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Comparative Pathological Studies on the Healing Effect of Natural (*Terfezia claveryi*) and Synthetic (Vigamox) Antimicrobials on Corneal Ulcers in Rabbits

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ABSTRACT

This study aimed to evaluate the role of *Terfezia claveryi* crude extract in the healing of induced corneal ulcers in rabbits in comparison with Vigamox through histopathological examinations. Corneal ulcer were induced in rabbit eyes through stromal injection with *Staphylococcus aureus*. The healing response due to topical application of *Terfezia claveryi* (1.5, 3 & 5%) extract was compared with using synthetic antibiotic (Vigamox) by clinical findings and histopathological investigation. The topical application of *Terfezia claveryi* in test group were reduced the corneal ulcer within 9 days. 1.5% of terfezia significantly reduced the corneal ulcer while the 3% delayed the healing process but 5% of terfezia were toxic and complicated the corneal ulcer. Application vigamox 0.5% improved the signs of corneal ulcer and healed within 3-5 days.

Histopathologically, corneal ulcer of rabbit treated topically with 1.5% and 3% *Terfezia claveryi* showed re-epithelialisation within 1 and 2 week; respectively. Corneal ulcer treated with 5% *Terfezia claveryi* showed improper epitheliazation with defective angiosis and loss of both keratocytes and stromal substance. Corneal ulcer treated with 0.5% Vigamox showed re-epithelialisation within 3-5 days with slight inflammatory infiltrate and marked vascularisation.

It could be concluded that, aqueous extract of *Terfezia claveryi* exhibited antibacterial activity and healed the corneal ulcer in rabbit eyes. Although vigamox is more promising, a serious side effects is expected due to development of antimicrobial resistance.

KEY WORDS*:* Pathological- Terfezia claveryi- Vigamox- corneal ulcers.

INTRODUCTION

Unique location of cornea, at the outermost surface of the eye, makes it vulnerable to damages from ultraviolet light exposure, by physical wounding, and bacterial or fungal infections. So corneal epithelium wound healing is an important process for maintaining the homeostasis of the cornea.

Corneal infection is one of the most common ocular disease in both humans and animals and can lead to blindness or loss of the eye [1,2]. There are various pathogenic organisms, like *Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus pyogenes, Streptococcus, Staphylococcus epidermis,* reported to cause corneal infection ([3, 4, 5].Corneal ulceration is often observed after ocular exposure to infectious agents such as *Staphylococcus aureus*[6].The condition is characterized by dissolution of the extracellular matrix (ECM) components of the corneal stroma, often leading to extensive corneal scarring and perforation.

Desert Truffles, a term used to refer to members of the genera Terfezia and Tirmania, are considered to be one of the oldest food-stuffs known for their nutritional value and medicinal properties for a variety of ailments [7, 8]. Desert truffles are seasonal, socio-economically and medicinally important fungi. The truffles usually appear in the deserts following the rainy season between February and April[9].Most desert truffles are a rich source of protein, amino acids, fatty acids, minerals and carbohydrates[10]. *Terfezia claveryi* aqueous extract was also proved as an antimicrobial activity in vitro [11].

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Many antibacterial preparations are used to treat eye infections such as, chloramphinecol, fluoroquinolone, neomycin aminoglycosides [12]. Moxifloxacin hydrochloride ophthalmic solution 0.5% is the ocular formulation/adaptation of moxifloxacin, an 8-methoxy fluoroquinolone, broad spectrum, anti-infective. It was introduced in 2003 as Vigamox® (Alcon Laboratories, Inc, Fort Worth, TX,USA) for the treatment of susceptible microorganisms recovered from patients with bacterial conjunctivitis. It is used more frequently off label for treatment of keratitis and as a prophylaxis agent in cataract and refractive surgeries [13, 14, 15].Moreover, the increasing resistance of many bacteria and the side effects to the currently used antibiotics are documented[16, 17].

The present study aimed to investigate the therapeutic effects of *Terfezia claveryi* crude extract on the corneal ulcers in comparison with Vigamox as a synthetic antibiotic through clinical and histopathological examinations.

MATERIALS AND METHODS

Animals.

This experimental study was conducted on 36 rabbits. The rabbits were obtained from Lab animals at Qassim University of same size and age. The rabbits were acclimatized for 7 days in the animal house conditions before starting the experiment. The experimental animals were housed in air conditioned rooms at 21-23^{o}C and 60-65% relative humidity and kept on 12 hour light/dark cycle. The animals were healthy and weighing between 2 and 2.5kg. They were housed in standard aluminum cages and fed with standard rabbit diet and normal tap water and examined to be free from infection. They were handled as per the international rules implemented in the experimental laboratory animals at Qassim University.

Bacteria

Staphylococcus aureus ocular strain, isolated from a human corneal samples, was used to induce experimental keratitis. The bacteria were propagated on Mueller-Hinton agar plates and incubated at 37°C for 18 hours. Several bacterial colonies were pooled and suspended in saline to adjust the turbidity to 0.5 McFarland units(equivalent to 5 x 10^8 colony-forming units [CFU]/mL). The suspension was then adjusted to a final concentration of 10^5 CFU/mL, as verified by a quantitative bacterial count on Mueller-Hinton agar plates.

Preparation of crude truffle Terfezia Claveryi extract.

Terfezia claveryi (Kamma) was purchased from the market of Riyadh city, KSA. 75 gram of peeled *Terfezia claveryi* was homogenized with 100 ml pre-cooled 50mM sodium phosphate buffer pH 7.0 in a tissue homogenizer for 3min. The extract was passed through four layers of cheese cloth to remove the major debris. The filtrate was centrifuged at 10,000 rpm for 15 min at 4^{o}C. The supernatant was considered as crude extract of *Terfezia claveryi* and was further diluted when used.

Induction of experimental *Staphylococcus aureus* ulcer.

Rabbits were anesthetized by subcutaneous injection of a mixture of xylazine(100 mg/mL; Butler Company, Columbus, OH) and ketamine hydrochloride (100 mg/mL; Fort Dodge Animal Health, Fort Dodge, IA). Proparacaine hydrochloride was topically applied to each eye with a 30-gauge needle and a 1-mL tuberculin syringe under a stereoscopic surgical microscope (Wild M690; Wild Heerbrugg, Gais, Switzerland),we injected 0.05 mL freshly prepared *Staphylococcus aureus*(10^5 CFU/mL) into the stroma at the center of the right cornea and the eye were examined daily for the development of corneal ulcer using hand held portable Slit- Lamp biomicroscope after staining with paper strip flourescein dye.

