

## ORIGINAL ARTICLE

# Antifungal effect of Gatifloxacin and copper ions combination

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It is a well-known fact that to bring a new molecule it may take more than a decade. The existing drugs, which are known for their adverse reaction or toxicity, if utilized and allowed in different formulation, the new effective formulation may be discovered and developed. This may help in reducing various side effects, time and costs. In this study, fungal infection was inoculated superficially over the skin of guinea pigs and treated with the broad-spectrum antimicrobial (gatifloxacin) in combination with non-toxic and effective amount of copper ions. MIC of copper (0.20%) was also determined. Concentration of gatifloxacin ( $100 \mu\text{g ml}^{-1}$ ) with the combination of copper ions (MIC) at which it inhibits the visible growth of fungal strains was also evaluated. Hematological parameters, such as total leukocyte count and differential leukocyte count, were evaluated. The results have shown increase in these parameters after fungal infection, which reaches its normal value after treatment with the combination of gatifloxacin and copper ions. Outcomes of the research concluded that gatifloxacin  $100 \mu\text{g ml}^{-1}$  can be used by 0.20% of copper ions to prevent growth of some fungal strains (*Candida albicans* and *Aspergillus niger*), which causes skin infections with more potency.

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## INTRODUCTION

Superficial fungal infections are common worldwide. These infections occur in both healthy and immunocompromised patients.<sup>1,2</sup> They are believed to affect 20–25% of the world's population, and the incidence continues to increase. They are predominantly caused by dermatophytes, yeasts and moulds and the causative species vary by geographic region. The prevalence of fungal infections has been suggested for the increase with age and to be present at a rate of about 5% in people aged 55 years and older.<sup>3,4</sup> However, the increasing administration of antifungal agents to treat fungal infections has led to the development of fungal resistance. The emergence of resistance shows the necessity of discovering new antifungal agents with broader antifungal spectra, low toxicity and higher therapeutic indexes.<sup>5,6</sup> Copper is an essential trace element for normal plant growth and development. However, an excessive amount of copper in soil is highly toxic to both higher plants and microorganisms.<sup>7,8</sup> If these ions are combined with the existing antimicrobial agents in limited quantity, then the combination can be used as an antifungal drug. Monotherapy is the usual treatment for invasive fungal infections, owing to lack of safe, effective combination of antifungal drugs. However, the benefits of a well-tolerated, synergistic combination would be great—the enhanced efficacy would improve clinical outcome, reduce the need for prolonged courses of treatment and

prevent the emergence of antifungal drug resistance. Combination therapy is therefore being suggested as a means of combating resistance and improving clinical outcome, just as it is for serious bacterial infections. Antifungal antibodies would be a natural partner in a combinatorial approach to antifungal therapy.<sup>9–11</sup>

DNA gyrase is a member of the group of enzymes known as DNA topoisomerases that catalyze changes in DNA topology.<sup>12</sup> Gyrase is the only member of this group that can introduce negative supercoils into DNA at the expense of ATP hydrolysis.<sup>13</sup> DNA gyrase is essential in bacteria and is the target of a number of antimicrobial agents.<sup>14</sup> Gatifloxacin is a broad-spectrum anti-infective agent of the fluoroquinolone class. It is most active against aerobic Gram-negative organisms including enteric pathogens. Gatifloxacin is bactericidal via inhibition of DNA gyrase, an enzyme responsible for counteracting the excessive supercoiling of DNA during replication or transcription.<sup>15</sup>

$\text{Cu}^{2+}$  ions are transported by the uptake system for essential metal ions to the cell where they can accumulate and exert toxic effects at high concentrations.<sup>16</sup> Copper's initial site of action is considered to be at the plasma membrane. It has been shown that exposure of fungi and yeast to elevated copper concentrations can lead to a rapid decline in membrane integrity. This generally manifests itself as leakage of mobile cellular solutes (for example,  $\text{K}^+$ ) and cell

