



In Vitro Antimicrobial Activity of Diacerein on 76 Isolates of Gram-Positive Cocci from Bacterial Keratitis Patients and *In Vivo* Study of Diacerein Eye Drops on *Staphylococcus aureus* Keratitis in Mice

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ABSTRACT Bacterial keratitis is an aggressive infectious corneal disease. With the continuing rise in antibiotic resistance and a decline in the discovery of new antibiotics, new antimicrobial drugs are now required. In the present study, we determined the antibacterial activity of diacerein, an anti-inflammatory drug, against 76 Gram-positive cocci isolated from bacterial keratitis patients *in vitro* and anti-*Staphylococcus aureus* activity in a mouse bacterial keratitis model *in vivo*. The MICs of diacerein were tested using the broth microdilution method *in vitro*. A BALB/c *Staphylococcus aureus* keratitis animal model was selected and the corneal clinical observation, viable bacteria, and hematoxylin-eosin and Gram staining of infected corneas were measured to evaluate the antibacterial efficacy of diacerein eye drops *in vivo*. An *in vivo* eye irritation study was carried out by a modified Draize test in rabbits. Our *in vitro* results showed that diacerein possesses satisfactory antibacterial activity against the majority of Gram-positive cocci (60/76), including all 57 tested *Staphylococcus* spp. and 3 *Enterococcus* spp. The *in vivo* experiment showed that diacerein eye drops reduced bacterial load and improved ocular clinical scores after topical administration of diacerein drops on infected corneas. The ocular irritation test revealed that diacerein eye drop had excellent ocular tolerance. These results indicated that diacerein possesses *in vivo* anti-*Staphylococcus aureus* activity. We suggest that diacerein is a possible topically administered drug for *Staphylococcus aureus*-infected patients, especially those with ocular surface inflammatory disorders.

KEYWORDS *Enterococcus*, Gram-positive cocci, *Staphylococcus aureus*, bacterial keratitis, diacerein eye drop

Bacterial keratitis is an aggressive infectious corneal disease characterized by acute onset and rapid progression and can lead to perforation of the cornea and blindness without prompt and effective treatment (1, 2). Patients often end up with severe vision loss even after the infection is completely eradicated (3–5). Today, it remains the most common cause of microbial keratitis worldwide (6), and the incidence of bacterium-induced keratitis has recently increased (7, 8). The estimated prevalence varies from 6.3 to 710 per 100,000 people (1).

Bacterial keratitis is presently treated with antibiotics. However, the treatment of bacterial keratitis has become increasingly difficult due to antibiotic resistance. It is difficult to develop effective new antibiotics when a new resistance mechanism emerges. Due to this new challenge, there is a demand for the development of new antimicrobial drugs (9). Unfortunately, few studies have demonstrated their efficacy *in vivo*.

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The literature shows that many anti-inflammatory drugs possess a moderate to powerful antimicrobial effect or increase the activity of certain antibiotics, both *in vitro* and *in vivo*, in addition to their potent anti-inflammatory action (10–13). These studies have collectively led to the idea that investigations focused on old anti-inflammatory drugs may shed light on new directions for possible clinical uses of their combined action in the management of difficult-to-treat bacterial infections related to inflammatory conditions.

Diacerein is a medicine available in a number of countries worldwide. It is a slow-acting drug in the anthraquinone class used to treat joint diseases, such as osteoarthritis (14, 15), and could improve the metabolic and inflammatory profile among patients with type 2 diabetes mellitus (T2DM) under long-term treatment with glucose-lowering agents (16–18). It works by blocking the actions of interleukin-1 beta, a protein involved in inflammation. A previous *in vitro* study showed that diacerein has growth-inhibitory effects against various drug-resistant staphylococcal strains, and synergistic or additive effects with oxacillin and tetracycline were observed against all *Staphylococcus aureus* strains (19, 20). To date, there are no reports describing the therapeutic effect of diacerein for the treatment of bacterial keratitis.