Treatment groups.

Thirty six Rabbits were divided randomly into six equal groups. Group (1) was negative control injected into the stroma with saline , Gp (2) infected by *Staphylococcus aureus* , Gps 3 – 6 rabbits with corneal ulcer due to infection by *Staphylococcus aureus* where Gp 3 treated with *Terfezia claveryi* 1.5%, Gp (4) treated with *Terfezia claveryi* 3%, Gp 5 treated with *Terfezia claveryi* 5% and Gp 6 treated with Vigamox 0.5%. The recommended dosing frequency for the treatment of corneal ulcer is one drop 3 times a day for 5 days with topical application of supernatant crude extract of *Terfizia claveryi* in 3 different concentrations (1.5%, 3% and 5%) and synthetic antibiotic vigamox 0.5% (moxifloxacin hydrochloride)[13, 14, 18, 19].

Clinical Observations

External examinations of each eye were done once daily during the entire study. Detailed, double masked, slit-lamp examinations of each rabbit were performed every other day initially for the first 10 days and then every day following the onset of corneal ulceration. Corneas were examined for the presence of corneal defects, ulceration, perforation, vascularisation or infection.

Histopathological techniques:

Whole eye were removed and tissue sections from the cornea of experimental rabbits were taken and immediately fixed in 10% neutral buffered formalin, then dehydrated in increasing concentrations of ethyl alcohol, cleared in xylene, blocked in paraffin and sectioned as 5 µm using rotary microtome. The obtained tissue slides were stained with hematoxylin and eosin (H&E) [20].

RESULTS

The topical application of 1.5% of *Terfezia claveryi* were significantly reduced the corneal ulcer within 9 days and ultimately healed as nebular type of central cornea opacity (Fig. 1) but the group treated with 3% concentration of *Terfezia claveryi* induced healing within 12-14 days. While 5% of *Terfezia claveryi*, were toxic where the eyes became dry, developed hypopyon and ultimately perforate. In comparison, topical application of synthetic antibiotic moxifloxacin hydrochloride (vigamox, 0.5%) dramatically improved the signs of corneal ulcer with marked healing within 3-5 days and left a transparent cornea (Fig. 2).

Fig 1: Rabbit eye, with corneal ulcer after treatment with 1.5% Terfezia, showing nebula type corneal opacity.

Fig. 2: Rabbit eye, with corneal ulcer after treatment with vigamox, 0.5% showing almost transparent Cornea.

Histopathology:

Gp (1), rabbits serve as a negative control where rabbit's eyes showed no histopathological changes (Fig. 3,).

Gp (2), rabbits with corneal ulcer induced in rabbit's eyes by *Staphylococcus aureus,* marked vacuolar and ballooning degeneration were evident in the corneal epithelium. The subepithelial tissue and stroma exhibited severe edema, congestion and pleomorphnueclear leukocytes (Fig. 4*)*. Some cases showed superficial ulceration with edema and mononuclear leukocytes in the subepithelial tissue (Fig. 5) . Descemet's membrane showed edema and diffuse infiltration of polymorphonuclear (PMN) leucocytes. Loss of keratocyte and central corneal vessels were seen along the stroma and PMN leukocytic infiltrations were extended to the limbus at the iridocorneal angle.

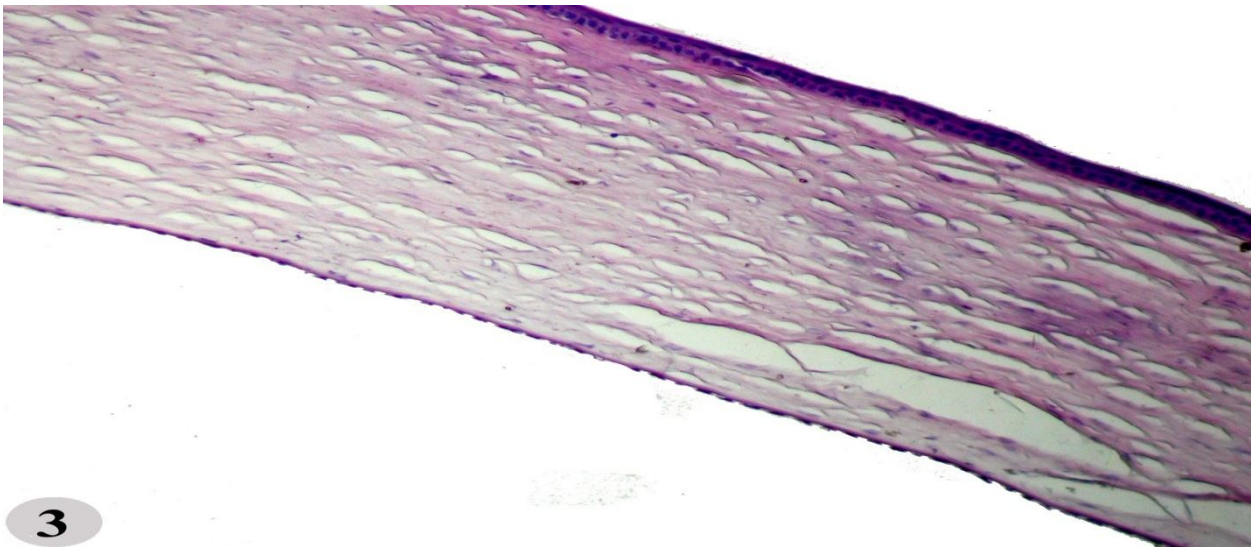

Fig. 3: Cornea, of control group, showing the 5 layers of cornea epithelium, basement membrane, stroma, Descement's membrane and endothelium. H & E stain, x 40

