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Comparative efficacy of marine *Streptomyces* formulation versus ciprofloxacin ophthalmic solution for treating *Staphylococcus aureus*-induced conjunctivitis in animal model

Femina Wahaab, Kalidass Subramaniam*, Morris Jawahar

Department of Biotechnology, School of Biotechnology and Health Sciences, Karunya University, Coimbatore, Tamil Nadu, India

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ABSTRACT

Objective: To report the efficacy of marine actinomycetes in controlling *Staphylococcus aureus* (*S. aureus*)-induced conjunctivitis in experimental rabbit.
Methods: The ethyl acetate extracts of the best isolates of actinomycetes from the soil samples of Rameswaram coastal regions, Tamil Nadu, India, were concentrated and re-constituted in buffer and used as actinomycetes ophthalmic suspension in this study. The anti-inflammatory efficacy of actinomycetes ophthalmic suspension was analysed in controlling *S. aureus*-induced conjunctivitis in rabbit in comparison with that of ciprofloxacin.
Results: Among the four best isolates, the RAM25C4 isolate had the highest antimicrobial activity in the secondary screening. Shelf life studies indicated that the activity can be retained for 75 days when it was stored at 8 ˚C and the optimum temperature for storage was between –20 ˚C and 30 ˚C. The animal model studies indicated that there was a profound anti-conjunctivitis effect. The actinomycetes ophthalmic suspension had better activity than ciprofloxacin ophthalmic solution.
Conclusions: This is the first time to report that *Streptomyces bacillaris* strain RAM25C4 has antimicrobial effect in controlling ophthalmic pathogen *S. aureus* under *in vitro* condition and the *in vivo* anti-inflammatory activity in controlling *S. aureus*-induced conjunctivitis.

1. Introduction

Bacterial conjunctivitis is called as pink eye, inflammation of the conjunctiva, which can be caused by many bacterial species, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* (*S. aureus*) and *Streptococcus pneumoniae*. Among these organisms, *Staphylococcus* is the most prevalent bacteria isolated from conjunctivitis-affected humans[1]. There are many ophthalmic solutions available in the global market. The side effects, resistance problem and the narrow spectrum of activity of the available drugs have made the researchers to find a novel one without these side effects from natural sources. The marine biosphere is one of the richest biospheres on the earth with innumerable habitats[2]. Today, both academic and industrial interest in marine microorganisms is increasing due to the presence of unique and biologically active metabolites in them. Among the marine microorganisms, marine actinomycetes have attracted considerable attention of researchers[3]. In this investigation, we therefore focused on analysing the anti-inflammatory activity of ethyl acetate extract of marine *Streptomyces* in comparison with that of ciprofloxacin ophthalmic solution by using experimental rabbit with *S. aureus*-induced conjunctivitis.

*Corresponding author: Dr. Kalidass Subramaniam, Associate Professor of Biotechnology, School of Biotechnology and Health Sciences, Karunya University, Coimbatore, Tamil Nadu, India.
Tel: 0422-2614 499, +91 9443022508
Fax: 0422-2615 615
E-mails: kalidass@karunya.edu, kallidass@gmail.com

All experimental procedures involving animals were conducted in accordance to CPCSEA guidelines and approved by Institutional Animal Ethics Committee.

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2. Materials and methods

2.1. Isolation and identification of actinomycetes

Marine soil samples were collected from Tamilnadu coastal regions. Selective isolation of actinomycetes from soil samples was carried out by spread plate technique using actinomycetes isolation agar medium. The isolation medium was supplemented with antibiotics to inhibit fungal and bacterial colonization, respectively. After incubation, actinomycetes colonies appearing on the agar medium were pure cultured by re-streaking in the same agar medium and used for further assays[2].

2.2. Bacterial culture

The ophthalmic pathogen *S. aureus* isolated from clinical specimen was collected from Clinical Microbiology Laboratory, Aravind Eye Hospital at Coimbatore, Tamil Nadu, India.

2.3. Antibiotic susceptibility of the tested organism

The log phase culture of the ophthalmic pathogen *S. aureus* was checked for its antibiogram pattern against the commercially available antibiotic discs (Himedia) by using the standard disc diffusion test[2].

