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Investigation of antifungal activity and histological effects of Myrrh on *Candida* corneal ulcers *in vivo*

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Abstract
Problem: Mycotic keratitis is commonly considered as a resistant ulcer and a main reason for vision impairment. Its treatment was long time challenge with commercial drugs and low safety of new trials.
Aim: the present work will search for antifungal activity of a natural extract while considering the safety and minimal side effects on the treated corneal tissues.
Methods: The most common causative agent was isolated and identified from human corneal ulcers. Aqueous extract was prepared for *Commiphora molmol*, its MIC was determined against the tested isolate, comparing with the commercial antifungal agent. GC-MS analysis investigated the active ingredients of the selected plant extract. Its antimicrobial activity and histological effects were investigated on rabbit eyes.
Results: *Candida albicans* was found to be the most common yeast corneal ulcer invader. It was greatly inhibited by the aqueous myrrh extract with MIC of 200 µg/ml. that can be supported by normal appearance and minimal side effects on the treated corneal tissues in comparison with fluconazole as a commercial agent. These combined antimicrobial and anti-inflammatory actions referred to the presence of 2-Pentanone, 4-hydroxy-4-methyl; Undecanal; Indan-1,3-diol monopropionate; Triacetin and other plant components.
Conclusion: *Commiphora molmol* aqueous extracts can be recommended for treatment of *Candida albicans* corneal ulcers with minimal side effects on corneal tissues as new safe remedy.

Keywords: *Candida albicans*, *Commiphora molmol*, myrrh, mycotic keratitis, histological safety

Introduction

About 1-45% of cases of microbial keratitis are caused by mycotic or fungal keratitis (MK/FK) (Sahay *et al*., 2019) [32]. While the use of contact lenses has emerged as a major risk factor for fungal keratitis in affluent nations, the most common risk factor in underdeveloped nations is trauma from contact with vegetable material or soil-contaminated objects (Maharana *et al*., 2016) [21]. *Aspergillus* species, *Fusarium* species, and *Candida* species are the three most common fungi that cause ocular pathology (Brown *et al*., 2021) [5].

The care of resistant or deep fungal keratitis required methods other than topical or oral administration of antifungal medications due to its poor prognosis and difficulties of treatment. First, the intracameral route was utilized to provide pharmaceuticals. Intrastromal injections of antimycotic medications quickly followed, classifying both of these routes under the umbrella of targeted drug delivery (Maharana *et al*., 2016) [21].

Targeted therapy is indicated for cases of resistance (not responding to normal treatment for at least two weeks) and deep mycotic keratitis (>50% of stromal depth). The age, size of the ulcer, mean interval between onset of symptoms and hospital admission, and presence and severity of hypopyon at the time of presentation were all examined in all clinical trials and case reports. Best spectacle-corrected visual acuity (BSCVA) following intervention, healing time, scar size, abscess size decrease, epithelial defect resolution, stromal infiltrates, and infection were the main outcomes (Raj *et al*., 2021) [30].

Pigmented rabbits (*Oryctolagus cuniculus*), New Zealand White rabbits, and Burgundy Fawn rabbits are among the frequently utilized strains of rabbits. The majority of FK research involving these animals is directed toward therapeutic testing and diagnostic screening. For instance, the effectiveness of WGA (Wheat germ agglutinin)-peroxidase as a stain for identifying fungal lectins in corneas infected with *Aspergillus fumigatus*, *Fusarium solani*, or *Candida albicans* was tested on rabbits (Ghosh *et al*., 2019) [16]. Information on all criteria, such as fungal burden, inflammation, and corneal architecture, can be obtained through histopathological assessment (Qin *et al.,* 2019) [29].

Records from the past demonstrate the usage of herbs and spices for culinary flavoring as well as therapeutic uses. Historical accounts indicate that the use of botanicals for flavor and medicinal purposes dates back to 6000 B.C. in China (Draughon, 2004) [10]. Approximately 1550 B.C., the ancient Egyptians employed spices not only for food preservation but also for embalming their deceased. The antimicrobial qualities of herbs, spices, and their components have been the subject of extensive scientific study on plant products in the 19th century, and interest in these compounds' qualities is still growing today. (Zaika, 1988) [37].