The most frequent pathogen in bacterial keratitis associated with non-contact-lens-related disease is the Gram-positive bacterium (8). We collected and isolated all bacteria from patients with bacterial keratitis between March 2017 and February 2018 at the Henan Eye Hospital in China and obtained 76 clinical isolates of Gram-positive cocci, including 6 common and 9 uncommon species. The *in vitro* antimicrobial activity of diacerein against all isolated Gram-positive cocci was evaluated. Diacerein showed potent antimicrobial activity against *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Staphylococcus intermedius* from bacterial keratitis.

The topical administration of antimicrobial agents for treating corneal infections has many advantages over systemic administration, including avoiding adverse systemic side effects, increasing the drug concentration at the target site of infection, and reducing the probability of developing drug resistance (21). In a previous study, we prepared diacerein eye drops and studied the pharmacokinetic behavior in the cornea (22, 23). Therefore, in this study, we examined the effectiveness of diacerein eye drops in a murine model of *Staphylococcus aureus* keratitis and compared it with levofloxacin, which is the first-line treatment drug. This investigation is the first to evaluate the *in vitro* antimicrobial activity of diacerein on 76 Gram-positive cocci isolates from bacterial keratitis patients and the *in vivo* study of diacerein eye drops on murine *Staphylococcus aureus* keratitis. The present study indicates that diacerein may be a potential drug to treat patients suffering from bacterial keratitis, especially those with ocular surface inflammatory disorders.

RESULTS

***In vitro* antibacterial activity of diacerein and its metabolite rhein against Gram-positive cocci isolates.** MICs of diacerein and rhein against 65 common clinical ocular Gram-positive bacterial isolates from ocular surface infection patients are displayed in Table 1. The data showed that there is no difference between diacerein and rhein against 3 *Staphylococcus* spp., 1 *Enterococcus* spp., and 2 *Streptococcus* spp. The MIC of diacerein and rhein against *Staphylococcus* spp. (*Staphylococcus epidermidis*, *Staphylococcus intermedius*, and *Staphylococcus aureus*) and *Enterococcus* sp. were lower than *Streptococcus* spp. (*Streptococcus sanguinis* and *Streptococcus pneumoniae*). Diacerein and rhein were more potent against *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Staphylococcus intermedius* than against *Streptococcus sanguinis* or *Streptococcus pneumoniae* ($P < 0.05$). Levofloxacin was found to have a lower MIC than diacerein and rhein against *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Staphylococcus intermedius*, and *Streptococcus sanguinis* ($P < 0.05$). Compared with amikacin, diacerein and rhein showed no significant difference, with the exception of *Staphylococcus epidermidis*.

TABLE 1 MIC range, geometric mean, MIC mode, MIC₅₀, and MIC₉₀ of diacerein and its metabolite rhein against 65 common clinical ocular Gram-positive bacterial isolates

Antibacterial agent by bacterial species (n)	MIC (μg/ml) ^a				
	Range	GM	Mode	MIC ₅₀	MIC ₉₀
<i>Staphylococcus epidermidis</i> (38)					
Diacerein	1–16	6.54 ^{b,c}	8	8	16
Rhein	2–16	8.61 ^{b,c}	8	8	16
Levofloxacin	0.125–16	0.75	0.5	0.5	4.40
Amikacin	0.25–16	1.44	0.5	1.50	8
<i>Staphylococcus aureus</i> (7)					
Diacerein	4–32	11.89 ^b	8	8	32
Rhein	4–16	8.83 ^b	16	8	16
Levofloxacin	0.125–2	0.41	0.25	0.25	2
Amikacin	0.25–16	2.69	1	4	16
<i>Staphylococcus intermedius</i> (6)					
Diacerein	4–16	8 ^b	8	8	16
Rhein	8–32	12.70 ^b	8	12	32
Levofloxacin	0.125–0.5	0.22	0.25	0.25	0.5
Amikacin	0.25–16	1.59	0.25	1.5	16
<i>Enterococcus</i> spp. (3)					
Diacerein	8–32	20.16	32	32	32
Rhein	8–32	16	8	16	32
Levofloxacin	0.25–4	0.63	0.25	0.25	4
Amikacin	0.5–8	3.17	8	8	8
<i>Streptococcus sanguinis</i> (8)					
Diacerein	32–128	69.79 ^b	64	64	128
Rhein	64–128	76.11 ^b	64	64	128
Levofloxacin	0.25–8	0.84	0.5	0.75	8
Amikacin	0.125–128	29.34 ^b	128	128	128
<i>Streptococcus pneumoniae</i> (3)					
Diacerein	64–128	101.59	128	128	128
Rhein	64–128	101.59	128	128	128
Levofloxacin	0.5–1	0.63	0.5	0.5	1
Amikacin	1–128	12.70	1	16	128

^aAll MICs were determined visually; the MIC₅₀ and MIC₉₀ values represent the concentrations required to inhibit 50% and 90% of the tested strains. GM, geometric mean.