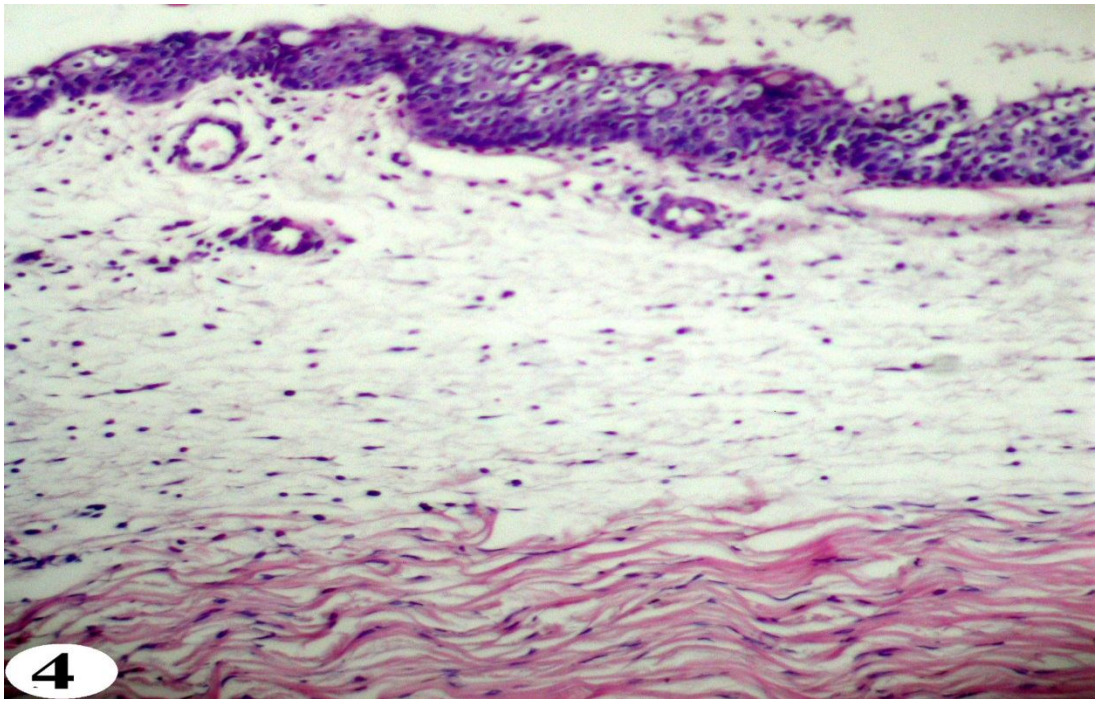

Fig. 4: Rabbit eye infected with *Staphylococcus aureus, showing* marked vacuolar and ballooning degeneration in the corneal epithelium with stromal edema, congestion and pleomorphnueclear leukocytes. H & E stain, x100.

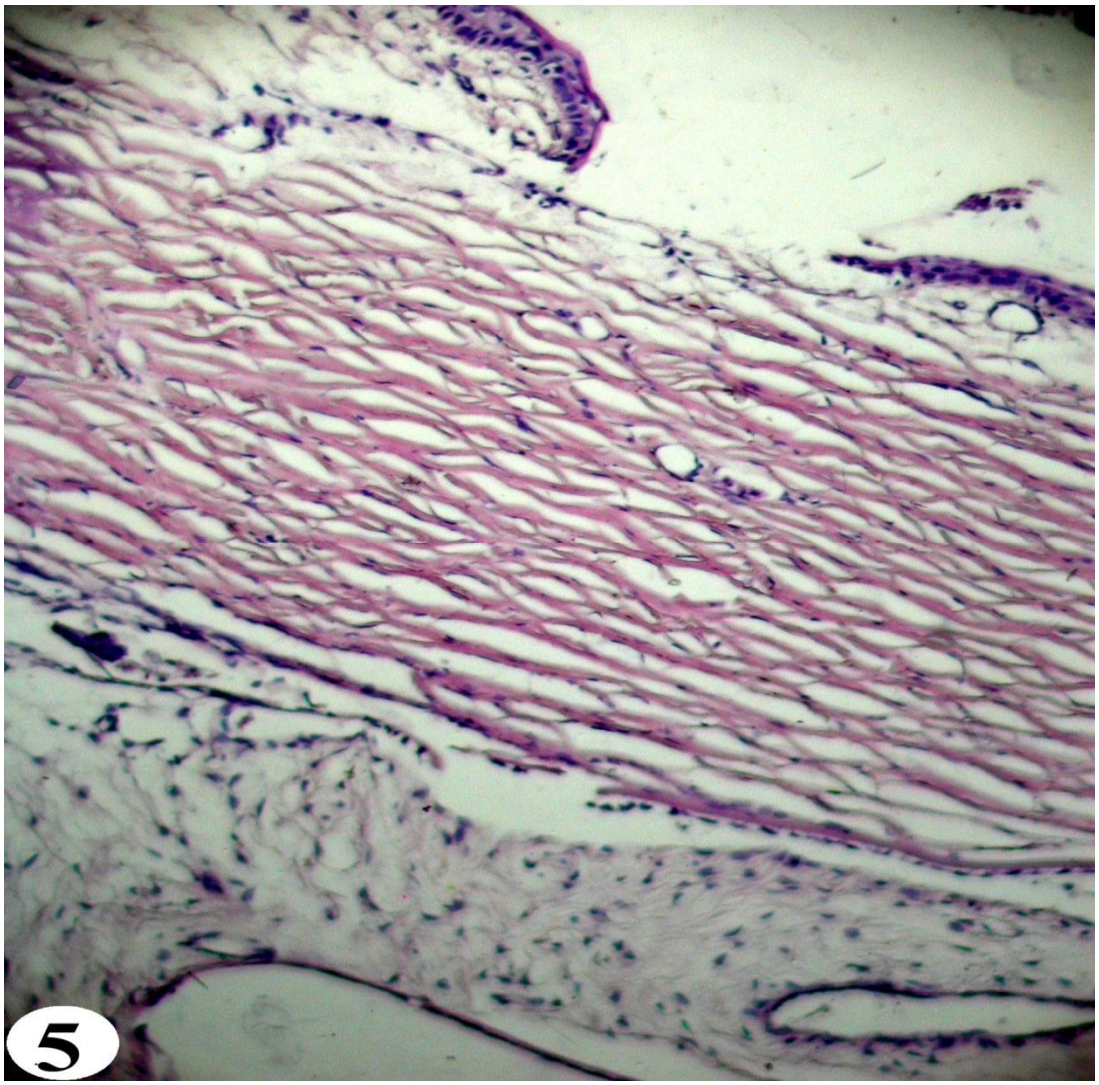

Fig. 5: Rabbit eye, infected with *Staphylococcus aureus*, showing ulceration with edema and mononuclear leukocytes in stroma. H & E stain, x 100.

