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Full Length Research Paper

Novel antibacterial activity of *Terfizia claveryi* aqueous extract against clinical isolates of corneal ulcer

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***Terfizia claveryi* was examined for *in vitro* antibacterial activity using the disc diffusion, well diffusion method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). *T. claveryi* exhibited excellent antibacterial activity against all clinical isolates of corneal ulcer tested, especially against *Pseudomonas aeruginosa* which showed the maximum antibacterial activity with mean zone of inhibition 20.33 mm at concentration of 100 mg/ml. The MIC for *Staphylococcus aureus* ranged from 0.040-1.250 mg/ml and MBC for *Escherichia coli* was 75 µl/ml. In the present study, the MIC value of the active aqueous extract were lower than the MBC values suggesting that, *T. claveryi* aqueous extracts were bacteriostatic at lower concentration but bactericidal at higher concentration. Also, the bacterial zone of inhibition increased with the increasing concentration of *T. claveryi* aqueous extract. To the best of our knowledge, this is the first report for the novel antibacterial activity of *T. claveryi* aqueous extract. This active compound may be used as alternative therapeutic drug for the control of corneal infections. However, further research is needed to examine its *in vivo* mechanism of action, toxicity, and therapeutic effect.**

Key words: Bacteria, corneal infection, antimicrobials, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), *Terfizia claveryi.*

INTRODUCTION

In the 21st century antibiotic resistance of clinical bacterial isolates are increasing drastically, the search for new and safe anti-bacterial compounds are important and natural medicinal products seems to be a logical and effective source for seeking new antimicrobial agents. In Saudi Arabi, the traditional medicinal practices have been known since ancient time for their unique properties and obvious therapeutic potential in treating a variety of diseases (Al-Bukhari and Al-Bukhari, 1996).

Terfizia claveryi, as brown desert truffles, are considered to be one of the oldest food stuffs known for their nutritional value especially when compared with meat and fish (Abu-Rabia, 1983). Truffle aqueous extract is used as a folk medicine in Gulf countries (Iraq, Saudi Arabia and Eastern Jordan) to treat eye infections (Bokhairy and Parvez, 1993). *T. claveryi* ascocarps contain 16% protein, 28% total carbohydrates, 4% total crude fiber, 2% total crude fat and rich in mineral as well as carbohydrate contents, with nine saturated and four unsaturated fatty acids and 29 amino acids, unique flavor, nutritional value and medicinal properties for a variety of ailments (Al-Delaimy, 1977). It is also used as a

Abbreviations: MIC, Minimum inhibitory concentrations; **MBC,** minimal bactericidal concentration.

nourishing and invigorating preparation for convalescents in Mediterranean countries (Janakat et al., 2004). Corneal infection is one of the most common ocular diseases in both humans and animals and can lead to blindness (Olivier, 2003; Jatoi et al., 2002). There are various pathogenic organisms, like *Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus spp.* and *Staphylococcus epidermis*, reported to cause corneal infection (Wahl et al., 1991; Dart, 1988; Charteris et al., 1994; Leeming, 1999). Many antibacterial preparations are used to treat eye infections such as, chloramphenicol, fluoroquinolone, neomycin and aminoglycosides (Goldstein et al., 1999). However, the increasing resistance of many bacteria and the side effects to the currently used antibiotics are documented (Lancaster and Swart, 1998; Ostier, 1993; Vaughan and Asbury, 1980; Skies et al., 2007). The medicinally important *T. claveryi* fruit was selected in this study to investigate whether having antimicrobial activity against clinical isolates of corneal ulcer.

MATERIALS AND METHODS

Collection of *Terfizia claveryi*

"Kamma" the local name of *T. claveryi*, brown desert truffles was purchased in April-2012 from the local market in Riyadh, Kingdom of Saudi Arabia. The desert truffle fruit material identification was confirmed by Department of food and Agriculture, Qassim University, Kingdom of Saudi Arabia.