According to Alshibly *et al.* (2022) [3], myrrh is one of the oldest known medications that the ancient Egyptians utilized extensively. In Chinese traditional medicine, myrrh and resinous Frankincense are well-known for treating inflammatory conditions, blood stagnation (together referred to as "blood moving medicine"), as well as pain and swelling (Fatani *et al*., 2016) [12]. According to recent reports, myrrh has antipyretic, analgesic, and antiseptic properties (Shalaby and Hammouda, 2014) [34].

The present study will aim to evaluate the antifungal activity against the fungal infection; and histological effects on the infected corneal tissues of the selected plant extract to elucidate its safe usage as a natural remedy for mycotic keratitis.

Materials and Methods

Culture

The patient eyes were anesthetized by a topical eye drop (Boxinate, Alex. Pharma, Egypt), that was tested for non-antimicrobial activity. All samples were taken using a flamed "kimura" platinum spatula (Alcon Couvreur, Belgium) for hard tissues or a single-use ethylene gas-sterilized cotton swab (BioMed, China) for soft wounds. Betadine (Nile Pharma, Cairo, Egypt) was administered as a mild cleaning around infected eyes; samples were also isolated in a closed controlled room in the hospital laboratory. The conjunctiva and eye lids were avoided during corneal scraping. Next, C-streaks were created on culture plates in order to differentiate between other plate impurities and microbial growth originating from corneal scraping. A medium known as Sabouraud's dextrose agar (SDA) was injected with the scrape. A 0.05% concentration of chloramphenicol was added to SDA in order to prevent bacterial growth and enable a more precise and selective isolation of *Candida albicans* (Margo and Brinser, 1987) [24].

Ethical consideration

All procedures involving human participants and animal models performed in this study were in accordance with the ethical standards of Tanta University's ethical committee and its later amendments or comparable ethical standards, approval with the code (IACUC-SCI-TU-0101).

Determination of MIC of *Commiphora molmol* aqueous extract

In order to verify the effective antimicrobial activity of the chosen plant cold-water extract against the microbial growth of the most prevalent fungal isolate among corneal ulcers in the current study, a minimum inhibitory concentration (MIC) test was performed. This could be indicated by the lowest percentage of surviving cells with noticeably low concentration of the plant extract. The MIC test was carried out in accordance with Baker *et al*. (2005) [4]; The liquid cultures were made by mixing 9 ml of liquid medium with 0.5 ml of spore or cell suspension ($6x10^6$ cells/ml) and 0.5 ml of plant aqueous extract at various dilutions (0.5 to 500 µg/ml). For 24 hours, the prepared combinations were incubated at 35°C. Following the calculation of the percentages (%) of surviving cells for each sample using a spectrophotometer set to 620 nm, the MIC was graphically displayed.

$$\% \text{ of surviving Cells} = [(A_{0.0} - A_{conc.})/A0.0] \times 100.$$

While: $A_{0.0}$= The spectrophotometer reading of untreated sample (concentration of the plant extract = 0.0mg/ml).
$A_{conc.}$= The spectrophotometer reading of a certain concentration of the plant extract.

Characterization of the active ingredients in the tested plant water extract

The active components in the examined extract were identified using gas chromatographic mass spectrometry at Tanta University's Scientific Research Center & Measurements (SRCM); utilizing a heated FID-equipped Perkin Elmer Clarus 580/560S kind of equipment. The following are the conditions: Oven: initial temperature of 50°C for 4 minutes, ramp 10°C/min to 140°C, hold for 5 minutes, ramp 10°C/min to 270°C, hold for 3 minutes; injection temperature of 270°C; volume of 1 µL; split ratio of 20:1, carrier gas of He; solvent delay of 4.00 minutes; transfer temperature of 180°C; source temperature of 200°C. Scan: Column (Elite-5MS, 30 m, 0.25 mm ID), 50 to 550 Da. The relationship between their biological activities and the current effective ingredients was ascertained (Manzan *et al*., 2003) [23].

