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Antifungal Activity of Clove (*Syzygium aromaticum*) Essential Oil Extract against Induced Topical Skin Infection by *Candida albicans* in Mice *in vivo*

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ABSTRACT

Introduction: Fungal infection is a serious health problem, involved with the life threatening mycosis and mortality. Emerging of resistance and limited antifungal drugs against most antifungal agents lead to the requirement for development of effective and alternate strategies to fight fungal infections, like herbal medicine such as clove oil which is used as antifungal agent against different strains like *Candida albicans.*

Objective: *In vivo* investigation of antifungal activity of clove oil extract against topical induced infection by *Candida albicans* in mice.

Methods: Forty mice were used to induce skin infection by making a scratch until the skin injuries was formed then inoculate 1×10^6 *C.albicans* suspension on scratched skin. Then observe the gross lesion, measure the level of IL-6 and histopathological changes.

Result: The development of lesions in mice after induced infection with *C.albicans* required a period of 5 days in all infected groups. Group treated with clove oil 5 mg/ml showed absence of lesion at day 4 and return to normal thickness and regrowth of hair at day 7 of treatment with significant decrease in level of IL-6 compared to group treated with clotrimazole 2%, which showed presence of dark patch on infected area at day 4 and return to normal but without regrowth of hair at day 7 with decrease in IL-6 but still high compared to negative control.

Conclusion: Eugenol has a strong activity against *C. albicans in vivo. So,* has potential therapeutic effect.

Keywords: *C. albicans***,** Clove oil, Eugenol, Gross examination, Interleukin-6, Histophathological changes.

INTRODUCTION

There are many different types of fungi, including yeasts, molds, and some that can be both a mold and a yeast, *Candida albicans* is the most common fungus that give individuals fungal infections, especially oral or vaginal thrush, which often happens after they take antibiotics [(1)] .

Fungi are classified according to the virulence into two kindes: Primary and opportunistic, Primary pathogens can cause infections in immune-competent hosts, such as *Cryptococcosis*, *Blastomycosis* and *Histoplasmosis* [(2)]**.**

While, Aspergillosis, candidiasis and cryptococcosis are examples of opportunistic fungal infections that are mostly characterized in immune-compromised hosts [(3)].

A kind of infection called superficial mycosis only affects the top layers of skin and its appendages, such as the nails and hair and the main agents of these infections are dermatophytes, non-dermatophytes moulds, yeast-like fungi and yeast [(4)]. In addition, subcutaneous mycoses, are implantation of fungal infections that form beneath skin and typically spread from the initial infected location to impact muscle, deep fascia, and sometimes bone such as *Chromomycosis, sporotrichosis* and *Lobomycosis* [(5)].

There are five major groups of antifungal drugs, including azoles, polyenes, echinocandins, allylamines, and pyrimidine analogues, that can be used for both systemic and topical antifungal treatments [(6)]. Azoles like clotrimazole stop an enzyme called sterol 14-demethylase from making sterol. They also change lanosterol into ergosterol, which is important for keeping fungal cell membranes flexible and stable [(7)] .

A new clinical issue in antifungal therapy is that fungal pathogens are becoming resistant to the drugs that are available to treat them [(8)]**.**

One alternative to synthetic drugs for treating fungal infections is to use medicinal plants and herbal products. These are safe, don't have any harmful side effects, are cheaper, and are often easy to find. Clove is one of the spices that can be used instead of chemical preservatives in many foods, especially when making meat because it has antioxidant and antimicrobial characteristics. In the commercial world, they are used for making medicines and in the fragrance industry [(9)]. Analysis of the structures of eugenol suggests that its ability to eradicate fungi may depend on the existence of an aromatic ring and the fact that it is still there [(10)]**.**

MATERIALS AND METHODS

Experimental Animals

For each test, 40 Albino Swiss female mice (Mus musculus) were used. They were put in a plastic cage by themselves. The animals were kept in a room with a temperature between 23 and 25 °C. They had free access to standard pellets and water (ad Librium).

