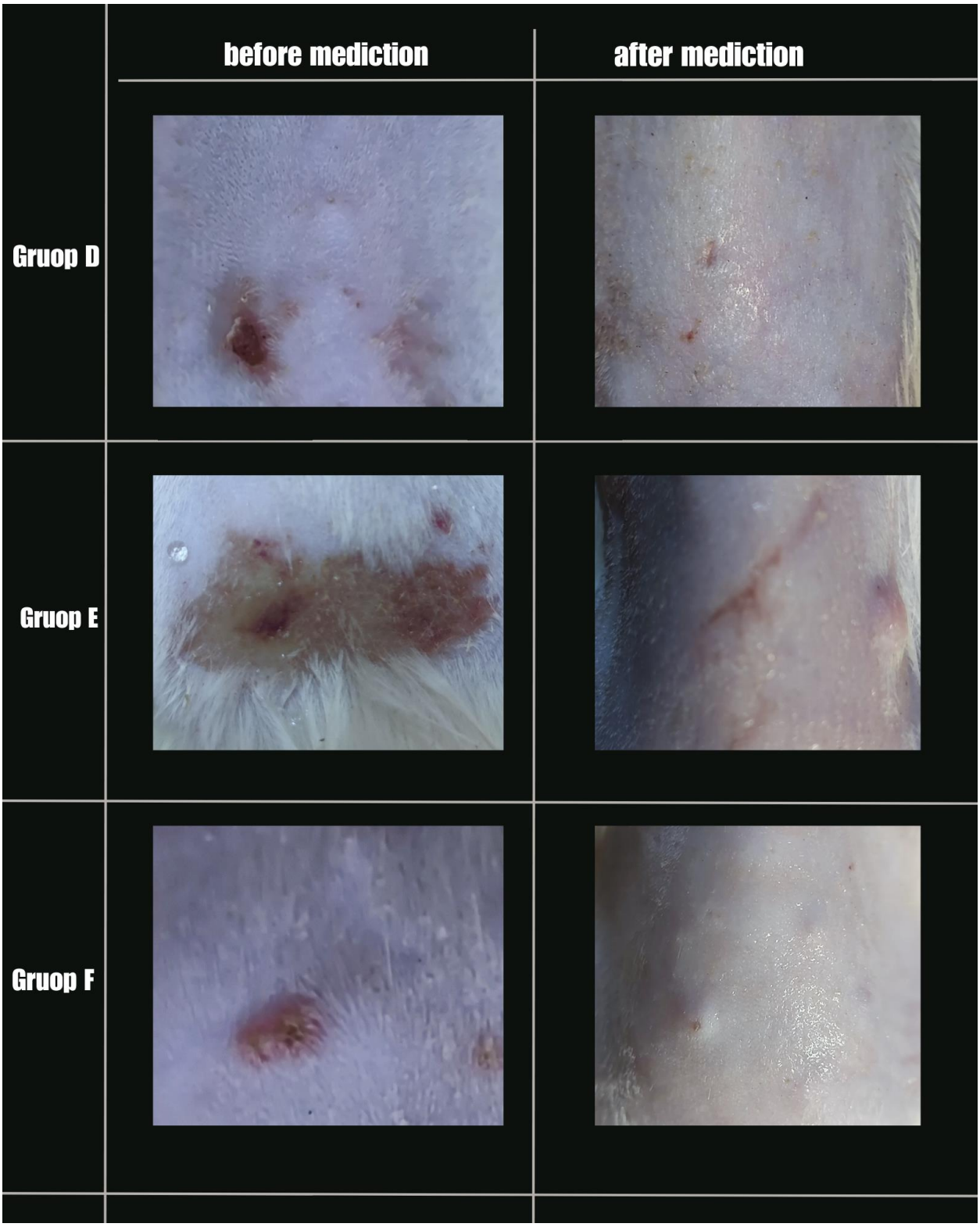

Figure (4)

Rat skin before and after treatment fungal infection with Candida albicans.

In vivo Testing for effectiveness against Candida species.

Wound healing remarkably varies shealing were able to spot between *candida*-**inoculated** groups. This control group was treated with only vehicle, a group with **Ketoconazole** only, and four other groups were treated with different concentrations of Combining the benefits of vitamin E with **Ketoconazole**.