Histopathological examination

Histopathological examination was carried out for 4 rabbit groups' corneal tissues; the first one represented the healthy corneal tissue as negative control (group A); the second one represented the non-treated infection with *Candida albicans* (group B); the third reported the treated corneal infection with fluconazole antifungal agent (Diflocan®) (group C); and the fourth represented corneal tissue after treatment the selected plant aqueous extract (group D). This was to elucidate the safety and effectiveness of the selected extract to be applied topically on the eyes according to the procedure of Aldebasi *et al.* (2014) [2] as follows:

i. Selection and treatment of experimental animals

In accordance with the ARVO policy for the use of animals in ophthalmic and vision research, four groups of New Zealand albino rabbits weighing between 1500 and 2000 g were prepared for laboratory settings in separate specific cages for three days prior to work, receiving green food exclusively.

ii. Inoculation and treatment Procedure

The microorganisms used for corneal inoculation were prepared by suspending fungal cells in sterile normal saline, adjusting the turbidity to Mcfarland number (1.05×10^5 cells/ml), which was then injected tangentially to the corneal surface into the central corneal stroma. Two days after the microbial suspension injection, when stromal keratitis had started to show symptoms, therapy was initiated separately for each group.

Group A neither infected nor treated, other hand Group B of rabbits infected with fungal growth without any treatments. Group C infected with the fungal growth and were treated with standard topical eye drops (Diflocan®) every 3 hours. The water plant extract was prepared in the form of topical eye drops by suspending its MIC in sterile distilled water, a volume of 0.2 ml of the extract MIC was topically dropped every 3 h. into the infected right eye of each rabbit in group D.

iii. Separation of intact corneal tissues

After three hours of the last dosage, albino rabbits were slaughtered (without the use of anesthesia). 150 ml of 80% ethanol, 60 ml of 40% formalin, 15 ml of glacial acetic acid, and one gram of picric acid crystals were combined to create the alcoholic Bouin fixative solution. In order to preserve the endothelium intact, a 27 gauge needle (MASCO, Egypt) was used to inject 0.1 ml of the fixative solution into the anterior chamber of the eye. On the surface of the eye, an additional 0.1 ml of fixative solution was applied (to retain the intact of epithelium of cornea).

To prevent corneal damage both treated and control corneas were removed from dissected eyes in an aseptic manner with a rim of sclera by making a 360° incision directly behind the limbus using a sterile Alcon short cut pointed surgical blade (size no.:11). Samples of excised cornea were soaked in the alcoholic Bouin fixative solution right away and left for a full day.

Preparation of corneal sections

Corneal samples were water-washed, immersed in serial dilutions of ethanol (30, 50, 70, and 80%) to dehydrate them, soaked in xylene for five minutes, placed in a molten soft paraffin bath. Next, paraffin blocks were instantly submerged in cold water to solidify them. Sections with a thickness of 5 µm were cut from paraffin blocks containing corneal samples by a rotary microtome (Alcon-Couvreur, Belgium). Toluene was used to thoroughly dissolve the paraffin, then hematoxylin solution was used to stain deparaffinized slides for a duration of 20 minutes. Slides were water-washed, alcohol-dehydrated. Eosin solution was used to counterstain dehydrated slides for five minutes, cleared with xylene. Aqueous Hoyer mounting media (30 g gum Arabic + 200 g chloral hydrate + 16 ml glycerol + 50 ml distilled water) was soaked over stained sections to permanently mount them. Overnight, a glass cover was adhered on and kept heated.

Slides of corneal sections were examined, using light microscope (Celestron, PentaView, USA). Sharp images were photographed with magnification power of 400X by Celestron built in digital camera.

Results

Isolates from patient's corneal ulcers were inoculated on (Sabouraud's dextrose agar) in the form of C-streaks. Only microbial growth on C-streaks was considered significant (Figure 1); that was identified as *Candida albicans* by using VITEK-2 compact system technique. *Commiphora molmol* (myrrh) aqueous extract at concentration of 500 µg/ml exhibit the highest inhibition zone against *Candida albicans* (30.5±1.6 mm), in comparison with the commercial fluconazole with concentration of 150 mg/ml had inhibition zone of 25.1±0.9 mm against *Candida albicans* (Figure 1). Figure (2) represents the MIC of *Commiphora molmol* aqueous extract against *Candida albicans*, that was recorded at 200 µg/ml.

a. Growth of *Candida albicans* isolated on Sabouraud's dextrose agar with C-streaks.

b. Inhibition zone of *Candida albicans* growth by MIC (200 µg/ml) of *Commiphora molmol* aqueous extract.

Fig 1: Representative plates of *Candida albicans* isolation and inhibition.