Inducing Skin Infection

Female mice were anesthetized by using chloroform, their back hair have been clipped and shaved, and the cleansing iodine was applied on their exposed skin. 25 mm diameter scratching until the skin injuries was formed. Directly following the injury, *C. albicans* was inoculated on the scratched skin by using a 1×10^6 cell

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suspension of the fungus prepared in PBS. Mice were housed individually to forbid the crossed infection.

Experimental designs

Forty female mice weighing 20-25 gm were used to perform the experiment of induced skin infection divided equally into four groups, each group ten animals. Applications of treatment were performed after appearance of lesion.

Group A: healthy mice left without infection as negative control.

Group B: infected mice left without treatment as positive control.

Group C: mice infected and treated topically with 5 mg of clove oil extract for 7 days.

Group D: mice infected and treated topically with 2% of Clotrimazole daily for 7 days.

3.8.5. Parameters:

- ✓ **Gross pathological Examination**
- ✓ **Histopathological examination of skin (day before infection, day after appearance of lesion and 7 days of treatment).**
- ✓ **skin section for Interlukin-6 (day before infection, day after appearance of lesion and 7 days of treatment).**

Gross examination

Monitor healing, photographs were taken after appearance of lesion and during the periods of treatment (day 4 and 7).

Histopathological Studies: Mice were placed to sleep, and tissue samples were taken. The tissues were washed with PBS, fixed in 10% neutral buffered formalin, and covered with gauze. After the tissues were fixed, they were dehydrated by passing them through 70%, 80%, 90%, and 100% ethyl alcohol twice each for 2 hours, and then they were cleaned with xylene for 1/2 hour. Samples were filled with paraffin wax at 58–60 °C and then covered with more paraffin wax to make paraffin blocks. Using a rotary microtome, sections 5-6 um thick were cut, soiled with eosin and hematoxylin stains, and then looked at under a microscopic examination [11,12].

Measurement of tissue IL-6:

Mice were euthanized and tissue biopsies have been taken then tissue was placed in a fridge for freezing for 24 hrs. Tissue homogenate has done by Eliza to measure IL-6, which is performed by the following steps: according to [13].

1. Remove the tissue from freezer and put it immediately in an ice place.
2. Cut a tissue into pieces by using a razor blade.
3. Weigh the pieces and then put the sample on ice again to keep it cold.
4. Chop the sample in to small pieces by using a blade.
5. Put the tissue in a glass tube homogenizer and add amount of cell lysing buffer solution.
6. Then grinding with pestle for several minutes until no more chunks are available.
7. Transfer the ground material to falcon tubes and place in ice.
8. Sonicate for 5-10 cycles by using sonifier 450 apparatus until foaming happened.
9. Incubate on ice for 10 minutes for cell lysis.
10. 10-Centrifugation for 10 minutes, then collect the supernatant in a PCR tube.

Ethical consideration:

The Ethics Board at Baghdad University approved the study, and each person who took part in the study gave their written approval after being told about the study. This work was done by following the World Medical Association's Code of Ethics (Declaration of Helsink) for studies that involve individuals.

Statistical analysis

Statistical analysis system-SAS (2012) was used to determine how different things affected the study parameters. In this study, the Least Significant Differences (LSD) test (ANOVA) was used to compare the means significantly.