Preparation of crude extract of *T. claveryi*

The collected *T. claveryi* fruits were cleaned and cut into small pieces and dried under shade at room temperature. The dried material was ground to fine powder using a mechanical blender and passed through 24 mesh sieve. *T. claveryi* powder (100 g) was extracted with 50 mM sodium phosphate buffer (pH 7.0) at 37°C. The extract was filtered through cheese cloth to remove the major debris and the filtrate was centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was considered as crude aqueous extract of *T. claveryi* and stored at 4°C for experimental use.

In-vitro antibacterial activity

Test microorganisms

Microbial cultures of eight different strains of both Gram positive and Gram negative bacteria were used for the determination of antibacterial activity. Gram-positive *(S. aureus, S. epidermidis, Staphylococcus faecalis)* and Gram-negative *(Escherichia coli, P. aeruginosa, Pseudomonas vulgaris, Klebsiella pneumonia),* clinical bacterial isolates were used. All the bacterial strains were sub-cultured at 37°C on Mueller-Hinton agar (Oxoid, Hampshire, UK) slants every 15 days and stored at 4°C. The bacterial isolates were obtained during parallel studies from clinical cases that suffered corneal infections and subjected to several hospitals at Qasssim region during 2012. Sampling, culturing, isolation and identification were done in the Department medical laboratory at College of Applied Medical Sciences, Qassim University using the standard Microbiology techniques (Collee et al., 1996).

Antibiotic susceptibility testing

The microorganisms were tested for their sensitivity against the antibiotics including: Ciprofloxacin (5 µg), tetracycline (30 µg), gentamycin (10 µg), tobramycin (10 µg), erythromycin (15 µg), moxifloxacin (5 µg), cefoxitin (30 µg), oxacillin (1 µg), clotrimazole (10 µg). The susceptibilities of the isolated pathogens were determined by the modified Kirby-Bauer disc diffusion method with Muller Hinton agar plates (Bauer et al., 1996). All the media used in the present investigation were obtained from Oxoid, Hampshire, UK.

Agar well diffusion method

Antibacterial activity of *T. claveryi* was determined by agar well diffusion method (Bauer et al., 1996). One hundred microliter (100 µl) of standardized inoculum (0.5 Mac-Farland) of each test bacterium were inoculated on molten Mueller-Hinton agar, homogenized and poured into sterile plates. Standard cork borer of various diameter (6, 16 and 20 mm) were used to make uniform wells into which different amounts of aqueous extract of *T. claveryi* (100, 300 and 500 µl) were added. Standard antibiotic ciprofloxacin was used as positive control and 50 mM sodium phosphate buffer alone was used as negative control. The plates were then incubated at 37 ± 1°C for 24 h. The experiments were carried out in triplicates and the zone of inhibition was measured with the help of standard scale.

Determination of minimum inhibitory concentrations (MIC)

The MIC of *T. claveryi* was determined by macro dilution method (Weckesser et al., 2007). Several dilutions of *Terfizia* aqueous extracts ranges (0.040-1.250 mg/ml) and standard antibiotic ranges (0.024-0.240 mg/ml) were prepared from stock solutions by serial dilution technique. Each sample dilution were mixed properly with 20 ml of sterile molten Muller Hinton agar and poured into 90 mm plates and allowed to cool under laminar air flow before streaking with 10 µl of 0.5 McFarland standards. The lowest concentration which did not show any macroscopic growth of tested microorganism was identified as the MIC.

Determination of minimal bactericidal concentration (MBC)

The MBC of the *T. claveryi* extracts were determined by a macro broth dilution method (Perez et al., 1990). Each set contains 8 tubes as follows: Positive control, negative control, positive control with 50 mM potassium phosphate buffer pH 7 where 50, 75, 100, 125, 150, 175 and 200 µl of *T. claveryi* aqueous extract were added for each bacterial species. 7 tubes out of eight were inoculated with 10 µl of single bacterial species. The plates were then incubated at 37°C overnight and the lowest dilution that yielded complete inhibition of bacterial growth was taken as the MBC. Each of the extract was tested in triplicate and the average values were obtained for two repeated experiments.