Gp 3 rabbits with corneal ulcer due to infection by *Staphylococcus aureus* and treated with 1.5% *Terfezia claveryi*. The treated corneas showed re-epithelialisation within 1 week. There were, relatively few inflammatory cells present in-treated corneas, and predominately located in the anterior half of the stroma (panel D) (Fig. 6). No inflammatory cells were observed along Descement's membrane. Cells that had the appearance of fibroblasts (keratocyte) were observed throughout the stroma. The stromal matrix appeared intact and retained the lamellar organization of collagen fibers that is characteristic of normal corneas. Markedly smaller and less congested blood vessels were seen.

Gp (4), rabbits with corneal ulcer due to infection by *Staphylococcus aureus* and treated with 3% *Terfezia claveryi*. Corneal re-epithelialisation was occurred within 2 week. The treated corneas revealed a moderate inflammatory cell infiltrate. The vascularisation was present throughout the anterior stroma. The Polymorphonuclear leukocytes were restricted to the anterior part of the stroma, at the edge of the ulcer, and in a band above Descement's membrane (Fig. 7).Complete epithelial regeneration and proliferation of stromal keratocytes with marked angiosis. The stroma showed a few loss of normal contour but essentially no loss of stromal substance (Figs. 8 & 9).

Gp(5), rabbits with corneal ulcer due to infection by *Staphylococcus aureus* and treated with 5% *Terfezia claveryi*. Marked epithelization with persistent epithelial defects were present. Focal detachment of epithelial sheet was also noticed (Fig. 10). The anterior stroma and mid-stroma surrounding the ulcerations and perforations, were

infiltrated with inflammatory cells. Severe edema in the stroma with defective angiosis and loss of both keratocytes and stromal substance were noticed (Fig. 11).

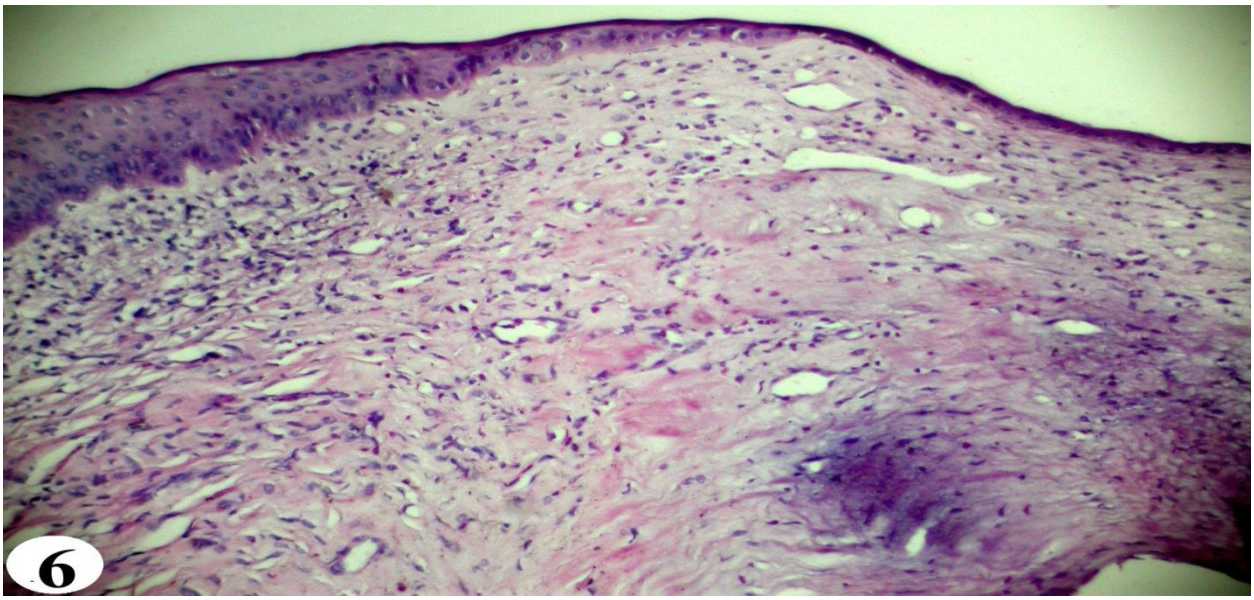

Fig. 6: Rabbits eye, with corneal ulcer due to infection by *Staphylococcus aureus* and treated with 1.5% *Terfezia claveryi,* showing re-epithelialisation with relatively few inflammatory cells in the anterior half of the stroma. H & E stain, x 100.