^b*P* < 0.05 compared with levofloxacin.

^c*P* < 0.05 compared with amikacin; analyzed using one-way ANOVA.

MICs of diacerein and rhein against 11 uncommon clinical ocular Gram-positive bacterial isolates (1 to 2 isolates each) are displayed in Table 2. We provided only the MIC ranges (2 isolates) or MIC (1 isolate each) because the number of strains per genus/species was low. These data may still be useful for understanding the antibac-

TABLE 2 MICs of diacerein and rhein against 11 uncommon clinical ocular Gram-positive bacterial isolates

Isolate	n	MIC range or MIC (μg/ml) ^a by antibacterial agent			
		Diacerein	Rhein	Levofloxacin	Amikacin
<i>Staphylococcus haemolyticus</i>	2	4–16	4–16	0.125–0.5	2–4
<i>Staphylococcus xylosus</i>	2	2–16	2–16	0.5–4	2–2
<i>Staphylococcus hominis</i>	1	8	8	1	0.25
<i>Staphylococcus capitis</i>	1	32	32	1	0.25
<i>Gemella haemolysans</i>	1	128	128	0.125	0.25
<i>Enterococcus durans</i>	1	128	128	8	0.125
<i>Streptococcus salivarius</i>	1	128	128	0.5	128
<i>Streptococcus mitis</i>	1	128	128	2	128
<i>Micrococcus luteus</i>	1	128	128	0.5	64

^aAll MICs were determined visually. Due to one strain tested for some bacteria, only MIC is presented.

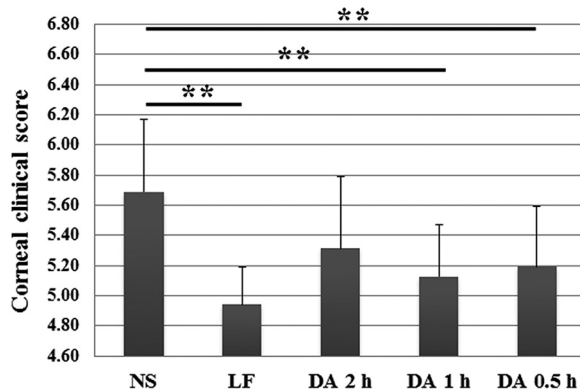


FIG 1 Corneal clinical scores in *Staphylococcus aureus*-infected BALB/c mice treated with normal saline (NS), levofloxacin (LF), and diacerein (DA) eye drop administered with 2-h, 1-h, and 0.5-h intervals (DA 2 h, DA 1 h, and DA 0.5 h) for 12 h. Eyes treated with LF, DA 1 h, and DA 0.5 h had significantly lower scores than those in NS-treated eyes, as indicated by an asterisk (**, $P < 0.01$). Error bars indicate standard error of the mean (SEM).

terial characteristics of diacerein and rhein. The data show that diacerein and rhein are more susceptible to *Staphylococcus* spp. (MIC range, 2 to 32 $\mu\text{g}/\text{ml}$). These results were similar to those displayed in Table 1.

Effect of diacerein eye drop on treatment of *Staphylococcus aureus* keratitis in a mouse model. (i) Corneal clinical scores. The corneal clinical scores in BALB/c mice eyes infected with *Staphylococcus aureus* treated with normal saline (NS), levofloxacin (LF), or diacerein (DA) (administered diacerein eye drops with 2-h, 1-h, and 0.5-h interval) for 12 h are summarized in Fig. 1. At 30 h postinfection (p.i.), eyes treated with LF and DA 1 h and DA 0.5 h for 12 h had significantly lower scores (4.94 ± 0.25 , 5.13 ± 0.34 , and 5.19 ± 0.40 , respectively) than normal saline-treated eyes (5.69 ± 0.48 , $P < 0.01$). There were no significant differences between LF and DA 2 h, DA 1 h, and DA 0.5 h.