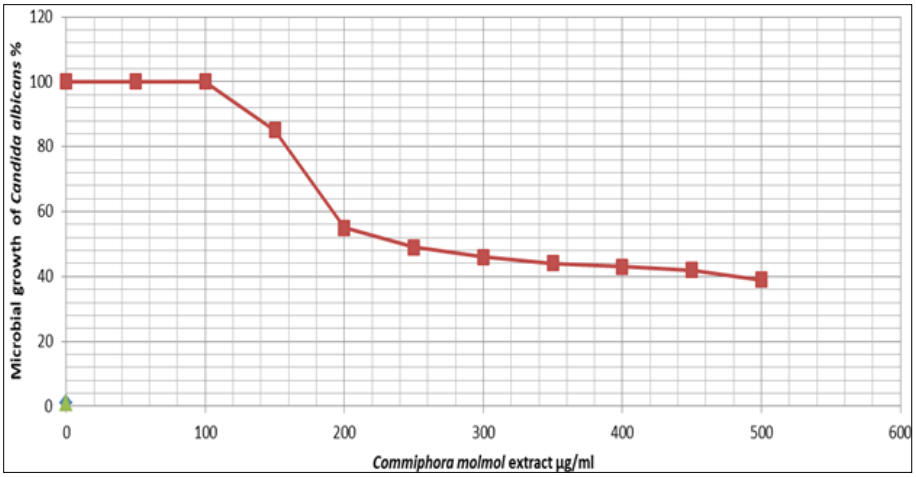


Fig 2: Investigation of MIC of *Commiphora molmol* aqueous extract against the surviving ratio of *Candida albicans*.

GC-MS parameters were developed and used to examine the phytochemical composition of myrrh extract. The resultant gas chromatogram is presented in Figure (3), indicating the retention time, peak area percentage value and Height. GC-

MS chromatogram showed many chemical components possessing wide range of activities in *Commiphora molmol* extract, like antimicrobial, analgesic and anti-inflammatory effects (Table 1).

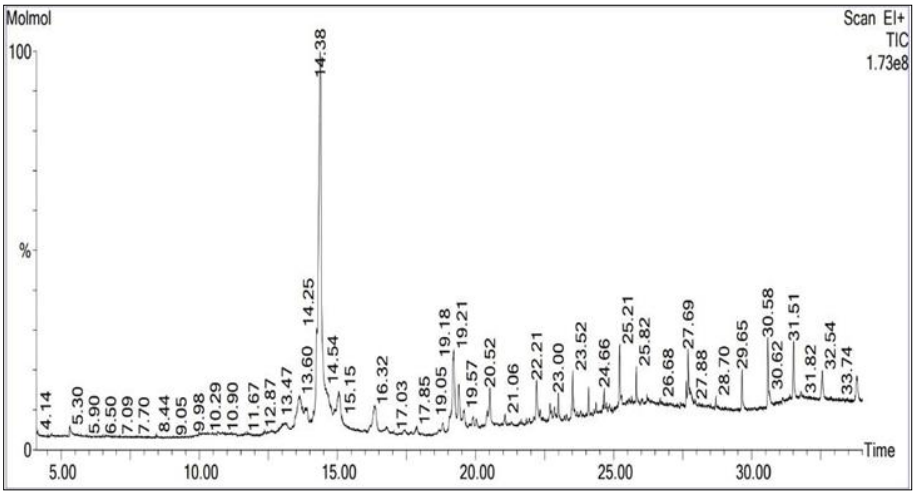
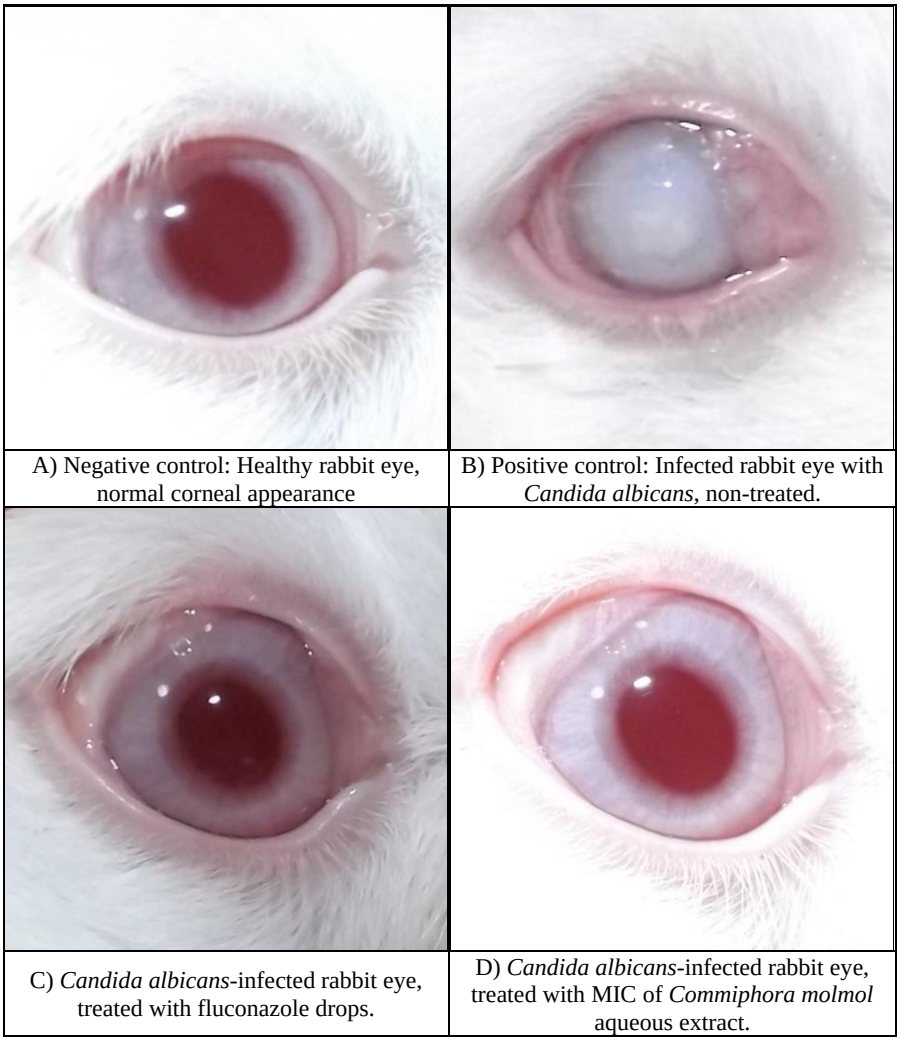