Statistical analysis

These parameters were tested in triplicates. The values were expressed as mean ± standard deviation (SD), mean value ± standard error of the mean (SEM) of growth inhibition zones diameters obtained with aqueous extract which amount was sufficient to perform repetitions. Statistical differences between the two variants of diffusion method were detected by analysis of variance (ANOVA) followed by Duncan test, the statistical analysis was performed using SPSS statistical software.

Table 1. Mean zone of inhibition (mm) of *T. claveryi* aqueous extracts against bacterial isolates in comparison with standard antibiotic.

Bacterial isolate	Mean zone of inhibition (mm) *(mean ± SD)*	
	***T. claveryi* (100 mg/ml)**	**Standard antibiotic (ciprofloxacin 5 mg/disc)**
Staphylococcus aureus	19.00± 1.00	27.33±0.57
Staphylococcus epidermidis	18.00± 1.00	29.00±0.57
Streptococcus faecalis	17.00± 1.00	28.66± 1.52
Escherichia coli	15.33± 0.57	30.33± 1.52
Pseudomonas aeruginosa	20.33± 1.00.	26.00± 1.52
Proteus vulgaris	15.33± 0.57	27.66± 0.57
Klebsiella pneumonia	14.66± 0.57	28.33± 1.52

RESULTS

Mean zone of inhibition

In the present study, all the tested bacteria were sensitive to *T. claveryi* aqueous extract. Among the Gram positive and Gram negative; S*. aureus, P. aeruginosa* exhibited highest rate of sensitivity to *T. claveryi* aqueous extract. This study also reveals that the *P. aeruginosa* was highest susceptible bacteria with 20.33 mm zone of inhibition followed by *S. aureus* (19.00 mm), *S. epidermidis* (18.00 mm), *S. faecalis* (17.00 mm), *E. coli* (15.33 mm), *P. vulgaris* (15.33 mm) and *K. pneumonia* (14.66 mm) at the test concentration of 100 mg/ml, which was comparable to standard antibiotic ciprofloxacin 5 mg/disc (Table 1).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Our results exhibited the broad spectra of antibacterial activity of *T. claveryi* aqueous extract (Figure 1). The MIC and MBC of *T. claveryi* aqueous extract against clinical isolates has been listed in (Tables 2 and 3). The MIC ranged between 0.40 to 1.25 mg/ml and the MIC of ciprofloxacin ranged between 0.024 to 0.240 mg/ml. Among the Gram positive bacteria strain, *S. aureus* isolates were found to be more sensitive than Gram negative *K. pneumonia* strains. *T. claveryi* extracts exhibited the greatest antibacterial activities as determined by the MBC. The MBC ranged between 75 to 120 µl/ml.

The lowest MBC value was observed towards *S. aureus* (75 µl/ml) and *K. pneumonia (*85 µl/ml). The inhibition of tested bacteria was increased by the increase in the amount of the *T. claveryi* aqueous extract (Table 4 and Figure 2).

In all clinical bacterial isolates, antibiotic susceptibility to eight antibiotics was accessed by Kirby-Bauer disc diffusion method (Table 5). All bacterial isolates were sensitive to ciprofloxacin while *S. aureus* and *S. faecalis* were resistant to erythromycin.

DISCUSSION

Saudi Arabia has old history in the use of diverse herbal medicines for traditional healing. Literature overview shows that, the most common species of the genus Terfizia especially *Terfizia claveryi* are round, tan to brown like small sandy potatoes, have unique flavor, nutritional value and medicinal properties. It is used for treatment of variety of ailments like eye infections, open cuts, stomach ailment among others (Goldstein et al., 1999; Lancaster and Swart, 1998). In Gulf countries, Truffle is used in the form of flour or juice for curing various infections in folk medicine.

