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Isolation of 1,4-naphthalenedione, an antibacterial principle from the leaves of *Holoptelea integrifolia* and its activity against β-lactam resistant *Staphylococcus aureus*

N V Vinod, M Haridas and C Sadasivan*

Department of Biotechnology and Microbiology
Kannur University, Thalassery Campus, Palayad P O
Kerala 670 661, India

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Antimicrobials derived from plants have been receiving increasing attention in recent years. Antimicrobial activities of a number of phytochemicals have been reported. Many present day antibiotics are ineffective against several pathogenic organisms. About 90% of *Staphylococcus aureus* isolates from clinical specimens is reported to have resistance against β-lactam antibiotics. In the present study, the effect of hexane, diethyl ether, acetone and water extracts of leaves of a medicinal plant *Holoptelea integrifolia* has been tested against β-lactam resistant strain of *S. aureus* in presence of antibiotics such as ampicillin, amoxicillin, cefotaxime and ceftriaxone. The diethyl ether extract has shown the maximum antibacterial activity and the active principle is found to be 1,4-naphthalenedione which is characterized by GC-MS and FTIR spectroscopy. The minimum inhibitory concentration (MIC) of the compound is found to be 4 mg/ml. Structural similarity of this compound with a functional group of a β-lactamase-resistant antibiotic indicates that 1,4-naphthlenedione may be acting as an inhibitor to β-lactamase.

Keywords: *Staphylococcus aureus*, Antibacterial activity, *Holoptelea integrifolia*, β-Lactam antibiotics, GC-MS, FTIR

Plants have been valuable source of natural products for maintaining human health[1]. According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs[2]. About 80% of population of developed countries use traditional drugs derived from the plants[3]. Medicinal properties of such plants need to be explored for developing better and more efficient medicines with fewer side effects.

The use of phytochemicals as natural antimicrobial agents is gaining popularity. A number of studies proving the antibacterial properties of plant extracts have been reported[4]. Many plant secondary metabolites have found applications as natural antimicrobial agents[5]. However, very little is known about the molecular level mechanism of their actions. The antimicrobial compounds reported from medicinal plants include proteins, peptides, and phenolic compounds[6]. Antimicrobial properties of tannins, glycosides and essential oils from several plants have been reported[7]. The essential oils and their components are known to be active against a wide variety of microorganisms, including both Gram-negative and Gram-positive bacteria[8]. Their high antimicrobial activity is assigned to different terpenoids and phenols[9].

The problem of antibiotic resistance has been reported from many parts of the world[10]. Bacteria have genetic ability to acquire and transmit the resistance[11]. About 90% of isolates of *Staphylococcus aureus* from clinical specimens have been reported as resistant to β-lactam antibiotics[12].

In the present study an antibacterial compound has been isolated from the leaves of *Holoptelea integrifolia* (Family: Ulmaceae) and tested against a β-lactam resistant strain of *S. aureus* in presence of antibiotics such as ampicillin, amoxicillin, cefotaxime and ceftriaxone. The plant is commonly found in deciduous forests throughout India and is known as 'Aval' in local language, Malayalam. The bark and leaves of the plant are also reported to be anti-inflammatory, carminative, anthelmintic, depurative and urinary astringent. They are useful in vitiated conditions like dyspepsia, vomiting, skin diseases and diabetes[13]. However, this is the first report of a compound from *H. integrifolia* showing antibacterial property.

Materials and Methods

Bacterial strain and reagents

S. aureus strain was obtained from the culture collection of the Institute of Microbial Technology (IMTECH), Chandigarh, India. The cells were found to be resistant to penicillin G and chloramphenicol. They were sub-cultured repeatedly in tryptone soy broth medium containing different β-lactam

*Author for correspondence:
E-mail: c_sadasivan@rediffmail.com
Tel: +91 490 2347394

antibiotics such as amoxicillin, ampicillin, ceftriaxone and cefotaxime. The concentration of antibiotics was gradually increased up to 5 µg/ml of broth. The bacterial cell concentration in the tryptone soy broth medium was adjusted to 10^8 CFU/ml by using 0.5 McFarland turbidity standards. Each bacterial suspension was plated on Muller-Hinton agar (MHA) medium. The antibiotic resistance was tested also using standard antibiotic discs[14]. The cells were found to be resistant to antibiotics, such as ampicillin, amoxycillin, ceftriaxone and cefotaxime. The media were purchased from Sisco Laboratories and the antibiotic discs were from Himedia.

Extraction of active principle

The leaves of *Holoptelea integrifolia* were collected from Kannur district of Kerala, India in June, 2007. The plant was authenticated by an expert in the field of Botany and the specimen was deposited in the Calicut University Herbarium (CALI), University of Calicut, Kerala. The leaves were crushed and extracted with hexane, diethyl ether, acetone and water using a Soxhlet apparatus. The solvents were evaporated to obtain the dry fractions and the yield was found to be 0.8, 1.0, 1.7 and 2.4%, respectively.