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death.<sup>17,18</sup> Similar effects reported in higher organisms have now been largely attributed to the redox-active nature of copper and the ability of copper to catalyze the generation of free radicals and promote membrane lipid peroxidation.<sup>19</sup> Copper may damage many proteins. This may occur via displacement of essential metals from their native binding sites in the proteins, or via direct interactions with the proteins. Copper also may mediate free radical attack of amino acids, especially of histidine and proline, causing substantial protein alterations and even protein cleavage.<sup>20–22</sup> Copper ion has a specific affinity for DNA and can bind with helical structure of DNA, leading to cross-linking the strands. Copper reversibly denatures DNA in low ionic strength solutions competing with the hydrogen bonding present within the DNA molecule. Thus, copper binding to DNA implies that nucleic acid degradation by generating several OH<sup>-</sup> radicals near the binding site causing multiple damage to the nucleic acids.<sup>23,24</sup>

Our hypothesis was based on the fact that gatifloxacin inhibits DNA gyrase in bacteria and it cannot penetrate the fungal membrane because of the presence of ergosterol on fungal membrane. Copper ions penetrate the membrane; the pore may be created on fungal membrane. In this study, copper ions are combined with gatifloxacin in a very small amount. These copper ions will create pores on the fungal membrane by disrupting the ergosterol present on the membrane and hence will help gatifloxacin in penetrating the fungal membrane where it will bind with DNA topoisomerases that are present inside the fungus and will inhibit it. This combination can be used in the treatment of fungal infection.

## MATERIALS AND METHODS

### Animals

Four healthy male guinea pigs weighing approximately 500 g were obtained from the animal house facility at Siddhartha Institute of Pharmacy, Dehradun, India. They were housed in individual ventilated cages under the conditions of 22–25 °C and a 12-h light–dark cycle, with free access to food and water. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC approval number-SIP/IAEC/2/A/2011) as per the guidance of Committee for the purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India.

### Chemicals and drugs

Gatifloxacin was procured from Cipla, Sikkim, India, as a gift sample for study. Fungal strains (*Aspergillus niger* and *Candida albicans*) were obtained from Sardar Bhagwan Singh College of Pharmacy with strain no. MTCC (282,183), Dehradun, India.

### In vitro study

**Determination of MIC of copper.** MIC was determined by incorporating various concentrations of copper (0.05, 0.10, 0.15 and 0.20%) in Sabouraud Dextrose broth. In all, 100 µl of fungal inoculum was added to each tube and incubated at room temperature for 7 days. The MIC is regarded as the lowest concentration that does not permit any visible growth after 7–21 days of inoculation.

**Determination of antifungal susceptibility of gatifloxacin.** Culture media for fungus were prepared by dissolving 6.5 g of Sabouraud Dextrose Agar in a conical flask by adding 50 ml of distilled water, autoclaved at 15 lbs (121 °C) for 15 min and cooled. The MIC of copper obtained was added in each flask. Sterilized media were transferred aseptically (by using laminar air flow) into the sterilized glass Petri dishes. When the media were solidified, Petri dishes were cultured with different strains of fungus (*Candida albicans* and *Aspergillus niger*). Different concentration of gatifloxacin (20, 50 and 100 µg ml<sup>-1</sup>) was added on half the portion of each Petri plate and kept in the incubator at 28 °C observed after 1st, 3rd, 5th, 7th, 9th and 10th day.

**Determination of antifungal susceptibility of combination.** Combination of 25 µg ml<sup>-1</sup> gatifloxacin with 0.20%, 50 µg ml<sup>-1</sup> gatifloxacin + 0.20% and 100 µg ml<sup>-1</sup> gatifloxacin + 0.20% copper was used to determine fungal growth inhibition on culture media. In all, 100 µg ml<sup>-1</sup> gatifloxacin + 0.20% copper was effective in inhibiting fungal growth effectively. Hence, this dose combination was selected for further study on guinea pigs.

### In Vivo study on guinea pig

**Induction of fungal infection.** Animals were divided into two groups, each having two animals. The first group was normal control. Second was treatment control group. In the second group, fungal spores were inoculated superficially and treatment was done with the combination of copper ions and gatifloxacin. The lesions were clinically followed-up daily until resolution was observed. After the 15th day of infection, animals were treated.

Animals were inoculated according to the modified method of Shimamura *et al.*<sup>25</sup> The hair in the posterior dorsal region of the animals was removed with a sterile scalpel blade. Fungal strains were suspended in sterile water and were inoculated at the site, which was covered with a polyethylene film and kept in place with a high elastic bandage for 24 h. After 24 h, the bandage was removed, the area was cleared and sterilized with ethyl alcohol and water and the animals were cased individually for 15 days with adequate supply of food and water.