Numerous studies have been reported in the past, focusing on antimicrobial activity of aqueous extract of *Asparagus racemosus1*, *Asphodelust enuifolius, Balanites aegyptiaca, Eclipta alba, Pedalium murex, Ricinuscommunis, Trigonellafoenumgraecum, TrianthemadecandraL, Argemone mexicana, Tinosporacordifolia* and *Cassia fistula* against various bacteria and fungi by using well diffusion and disc diffusion methods (Satavat and Gupta, 1987; Kaul (1997); Valsaraj et al., 1997; Khafagy and Ishrak, 1999; Mandal et al., 2000; Geethalakshmi et al., 2010; Rahman et al., 2011; Upadhyay et al., 2011). The antimicrobial analysis using the agar well diffusion method and MIC value had been used by many researchers (Arora and Kaur, 2007; Gurudeeban et al., 2010; Pavithra et al., 2010). However, since no studies have been reported on the antimicrobial activity of *T. claveryi* extracts against bacterial isolates causing corneal ulcer, we made this attempt.

Several herbal medicines like *Pothomorphe umbellate* extract and Chinese herbal medicine (emodin) have been reported to play an important role in the therapeutic activities of corneal ulcerative problems (Barros et al., 2007; Kitano et al., 2007). The effects of *S. jalambrensis* preparation on ocular inflammation have been explored by *in vivo* topical administration. The scientific research on the antimicrobial potency of many of the plants and herbs used for medicinal purposes in Saudi Arabia is lacking.