(ii) Viable bacterial counts. The average CFUs per cornea (Log_{10}) of *Staphylococcus aureus*-infected BALB/c mice treated with NS, LF, and DA eye drops (administered with 2-h, 1-h, and 0.5-h interval) for 12 h are presented in Fig. 2. DA 2 h-, DA 1 h-, DA 0.5 h-, and LF-treated eyes exhibited a significant decrease in the corneal *Staphylococcus aureus* burden compared with eyes treated with NS ($P < 0.01$), and LF-treated eyes produced a significant reduction in corneal bacteria compared with DA 2 h, DA 1 h, and DA 0.5 h ($P < 0.01$). Eyes treated with DA 1 h and DA 0.5 h produced a significant reduction in bacteria compared with DA 2 h ($P < 0.01$). There was no significant difference between DA 1 h and DA 0.5 h. These results indicated that the diacerein exerted antibacterial activity *in vivo*; moreover, the diacerein administered with a 1-h and 0.5-h interval showed more efficacy than that with 2 h; however, its antibacterial activity was no more powerful than LF.

(iii) Histopathologic analysis. The typical histological features treated with NS, LF, and DA 1 h are shown in Fig. 3. Eyes treated with NS (Fig. 3A) demonstrated focally severe polymorphonuclear leukocyte (PMN) accumulation throughout the cornea, severe edema, and mass Gram-positive cocci in the anterior stroma. Treatment with LF (Fig. 3B) demonstrated minimal PMN infiltration, mostly in the anterior stroma, mild edema and a small Gram-positive cocci in the anterior stroma; and treatment with DA 1 h (Fig. 3C) demonstrated moderate PMN infiltration in the anterior and middle stroma, moderate edema, and medium bacterial load in the anterior stroma. These results corresponded to corneal clinical scores.

(iv) Ocular irritation. The results of the Draize testing with diacerein and normal saline in rabbit eyes are shown in Table 3. The irritation mean total scores for both groups was less than 1. These results indicated that diacerein eye drop had excellent ocular tolerance.

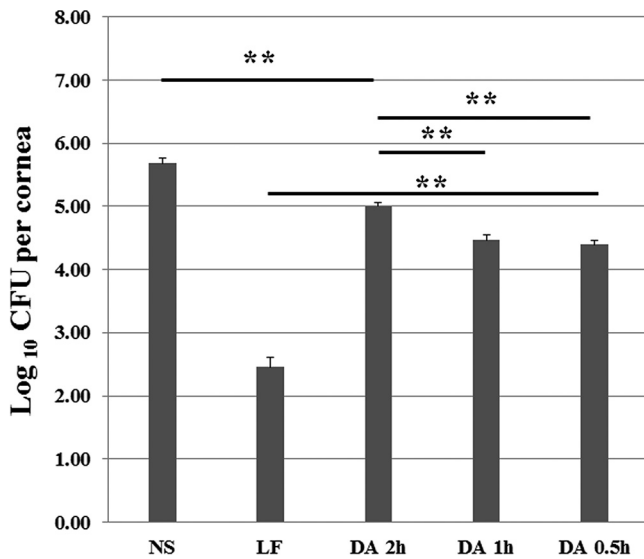


FIG 2 Average CFU per cornea (\log_{10}) in *Staphylococcus aureus*-infected BALB/c mice treated with normal saline (NS), levofloxacin (LF), and diacerein (DA) eye drops administered with 2-h, 1-h, and 0.5-h intervals (DA 2 h, DA 1 h, and DA 0.5 h) for 12 h. Corneas treated with LF and DA 2 h, DA 1 h, and DA 0.5 h had significantly lower CFU than NS-treated corneas, as indicated by an asterisk (**, $P < 0.01$). LF-treated eyes produced a significant reduction in corneal bacteria compared with DA 2 h, DA 1 h, and DA 0.5 h (**, $P < 0.01$). DA 1 h- and DA 0.5 h-treated eyes produced a significant reduction in corneal bacteria compared with DA 2 h ($P < 0.01$). The number of viable *Staphylococcus aureus* per cornea was quantified and expressed as base 10 logarithms \pm standard deviations (indicated by error bars). Data are from eight independent samples.