### Treatment

Treatment was done with microemulsion. For preparing the microemulsion, 40% oil, 10% surfactant (Tween80) and 50% water were mixed together with the concentration of gatifloxacin at which it inhibits the visible growth of fungus (100 µg ml<sup>-1</sup>) and the MIC of copper (0.20%), homogenized with the help of homogenizer and are stored in glass vials until use. Induction of infection was confirmed on the 15th day of inoculation. The treatment of the animals was started after confirmation of the infection. The infected areas were applied with microemulsion twice daily in the morning and afternoon. Treatment continued regularly at the same time until complete recovery was achieved.

### Hematological evaluation

Whole blood was collected from the animals into EDTA bottles and assayed for the total leukocyte count (TLC) and differential leukocyte count (DLC) using standard laboratory technique.

## RESULTS

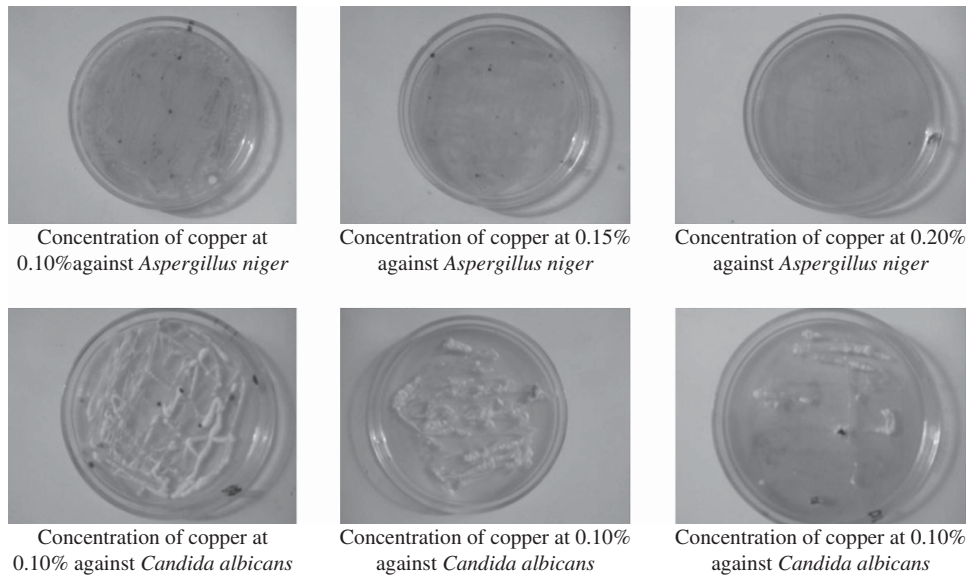
### MIC of copper

*Aspergillus niger* and *Candida albicans* were able to tolerate copper metal ion in the growth medium up to 0.10% and 0.15%, respectively, but inhibited to grow at 0.20%. Fungal growth was increased at 0.10% (Table 1 and Figure 1) but then significantly decreased with increased copper concentrations in the medium (Tables 2 and 3 and Figure 1). The data shown in the tables below depict that copper ions in the concentration of 0.10% and 0.15%, respectively, were not effective for *Aspergillus niger* and *Candida albicans*. The data depict that copper ions in the concentration of 0.20% were sufficient to prevent the growth of both fungal strains. Hence, we considered it as the MIC of copper ion for preventing the growth of all strains of fungus.

**Table 1 Fungal strain growth pattern for copper at 0.10%.**

Fungal strain	Day 1	Day 3	Day 5	Day 7	Day 9
<i>Aspergillus niger</i>	–	–	+	+	+
<i>Candida albicans</i>	–	+	+	+	+

–, No growth; +, growth of fungus.



**Figure 1** Concentration of copper at 0.10, 0.15 and 0.20% against growth of *Aspergillus niger* and *Candida albicans*. A full color version of this figure is available at the *Journal of Antibiotics* journal online.