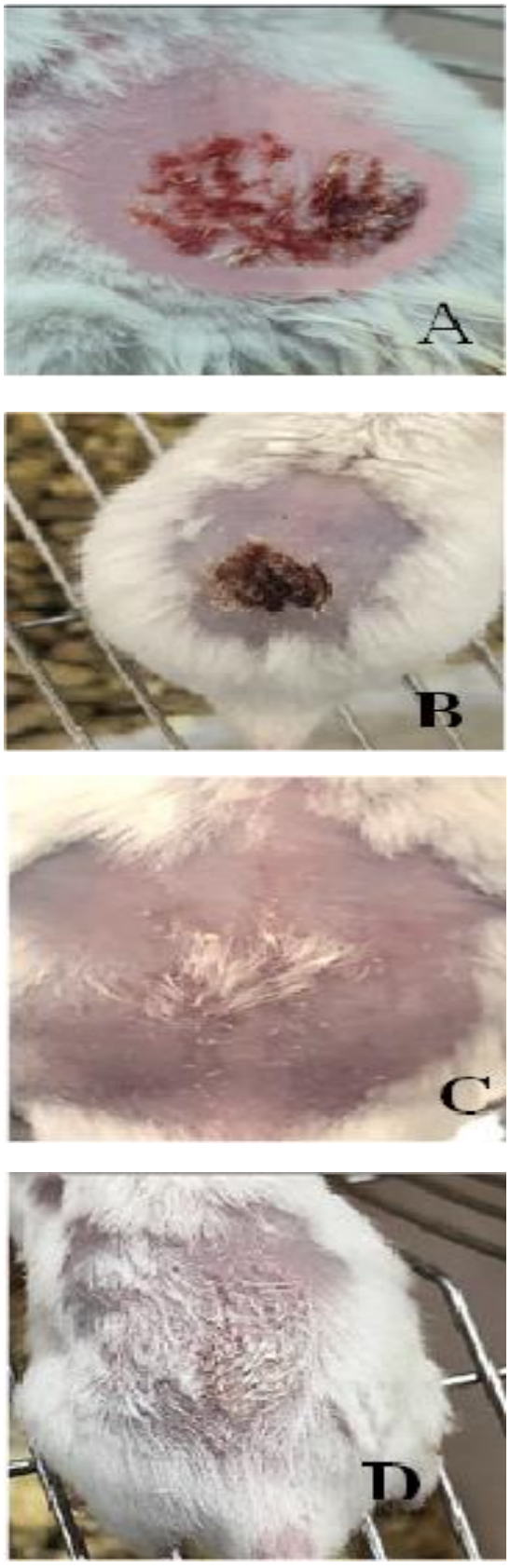
RESULTS

Gross examination

The development of lesions in mice after induction of infection with *C.albicans* required a period of 5 days in all infected groups (Figure 1- A), which characterized by different signs such as erythema, increase thickening of skin after 7 days of infection. On day 4, the skin lesion of animal treated with *S. aromaticum* oil extract 5 mg/ml looked partial healing by gross examination and showed absence of lesion and regenerated hair encircling the scratches (Figure 1- C). On day 7, showed exudates dried, with complete skin healing and returned to normal thickness with the regrowth of hair compared to untreated control group, which showed remaining the inflammation, redness, hyperkeratosis of the skin and exudates in the scratches (Figure 1- B).

When clove oil was used to treat *C. albicans*-induced experimental lesion induced inflammation, the severity of the skin lesion and local inflammation was significantly reduced. Clove bud essential oil is widely utilized and renowned for its therapeutic benefits (Figure 1- D). The group treated with Clotrimazole 2% at day 4 of treatment there was no any evidence for healing and the scratches still covered with scab, and clear signs of inflammation such as erythema and increased thickening of skin (Figure 1-

E). While, on the seventh day of treatment showed normal thickness of skin and absence of signs of inflammation (Figure 1- F).



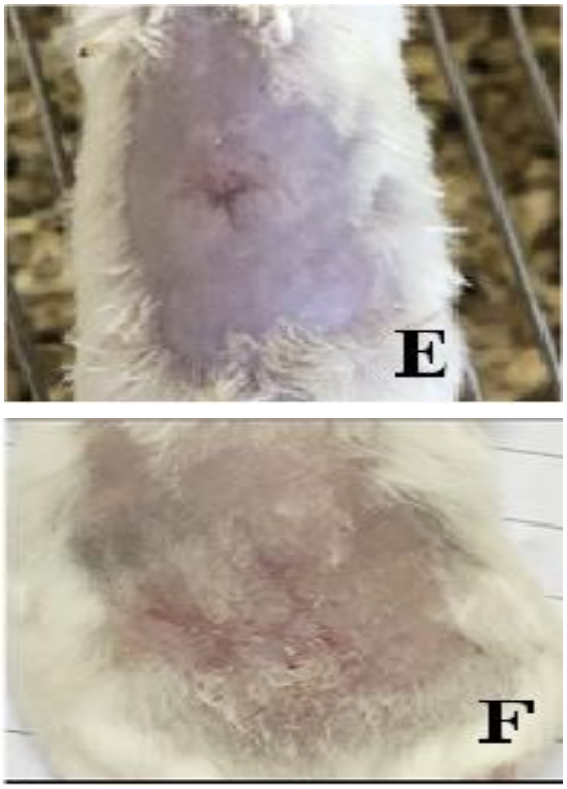


Figure (1): A: infected mice with *C.albicans* showed distinctive clinical signs: scabs, cracked, erythematous lesion and loss of hair.