DISCUSSION

Due to the continuing rise in antibiotic resistance and a decline in the discovery of new antibiotics, novel treatments for infectious diseases are now required (24, 25). In the present study, we investigated whether diacerein, an anti-inflammatory drug, possesses potent antibacterial activity against Gram-positive cocci. We determined the

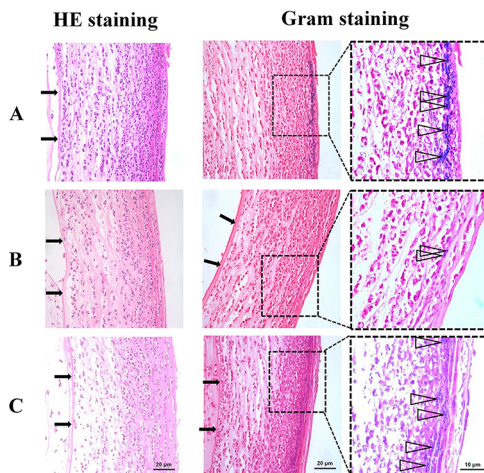


FIG 3 The typical corneal histopathology images of *Staphylococcus aureus*-infected BALB/c mice treated with normal saline (NS), diacerein with administered at 1-h interval (DA 1 h), and levofloxacin (LF) eye drops for 12 h. Sections were stained with hematoxylin and eosin and Gram. Treatment with NS (negative control, A) demonstrating focally severe PMN accumulation throughout cornea, severe edema, and mass Gram-positive cocci in the anterior stroma. Treatment with LF (positive control, B) demonstrated minimal PMN infiltration mostly in anterior stroma, mild edema, and small Gram-positive cocci in the anterior stroma; treatment with DA (C) demonstrated moderate PMN infiltration in the anterior and middle stroma, moderate edema, and medium bacterial load in the anterior stroma. The arrows indicate the endothelium. The triangle indicates Gram-positive *Staphylococcus aureus* (dark purple). Scale bars: H&E, 20 μ m; Gram, 20 μ m (left) and 10 μ m (right).

TABLE 3 The irritation mean total scores of the Draize test with diacerein and normal saline in rabbit eyes^a

Time (h)	Mean score of Draize test by treatment	
	Diacerein	Normal saline
1	0	0
2	0	0
4	0.25	0.5
24	0.25	0.5
48	0	0
72	0.25	0

^a*n* = 6.

antibacterial activity of diacerein against 76 Gram-positive cocci isolates from bacterial keratitis patients *in vitro* and anti-*Staphylococcus aureus* activity in a mouse bacterial keratitis model *in vivo*. Our results showed that diacerein possessed effective antibacterial activity against *Staphylococcus* spp. and *Enterococcus* spp. *in vitro* and reduced bacterial load and improved ocular clinical scores in a murine *Staphylococcus aureus* keratitis model *in vivo*. The literature reports that diacerein is entirely converted into rhein before reaching systemic circulation (26), which is similar with our results that diacerein transfers into rhein quickly in the cornea (22). In the *in vitro* study, we tested the antibacterial activity of diacerein and its metabolite rhein against all strains of Gram-positive cocci isolated from bacterial keratitis patients between March 2017 and February 2018 at our institute. Our results showed that diacerein and rhein possessed the same satisfactory *in vitro* antibacterial activity against the majority of Gram-positive cocci (60/76), including all 57 tested *Staphylococcus* spp. and 3 *Enterococcus* spp. bacteria. The 57 *Staphylococcus* species included 38 *Staphylococcus epidermidis* isolates, 7 *Staphylococcus aureus* isolates, 6 *Staphylococcus intermedius* isolates, 2 *Staphylococcus haemolyticus* isolates, 2 *Staphylococcus xylosus* isolates, 1 *Staphylococcus hominis* isolate, and 1 *Staphylococcus capitis* isolates. MIC results have been applied to classify bacteria as susceptible (often called sensitive), intermediate, or resistant to a particular antimicrobial by using breakpoints. The setting of breakpoints involves several factors, including clinical results from wild-type MIC distributions for relevant species of organisms, antimicrobial doses, and pharmacokinetic and pharmacodynamic considerations. Based on MIC breakpoints of levofloxacin for *Staphylococcus* spp. (27), 26 isolates are susceptible and 12 isolates are unsusceptible to levofloxacin. Diacerein is a novel antibacterial agent; the antibacterial breakpoint could not be found in the literature or in standards. In our previous study, we measured corneal diacerein concentrations, and the results showed that the diacerein concentration in the cornea (22, 23) was much higher than the MIC against *Staphylococcus* spp. The MIC₅₀ and MIC₉₀ of diacerein were much higher than those of levofloxacin and amikacin *in vitro*. However, the diacerein concentration in the cornea was much higher than the MIC against *Staphylococcus* spp. Therefore, our work is meaningful that as an anti-inflammatory drug, diacerein is also a potential topically administered drug for *Staphylococcus* spp.-infected patients, especially those with ocular surface inflammatory disorders.

