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# Therapeutic effects of ethanol extract of propolis on experimental cutaneous candidiasis in horse

**Sayyed Zakaria Mahmoodi[1], Vahid Najarnezhad[2]*, Ghader Jalilzadeh-Amin[2], Abdolghaffar Ownagh[3], Navideh Mirzakhani[4]**

*[1] DVM Graduate, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [2] Department of Internal Medicine, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [3] Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [4] Department of Pathobiology, Faculty of Veterinary Medicine, Amol University of Special Modern, Amol, Iran.*

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## Abstract

*Candida albicans* is a commensal opportunistic yeast colonizing the skin and mucosal surfaces of humans and animals, which, under specific predisposing conditions, can proliferate excessively and lead to clinical manifestations of candidiasis. The widespread prophylactic and therapeutic use of antifungal agents has led to the emergence of drug-resistant strains, necessitating the exploration of novel therapeutic alternatives. Considering the clinical and conformational significance of equine skin health, the present study aimed to evaluate the therapeutic efficacy of ethanol extract of propolis in an experimental model of cutaneous candidiasis in horses. In 2022, two clinically healthy, six-year-old female horses (approximately 400 kg) were selected for the study. Immunosuppression was induced using dexamethasone and four intradermal inoculation sites were created on the shaved thoracic and flank regions of both lateral aspects using *C. albicans* (PTCC: 5027) suspension ($5 \times 10^6$ CFU $mL^{-1}$). The experimental sites on the right thorax were treated with ethanol extract of propolis while those on the left thorax received topical nystatin. Lesions on the right flank were treated with glycerin as a vehicle control, and lesions on the left flank were left untreated. Cutaneous candidiasis was successfully induced within five days post-inoculation. Clinical resolution was observed following five days of treatment with ethanol extract of propolis, whereas, nystatin required eight days to achieve complete lesion resolution. The findings of this study suggested that topical ethanol extract of propolis demonstrated superior efficacy in accelerating the resolution of *C. albicans*-induced cutaneous lesions in horses compared to nystatin.

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## Introduction

Candidiasis is a mycotic infection caused by yeasts of the genus *Candida*, with *Candida albicans* being the most prevalent pathogenic species in both humans and animals. As an opportunistic pathogen, *C. albicans* resides on the skin and mucosal surfaces, typically in a commensal state. However, in immunocompromised hosts or under specific predisposing conditions, such as deficiencies in iron, zinc, vitamin B12, and folate, prolonged glucocorticoid administration, or extensive antibiotic use, *C. albicans* can transition into a pathogenic form, leading to cutaneous or systemic infections. Cutaneous candidiasis has been documented in various species including humans, horses, goats, dogs, cats, poultry and aquatic animals.[1-3] Moreover, cutaneous manifestations of candidiasis may arise secondary to systemic infection.[4,5]

The primary therapeutic approach for candidiasis involves antifungal agents such as nystatin, amphotericin B, natamycin, ketoconazole and fluconazole.[6-8] However, these agents are associated with considerable adverse effects including nephrotoxicity, gastrointestinal disturbances, neurological symptoms, hematologic abnormalities and cardiovascular complications. Also, high-dose prophylactic and therapeutic administration has led to the emergence of drug-resistant *Candida* strains and recurrent infections.[9-12] Consequently, there is a growing impetus to explore alternative, natural therapeutic modalities to mitigate these challenges.

Propolis, a resinous substance produced by bees from botanical sources, has been extensively investigated for its therapeutic potential. Its primary constituents include resin (50.00 - 70.00%), wax and oils (30.00 - 50.00%), pollen (5.00 - 10.00%), bioactive compounds such as

***Correspondence:**

Vahid Najarnezhad. DVM, DVSc
Department of Internal Medicine, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
**E-mail**: v.najarnezhad@urmia.ac.ir

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flavonoids, phenolic acids, vitamins (B, C, and E) and aromatic compounds.[13] Propolis exhibits a broad spectrum of pharmacological activities, including antibacterial, antifungal, antiviral, antiparasitic, anti-inflammatory, antitumor, and organoprotective effects.[14-19] Furthermore, it has been shown to potentiate macrophage activity and enhance the cytotoxic function of natural killer cells against tumor cells.[20] Clinically, propolis has been employed in wound healing, post-thyroidectomy care and the treatment of chronic ulcerative lesions.[21]