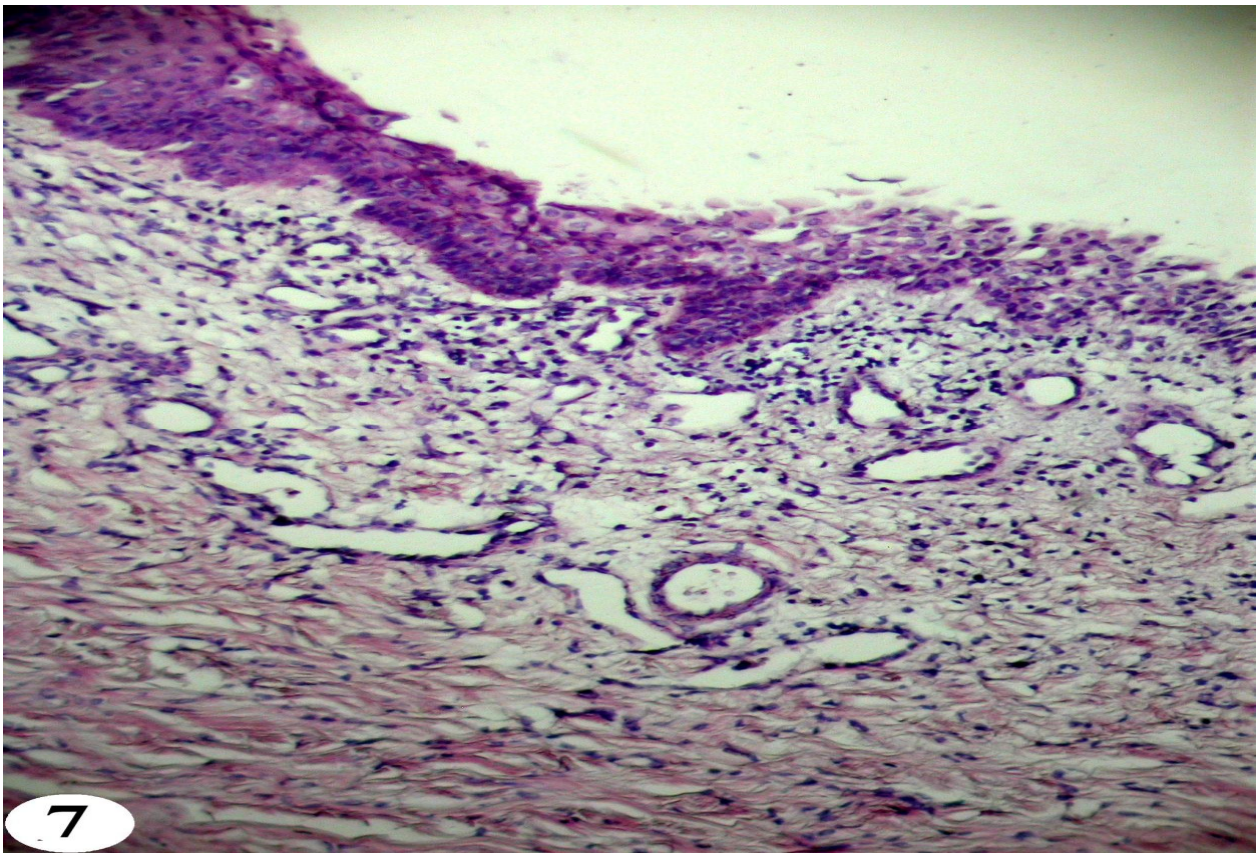

Fig. 7: Rabbits eye, with corneal ulcer due to infection by *Staphylococcus aureus* and treated with 3% *Terfezia claveryi, showing* re-epithelialisation with vascularization and a moderate inflammatory cell restricted to the anterior part of the stroma and at the edge of the ulcer. . H & E stain, x 100.

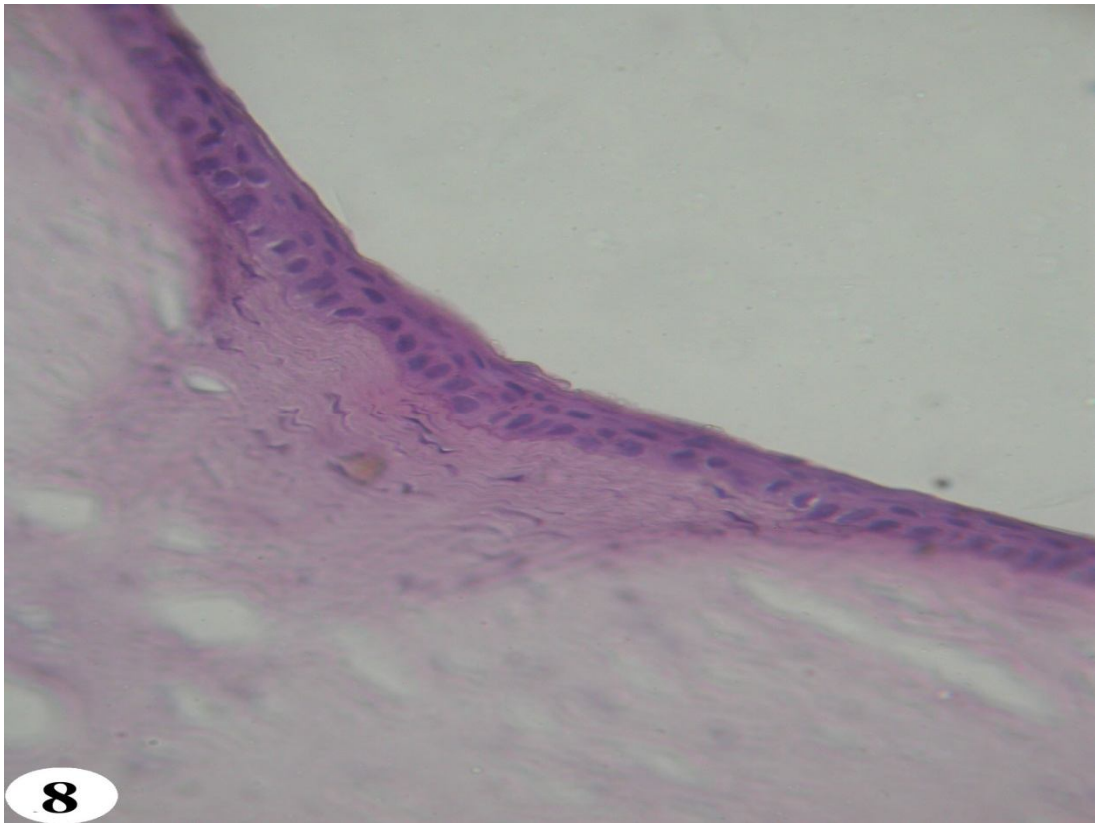

Fig. 8: Rabbits eye, with corneal ulcer due to infection by *Staphylococcus aureus* and treated with 3% *TerfeziaClaveryi*, *showing* complete epithelial regeneration and proliferation of stromal keratocytes with marked angiosis. . H & E stain, x 100.