Following infection, the wounds of the rats were severe, suppurative, and bright red.

In the 0–15-day treatment, our observation stated that the groups with different Vitamin E concentrations combined with Ketoconazole underwent an outstanding healing process. In contrast, the group of rats treated with **Ketoconazole** only healing was marginal and experienced moderate weight loss, while the group of untreated rats (control) recovery was not present in conjunction with excessive weight loss, leading to death.

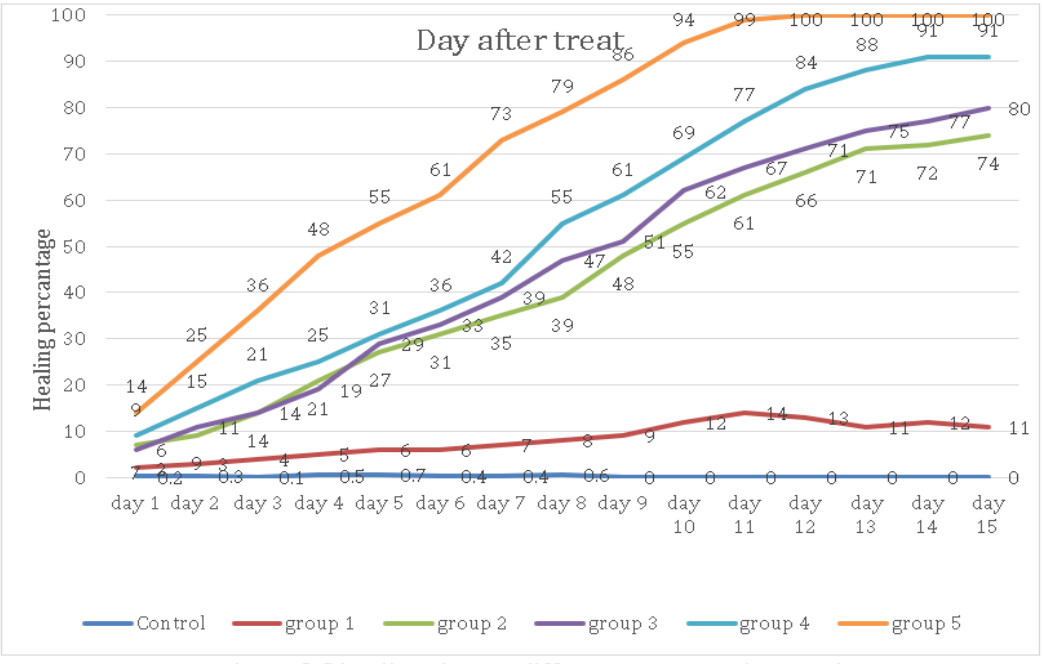

Figure [5] healing time at different concentrations and groups.

Group (1) was treated with a Solution-Ketoconazole (0.2) g only.

Group (2) was treated with a Solution of (0.2) g Vit. E with (0.2) g of Ketoconazole.

Group (3) was treated with a Solution of (0.1) g Vit. E with (0.2) g of Ketoconazole.

Group (4) was treated with a Solution of (0.05) g Vit. E with (0.2) g of Ketoconazole

Group (5) was treated with a Solution of (0.025) g Vit. E with (0.2) g of Ketoconazole.

Azole antifungal medications have become increasingly popular With the incidence of invasive fungal diseases like candidiasis during the past two decades [11, 12]. Clinically significant toxic Triazole antifungals like fluconazole and imidazole's like ketoconazole can cause skin rash, nausea, increased liver enzyme, gynecomastia, adrenal insufficiency, and hepatotoxicity.[13]. Resistance to azoles has increased over time, reducing their effectiveness in various therapeutic contexts [14].

In this study, using standardized susceptibility tests, the *C. albicans* as the organism to test its resistance to ketoconazole. We found that **ketoconazole** did not have any antimicrobial effect on resistance.

C. albicans in contact tests, even in a rat model. We hypothesized that antioxidants might alter the membrane function (6), which could influence the sensitivity to **ketoconazole** (7).