Recent research has particularly emphasized the antifungal efficacy of ethanol extract of propolis.[22-24] Koc *et al.*, demonstrated superior antifungal activity of ethanol-extracted propolis compared to ketoconazole against *Trichophyton spp*.[25] Similarly, Ownagh *et al.*, reported significant inhibitory effects of ethanol-extracted propolis from West Azarbaijan on both dermatophyte and non-dermatophyte fungi *in vitro*.[26] In animal studies, ethanol-extracted propolis has been successfully utilized in the treatment of *Candida*-induced keratitis in rabbits[27] and experimentally induced cutaneous candidiasis in goats.[28]

Given the significance of dermatological health in equine conformation and performance, this study was designed to evaluate the therapeutic efficacy of ethanol extract of propolis in experimentally induced cutaneous candidiasis in horses. An experimental model for equine cutaneous candidiasis was developed allowing for precise clinical and histopathological monitoring of lesion progression. This research aimed to provide novel insights into the potential application of propolis as an alternative antifungal therapy in equine medicine, addressing the limitations of conventional antifungal agents and contributing to the advancement of veterinary dermatology.

## Materials and Methods

**Organism preparation.** The *C. albicans* strain (Persian Type Culture Collection [PTCC]: 5027;) was obtained from the fungal culture archives of the Department of Mycology, Faculty of Veterinary Medicine, Urmia University. The fungal colonies were cultured in Sabouraud Dextrose Broth (Merck, Darmstadt, Germany) at 25.00 ˚C for 7 days to achieve optimal growth. A standardized fungal suspension with a concentration of $2.50 \times 10^6$ colony forming unit *per* mL was prepared by resuspending the cultured colonies in sterile normal saline (Merck) to match the turbidity of a 0.50 McFarland standard.[29]

**Animals preparation**. Two clinically healthy, 6-year-old female horses, each weighing approximately 400 kg were selected for the study. The animals were housed individually in dedicated isolation facilities within the Veterinary Teaching Hospital of Urmia University, under conditions approved for infectious disease research. The horses were provided with alfalfa hay twice daily with *ad libitum* access to fresh water throughout the study period. A comprehensive health evaluation, including physical examination and laboratory diagnostics, confirmed the absence of any pre-existing cutaneous disorders. To eliminate potential parasitic confounders both animals were prophylactically treated with febantel (Zagros Pharmed Pars Co., Borujerd, Iran) at a dose of 10.00 mg $kg^{-1}$, orally for 5 consecutive days. Additionally, penicillin G (NASR Pharmaceutical Co., Mashhad, Iran) was administered at 22,000 IU $kg^{-1}$, intramuscularly, once to prevent bacterial skin infections, ensuring a sufficient washout period before experimental inoculation. To induce immunosuppression, dexamethasone (Rooyan Darou Pharmaceutical Co., Tehran, Iran) was administered at a dosage of 2.00 mg $kg^{-1}$ intramuscularly once daily for 4 consecutive days. For experimental infection, symmetrical regions on both flanks and the thoracic region were prepared by shaving an area of 30.00 $cm^2$ *per* site. To ensure reproducibility of therapeutic effects, four equidistant intradermal inoculation sites (5.00 cm apart) were designated within each region, and 0.40 mL of the prepared *C. albicans* suspension was injected intradermally at each site.[30] All experimental procedures were reviewed and approved by the Animal Research Ethics Committee of the Faculty of Veterinary Medicine, Urmia University, Iran, (IR-1559, 2022) in accordance with institutional and national guidelines for the care and use of laboratory animals.

**Treatment protocol**. Following the successful induction of cutaneous candidiasis, treatment was initiated and continued daily until complete resolution of lesions. The study design incorporated four treatment groups: 1) Right thoracic region: Ethanolic extract of propolis (1,000 µg $mL^{-1}$) was dissolved in glycerin and was topically applied.[31] 2) Left thoracic region: Standard antifungal treatment with nystatin ointment (Jaber Ebne Hayan Pharmaceurical Co., Tehran, Iran; 10.00 mg *per* 100 g) was applied. 3) Right flank: Glycerin (Neutron Pharmachemical Co., Tehran, Iran) alone was applied as a vehicle control to confirm the inertness of the solvent. 4) Left flank: No treatment was applied, serving as an untreated control.