B: image showed the progression of lesion of infected skin with *C.albicans* after 14 days of infection.
C: image showed absence of lesion and regenerated hair encircling the scratches of treated group with clove oil at day 4 of treatment.
D: image showed exudates dried, with complete skin healing and regrowth of hair of treated group with clove oil at day 7 of treatment.
E: image showed the presence of inflammation and dark patches of group treated with clotrimazole.
F: image showed absence of inflammation and skin return to normal thickness of treated group with clotrimazole at day 7 of treatment.

Measurement of tissue IL-6

As shown in table (1) and figure (2), the concentration of IL-6 was calculated in pg/ml. There were no significant differences among all groups before infection and significantly increase after 7 days of inducing infection compared to negative control group. Treated group with *Syzygium aromaticum* oil extract after 7 days showed significant decrease ($P \leq 0.01$) in the concentration of IL-6 compared to treated group with clotrimazole and infected untreated group while non-significantly with negative control group. Animals treated with clotrimazole showed decrease in IL-6 value after seven day of treatment but still high than negative control value.

Table (1): Topical IL-6 level (pg/ml) in mice infected with *C.albicans* and treated topically with clove oil extract and clotrimazole once daily for 7 days

Groups	Mean ± SE of Topical IL-6 (pg/ml)			LSD value
	Before infection	After 7 days of infection	After 7 days of treatment	
Negative Control	8.45 ±1.76 A a	7.02 ±1.26 B a	6.22 ±1.38 C a	4.760 NS
Positive Control	7.45 ±1.12 A b	24.35±1.87 A a	21.45±1.23 A a	4.641 **
Clove oil 5 mg/ml	7.40 ±0.83 A b	20.20±0.63 A a	5.52 ±1.67 C b	3.643 **
Cotrimazole 2%	6.82 ±1.02 A c	22.25±1.41 A a	15.02±2.14 B b	5.104 **
LSD value	3.804 NS	4.234 **	5.071 **	---

- Values represent mean ± S.E
- Different capital letters mean significant (P<0.01) between groups
- Different small letters mean significant (P< 0.01) between periods