The dry fractions were made into a suspension using 10% dimethylsulphoxide (DMSO) in distilled water. The concentration of the material was made 1 mg/ml. The antibacterial activity of the extracts was studied by disc diffusion assay. The filter paper (Whatman No. 1) discs of 6 mm diameter were soaked in 20 µl of the extracts and dried in an incubator at 40°C to remove the solvent. MHA plates containing ampicillin, amoxicillin, ceftriaxone and cefotaxime were prepared. The concentration of antibiotics in the plate was 4 µg/ml. The plates were inoculated with the bacterial cell culture of concentration 10^8 CFU/ml as described previously[14]. Paper discs soaked in 10% DMSO and dried in an incubator at 40°C were used as the control. For each extract, six separate discs loaded with about 50 µg per disc were used and the average value of the diameters of inhibition zones was taken and the results are given in Table 1. The diethyl ether extract showed the maximum resistance and was fractionated further to identify the antibacterial compound.

Silica gel chromatography was performed to separate different components of the extract and the mixture of hexane and ethyl acetate of varying ratio was used to elute the fractions. Purity of the components was tested using thin layer chromatography (TLC). Each fraction was tested for antibacterial activity using disc diffusion method as described above and the compound in the fraction showing the maximum activity was characterized.

Determination of MIC

Minimum inhibitory concentration (MIC) of active principle was determined by serial dilution method. Test-tubes containing tryptone soy broth medium and the active principle of varying concentrations from 128 to 0.125 mg/ml were prepared. 50 µl of the standard test bacterial broth culture was added to each of the test-tubes and the tubes were incubated at 37°C for 24 h. A positive control tube containing only the growth medium and the organism was also set-up. The MIC was found to be 4 mg/ml.

Identification of active principle

GC-MS and FTIR spectroscopic analysis were carried out to characterize the active principle. GC-MS analysis was carried out on a HP Chem Gas Chromatogram fitted with a DB5 silica column interfaced with quadrupole mass selective detector operated by software. The compound was identified based on comparison of the mass spectra with WILEY 275.L database. The spectrum and the library database showed that the compound was 1,4-naphthalenedione. The dry samples were milled with potassium bromide and an FT-IR was taken on a Shimadzu spectrometer to confirm the result.

Results and Discussion

Antibacterial activity of different solvent extracts of the leaves is given in the Table 1. The diethyl ether and acetone extracts showed the maximum activity. Maximum inhibition zone was given by the diethyl ether extract, while water and hexane extracts were found to be ineffective. The purified active principle showed larger inhibition zones in presence of amoxicillin, ampicillin, cefotaxime and ceftriaxone, which might be due to its synergistic effect with the antibiotics. In the absence of antibiotics, the average inhibition zone was only slightly larger than that of the extract. This might be due to presence of negligible quantities of other compounds than the active principle in the extract. The MIC of purified

Table 1—Average diameter of growth inhibition zone (in mm) of different solvent extracts as well as purified antibacterial compound from *Holoptelea integrifolia* against *S. aureus*
[The cells were grown on a MHA plate containing different antibiotics (concentration is given in parentheses)]

	Amoxycillin (4 µg/ml)	Ampicillin (4 µg/ml)	Cefotaxime (4 µg/ml)	Ceftriaxone (4 µg/ml)	No antibiotics
Hexane extract	14	-	-	-	-
Diethyl ether extract	23	21	18	19	20
Acetone extract	15	15	09	08	12
Water extract	-	-	-	-	-
Purified active principle	25	23	24	25	21

active principle was found to be 4 mg/ml, which was comparable to many other antibacterial compounds reported[15]. The active principle was characterized using spectroscopic analysis such as GC-MS and FTIR. The m/z value of the major peak in the GC-MS spectrum was found to be 158 and using the database the compound was identified as 1,4-naphthalenedione. IR spectra also confirmed the result.

This is the first report of the antibacterial principle from the *Holoptelea integrifolia* against β-lactam-resistant *S. aureus*. The study also shows that 1,4-naphthalenedione is contributing to its antibacterial activity. A number of studies have reported the antibacterial activities of naphthalenedione derivatives from plants[16,17]. Nafcillin, a synthetic β-lactamase-resistant antibiotic of the penicillin class possesses a naphthalenyl group attached to β-lactam ring[18]. This indicates that 1,4-naphthalenedione may also act as an inhibitor to β-lactamase, due to the structural similarity and explains the activity of the compound against β-lactam-resistant *S. aureus*.

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