**Preparation of ethanolic extract of propolis.** Propolis samples were collected from beehives in the West Azarbaijan region, Iran. Thirty grams of raw propolis was finely powdered and macerated in 300 mL of 96.00% ethanol (Merck) at 25.00 ˚C for 48 hr. The extract was filtered twice using Whatman No. 42 filter paper, followed by solvent evaporation under reduced pressure using a rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland). The resulting concentrate was dissolved in glycerin (Neutron Pharmachemical Co.) at a final concentration of 1,000 µg $mL^{-1}$. To ensure sterility, the solution was passed through a 0.20-µm membrane filter (Millipore, Burlington, USA) and stored at 4.00 ˚C until use.

**Gas chromatography-mass spectrometry analysis.** The chemical composition of the propolis sample was analyzed using Gas Chromatography-Mass Spectrometry. A Hewlett Packard HP 5890 Series II Gas Chromatograph coupled to a mass spectrometer and equipped with an HP-5MS capillary column (30.00 m × 0.25 µm film thickness, 0.50 µm internal diameter; Agilent Technologies, Santa Clara, USA) was employed. The system was operated in electron ionization mode (70.00 eV). Helium served as the carrier gas at a constant flow rate of 0.80 mL *per* min, with a split ratio of 1 : 10. The injector temperature was set at 280 ˚C. The oven temperature program was initiated at 60.00 ˚C, increased at a rate of 5.00 ˚C *per* min to 300 ˚C, and held for 10 min. This analytical setup enabled effective chromatographic separation and accurate mass spectral detection, providing a comprehensive profile of the chemical constituents present in the propolis sample.

**Evaluation of treatment efficacy**. The progression of cutaneous lesions was monitored daily through detailed clinical examination and photographic documentation from the day of inoculation until complete healing. Lesion size, erythema, ulceration, exudation and other clinical parameters were recorded systematically to assess the therapeutic response in each treatment group.

**Histopathological analysis**. Skin biopsy specimens were collected from lesion sites under mild sedation using a 5.00-mm biopsy punch (Kai Europe GmbH, Solingen, Germany). Prior to the procedure, sedation was induced by intramuscular injection of xylazine (ChemiDarou Company, Tehran, Iran) at a dosage of 2.00 mg $kg^{-1}$. Tissue samples were fixed in 10.00% neutral-buffered formalin (Sigma-Aldrich, St. Louis, USA) for 10 days before paraffin embedding. Two serial sections were prepared from each sample: 1) Azan trichrome staining: Performed to evaluate inflammatory response and connective tissue remodeling. 2) Grocott's methenamine silver staining: Used to visualize fungal elements including *C. albicans* hyphae, within the affected tissue. All histological sections were examined under a light microscope (BX53; Olympus, Tokyo, Japan) to assess the extent of fungal invasion, tissue reaction and therapeutic efficacy. This experimental model represented a novel approach for studying the clinical progression and therapeutic interventions for cutaneous candidiasis in equine species.

## Results

**Chemical composition of propolis.** A total number of nine distinct flavonoid constituents and ten volatile organic compounds were unequivocally characterized through comprehensive gas chromatography analysis of the propolis sample. The quantitative profiling of the identified phenolic and flavonoid derivatives together with the catalogued volatile organic fraction is systematically presented in Tables 1 and 2, respectively.

**Table 1.** The amounts of phenolic and flavonoid compounds in the propolis studied (ppm).

| Compounds | ppm (µg $mL^{-1}$) |
|---|---|
| **Cinamic acid** | 27,194.90 |
| **Comaric acid** | 7,019.40 |
| **Rutin** | 4,190.60 |
| **Apigenin** | 3,192.60 |
| **Caffeic acid** | 2,220.50 |
| **Quercetin** | 1,840.50 |
| **Rosemaric acid** | 1,398.30 |
| **Gallic acid** | 1,108.70 |
| **Chlorogenic acid** | 401.10 |