Fig 3: GC-MS chromatogram of the *Commiphora molmol* aqueous extract

Table 1: The biological activities of main compounds in *Commiphora molmol* aqueous extract:

Ingredient name	Area %	Biological activity	Reference
2-Pentanone, 4-hydroxy-4-methyl	0.412	Antibacterial, Antifungal, Antinociceptive, Analgesic	(Ranjbar *et al.*, 2022)
1,2-15,16-Diepoxyhexadecane	0.320	Antitumor, anti-inflammatory, Antifungal, Antioxidant	(Pigot *et al.*, 2022)
Undecanal	2.011	Antimicrobial, Anti-Allergic, Anti-Inflammatory	(Choi *et al.*, 2020)
Triacetin	31.392	Antifungal, Antibacterial, Ophthalmic humectants, antioxidants	(Lertsuphotvanit *et al.*, 2023)
4-terta-Butylcyclohexyl acetate	5.158	Antifungal, Antibacterial, Antiviral, Antioxidant	(Sıcak *et al.*, 2019)
Phenol, 2,4-bis (1,1-dimethylethyl)	0.419	Antimicrobial	(Devi *et al.*, 2021)
Indan-1,3-diol monopropionate	3.615	Antitumor, Antibacterial, Anti-inflammatory	(Pigot *et al.*, 2022)
Dithiocarbamate, S-methyl-, N-(2-methyl-3-oxobutyl)	0.568	Anticancer	(Choi *et al.*, 2020)
1-Heptatriacotanol	0.231	Antioxidant, anticancer, anti-inflammatory	(Hadi *et al.*, 2016)
Benzaldehyde, 3-benzyloxy-2-fluoro-4-methoxy	1.188	Antioxidant, anticancer activities	(Sıcak *et al.*, 2019)
Hexadecanoic acid, methyl ester	0.759	Antimicrobial, Neuroprotective agent	(Lee *et al.*, 2019)
cis-11-Eicosenoic acid	0.226	Antifungal, antibacterial	(Moni *et al.*, 2021)
E,E,Z-1,3,12-Nonadecatriene-5,14-diol	0.969	Antibacterial	(Hadi *et al.*, 2016)
9,12-Octadecadienoyl chloride, (Z,Z)	1.269	antihistaminic, and anticancer	(Soltan *et al.*, 2023)
Heptacosane	1.808	Anti corrosive, Antioxidant Antibacterial	(Moni *et al.*, 2021)
2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl	1.123	Anti-bacterial, nematocidal, Antioxidant activity	(Lee *et al.*, 2016)
d-Mannitol, 1-decylsulfonyl	1.100	Antimicrobial	(Mohan, 2012)