The culture broth was seeded in Muller Hinton agar plate by sterile swab. The following antibiotic discs were placed in equidistant with uniform contact between the antibiotic discs and on the surface of the Muller Hinton agar plates: amoxyclav (30 μg), ceftazidime (30 μg), chloramphenicol (30 μg), gentamicin (10 μg), nalidixic acid (30 μg), nitrofurantoin (30 μg), norfloxacin (10 μg), tetracycline (30 μg), kanamycin (30 μg), methicillin (15 μg) and ciprofloxacin (15 μg). The plates were then incubated at 37 ℃ in upright position for overnight. Diameters of the inhibition zones were measured and tabulated.

2.4. Screening of actinomycetes for antimicrobial activity

All the 131 actinomycetes isolates were streaked perpendicularly to the ophthalmic pathogen *S. aureus* with four isolates per Petri dish. The zones of inhibition were measured and tabulated[4]. The actinomycetes isolates which exhibited good antimicrobial effect against *S. aureus* were pure cultured respectively in culture flask. The culture was extracted with solvent extraction method by using ethyl acetate as the solvent[5]. The *Streptomyces* culture extract was secondarily screened for their antimicrobial activity against ophthalmic pathogen. The secondary screening was done by well diffusion method.

2.5. Determination of stability and shelf life of the best Streptomyces ethyl acetate extract

The best *Streptomyces* culture extract which had the maximum antimicrobial activity against ophthalmic pathogen was subjected to stability and shelf life test. The extract was aliquot into 7 screw cap vials with 5 mL of each. These vials were incubated for an hour at different temperatures (–20, 8, 30, 40, 50, 60 and 70 ℃) in deep freezer, fridge and in water baths with the above mentioned temperatures, respectively. After the incubation period, the extracts were checked for their antimicrobial activity by well diffusion method. Similarly for the shelf life expectancy study, 2 sets of 6 aliquots of 5 mL extract in vials were stored in refrigerated condition (8 ℃) and at room temperature (30 ℃) for 1 h. Each aliquot was checked for its antimicrobial activity starting from 0 day to 75 days at every 15 days' time interval[6].

2.6. Actinomycetes extract preparation

The marine actinomycetes isolates were inoculated in 50 mL of ISP2 broth and incubated at 30 ℃, 150 r/min in orbital shaking incubator for two days. After incubation period, 20 mL of the actinomycetes cultures were inoculated in 300 mL of ISP2 broth in shake flask and incubated at 30 ℃ in an orbital shaker at 150 r/min for 7 days. The grown culture was centrifuged at 5000 r/min for 10 min and the culture filtrate was subjected to solvent extraction for recovery of secondary metabolites. The solvent ethyl acetate was added to the filtrate in the ratio of 1:1 (v/v) and shaken vigorously for complete extraction. Two layers, namely, aqueous and the organic layers were separated, and the solvent ethyl acetate layer was collected and the aqueous layer discarded. Extraction was continued up to three times with the same solvent and the solvent layer was accumulated and concentrated by rotary vacuum evaporator. The residue is weighed and resuspended in buffer. This was used as ophthalmic suspension[7].

2.7. In vivo animal model

To analyse the anti-inflammatory efficiency of the *Streptomyces* extract, the anti-conjunctivitis study was done in the eye balls of young New Zealand albino male rabbits weighing between 2–2.5 kg. The experiments of conjunctivitis animal model studies were conducted in accordance to the CPCSEA guidelines and aprroved by Institutional Animal Ethical Committee (IAEC/KU/BT /13/10). The study had 4 groups with 2 animals in each group and used both eyes of each animal. From the acclimatized ten rabbits housed for study, two were caged uninfected and named Group I (uninfected). The other eight animals were individually infected on both eyeballs with *S. aureus* log phase culture. The animals were held at ease and the lower eyelid was gently made as cavity, into which 50 μL of 8-hour old log phase culture of *S. aureus* containing 100 CFUs was instilled and held for few seconds, ensuring the culture spread on the entire eyeball and not spilled out.

After 24 h, the 8 infected rabbits' eyeballs were examined, and six animals who's both eyeballs had inflammation (reddening) were chosen and randomized into 3 groups[8].

Treatment was initiated after this infection development (Day 2). Animals in Group II were treated with normal sterile saline (negative control/untreated group); animals in Group III were treated with commercial eye drop containing ciprofloxacin (0.3% w/v) (positive control) and animals in Group IV were treated with *Streptomyces* extract resuspended in buffer (test group).

All these 3 groups received 50 μL treatment solution/eyeball, respectively. From Day 2 to Day 5 before administration of drug, the rabbits' eyeballs were observed daily for ocular inflammation. Scoring was done by observing the symptoms, such as mucopurulent discharge, chemosis, conjunctival congestion and palpebral fissure.