**Clinical observations**. Following the intradermal inoculation of *C. albicans* suspension, the inoculation sites exhibited mild localized swelling relative to the surrounding skin (Fig. 1A). By the 1st day post-inoculation, the swelling became more pronounced (Fig. 1B). On the 2nd day, the affected sites demonstrated increased prominence and slight discoloration of the overlying skin (Fig. 1C). By the 3rd day erythema and swelling was intensified accompanied by the emergence of serous exudate on the surface of some lesions (Fig. 1D). On the 4th day, the lesions were markedly edematous, vesiculated and exhibited tenderness on palpation (Figs. 1E and 1F). By the 5th day, some vesicles developed central pitting and a subset of lesions ruptured, releasing a thick and purulent exudate (Fig. 1G). Despite the localized inflammatory reaction, systemic parameters including body temperature, heart rate, respiratory rate and appetite were remained within normal physiological ranges throughout the study. Topical administration of ethanol extract of propolis resulted in significant improvement of *C. albicans*-induced lesions within 5 days of treatment (Fig. 2A). By the 5th day, the lesions exhibited complete clinical resolution with restoration of normal skin consistency and the absence of tenderness. However, hair regrowth was not observed within one month following lesion resolution. In contrast, daily application of nystatin ointment required 8 days to achieve complete resolution of the lesions (Fig. 2B). By the 8th day, the affected areas regained normal consistency and were non-tender upon palpation, yet hair regrowth was similarly absent for up to one-month post-treatment. Administration of glycerin over 8 days failed to produce any therapeutic effects on *C. albicans*-induced lesions. By the 8th day, lesions remained edematous, soft upon palpation and continued to exude a bloody-purulent discharge which persisted until the termination of the study on day 13 post-inoculation (Fig. 2C). Similarly, in the untreated control group, lesion progression mirrored that of the glycerin-treated group with sustained edema tenderness and persistent exudation throughout the study period (Fig. 2D). At the conclusion of the experiment, all untreated lesions were subjected to localized therapy with ethanol extract of propolis to facilitate resolution.

**Table 2.** The amounts of volatile organic compounds in the propolis studied (%).

| Volatile organic compounds | Sensitivity (%) | Amount (%) |
|---|---|---|
| **Propanedinitrile** | 52.00 | 13.14 |
| **1-Imidazole-1-yl-3-methylbut-2-en-1** | 50.00 | 10.88 |
| **2-Propen-1-one** | 97.00 | 7.31 |
| **Alpha-Atlantone** | 64.00 | 4.29 |
| **2-NaphtaleneMethanol** | 89.00 | 4.11 |
| **Alpha-Eudesmol** | 98.00 | 3.29 |
| **3,4-Dimethoxyphenylacetone** | 65.00 | 2.17 |
| **15-Tetracosanoic acid** | 67.00 | 1.69 |
| **2-Methyl-7-oxo-4,7-Dihydro-1,2,4,T** | 61.00 | 1.29 |
| **Aromadendrene** | 94.00 | 1.10 |

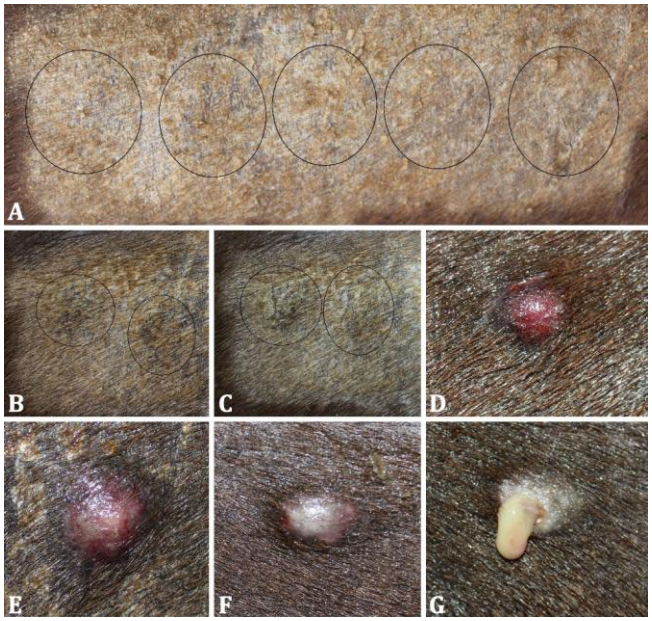


**Fig. 1. A)** The inoculation sites of *Candida albicans*. **B)** Slight swelling of the inoculation site, 1 day after the inoculation of *C. albicans*. **C)** Slight swelling and discoloration of the affected area on the 2nd day. **D)** Swelling and redness of the affected area on the 3rd day. **E** and **F)** Swelling of the affected area like a vesicle on the 4th day. **G)** Ruptured bulge with draining thick, creamy discharge.