Despite a previous *in vitro* study describing promising growth-inhibitory effects of diacerein against various drug-resistant staphylococcal strains (19), the synergistic anti-*Staphylococcus aureus* effects of its active metabolite rhein with ampicillin and oxacillin were also discovered (20). No data have reported the results of the use of diacerein against Gram-positive bacteria in ophthalmology or against strains such as *Enterococcus* spp., *Staphylococcus intermedius*, *Staphylococcus haemolyticus*, *Staphylococcus xylosus*, *Staphylococcus hominis*, and *Staphylococcus capitis*. To our knowledge, this is the first report of diacerein having potential *in vitro* antibacterial effects against 60 Gram-positive cocci isolates from 76 patients with infected corneas.

Staphylococcus aureus is the predominant pathogen isolated from ocular infections (7, 8), and BALB/c mice are considered to be more susceptible to *Staphylococcus aureus* keratitis than C57BL/6 mice (28, 29). Therefore, in our study, we selected a BALB/c

Staphylococcus aureus keratitis animal model to evaluate the antibacterial efficacy of three doses of diacerein eye drops *in vivo*. The results showed that diacerein reduced bacterial load and improved ocular clinical scores after topical administration of diacerein drops on infected corneas, and the dose of the diacerein eye drops administered with a 1-h interval is the best dosing schedule. Moreover, hematoxylin and eosin (H&E) microscopy and Gram staining revealed that PMN infiltration, edema, and the bacterial load in the diacerein-treated cornea were lower than that normal saline-treated negative control. The reduced number of infiltrating PMNs and the lower bacterial burden explained the lower clinical score in diacerein-administered cornea. These results indicated that diacerein possesses *in vivo* anti-*Staphylococcus aureus* activity.

Until now, there have been no reports of diacerein or its metabolite, rhein, that possess *in vivo* antimicrobial action, perhaps because the maximum plasma concentrations (C_{max}) of diacerein metabolite were no more than 5 mg/liter (29, 30) which was lower than the MIC. In our *in vivo* experiment, the diacerein eye drops were topically applied every 2 minutes three times, and then at 1-h intervals for 12 h after 18 h p.i. Therefore, diacerein concentrations in the cornea in this study would be much higher than the applicable MIC₉₀ (32 µg/ml), which can explain the *in vivo* antimicrobial action of diacerein against *Staphylococcus aureus*.

Diacerein has not been previously applied in ophthalmology. The safety of diacerein eye drops is unknown. To assess the safety of diacerein eye drops at the corneal surface, the Draize test was performed. The irritation score was less than 1. Hence, we conclude that diacerein eye drops are suitable and safe for ophthalmic application. Moreover, in our *in vivo* dose-response experiment, no obvious eye irritation was detected even at the dose of the diacerein eye drops with a 1-h and 0.5-h interval. In conclusion, based on the *in vitro* and *in vivo* antibacterial activity of diacerein, especially the *Staphylococcus aureus* activity, we suggest diacerein as a new promising antibacterial agent for ocular infection. Diacerein is an anti-inflammatory drug recommended for the treatment of osteoarthritis (14, 15); therefore, we suggest that diacerein is a possible topically administered drug for *Staphylococcus aureus*-infected patients, especially those with ocular surface inflammatory disorders.