**Table 2** Fungal strain growth pattern for copper at 0.15%.

Fungal strain	Day 1	Day 3	Day 5	Day 7	Day 9
<i>Aspergillus niger</i>	–	–	–	+	+
<i>Candida albicans</i>	–	–	–	+	+

–, No growth; +, growth of fungus.

**Table 3** Fungal strain growth pattern for copper at 0.20%.

Fungal strain	Day 1	Day 3	Day 5	Day 7	Day 9
<i>Aspergillus niger</i>	–	–	±	±	±
<i>Candida albicans</i>	–	–	±	±	±

–, No growth; ±, inhibition of growth.

**Table 4** Combination of 25 µg ml<sup>-1</sup> gatifloxacin + 0.20% copper against growth of fungal strains.

Fungal strain	Day 1	Day 3	Day 5	Day 7	Day 9
<i>Aspergillus niger</i>	–	±	±	±	±
<i>Candida albicans</i>	–	–	±	±	±

–, No growth; ±, inhibition of growth.

**Table 5** Combination of 50 µg ml<sup>-1</sup> gatifloxacin + 0.20% copper against growth of fungal strains.

Fungal strain	Day 1	Day 3	Day 5	Day 7	Day 9
<i>Aspergillus niger</i>	–	–	±	±	±
<i>Candida albicans</i>	–	–	±	±	±

–, No growth; ±, inhibition of growth.

### In vitro antifungal susceptibility test of combination of drug

As shown in Tables 4 and 5 and Figure 2, combination of 25 µg ml<sup>-1</sup> gatifloxacin with 0.20% copper and combination of 50 µg ml<sup>-1</sup> gatifloxacin + 0.20% copper were not effective to inhibit the growth of fungus, but the data shown in the Table 6 shows that combination of 100 µg ml<sup>-1</sup> gatifloxacin + 0.20% copper was sufficient to inhibit the strains of fungus under observation. Hence, from the above data, we consider that 100 µg ml<sup>-1</sup> gatifloxacin can be combined with 0.20% copper ions to prevent growth of fungal strains.

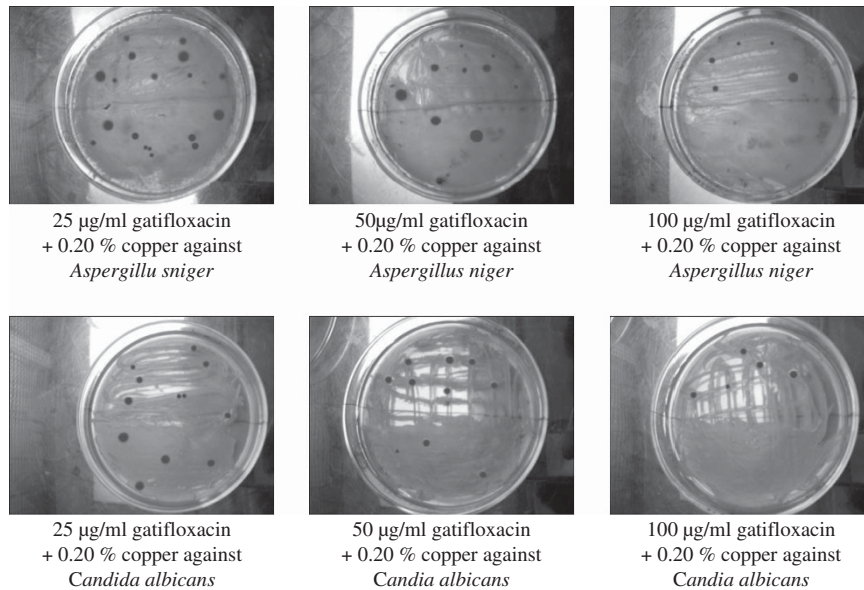
### Guinea pig model of fungal infection

Experimental infection of guinea pigs with *Aspergillus niger* and *Candida albicans* resulted in lesions in animals. The first signs of infection were observed on the 5th day after inoculation, which are manifested in the form of edema, erythema and mild shedding. These alterations became more evident around the 10th day. The lesions progressively increased and were found to be covered with white-yellow crusts strongly adhered to the epidermis between the 8th and 15th day.