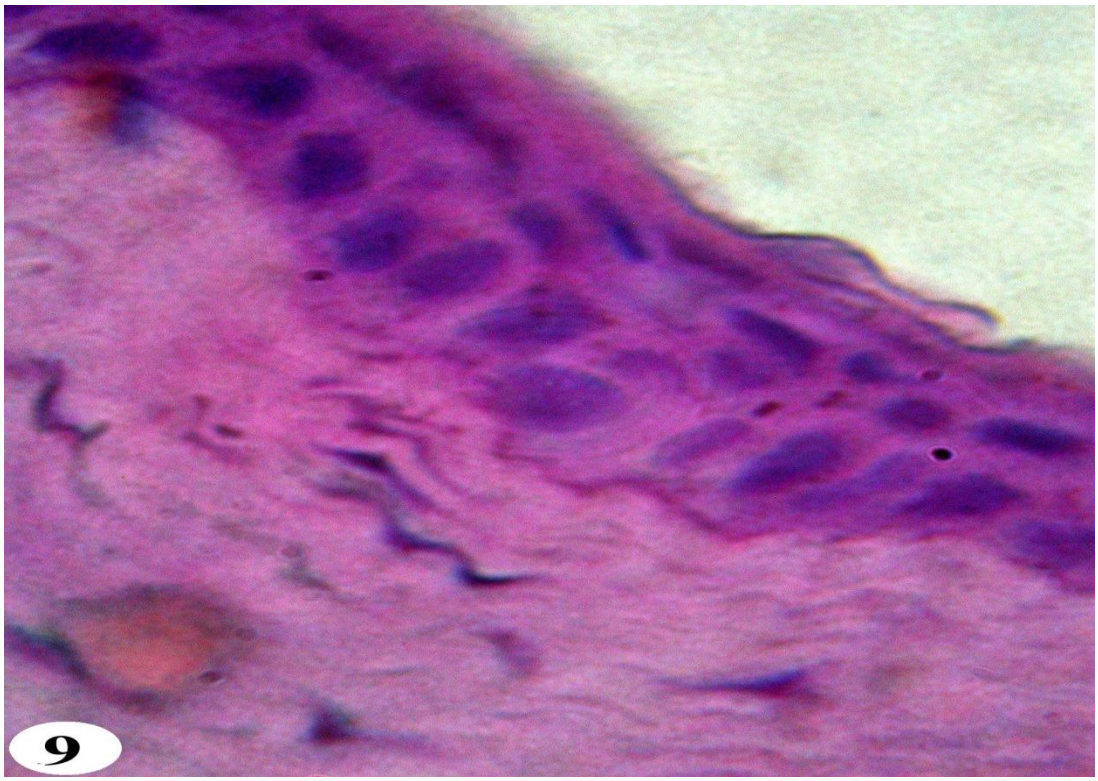

Fig. 9: Higher magnification of Fig. 8. . H & E stain, x 1000.

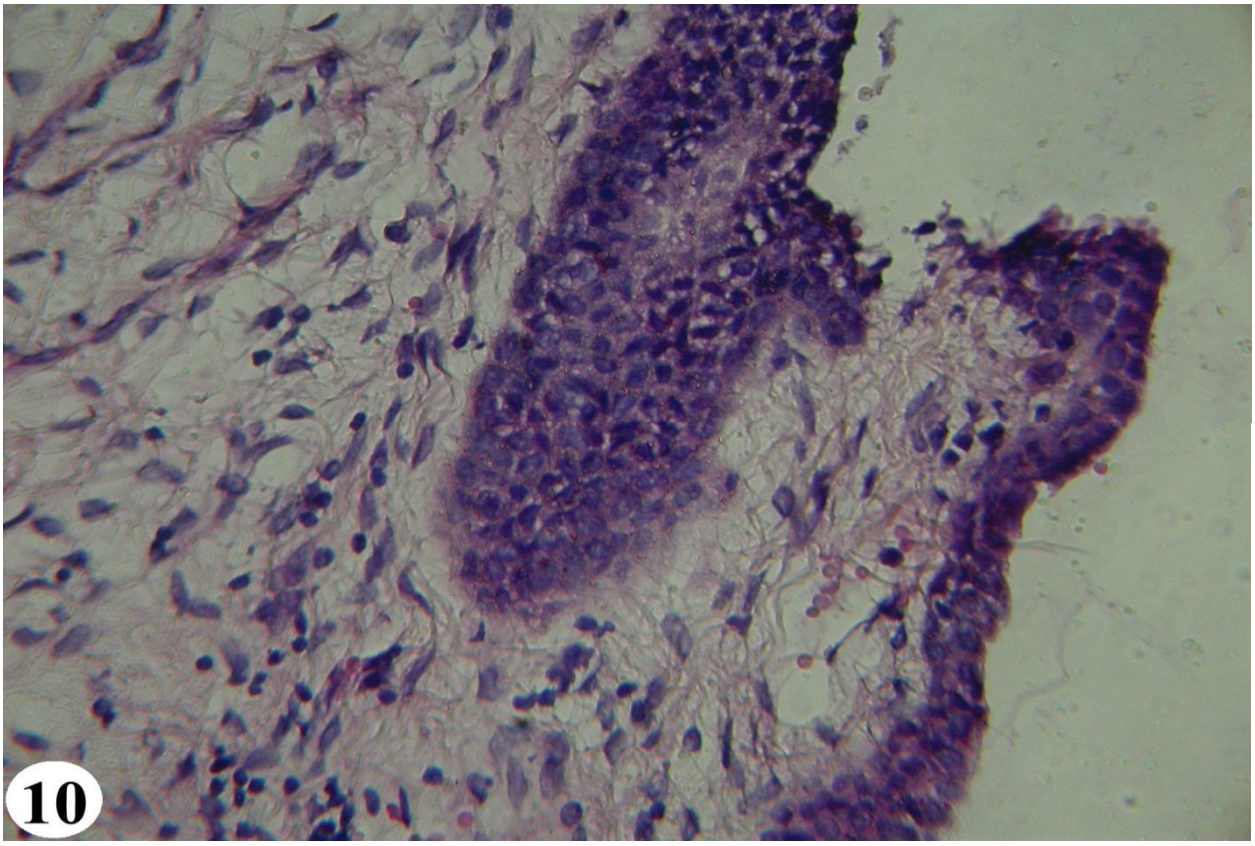

Fig. 10: Rabbits eye, with corneal ulcer due to infection by *Staphylococcus aureus* and treated with 5% *TerfeziaClaveryi*, showing marked defective epithelization with stromal edema. . H & E stain, x 250.