**Histopathological findings.** Skin biopsy specimens were collected on days five, 10 (corresponding to day five of treatment) and 13 (day eight of treatment) post-inoculation of *C. albicans*. Histopathological analysis on day five post-inoculation revealed extensive dermal inflammation and necrosis (Fig. 3A). Following 5 days of ethanol extract of propolis treatment, a marked reduction in inflammatory cell infiltration was observed in the dermis (Fig. 3B). By day eight of treatment, histopathological evaluation confirmed the complete resolution of infection (Fig. 3C). Conversely, 5 days of nystatin treatment resulted in moderate epidermal inflammation and necrosis (Fig. 3D), while histopathological examination on day eight demonstrated lesion resolution with evidence of dermal repair (Fig. 3E). In the glycerin-treated group, histopathological examination on day five showed severe inflammatory cell infiltration and extensive dermal necrosis (Fig. 3F). By day eight, persistent inflammation, folliculitis and intradermal abscess formation were evident due to secondary bacterial invasion (Fig. 3G). Similarly, by day 13 post-inoculation, the untreated group exhibited sustained dermal necrosis, epidermal disruption and profound inflammatory cell infiltration (Fig. 3H).

Grocott's methenamine silver staining demonstrated fungal hyphal presence within the epidermis. By day five post-inoculation, dense accumulations of fungal hyphae were observed (Fig. 4A). No fungal elements were detected in the ethanol extract of propolis-treated group on either day five or eight (Figs. 4B and 4C).

In the nystatin-treated group, sparse fungal hyphae were noted on day five with complete clearance by day eight (Figs. 4E and 4E). However, in the glycerin-treated group, dense accumulations of fungal hyphae persisted within the epidermis on both days five and eight (Figs. 4F and 4G). Similarly, in the untreated control group, persistent fungal infiltration was observed in epidermal sections on day 13 post-inoculation (Fig. 4H).

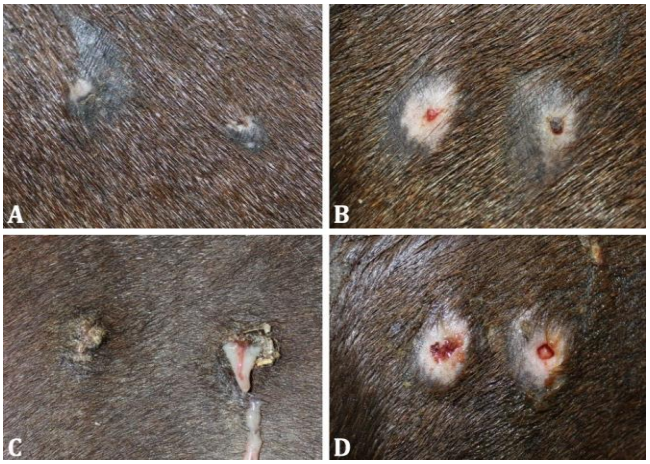


**Fig. 2. A)** Improvement of skin lesions following 5 days administration of ethanol extract of propolis. **B)** Improvement of skin lesions following 8 days administration of nystatin. **C)** Ruptured bulge with draining of bloody-creamy discharge following 8 days administration of glycerin. **D)** Ruptured bulge with draining of bloody-creamy discharge on the last day of the survey (13 days after the inoculation of *Candida albicans*) in the untreated group.