2.8. Data analysis

The ophthalmic scores were expressed as mean ± SD. The data was evaluated by One-way ANOVA followed by Dunnett's *t*-test for comparison of multiple variations by using SPSS/16 software. The values of $P < 0.05$ and $P < 0.01$ were considered as significant.

3. Results

3.1. In vitro antimicrobial activity

From the preliminary screening, 4 actinomycetes (RAM25C4, RAM24C2, RAM25B4 and RAM23C1) which exhibited the maximum antimicrobial activity against *S. aureus* were taken for further secondary screening. In secondary screening, among the four actinomycetes extracts, ethyl acetate extract of RAM25C4 identified as *Streptomyces bacillaris* (*S. bacillaris*) showed good zone of inhibition (34 mm) against *S. aureus,* followed by RAM24C2 (14 mm), RAM23C1 (13 mm) and RAM25B4 (7 mm) and controlled the growth of *S. aureus*. Especially RAM25C4 extract had better activity than the commercial antibiotic ciprofloxacin.

3.2. Antibiotic susceptibility of the ophthalmic pathogen S. aureus

The *S. aureus* was tested against eleven different antibiotics. It is found sensitive to the ciprofloxacin, chloramphenicol and tetracycline and showed intermediate response to the amoxyclav, ceftazidime, gentamicin, norfloxacin and nitrofurantoin. But it is resistant to the antibiotics methicillin, nalidixic acid and kanamycin (Table 1). Ciprofloxacin was used as the standard (positive control) to compare the results of actinomycetes antimicrobial activity.

3.3. Determination of stability and shelf life of the RAM25C4 ethyl acetate extract

The RAM25C4 ethyl acetate extract showed different degree of antimicrobial activity when subjected to storage at different temperatures. The RAM25C4 extract was stable till 40 ℃, but the maximum activity was observed when stored at –20 ℃ to 30 ℃. There was no inhibitory activity when stored above 40 ℃. The results showed that increasing the temperature above 30 ℃ decreased the activity (Figure 1). The extracts stored at fridge and room temperature

retained their activity for the entire duration of testing, but when stored at room temperature, reduction in activity with days was observed. The extract stored at 8 ˚C retained the activity for the entire testing period, but after 15 days the activity was slightly reduced. The activity started reducing after 15 days onwards for the extract stored at 30 ˚C. The stability of the extract was higher when stored at 8 ˚C than 30 ˚C(Figure 2).

Table 1

Antibiotic susceptibility of ophthalmic pathogen *S. aureus*.

Antibiotic discs	Zone of inhibition (mm)	Susceptibility
Amoxyclav	16	Intermediate
Ceftazidime	17	Intermediate
Chloramphenicol	22	Sensitive
Gentamicin	15	Intermediate
Nalidixic acid	0	Resistant
Methicillin	0	Resistant
Nitrofurantoin	16	Intermediate
Norfloxacin	15	Intermediate
Tetracycline	21	Sensitive
Kanamycin	0	Resistant
Ciprofloxacin	19	Sensitive

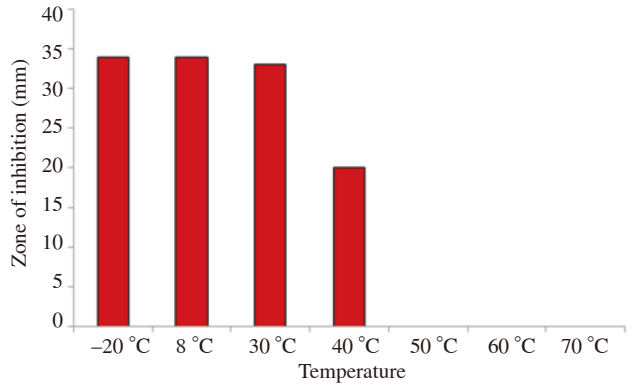

Figure 1. The antibacterial activity of RAM25C4 ethyl acetate extract at different temperatures.