Group A of animal models was a negative control (healthy); Group B, C and D were inoculated with *Candida albicans* suspension of 5×10^5 cells/ml. After inoculation and establishment of the *Candida albicans* infection to the corneal tissues, all eyes appeared to be inflamed with redness and swelling due to proliferate of candida cells in epithelial layer of the corneas. The photographs of the cornea in the first 48 h. showed the start of infiltration stage. Group A, which was not given any treatment or vaccination, exhibited no clinical symptoms at all during the trial. The photos of the eye and cornea showed no signs of inflammation, infiltration, ulceration, or opacification during the trial because the rabbits were neither infected nor treated. Groups B, C, and D showed severe clinical indications on the third day of infection. Severe symptoms persisted in group B (untreated) at the fifth day of infection, but they were less severe in groups C (fluconazole) and D (aqueous extract of *C. molmol*). Group B continued to show significant indications of keratitis at day 12, while group C showed milder signs at the same time. In groups D (aqueous extract of *C. molmol*), the least severe clinical symptoms were seen. As seen in Figure (4), on the 24th day of infection, the clinical indications had substantially decreased (recovery progressed) to a point that was nearly identical to the control group (A).



A) Negative control: Healthy rabbit eye, normal corneal appearance

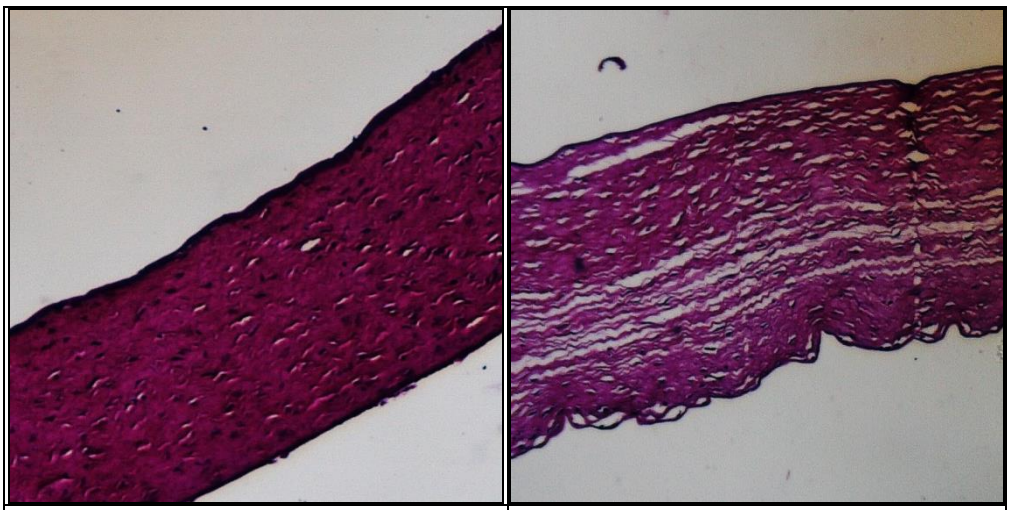
B) Positive control: Infected rabbit eye with *Candida albicans*, non-treated.

C) *Candida albicans*-infected rabbit eye, treated with fluconazole drops.

D) *Candida albicans*-infected rabbit eye, treated with MIC of *Commiphora molmol* aqueous extract.