MATERIALS AND METHODS

***In vitro* study. (i) Gram-positive cocci clinical isolates.** Seventy-six strains of Gram-positive cocci were isolated from patients with bacterial keratitis from March 2017 to February 2018 at Henan Eye Hospital and Henan Eye Institute in Zhengzhou, China. Representative bacterial colonies from each isolate were Gram stained and identified to strain based on an *in vitro* bacterial diagnostic reagent plate using a commercially available kit (Zhuohai DL Biotech Co., Ltd, Zhuhai, China). In total, 38 *Staphylococcus epidermidis* strains, 7 *Staphylococcus aureus* strains, 8 *Streptococcus sanguinis* strains, 6 *Staphylococcus intermedius* strains, 3 *Enterococcus* sp. strains, 3 *Streptococcus pneumoniae* strains, 2 *Staphylococcus xylosum* strains, 2 *Staphylococcus haemolyticus* strains, 1 *Enterococcus durans* strain, 1 *Gemella haemolysans* strain, 1 *Staphylococcus hominis* strain, 1 *Staphylococcus capitis* strain, 1 *Streptococcus salivarius* strain, 1 *Streptococcus mitis* strain, and 1 *Micrococcus luteus* strain were identified. Two *Staphylococcus aureus* reference strains (ATCC 25923 and ATCC 29213; a kind gift from the Department of Clinical Laboratory of Zhengzhou Yihe Hospital) were used as quality controls for the disk diffusion method and dilution susceptibility tests.

(ii) Determining the MIC of diacerein. The MIC of diacerein and its metabolite rhein on 76 Gram-positive cocci clinical isolates was determined by broth microdilution. Briefly, each bacterial strain was subcultured, and 100 µl of the bacterial diluent (1×10^6 CFU/ml) was added to each well on the 96-well plate. The diacerein and rhein stock solution contained 2,560 mg diacerein in 200 µl dimethyl sulfoxide (DMSO), and 800 µl Mueller-Hinton medium was prepared. One-hundred microliters of diacerein and rhein work solution was added to the bacterial well, and the diacerein and rhein test concentrations were 256, 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 µg/ml. Levofloxacin (Yangtze River Pharmaceutical [Group] Co., Ltd., Taizhou, China) and amikacin (Qilu Pharmaceutical Co., Ltd., Jinan, China) in a series of concentrations of 128, 64, 32, 16, 8, 4, 2, 1, 0.5, and 0.25 µg/ml were used as positive controls. The 96-well plates were incubated at 37°C for 24 h. The MICs were determined by concentrations at which there was no visible growth. Each experiment was repeated at least three times.

***In vivo* study. (i) Animals.** BALB/c mice (8 to 10 weeks of age) and Japanese big-ear rabbits (weighing 2.1 to 2.5 kg) were purchased from Henan Province Laboratory Animal Center (Zhengzhou, China) for evaluating antibacterial effectiveness and ocular irritation of diacerein *in vivo*. All of the animals were free of clinically observable ocular disease, and the procedures in the study were in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in

Ophthalmic and Vision Research. The murine care and experimental protocols were approved by the Ethical Committee of Experimental Animal Care of Henan Eye Institute.

(ii) Mouse *Staphylococcus aureus* keratitis model. All mice were anesthetized by intraperitoneal injection of pentobarbital sodium (80 mg/kg of body weight) (Sigma-Aldrich, USA). The corneal surface was anesthetized with 1% tetracaine hydrochloride eye drops. Under a dissecting microscope, a piece of filter paper (Sigma-Aldrich) was cut into round shapes with a diameter of 2-mm trephine, soaked with 1.5 μ l 0.3 M sodium hydroxide, and then applied to the center cornea for 1 minute. The eyes were then immediately irrigated with 50 ml of normal saline. Thirty minutes later, 5- μ l *Staphylococcus aureus* (ATCC 29213) inoculum (15×10^8 CFU/ml) was topically applied to the damaged cornea.