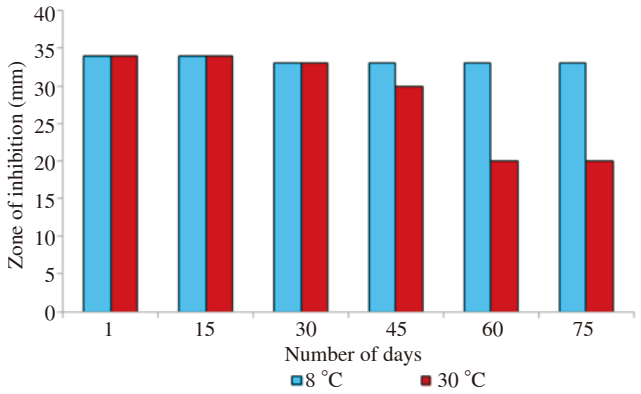

Figure 2. Antibacterial activity of RAM25C4 ethyl acetate extract in shelf life test.

3.4. Anti-inflammatory effect of Streptomyces formulation

On the second day of infection, the infected rabbits developed inflammatory symptoms such as conjunctival congestion, chemosis, palpebral fissure and exudates formations with eyelids sticking together (Figure 3). On the second day, there was no difference observed among the treatment groups. The treatment was started from the second day onwards and it continued for five days. Ocular inflammation was scored on Day 2 till Day 5 after infection. The inflammation scoring results based on the disease symptoms were recorded and shown in Figure 4.

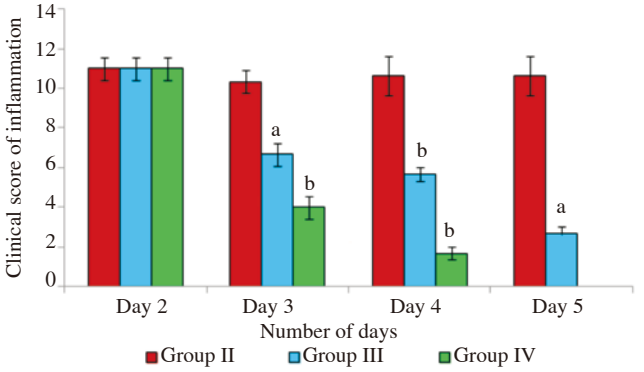

Figure 4. Clinical scores of inflammation and anti-inflammatory effect of *Streptomyces* formulation versus ciprofloxacin ophthalmic solution in *S. aureus*-induced conjunctivitis. Values are expressed as mean ± SD, $n = 4$. [a]: $P < 0.05$ compared with Group II; [b]: $P < 0.01$ compared with Group II.

The clinical score results revealed that on the first day after treatment, significant differences were observed between the treated and untreated groups. On the other hand, significant differences were observed between the treatment groups till the end of the treatment. Compared to the untreated group, the clinical cure effects increased day by day in the treated groups. It is also observed that the formation of exudates stopped considerably in the group treated with *Streptomyces* formulation after the first day of treatment. But till the end of the treatment, little exudates formation was observed in the saline-treated and ciprofloxacin-treated rabbits. After three days, the untreated group showed beefy red conjunctiva. However, the eyes treated with ciprofloxacin and *Streptomyces* formulation showed total absence of conjunctival congestion. The clinical signs such as redness, eyelid edema and mucopurulent discharge were reduced in the treated rabbits. *Streptomyces* formulation exhibited statistically significant increased efficacy against *S. aureus* after three days of treatment in rabbits (Figure 5).

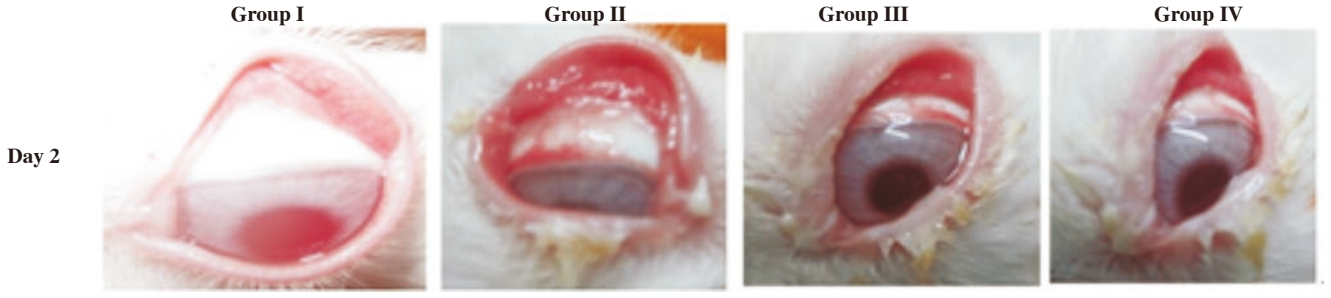

Figure 3. Development of bacterial conjunctivitis in different animal groups after infected with *S. aureus*.