Antioxidants and triazole greatly increased the inhibitory action of **ketoconazole** in susceptible isolates, with the highest MIC values recorded at the concentration of **Vitamin E 0.5 mg /0.05 ml with KTZ 1 mg /0.05 ml.**, (21.56) mm diameter against

C. albicans).[15],[16]

Previous Study shows **fluconazole** and vitamin E treatment significantly improves signs and symptoms, with even more improvements observed at higher doses due to antioxidant properties.[17]

Another researcher found that Vitamins have been shown to enhance antibiotic activity. In rat models, vit. E decreases inflammation and increases antibiotic effectiveness in methicillin-resistant S. aureus wounds. Vitamin E increases antibiotic concentration around bacterial cells by inhibiting lipocalin antibiotic binding. Vit. E may be used as an antibiotic adjuvant alongside antibiotics to treat multidrug-resistant bacteria infections.[18]

Study shows fluconazole and vit. E treatment significantly improves signs and symptoms, with even more improvements observed at higher doses due to antioxidant properties.[11]

The study did not examine the mechanism of action, but it shows that vitamin E and antifungals may work better against Candida species when combined.

Azole drugs inhibit lanosterol 14α-demethylase, which is crucial for ergosterol synthesis. Combining vitamin E with conventional antifungal drugs, such as ketoconazol*e*, reduces individual MICs and increases their efficacy against *Candida* species. Antifungal resistance is a major issue, prompting further research on new antifungal drugs. Researchers have tested various agents from natural or synthetic sources, with different mechanisms to stop fungal growth and infection.

Antibiotic effectiveness may be boosted indirectly by vitamin E. In experimental wounds infected with methicillin-resistant Staphylococcus aureus [19], pre-infection vitamin E therapy increases immunological indices and antibiotic efficiency. In a rat model of E. coli pyelonephritis [20] and Pneumococcal lung infection [21], vitamin E treatment reduces inflammation.) protein, revealing a unique relationship between bacterial lipocalin and vitamin E binding.

Vitamin E is the major antioxidant in biological membranes [22], and its stability of polyunsaturated fatty acids in membrane lipids helps regulate immunoregulation [23]. Due to its physical properties, vitamin E and its derivatives are good drug transporters [24], notably in the pulmonary system [25]. According to our findings, vitamin E may be added to nebulized antibiotics for cystic fibrosis patients. Vitamin E inhibits antibiotic binding to lipocalin, increasing the effective concentration of antibiotics around bacterial cells. We propose using vitamin E as an antibiotic adjuvant in combination with antibiotics to treat multidrug-resistant *Burkholderia* and other multidrug-resistant bacteria infections.

Antifungals rarely work against C. albicans biofilms. [26]

According to susceptibility studies, biofilms have been shown to be extremely resistant to antifungal medicines, in some cases thousands of times more resistant than planktonic cells of C. albicans. [27,28]

There is currently no effective antifungal medication for *C. albicans* biofilm-related infections. However, antifungal drugs should not be used in greater numbers due to their toxicity. To effectively battle biofilms, it would be wise to mix medications with different mechanisms of action that inhibit multiple cellular targets. The work of Khun et al. in 2002 demonstrated the efficacy of lipid formulations of amphotericin B and echinocandins against Candida biofilms.[29]

Since then, more studies have been undertaken in this exciting area, and a recent review article by Bink et al. provides a summary of these studies. [30]

Currently, no antifungal antibiotic has been shown to be effective against *C. albicans* infections linked to biofilms. Meanwhile, the toxicity of the antifungal medications means that higher quantities shouldn't be used. Therefore, it would be prudent to combine medicines using several mechanisms of action to block various cellular targets in order to eliminate biofilms. Against Candida biofilms, In 2002, thanks to the efforts of Khun et al., lipids formulation, of amphotericin. B, and echinocandins, were shown to be efficacious.[29]

Researchers linked Vitamin E's potency to wound healing using this way. Some writers suggest that topical antibiotic treatment is an effective way to eliminate microbial populations because it increases medication availability at the infected wound site and speeds wound healing [31].

Barku et al. characterize wound healing as a dynamic process that repairs damaged tissue's cellular structures and tissue layers to their normal state. Vitamin supplement ketoconazole contains anti-fungal properties that speed wound healing in rats. Effective microbial infection control improves wound care [32] findings were confirmed. Similar research suggests Candida-infected wounds heal slower [33]. Thus, reducing the infection's cause speeds healing [34].

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