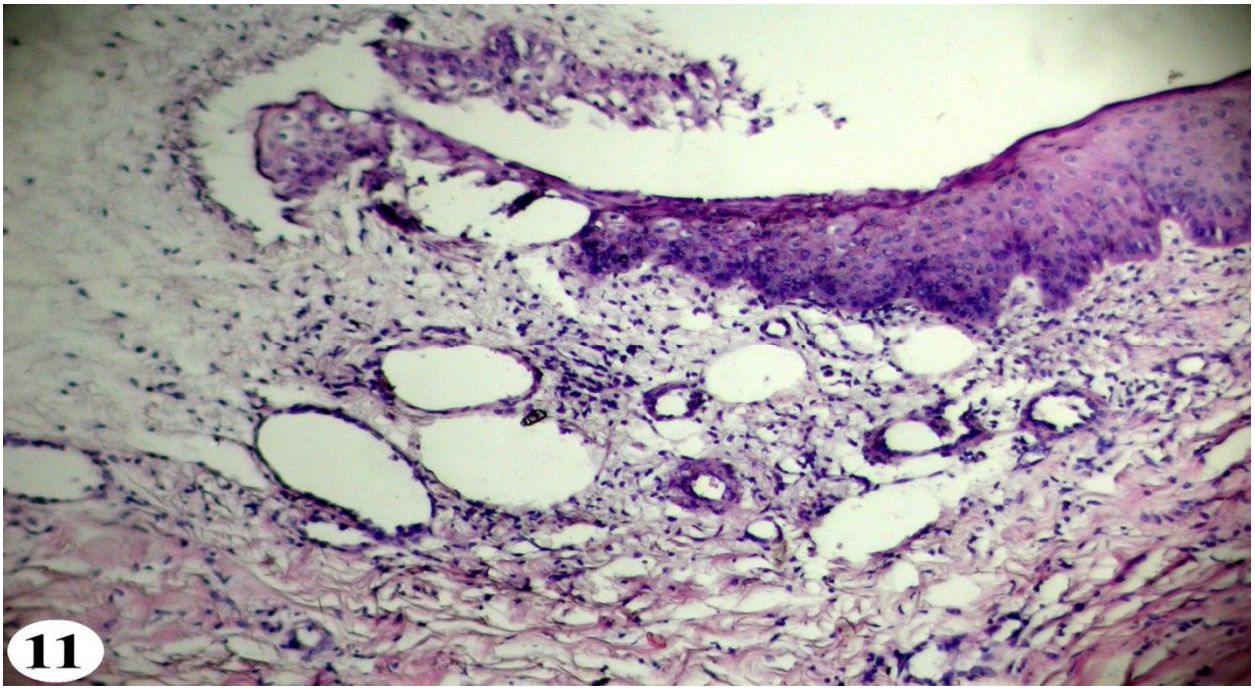

Fig. 11: rabbits with corneal ulcer due to infection by *Staphylococcus aureus* and treated with 5% *Terfezia Claveryi* showing marked defective epithelization with edema and pleomorphnuclear cells in the stroma along with loss of normal stromal contour and defective angiosis. . H & E stain, x 250.

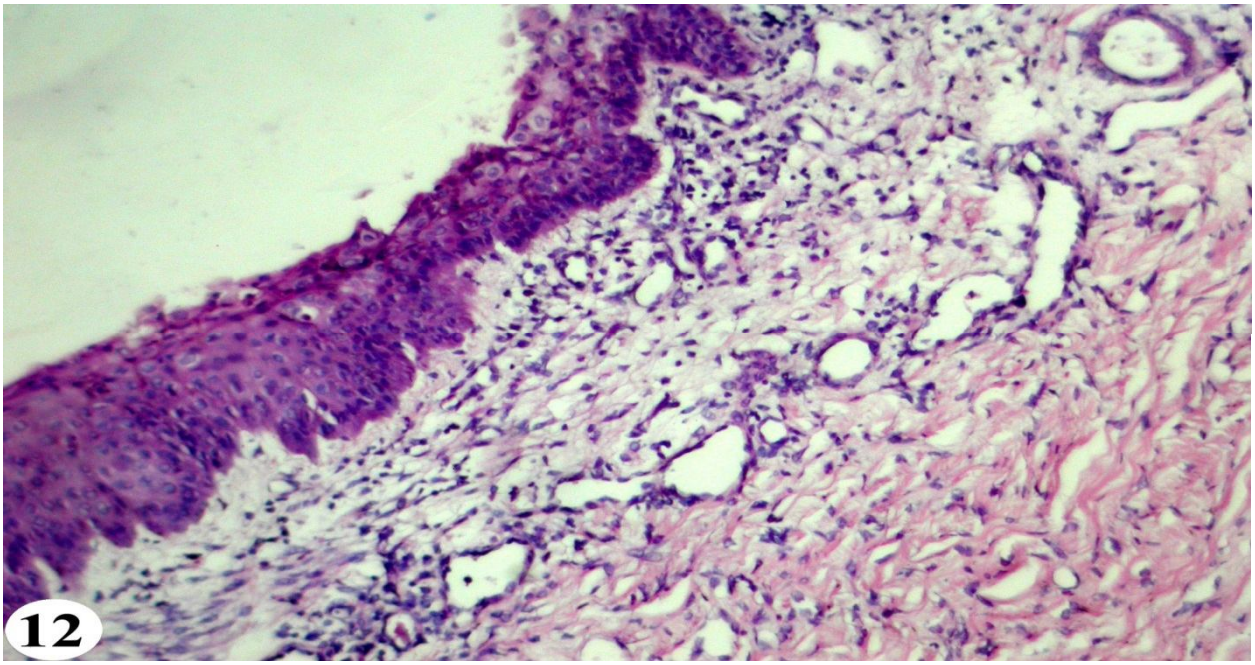

Fig. 12: Rabbits eye, with corneal ulcer due to infection by *Staphylococcus aureus* and treated with 0.5% Vigamox, showing corneal re-epithelialisation with less corneal inflammatory infiltrate, edema and vascularization. .H & E stain, x 250.

Gp(6), rabbits with corneal ulcer due to infection by *Staphylococcus aureus* and treated with 0.5% Vigamox. Corneal re-epithelialisation was occurred within 3-5 days with Slightly less corneal inflammatory infiltrate and edema in the mid-peripheral and in the peripheral cornea. The vascularisation present along the stroma(Fig. 12).

DISCUSSION

Keratitis by *Staphylococcus aureus* is one of the commonest forms of bacterial keratitis[21]. An important feature of *S. aureus* organisms is that in addition to fighting off the host's defence mechanisms and destroying healthy corneal tissue, they produce extracellular proteins, including enzymes that facilitate their multiplication and dispersion in the corneal tissue[22]. These proteins include hyaluronidase, which degrades extracellular ground substance, fibronectin, which facilitates migration; proteases, collagenases, and nucleases, which enhance bacterial pathogenicity; catalase, which reduces oxidative killing by neutrophils; leukocidin, which damages leukocyte membranes, causing the death of these cells; and coagulase, which helps prevent phagocytosis by macrophages and polymorphonuclear (PMN) leukocytes. Once bacteria have invaded the corneal stroma, an inflammatory response is initiated by the PMN leukocytes, which phagocytise the bacteria and destroy them by secreting proteolytic enzymes. However, these enzymes also produce toxic metabolites that may contribute to progressive destruction of the cornea [23].