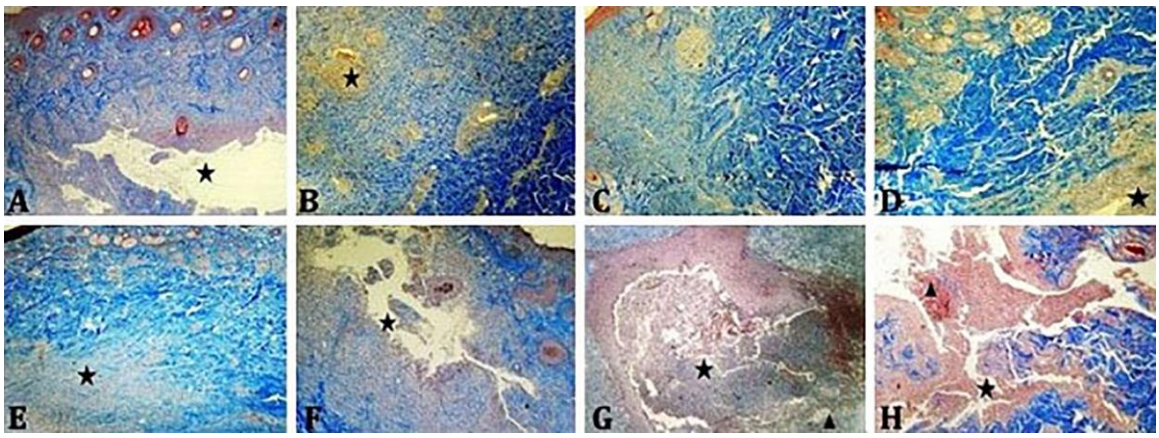


**Fig. 3. A)** Massive inflammation and necrosis in dermis (★), 5 days after inoculation of *Candida albicans*. **B)** Scant infiltration of inflammatory cells in dermis (★), on the 5th day of treatment with ethanol extract of propolis. **C)** Elimination of the infection, on the 8th day of treatment with ethanol extract of propolis. **D)** Moderate inflammation and necrosis of the epidermis (★), on the 5th day of treatment with nystatin. **E)** Resolution of the infection with the signs of healing in the dermis (★) on the 8th day of treatment with nystatin. **F)** Severe inflammatory cells infiltration and massive necrosis of dermis (★) on the 5th day of treatment with glycerin. **G)** Infiltration of inflammatory cells in the follicles and abscess formation in the dermis (★) due to hair follicle infection and disintegration (▲) on the 8th day of treatment with glycerin. **H)** Severe infiltration of different types of inflammatory cells (★) with dermal necrosis and disruption of epidermis (▲) on the 13 days after inoculation of *C. albicans* in the untreated group. (Azan trichrome staining; 100 ×).

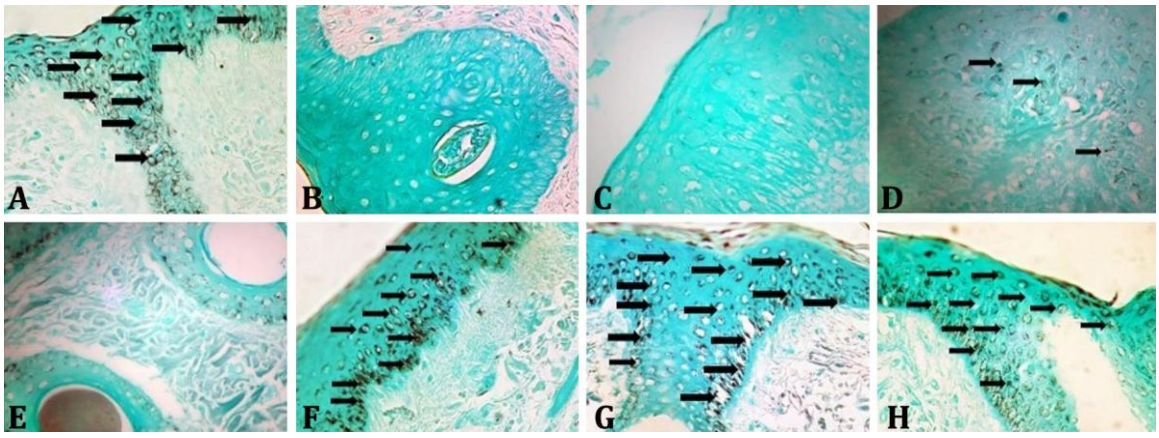


**Fig. 4.** The *Candida* hyphae is noted as black dots (arrows). **A)** A dense accumulation of fungal hyphae in the epidermis, 5 days after inoculation of *Candida albicans*. **B)** Elimination of fungal hypha 5 days after treatment with ethanol extract of propolis. **C)** No fungal hypha was seen 8th days after treatment with ethanol extract of propolis. **D)** Accumulation of few *candida* hypha in the epidermis on day five after nystatin administration. **E)** Complete elimination of *candida* hypha from the epidermis 8th days after nystatin administration. **F)** Dense accumulation of fungi hyphae in the epidermis on day 5 after glycerin administration. **G)** Dense accumulation of fungi hyphae in the epidermis 8th days after glycerin administration. **H)** Dense accumulation of fungi hyphae in the epidermis on day 13 after inoculation of *C. albicans,* in untreated group. (Grocott's methenamine silver staining; 400 ×).