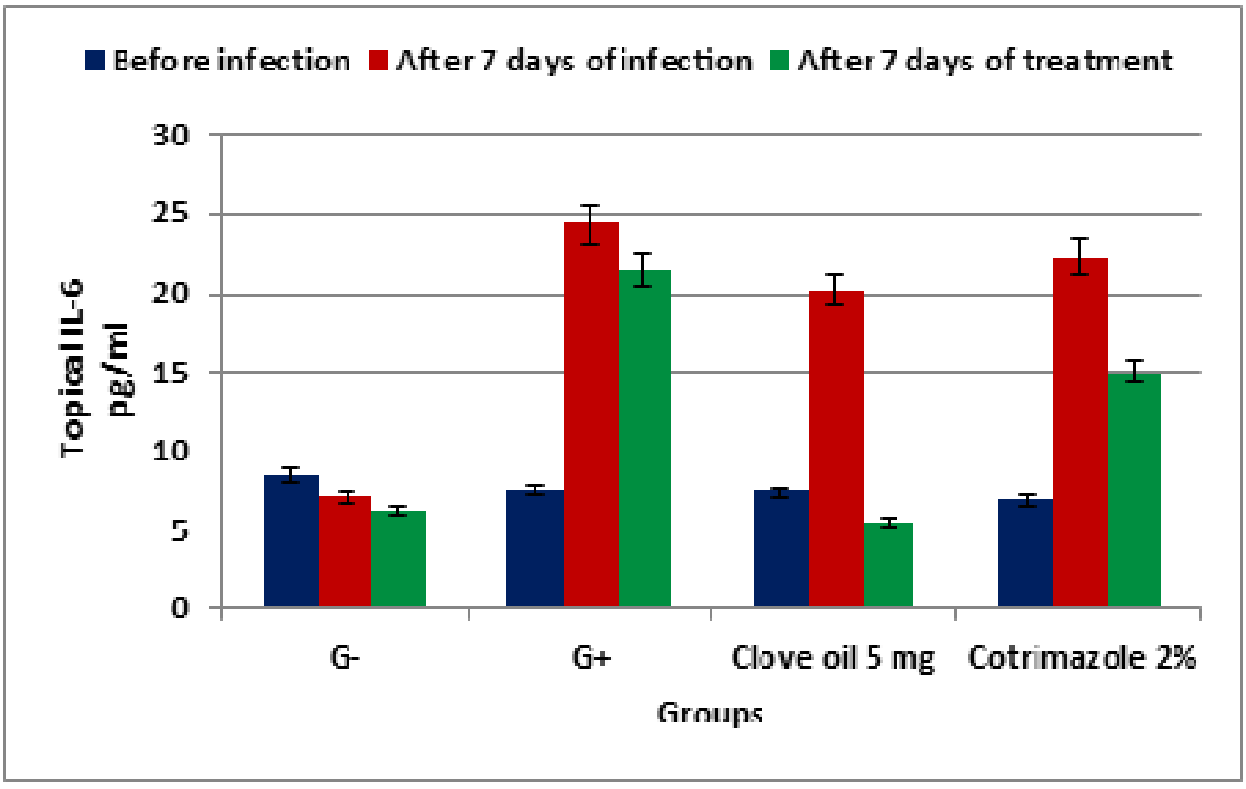


Figure (2): Tropical IL-6 values of mice infected with C. albicans and treated with Clove oil and Clotrimazole.

Microscopically changes

The histopathological section of skin in healthy mice showed normal epidermis and dermis of skin with hair follicles (Figure -3). While, the histopathological examination after 7 days of infection with *C albicans* showed epidermal ulceration, necrotic and cellular debris present with infiltration. Other section showed large abscess and necrotic tissue subcutaneously surrounded by zone of inflammatory cells. Also subcutaneous abscesses seen (Figure-4). The histopathological section of skin of treated mice with *Syzygium aromaticum* extract 5 mg/ml for 7 days showed marked proliferation of hair follicles and subcutaneous adipocytes accumulation, Keratin material also proliferated during 7 days of treatment (Figure-6). The group treated with the clotrimazole cream 2 % for 7 days showed necrotic of large hair follicle and presence of scab (Figure-7).

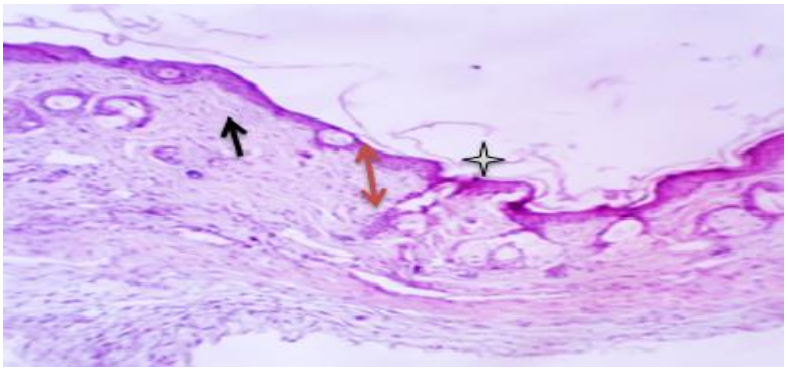


Figure (3): Histopathologic section of skin in negative group: normal epidermis (star) and dermis (red arrow) of skin with hair follicles (blackarrow) (H&E stain, 4X).