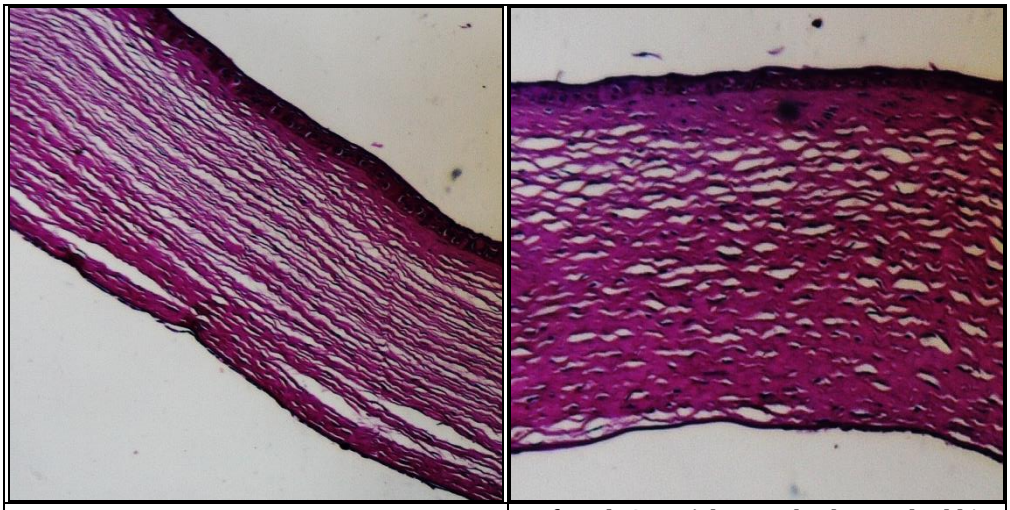
Fig 4: Appearance of rabbit eyes at the end of different treatments of Candida infections.

Histological examinations of rabbits' corneas were obtained after their treatment with the selected *Commiphora molmol* extract comparing with commercial antimicrobial ophthalmic preparations and healthy non-treated control rabbit's cornea. Healthy cornea possessed normal arrangement and size of epithelium, endothelium, stroma and normal bound collagen lamellae. *Commiphora molmol* extract showed nearly similar size of corneal layers with mild rare infiltrates within collagen lamellae, comparing non-treated cornea. On the other hand, fluconazole as a standard antifungal agent gave higher level of infiltration and loosing of collagen lamellae. This comparison encouraged the safe usage of *Commiphora molmol* extract on corneal tissues with normal layers arrangement, size and absence of any toxic precipitates, with only rare corneal infiltrates, that can be normally regenerated at the end of treatment of corneal ulcer (Figure 5).



Healthy corneal layers of non-infected, non-treated rabbit right eye.

Infected, non-treated rabbit right eye.



Infected, Fluconazole treated rabbit right eye.	Infected, *Commiphora molmol* treated rabbit right eye.

Fig 5: Histological effects of *Commiphora molmol* aqueous extract on the corneal tissues of experimental animals, infected with *Candida albicans*.

Discussion

The MIC of the aqueous extract of *Commiphora molmol* against *Candida albicans* was found to be 200 µg/ml in the current investigation. According to Alshibly *et al*. (2022) [3], *Commiphora molmol* extract exhibited antibacterial activity that resulted in a zone inhibition of 29 mm against *Escherichia coli*. This was followed by *Staphylococcus epidermidis*, *Candida albicans* and *Aspergillus brasiliensis*, measuring 27 mm and 16 mm, respectively. *Commiphora molmol* extract revealed significant amounts of sterols, isoprenoids, and tannins in phytochemical tests, having effects of on the cellular morphology of the pathogens by SEM examination.

The current study showed that the *Commiphora molmol* aqueous extract was able to inhibit the growth of *Candida albicans* isolates, and this may be due to the biological activities of compounds found in the extract, such as 2-Pentanone, 4-hydroxy-4-methyl (Al-Daghari *et al.*, 2020) [1], Undecanal (Santhi *et al*,. 2013) [33], Triacetin (Lertsuphotvanit *et al.*, 2023) [20], 4-tert-Butylcyclohexyl acetate (Sıcak and Erdoganeliuz, 2019) [35], Phenol, 2,4-bis (1,1-dimethylethyl) (Devi *et al.*, 2021) [8], Indan-1,3-diol monopropionate (Pigot *et al.*, 2022) [28], Hexadecanoic acid, methyl ester (Lee *et al.*, 2019) [19], cis-11-Eicosenoic acid (Moni *et al.*, 2021) [27], E,E,Z-1,3,12-Nonadecatriene-5,14-diol (Hadi *et al.*, 2016) [17], Heptacosane (Dandekar *et al.*, 2015) [7], 2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl (Lee *et al.*, 2016) [18] and d-Mannitol, 1-decylsulfonyl (Mohan, 2012) [26].