Treatment procedure was started after the 15th day. Microemulsion was applied topically over the fungal-infected site of the guinea pig twice at morning and afternoon. Lesions were formed where the skin was abraded and did not extend to other sites of the body. Treatment causes little removal of crusts, erythema and little gain in hair on the 8th day of treatment. The effects were more marked on day 10 with clearer skin and more hair. On day 14, the treatment showed complete removal of infected material (crusts, large skin flakes, shedding and erythema) with hair growth. After the treatment, skin looked clearer, moisturized, soft and elastic.

### Total leukocyte count

The TLC in fungal-infected guinea pigs was increased as compared with the normal group. After treatment with the combination of 100 µg ml<sup>-1</sup> gatifloxacin and 0.20% copper ion, TLC of guinea pigs reached to its normal value (Table 7 and Figure 3).



**Figure 2** Combination of 25, 50 and 100 µg ml<sup>-1</sup> gatifloxacin with 0.20% copper at MIC against growth of *Aspergillus niger* and *Candida albicans*. A full color version of this figure is available at the *Journal of Antibiotics* journal online.

**Table 6** Combination of 100 µg ml<sup>-1</sup> gatifloxacin + 0.20% copper against growth of fungal strains.

Fungal strain	Day 1	Day 3	Day 5	Day 7	Day 9
<i>Aspergillus niger</i>	–	–	–	–	–
<i>Candida albicans</i>	–	–	–	–	–

–, No growth.

**Table 7** TLC (expressed in per cu mm) of guinea pigs before inoculation, before treatment and after treatment.

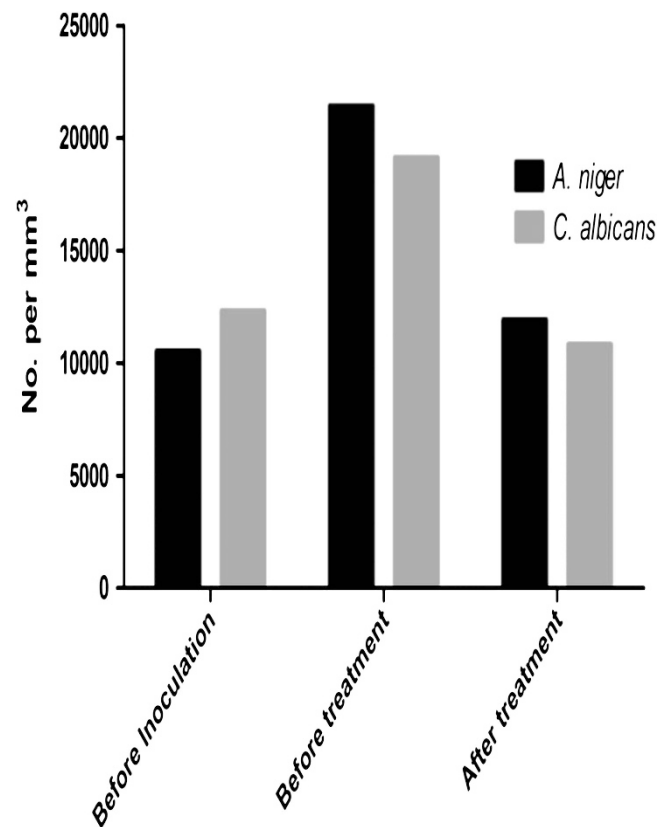
S. no.	Fungal strain	Before inoculation	Before treatment	After treatment
1.	<i>Aspergillus Niger</i>	10 500/cu mm	21 400/cu mm	11 900/cu mm
2.	<i>Candida Albicans</i>	12 300/cu mm	19 100/cu mm	10 800/cu mm

Abbreviation: TLC, total leukocyte count.

**Table 8** DLC parameters (expressed in %) of guinea pigs before inoculation, before treatment and after treatment.

	Eosinophil (%)	Monocyte (%)	Neutrophils (%)	Lymphocyte (%)
Before inoculation				
<i>Aspergillus niger</i>	2	6	37	46
<i>Candida albicans</i>	2	7	38	47
Before treatment				
<i>Aspergillus niger</i>	2.2	6.4	37.2	46.1
<i>Candida albicans</i>	2.3	7.2	38.1	47.2
After treatment				
<i>Aspergillus niger</i>	1.5	6.1	37	45.2
<i>Candida albicans</i>	2	6.9	37.6	46.3

Abbreviation: DLC, differential leukocyte count.