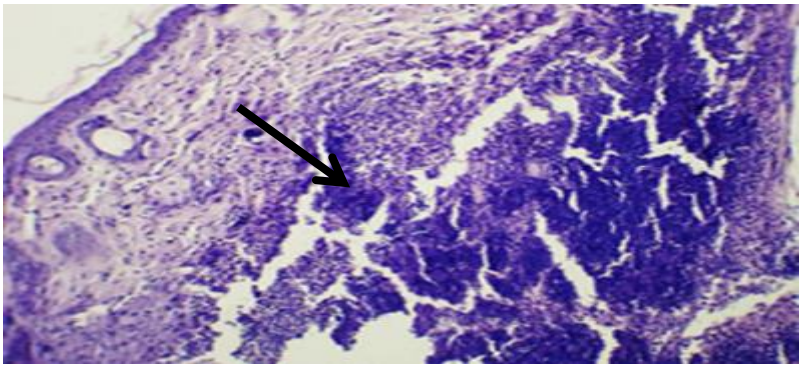


Figure (4): large abscess and necrotic tissue subcutaneously surrounded by zone of inflammatory cells. Also subcutaneous abscesses seen (H & E stain, 10X).

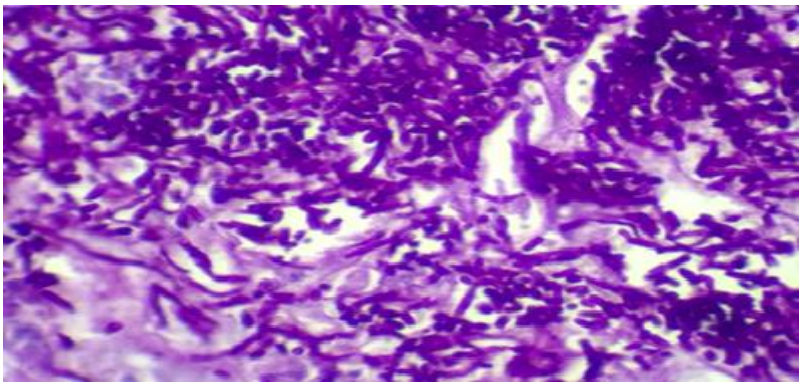


Figure (5): Histopathologic sectionof skin in positive group: the presence of the non-septated hyphae of *Candida* (PAS stain, 10X)

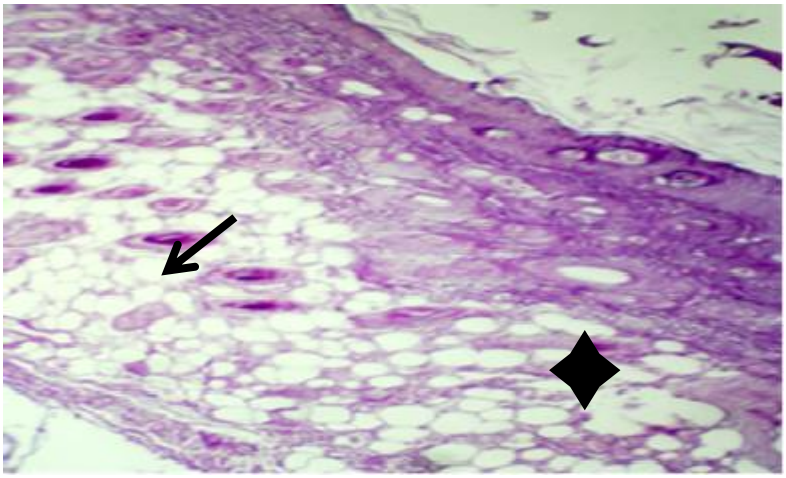


Figure (6): Histopathologi section of skin in treated group with Clove oil 5 mg: showed marked proliferation of hair follicles (black arrow) and subcutaneous adipocytes accumulation (star) (H & E stain, 10X).