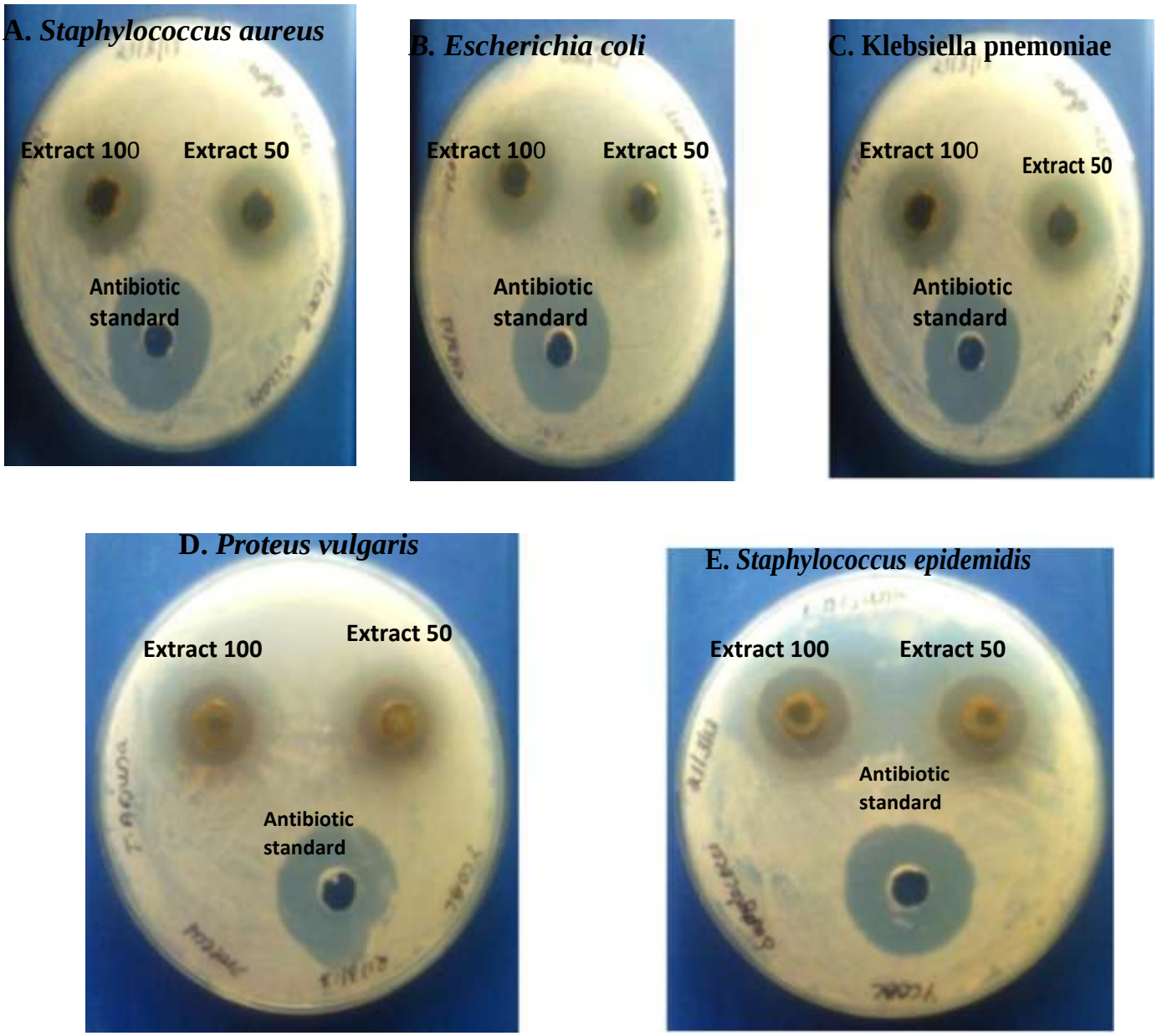

Figure 1. Efficiency of *Terfizia claveryi* aqueous extract against different bacteria compared with standard antibiotic (ciprofloxacin).

Table 2. Minimum inhibitory concentrations (MICs) of *T. claveryi* aqueous extract against bacterial isolates in comparison with standard antibiotic.

Bacterial isolate	MIC value (mg/ml)	
	T. claveryi	Standard antibiotic (ciprofloxacin)
Staphylococcus aureus	0.40	0.024
Staphylococcus epidermidis	0.40	0.024
Streptococcus faecalis	0.75	0.120
Escherichia coli	0.60	0.195
Pseudomonas aeruginosa	0.55	0.240
Proteus vulgaris	1.20	0.124
Klebsiella pneumonia	1.25	0.110

Table 3. Minimum Bactericidal concentrations (MBC) of *T. claveryi*a aqueous extract against bacterial isolates in comparison with standard antibiotic.

Bacterial isolate	MBC value (µl/ml)	
	T. claveryi	**Standard antibiotic (ciprofloxacin)**
Staphylococcus aureus	75	0.024
Staphylococcus epidermidis	75	0.024
Streptococcus faecalis	90	0.120
Escherichia coli	95	0.195
Pseudomonas aeruginosa	100	0.240
Proteus vulgaris	120	0.124
Klebsiella pneumonia	85	0.110

Table 4. Inhibition zone diameter by *T. claveryi* aqueous extracts against tested bacterial isolates.

Test organism	***T. claveryi* aqueous extract**		
	100 µl	**300 µl**	**500 µl**
	Well (6 mm)	**Well (16 mm)**	**Well (20 mm)**
Staphylococcus aureus	19	34	41
Staphylococcus epidermidis	20	33	40
Streptococcus faecalis	17	27	32
Escherichia coli	18	28	33
Pseudomonas aeruginosa	16	26	30
Klebsiella pneumonia	16	26	30
Proteus mirabilis	20	32	36