Our results showed that the healthy cornea possessed normal arrangement and size of epithelium, endothelium, stroma and normal bound collagen lamellae. *Commiphora molmol* extract showed nearly similar size of corneal layers with mild rare infiltrates within collagen lamellae, comparing with non-treated cornea. On the other hand, fluconazole as a standard antifungal agent gave higher level of infiltration and loosing of collagen lamellae. This comparison encouraged the safe usage of *Commiphora molmol* extract on corneal tissues with normal layers arrangement, size and absence of any toxic precipitates, with only rare corneal infiltrates; that can be referred to the anti-inflammatory, analgesic and antihistaminic properties of the GC-MS-detected *Commiphora molmol* extract components, that were confirmed by other studies about their biological sctivities, such as 2-Pentanone, 4-hydroxy-4-methyl (Ranjbar *et al.*, 2022) [31], 1,2-15,16-Diepoxyhexadecane (Ganesh and Mohankumar, 2017) [13], Undecanal (Choi *et al.*, 2020) [6], Indan-1,3-diol monopropionate (Pigot *et al.*, 2022) [28], 1-Heptatriacotanol (Hadi *et al.*, 2016) [17] and 9,12-Octadecadienoyl chloride, (Z,Z) (Soltan *et al.*, 2023) [36]. Additionally, Hexadecanoic acid methyl ester was founded to play role as neuroprotective agent (Lee *et al.*, 2019) [19], also Triacetin was used in ophthalmic formulations and act as humectants and antioxidants (Gawin-Mikolajewicz *et al.*, 2021) [14].

In the current study, the rabbit eyes treated with 200 µg/ml of *Commiphora molmol* extract had the best cure rate, followed by 150 mg/ml of fluconazole. The most common indications of infection were blepharitis, conjunctivitis, iritis, and corneal edema, which are symptoms of *Candida albicans* keratitis; and after treatment with *Commiphora molmol* extract, those signs were gradually cured faster than using fluconazole. The anti-inflammatory, anti-histaminic, analgesic, neuroprotection and antimicrobial properties of *Commiphora molmol* extract may be the cause to give minimal side effects on cornea during the period of treatment better than the commercial antifungal agent fluconazole against *Candida albicans* corneal ulcers in albino rabbits. This was in agreement with previous *in vivo* experiments on animal models to test the safety of *Commiphora molmol* by Dolara *et al.* (2000) [9], who investigated the effects of *Commiphora molmol* at 280 µg/ml in the conjunctival sac of male New Zealand albino rabbits, provided as eye drops (2% DMSO + phosphate buffer). The control was in the car. At 100 µg/ml, the impact was approximately half that of procaine. Germano *et al.* (2017) [15] investigated the analgesic properties of a myrrh (*Commiphora molmol*) extract in 89 Italian men and 95 Italian women in a clinical pilot research, experiencing no negative effects.

The clinical efficacy and safety of a myrrh-based oral mucoadhesive gels (0.5% w/w) has been studied in 30 patients in Saudi Arabia in the management of minor recurrent aphthous stomatitis (Mansour *et al.*, 2014) [22]. At days 4 and 6 of study entrance, changes in ulcer size, pain intensity, erythema, pain and exudation were reduced, with no side effects were reported.

No serious toxicities have been reported with myrrh. Myrrh is approved by the Food and Drug Administration for use in food and was given generally recognized as safe (GRAS) status as a flavoring agent (Massoud *et al.,* 2001) [25].

Conclusion

In vivo Histopathological investigation revealed that using aqueous *Commiphora molmol* extract had minimal side effects and considerable anti-microbial activity at MIC of 200 µg/ml against corneal ulcers infected with *Candida albicans* when compared with fluconazole.

Conflict of Interset

There is no conflict of interest.

Authors' Contributions

This work was carried out in collaboration among all authors. Authors EHA, MSE, MAA and ASE designed the research plan and wrote the manuscript. Authors MAA and ASE performed the experimental work. All authors read and approved the final manuscript.

Disclaimer (Artificial Intelligence)

Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during writing or editing of manuscripts **.**

Consent

It is not applicable**.**

Ethical Approval

This study was approved by the Research Ethics Committee of Tanta University with an approval code of (IACUC-SCI-TU-0101)**.**

Competing Interests

Authors have declared that no competing interests exist**.**

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