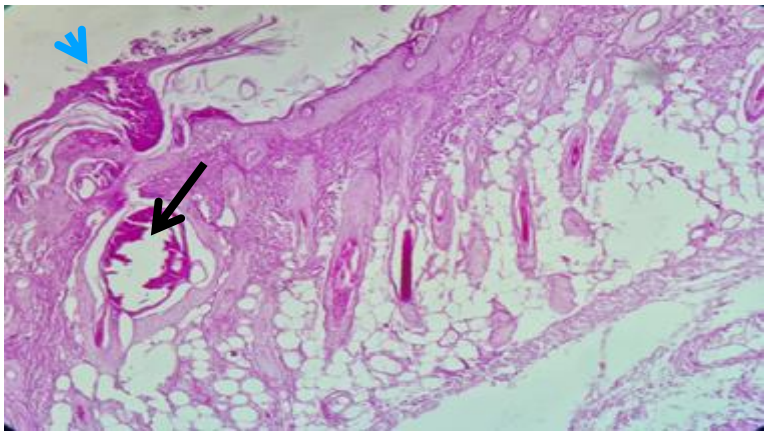


Figure (7): Histopathologic section of skin in treated group with clotrimazole 2 % showed necrotic of large hair follicle (black arrow) with the presence scab (arrow head). (PAS stain, 10X).

DISCUSSION

Skin act as a protective barrier so any cut or breakdown allow the overgrowth of *Candida* on skin and infection happens by the ability of yeast cells to adhere to the mucosal tissue then invasion of hyphae producing highly damage, fairly facilitated by secreted proteolytic enzymes. Eugenol is commonly regarded as the primary ingredient in clove oil, with smaller amounts of -caryophyllene and other ingredients [(14)].

The primary ingredient in clove oil, eugenol, has anti-inflammatory and fungicidal properties, which cause degradation of fungal cell walls. In addition to that, the ability of eugenol to inactivate ergosterol synthesis, which leads to fungal cell death by breaking down proteins and reacting with the phospholipid bilayer of cell walls changing the permeation of the walls and suppress the production of free radicals, which inhibit the inflammatory process and absence of inflammation signs like redness, increase in the thickness of skin, and other signs. This result is confirmed by **Khan *et al.*** [(15)]. When applied to the skin, eugenol encourages the growth of hair. In addition to other nutrients like potassium, salt, omega-3 fatty acids, iron, manganese, vitamins K and C, iodine and fiber that are good for skin healing and hair growth. This oil also contains antibacterial and anti-inflammatory compounds [(16)]. Clotrimazole works as anti-fungal action by decreasing ergosterol biosynthesis, which is a significant part of the fungal cytoplasmic membrane and, when inhibited, causes cell death by preventing the microsomal cytochrome P450 (CYP450)-dependent process 14-lanosterol demethylation, which is regarded as a critical step in ergosterol formation [(17)].

Whereas, the skin didn't show regrowth of hair on day 7 of treatment that may be due to the symptomatic improvement. Complete healing requires at least 2 -3 weeks [(18)]. Inflammatory macrophages are the primary source of interleukin-6, which is the main trigger in the acute stage of inflammation [(19)].

Treatment of mice with Syzygium aromaticum oil extract prevent macrophages from secreting IL-6. According to these outcomes, one may propose that eugenol was in charge of inhibiting IL-6 production by macrophages [(20)]. Due to their capacity to raise levels of reactive oxygen and glutathione, eugenol may have reduced the release of IL-6, which may have led to less NF-B pathway activation [(21)]. Studies have shown that eugenol can control the functions of macrophages and negatively regulate inflammation because they are immune system cells that are involved in the secretion of mediators (like pro-inflammatory cytokines and nitric oxide), which are crucial to vascular and cellular events during the progression of an inflammatory process [(22)].

Clotrimazole is a synthetic imidazole with wide-ranging anti-Candida Albicans and anti-other yeast species action. The azoles are thought to work by rupturing the fungal cell wall membrane, which results in cell death by inhibiting the biosynthesis of ergosterol, a sterol that is unique to fungi and is a significant component of the fungal cell membrane and in charge of maintaining cell integrity and function [(16)] in a concentration-dependent manner by preventing 14 alpha lanosterol's demethylation [(23)]. When ergosterol synthesis is hindered, the cell dies because it cannot build an intact and functional cell membrane [(24)].

CONCLUSION

The active constituents Eugenol of clove oil exposed a strong activity against topical skin infection induced by *C. albicans in vivo* hence has potential therapeutic effect.

Conflict of interest: According to the authors, there is no conflict of interest.

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Author contribution: In this study, all author contributed equally.

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