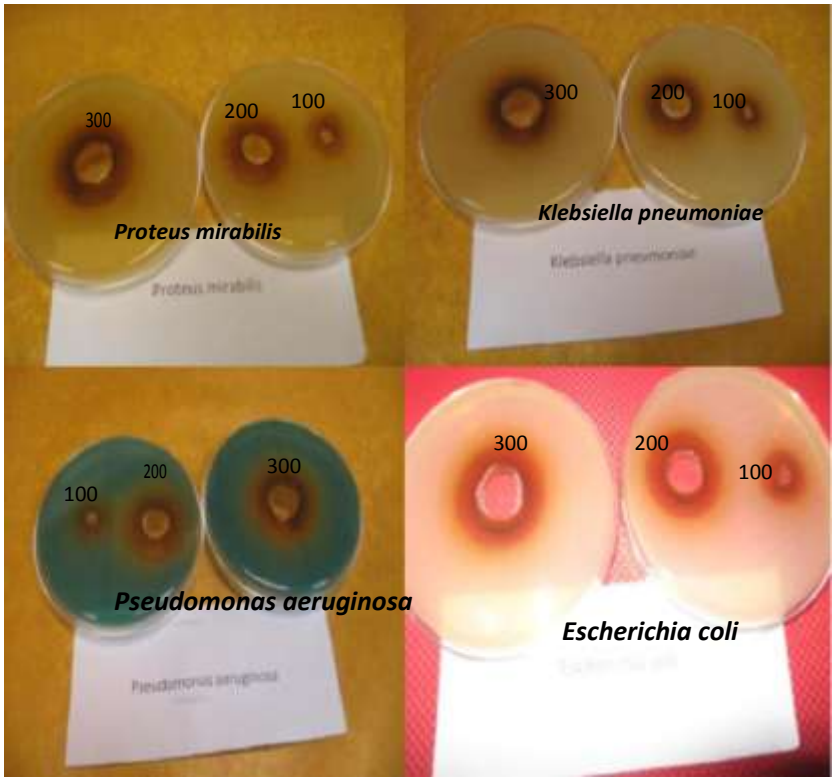

Figure 2. Effect of *Terfizia claveryi* aqueous extract against tested bacteria isolates.

Table 5. Antibiotic susceptibility pattern of Gram positive and Gram negative bacteria.

Test organism	CIP	CN	TE	TOB	E	MXF	FOX	OX	
Staphylococcus aureus	S	S	S	S		R	S	R	R
Staphylococcus epidermidis	S	S	S	S		S	S	S	S
Streptococcus faecalis	S	R	R	S		R	S	R	ND
Escherichia coli	S	S	S	S		S	S	S	ND
Pseudomonas aeruginosa	S	S	R	S		S	S	R	ND
Klebsiella pneumonia	S	S	S	S		S	S	S	ND
Proteus mirabilis	S	R	R	S		R	S	S	ND

ND, Not determined; R, resistance; S, sensitive; CIP, ciprofloxacin; CN, cefoxitin; TE, tetracyclin; TOB, tobramycin; MYX, moxifloxacin; FOX, gentamycin; OX, oxicillin.

So, the present investigation evaluates the *in-vitro* antimicrobial activity of crude extracts of *T. claveryi* against the clinical bacterial isolates causing corneal ulcer. In the present study, we found that, *T. claveryi* extracts showed good antibacterial activity against most of the clinical bacterial isolates from the corneal ulcer cases (Table 1 and Figure 1).

The results of the current study clearly demonstrate that, aqueous extracts of *T. claveryi* could inhibit the growth of several bacterial pathogens causing cornea ulcer. However, the effectiveness varied against the different tested bacterial isolates. We determined the potential antibacterial activity of *T. claveryi* aqueous extract against seven clinical bacterial isolates causing corneal ulcer. The zone of inhibition for the *T. claveryi* extracts obtained by disc diffusion were equal or larger than those of the eight antibiotics commonly used to treat corneal ulcer infections (Table. 5).

In the present study, the MIC value of the active aqueous extract were lower than the MBC values suggesting that, the *T. claveryi* aqueous extracts were bacteriostatic at lower concentration but bactericidal at higher concentration (Tables 2 and 3). The mean zone of inhibition as shown in (Table 4 and Figure 2) was increased by the increase in the amount of the *T. claveryi* aqueous extract (Maji et al., 2010). The current results highlights, the fact that *T. claveryi* aqueous extracts exhibited antimicrobial activity against Gram positive and Gram negative bacteria isolated from corneal ulcer. Considering that these *T. claveryi* are edible and are traditionally used for treatment of a number of ailments, their anti-bacterial activity is quite significant and could present alternative treatments for corneal ulcer.

Conclusion

To the best of our knowledge, this is the first report for the novel anti-bacterial activity of *T. claveryi* aqueous extract against bacterial isolates causing corneal ulcer. This active compound may be used as an alternative therapeutic drug for the control of corneal infections. Further research is needed to examine its *in-vivo* mechanism of action, toxicity, and therapeutic effect.

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