(iii) Diacerein eye drop treatment of *Staphylococcus aureus* keratitis. Eighteen hours after inoculation, the mice were divided into the following five treatment groups according to clinical score: the normal saline (NS) group (negative group), the levofloxacin eye drop (LF; Santen Pharmaceutical [China] Co., Ltd., Suzhou, China) group (positive control), and the three diacerein eye drop (DA; prepared by Henan Eye Institute, Zhengzhou, China) group. Immediately following grouping, 5 μ l normal saline or levofloxacin or diacerein eye drop was applied every time. The three diacerein eye drop group was treated 3 successive times with 2-min intervals, and then at 2-h (DA 2 h), 1-h (DA 1 h), and 0.5-h (DA 0.5 h) intervals, respectively, for 12 h after 18 h inoculation.

(iv) Slit lamp observation and cornea clinical score. The cornea clinical evaluation was carried out in a blind manner with the aid of a slit lamp microscope equipped with a camera (SLM-8E; Chongqing Kanghua Ruili Science Technology Co., Ltd, Chongqing, China) at 18 h and 30 h postinoculation (p.i.). The slit lamp examination scores were performed with the following criteria: (1) area of opacity with grade 1 (1% to 25% of total corneal area), grade 2 (26% to 50% of total corneal area), grade 3 (51% to 75% of total corneal area), and grade 4 (76% to 100% of total corneal area); (2) density of opacity with grade 1 (slightly misty opacity of cornea, relatively clear and pupil iris), grade 2 (opacity of corneal superficial layer, visible pupil and iris through the lesion), grade 3 (uneven opacity of whole corneal layer), and grade 4 (even and dense opacity); and (3) hypopyon with grade 1 (hypopyon not reached the paracentral cornea) and grade 2 (hypopyon reached the paracentral cornea).

(v) Quantification of viable bacteria from infected corneas. At 30 h p.i., the infected corneas (both controls and treatment) were collected after 12 h of treatment, and the number of viable bacteria was quantitated. Individual corneas were homogenized in sterile 0.9% saline. A 10- μ l aliquot of the homogenate was diluted and 10-fold serial dilutions were plated in duplicate onto Mueller-Hinton agar (MHA) plates. The plates were incubated overnight at 37°C for 48 h and the number of CFUs was counted. The results are expressed as the log₁₀ number of CFUs per cornea.

(vi) Hematoxylin-eosin and Gram staining. After euthanization, the mice corneas in NS, LF, and DA 1 h were excised and fixed in formaldehyde, alcohol and sodium chloride (FAS) fixation fluid (Wuhan Servicebio Technology Co., Ltd., Wuhan, China), followed by dehydration with a serial concentration of ethanol. After dehydration, the samples were embedded in paraffin and approximately 4- μ m thin slices were obtained and mounted on glass slides using routine procedures. The cornea sections were stained with hematoxylin and eosin (H&E) and Gram staining and observed with light microscopy (Nikon ECLIPSE 80i, Japan).

(vii) Ocular irritation test. An *in vivo* eye irritation study was carried out by a modified Draize test in rabbits. Rabbits were divided into two groups (six rabbits per group) and then topically treated 17 times a day (the first and the last time the doses were given 3 successive times with 2-minute intervals; the other doses were given every 1 h in the right eye with diacerein eye drops) for 10 days. The left eyes served as controls and were treated with natural saline. A total of 50 μ l of diacerein eye drops was instilled into the conjunctival sac per eye. The ocular condition was recorded before and 1, 2, 4, 24, 48, and 72 h after the last administration. According to the modified Draize test, ocular irritation scores for every rabbit were calculated (31). The eye irritation score was obtained by dividing the total scores for all rabbits by the number of rabbits.

Statistical analysis. All of the data are expressed as the mean \pm standard deviation (SD). Multiple pairwise comparisons of sample means were performed using one-way analysis of variance (ANOVA). If homogeneity of variance was present, the least significant difference (LSD) was used. In all other instances, a Tamhane's T2 test was performed. The chi-square test was used to analyze the count data. The MIC range and mode, MIC₅₀ and MIC₉₀ were determined for the isolates with the SPSS statistical package. For calculation, any high off-scale MIC was converted to the next higher concentration. A *P* value of less than 0.05 was considered significant.

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