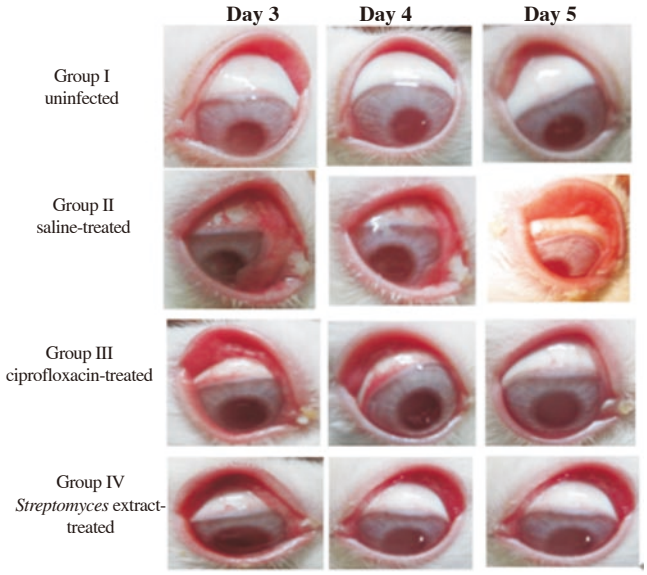

Figure 5. Stages of curing conjunctivitis in different groups.

4. Discussion

The present study reveals the efficacy of the marine actinomycetes RAM25C4 extract (*S. bacillaris*) in controlling infective conjunctivitis. Although many drugs are available to treat conjunctivitis[9], there is no report on the *S. bacillaris* extract against either *S. aureus* or *S. aureus*-induced conjunctivitis in rabbit model.

Here we compared the efficacy of marine actinomycetes *S. bacillaris* extract with that of ciprofloxacin in controlling *S. aureus*. *In vitro* antibacterial activity and *in vivo* anti-conjunctivitis effect on *S. aureus*-induced conjunctivitis in animal model were evaluated. The results demonstrated that RAM25C4 extract had potent antimicrobial activity against the ocular pathogen *S. aureus*. The antimicrobial activity exhibited by RAM25C4 extract is better than the other isolates extracts and the commercial antibiotics tested. The RAM25C4 extract was more effective than ciprofloxacin in controlling *S. aureus*.

The optimum storage temperature of the extract was identified between –20 ˚C and 30 ˚C and the maximum shelf life of the extract was found to be 75 days. The active principles may be heat-labile compounds which might be denatured by storage above 40 ˚C, and the active principles may be protected and shelf life prolonged by mixing the extract with some ophthalmic ointment base. The ophthalmic pathogen *S. aureus* was found to be resistant to methicillin, nalidixic acid and it showed intermediate resistance to other antibiotics while it was well inhibited by the RAM25C4 extract.

The infective conjunctivitis therapy includes the topical application of any effective antimicrobial agents or antibiotics against ocular pathogens[10]. The anti-conjunctivitis efficacy of the extract was evaluated by monitoring the symptoms and scoring of each group. The clinical score results revealed, on the first day after treatment, significant differences between the treated and untreated groups. The group treated with ciprofloxacin ophthalmic solution showed that the exudate formation gradually reduced from Day 3. But in the group treated with *Streptomyces* extract, there is a considerable change from Day 2, the day of extract administration. The extract significantly inhibited the ocular inflammation and the eyes became almost clear. Both in ciprofloxacin and *Streptomyces* treated groups the conjunctival congestion was absent. This indicated that the presence of some antibacterial principles in ciprofloxacin and *Streptomyces* extract has avoided further colonization, protecting from further damages to the tissues. The anti-inflammatory activity and the absence of exudates formation in the *Streptomyces* extract treated group indicated that there may be some anti-inflammatory and mucolytic principles in the extract[11].

In other studies, the topical application of ophthalmic antibiotic solution and test solution has been administered four times a day or five times a day[12,13], while in our study with the minimum administration of drug twice a day we could find complete recovery from inflammation on the second day in RAM25C4 treated group and on third day in the ciprofloxacin treated group.

In conclusion, the separated and purified active molecule responsible for the antibacterial and anti-inflammatory properties may serve as a rational agent for the treatment of infective bacterial conjunctivitis in human. Further extensive studies are warranted in various models.

Conflict of interest statement

We declare that we have no conflict of interest.

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