Our histopathological finding showed superficial ulceration, the anterior and deep layer of stroma were diffusely infiltrated with polymorphonuclear (PMN) leucocytes. Stromal edema and loss of architecture of the stroma and affinity for staining. Complete loss of keratocyte with Central corneal vessels seen similar resulted recorded by [6],who recorded that *S. aureus* or purified alpha or beta toxins induced greater corneal damage including epithelial erosions, iritis, scleral inflammation and stromal edema, this in accordance with[24].

In search for new therapeutic alternatives, and most importantly for modern medicine, truffles have an unlimited source of therapeutic compounds as anti-inflammatory, immune-suppressor, anti-mutagenic, anti-carcinogenic [25] and anti-microbial [26] properties.

Terfezia claveryi Chatin is a hypogeous mycorrhizal fungus belonging to the so-called "desert truffles" with a good record as an edible fungus, meaning that it is of considerable economic importance. The fungus establishes an ectendomycorrhizal symbiosis [27], with annual and perennial species of the genera *Cistus* and *Helianthemum* [28]. Recently, biotechnological methods, accompanied by improved plantation management, have been developed to cultivate this species [29]. *Terfezia claveryi* truffle extract is used as a nourishing and invigorating preparation for convalescents in Mediterranean countries [30].

The aqueous extracts of *Terfezia claveryi* was found to possess a very powerful antibacterial activity against both *S. aureus* and *P. aeruginosa* using agar well diffusion. Using 4% of the aqueous extracts of *T. claveryi* in the growth medium of *S. aureus* caused a significant inhibition of *S. aureus* growth by 86.48% and was found to cause a significant inhibition of the growth of *P. aeruginosa* by 71.11%.Therefore, *T. claveryi* can be considered a source of natural therapeutic agents that can be used to treat eye infections caused by resistant bacteria such as *S. aureus*[11]. In our results the supernatant crude extract in dilution of (1.5% and 3%) of *Terfezia claveryi* showed its antimicrobial activity in induced corneal ulcer in rabbits eyes. The corneal ulcer was gradually healed within 9-14 days and left behind central corneal opacity. The topical application of 5% of *Terfezia claveryi*, were toxic, the eyes became dry, developed hypopyon and ultimately perforated., Aqueous extract contains a potent antimicrobial agent that is protein in nature and may be used in the treatment of eye infections caused by *P. aeruginosa.*A promising antibiotic and antimicrobial activity of terfezia in-vitro reported by [26, 31], reported that, 5% aqueous extract of terfezia inhibited the growth of Staphylococcus aureous by 66.4%.

On the other hand, topical application of synthetic antibiotic moxifloxacin hydrochloride (vigamox) 0.5%, dramatically improved the signs of corneal ulcer and healed with almost left a transparent cornea within 3-5 days.These attributed to Moxifloxacin is a bactericidal, concentration dependent, anti-infective. It interferes with bacterial survival by binding to DNA gyrase (topoisomerase II) and topoisomerase IV, essential bacterial enzymes involved in the replication, translation, repair and recombination of deoxyribonucleic acid. DNA gyrase is encoded by the genes gyr A and gyr B, while topoisomerase IV is encoded by Par C (grl A) and pare (grl B). Inhibition of either enzyme leads to bacteria death [32, 33, 34, 35].Takei and colleagues using MIC ratios; classified moxifloxacin as a class three quinolone exhibiting dual activity against the two enzymes in *Staphylococcus aureus* [36].The safety and efficacy of moxifloxacin for the treatment of bacterial conjunctivitis, clinical cures were documented in 66%–69% of patients by day 4. Microbiological eradication occurred in 84%–94% of the patients. Patients were dosed 3 times a day for 4 days. No adverse events were reported in thisgroup [14].

Both of 1.5% and 3% of *Terfezia claveryi* and antibiotic treatment initiates a process of repair that, in combination with the host's inflammatory response, usually succeeds in arresting the infectious process. The cornea reacts to damage by releasing numerous substances, including cytokines, growth factors, proteases and neuropeptides, in order to restore anatomical integrity (re-epithelialisation, stromal repair, and re-innervation). In the absence of complications, re-epithelialisation occurs in a few days, while complete functional and morphological maturation of the epithelium takes longer. The keratocytes activated in this way undergo myofibroblastic transformation and produce collagen fibres and substances essential for the process of restoring full integrity to the stroma [37]. These were confirmed by our clinical and histopathological finding. Any change to the process of re-epithelialisation, whether this is a delay or a defect in epithelialisation, has a negative effect on the repair of the underlying stroma, thus significantly increasing the risk of corneal scarring and, therefore, of corneal opacity. New vascularisation and disorderly collagen resynthesis restore corneal integrity, albeit at the expense of degradation in optical clarity and in the refractive properties of the cornea. Fibrosis and angiogenesis are common causes of reduction in visual acuity. These phenomena was confirmed by our histopathological findings in group5 .

Although using antibiotics in treating corneal ulcers is more promising, a serious side effects is expected with the possibility of increasing resistance of many microorganisms to the currently used antibiotics, together with the high cost of production of synthetic compounds [38, 39].The concentration of *Terfezia claveryi* extract might have equal or less effective than topical fortified. It was attempted for the first time to conduct in-vivo studies on therapeutic effect of *Terfezia claveryi* extract as an alternative choice for antibiotics to be used for treatment of eye infection.

CONCLUSION

Aqueous extract of Terfezia claveryi contained peptide constituents, exhibited antibacterial activity and healed the corneal ulcer, induced in rabbit's eyes at given concentration of 1.5% to 3%. The extract of Terfezia may be work through either an effect on re-epithelialization of corneal epithelium during wound healing or via pathway of antioxidant, antiradical and antimicrobial activities. However, further research to understand the therapeutic role of *Terfezia claveryi* needs to be worked out.

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