## Discussion

Cutaneous candidiasis is a fungal infection predominantly caused by *C. albicans*, an opportunistic yeast capable of inducing a spectrum of diseases ranging from superficial mucocutaneous disorders to systemic infections. The clinical manifestation of candidiasis varies widely and is influenced by the immune status of the host.[3] Severe forms of the disease are predominantly observed in immunocompromised individuals such as those undergoing chemotherapy, immunosuppressive therapy or organ transplantation.[32]

Experimental models of cutaneous candidiasis have been widely developed in laboratory animals to investigate pathogenesis and therapeutic approaches.[33-35] The objective of this study was to evaluate the therapeutic efficacy of ethanol extract of propolis in an experimentally induced model of cutaneous candidiasis in horses.

In this study, the successful establishment of cutaneous candidiasis in dexamethasone-treated horses was achieved within 5 days following intradermal inoculation of *C. albicans* suspension ($2.50 \times 10^6$ colony forming unit *per* mL). Similar results were reported by Eslami Milanee *et al.*, who induced cutaneous candidiasis in goats.

However, lesion development in goats occurred over an 8-day period, likely attributable to interspecies histological differences in the skin. Clinical signs observed in this study including abscess formation and cutaneous perforation at the inoculation sites were consistent with previous reports of *Candida*-induced skin infections.[28] Histopathological examinations confirmed the presence of *Candida* hyphae, folliculitis, abscess formation and epidermal disruption. The ability of *Candida* species to invade tissues is attributed to their production of keratinases, acid proteinases and phospholipases which degrade the stratum corneum and facilitate tissue penetration.[36] The rapid infiltration of neutrophils at the site of infection results in abscess formation, follicular necrosis and epithelial destruction.[3] Additionally, *Candida* species have been implicated in equine arthritis and osteomyelitis[37] as well as systemic infections secondary to prolonged antibiotic or corticosteroid administration in livestock.[7]

A variety of antifungal agents, including nystatin, amphotericin B, imidazoles and triazoles, have been employed in the treatment of candidiasis.[38] Nystatin, a polyene antifungal, exerts its effect by binding to ergosterol within fungal cell membranes, altering permeability and inducing cell lysis.[39] Despite advances in antifungal pharmacotherapy, the increasing prevalence of fungal infections and the emergence of antifungal resistance remain significant challenges.[40]

Natural products with antimicrobial properties have garnered considerable interest due to their cost-effectiveness, availability and broad-spectrum antimicrobial activities.[41] Propolis, a resinous compound produced by honeybees, has demonstrated efficacy in wound healing, burn treatment and tissue regeneration.[42] The antifungal activity of propolis is attributed to its high content of flavonoids (*e.g.,* galangin) and phenolic acids (*e.g.,* caffeic acid) which have been shown to inhibit fungal cell division by interfering with DNA replication.[43] Additionally, propolis accelerates tissue repair by stimulating glycosaminoglycan synthesis, promoting granulation tissue formation and facilitating wound closure.[42] Its anti-inflammatory properties are linked to its ability to scavenge free radicals, inhibit cyclooxygenase activity, suppress prostaglandin biosynthesis and modulate cytokine production.[44]

Thee findings demonstrated that topical application of ethanol extract of propolis led to complete resolution of *C. albicans* infections in a shorter duration compared to nystatin. This was consistent with prior reports of propolis efficacy in the treatment of cutaneous fungal infections in goats.[28] The superior therapeutic outcome of propolis can be attributed to its antifungal, anti-inflammatory, antioxidant and immune-modulatory effects.[45] The prolonged adhesion of propolis to the skin, facilitated by its interaction with cutaneous oils and proteins, may have contributed to its sustained antifungal activity.[46]

This study provided the first evidence of the high efficacy of ethanol extract of propolis in the treatment of experimentally induced cutaneous candidiasis in horses. The results underscored the potential of propolis as a natural, cost-effective and safe alternative to conventional antifungal agents for managing cutaneous fungal infections in equines.

In conclusion the findings of this study indicated that ethanol extract of propolis effectively enhanced the healing of *C. albicans*-induced cutaneous lesions in horses, achieving therapeutic outcomes in a shorter duration compared to nystatin. Given its potent antifungal activity, anti-inflammatory properties and affordability, propolis represented a promising alternative to synthetic antifungal agents for the treatment of equine cutaneous candidiasis.

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## Conflict of interest

The authors declare no conflict of interest.

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