**Figure 3** Total leukocyte count (per cu mm) of guinea pigs before inoculation, before treatment and after treatment.

#### Differential leukocyte count

Eosinophils, monocytes, neutrophils and lymphocytes showed an increase in fungal-infected guinea pigs. After treatment with a combination of 100 µg ml<sup>-1</sup> gatifloxacin and 0.20% copper ion,

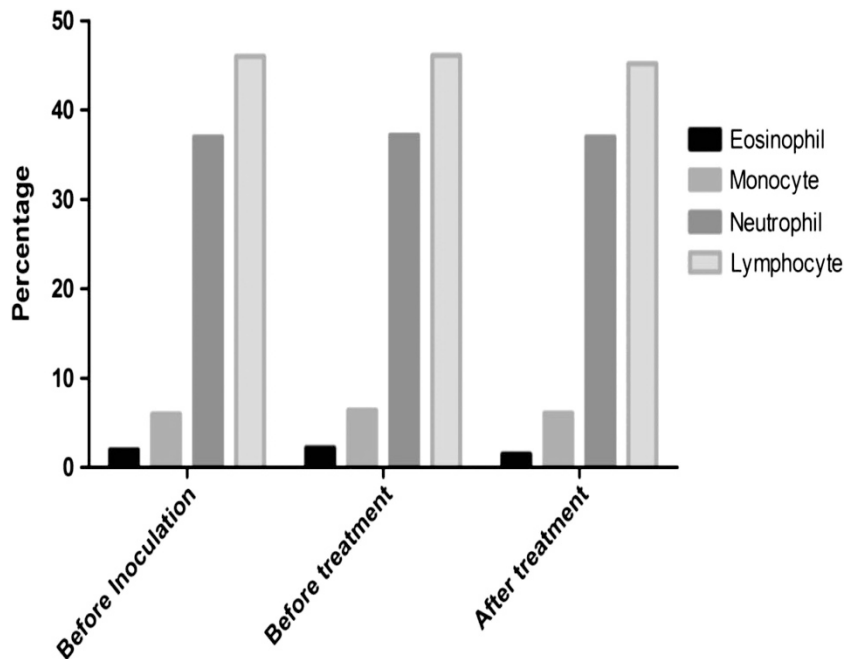


Figure 4 Differential leukocyte count (%) of guinea pigs before inoculation, before treatment and after treatment (*Aspergillus niger*).

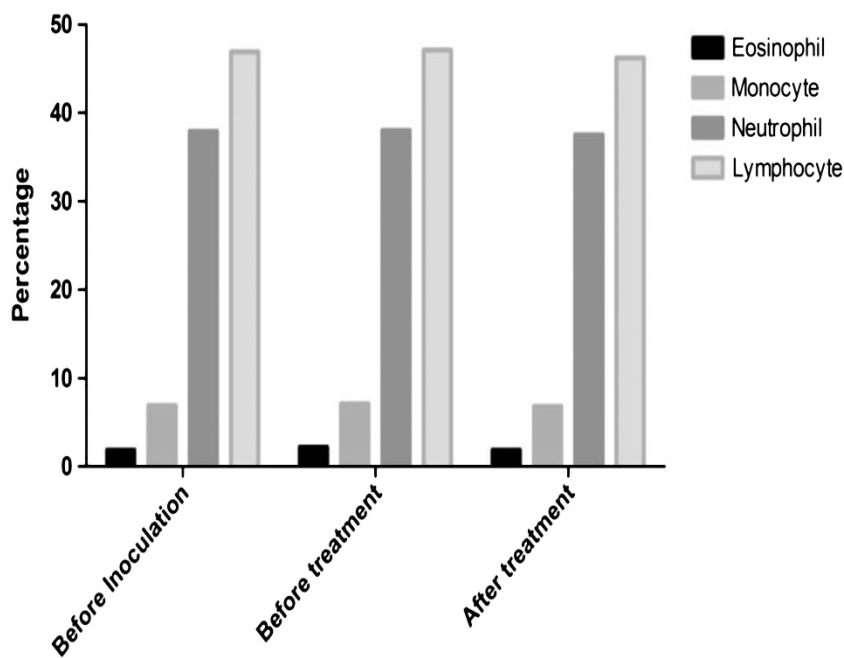


Figure 5 Differential leukocyte count (%) of guinea pigs before inoculation, before treatment and after treatment (*Candida albicans*).

eosinophils, monocytes, neutrophils and lymphocytes reached to their normal level (Table 8 and Figure 4).

#### DISCUSSION AND CONCLUSION

Summarizing the results, we can say that the present work on fungal infection in guinea pigs represents a relatively simple model to perform the study of the efficacy of the combination of gatifloxacin and copper ions as an antifungal agent. Over the past decade,

superficial mycoses of the glabrous skin are among the most prevalent of human infectious diseases seen in clinical practice.<sup>26</sup> There are lots of weaknesses in spectrum, potency, safety and pharmacokinetic properties of existing antifungal agents. When faced with antifungal drugs, fungal pathogens have, in principle, the capacity to overcome their inhibitory action through specific resistance mechanisms.<sup>11</sup> This significant clinical failure has led to the development of new antifungal agents, the tendency to increase



the administered dose of antifungal agent, and to the combination of two or more agents.<sup>10</sup>

Gatifloxacin is bactericidal via inhibition of DNA gyrase, an enzyme responsible for counteracting the excessive supercoiling of DNA during replication or transcription.<sup>15</sup>

Copper's initial site of action is considered to be at the plasma membrane. It has been shown that exposure of fungi and yeast to elevated copper concentrations can lead to a rapid decline in membrane integrity. This generally manifests itself as leakage of mobile cellular solutes (for example,  $K^+$ ) and cell death.<sup>17,18</sup>

From this viewpoint in this study, we have treated the fungal-infected guinea pigs with the combination of gatifloxacin and copper ions at low concentration in the form of microemulsion because of their stability and their considerable potential to act as drug delivery vehicles.<sup>27–29</sup> As gatifloxacin cannot penetrate the fungal membrane because of the presence of ergosterol on the fungal membrane, which serves as a bioregulator of membrane fluidity and asymmetry and consequently membrane integrity.<sup>30</sup> Hence, we used copper ions for the penetration of gatifloxacin inside the fungal membrane. The results suggest that copper ions at low concentration create micropores on the fungal membrane by disrupting the ergosterol in fungal cells by the leakage of transmembrane proteins, essential solutes from the fungal membrane and hence leads to the disruption of membrane integrity. This assisted gatifloxacin in penetrating the fungal cell wall through the micropores. Gatifloxacin after penetration inhibits DNA topoisomerase I and II that are present in the fungus<sup>28,31</sup> and hence prevents its replication. This causes the denaturation of DNA inside the fungus. This suggests its fungicidal action.

Previously, researchers reported that sensitization to *Aspergillus* increased eosinophils and total serum immunoglobulin E levels,<sup>32</sup> moreover some others found in their investigations that total eosinophil counts and lymphocyte counts of infected fishes decreased significantly (43% and 36%). When the DLCs were performed, the lymphocytes decreased in infected groups (9%, 11%) whereas neutrophils increased.<sup>33</sup>

TLC and DLC parameters are used as a specific marker during diagnosis in the early detection of fungal infection. From this study, it can be concluded that the exposure of fungal infection to guinea pigs leads to the increase in the TLC and DLC parameters significantly ( $P \leq 0.001$ ) (Tables 7 and 8) as compared with a normal control group. The TLC and DLC levels decreased significantly ( $P \leq 0.001$ ) and reached toward the normal range in the treatment group treated combination therapy of gatifloxacin and copper ions. Thus, from this study, this combination of copper and gatifloxacin prevents the fungal growth, which further helps in controlling the infections (Figures 3–5).

The data obtained from this study performed in a combination of gatifloxacin with copper ions to screen its antifungal activity, it is concluded that a combination of 0.20% of copper ions with  $100 \mu\text{g ml}^{-1}$  gatifloxacin was able to treat the fungal infection and decreased the hematological markers, which were increased during fungal infection. This will open new perspectives that this combination therapy of gatifloxacin with copper ions will prevent, slow or treat the occurrence of fungal infection.

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