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Antibacterial activity of 3,3′,4′-Trihydroxyflavone from *Justicia wynaadensis* against diabetic wound and urinary tract infection

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ABSTRACT

The present investigation was designed to study the effect of an active compound isolated from *Justicia wynaadensis* against multi drug resistant organisms (MDRO's) associated with diabetic patients. The drug resistant pathogens implicated in wound and urinary tract infection of diabetic patients were isolated and identified by molecular sequencing. Solvent–solvent fractionation of crude methanol extract produced hexane, chloroform, ethyl acetate and methanol–water fraction, among which chloroform fraction was found to be potent when compared with other three fractions. Further, chloroform fraction was subjected to preparatory HPLC (High-Performance Liquid Chromatography), that produced four sub-fractions; chloroform HPLC fraction 1 (CHF1) through CHF4. Among the sub-fractions, CHF1 inhibited the pathogens effectively in comparison to other three sub-fractions. The purity of CHF1 was found to be >95%. Therefore, CHF1 was further characterized by NMR and FTIR analysis and based on the structure elucidated, the compound was found to be 3,3′,4′-Trihydroxyflavone. The effective dose of this bioactive compound ranged from 32 μg/mL to 1.2 mg/mL. Thus, the present study shows that 3,3′,4′-Trihydroxyflavone isolated from *J. wynaadensis* is an interesting biopharmaceutical agent and could be considered as a source of antimicrobial agent for the treatment of various infections and used as a template molecule for future drug development.

Introduction

Infectious diseases are among the leading causes of death globally and are more frequent in patients with diabetes mellitus. The recurrence of microbial complications in diabetic patients is brought about by the hyperglycaemic environment that favours immune dysfunction, neuropathy, reduced antibacterial action of urine, urinary dysmotility and prominent usage of various medications.[1] Common pathologies among diabetes are wound and urinary tract infection (UTI). Wound in diabetic patients is associated with peripheral

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vascular disease, foot ulceration and gangrene that lead to limb amputation.[2] While, UTI is allied with acute pyelonephritis (upper UTI) and emphysematous pyelonephritis necessitating hospital admission.[3] The first line of treatment in these patients is the usage of antibiotics. The choice of antibiotic depends on several factors such as severity of infection, antibiotics used previously and the resistance to antibiotics by the infecting microorganism. However, excessive use of antibiotics is of concern as their overall effect on the patient is unclear. The increasing incidence of antibiotic resistance among human pathogens against conventional antibiotics demands search for alternate modes of treatment.[4]

Medicinal plants have been found to be effective in treating microbial infections associated with diabetes. Bioassay with activity-guided fractionation has provided directions to isolate new bioactive compounds with novel targets to overcome drug resistance among the pathogenic microorganisms.[5] The search for more potent and safer biomolecules has continued to be an important area of active research. Therefore, in this study, we selected *Justicia wynaadensis*, which is known traditionally to possess vital bioactive molecules having various medicinal properties.[6] *Justicia*, the largest genus of Acanthaceae, has approximately 600 species that are found in pantropical and tropical regions.[7] *J. wynaadensis* is endemic to the rainforest region of the Western Ghats.[8] To the best of our knowledge, there are no reports available, that describes the isolation and characterization of any antimicrobial compound from this plant.

In the present study, an attempt has been made to isolate a bioactive compound with potent antibacterial activity against drug resistant microorganisms isolated from wound and UTI of patients with diabetes.

Materials and methods

Multidrug resistance organisms

Isolation and identification of MDRO's from diabetic wound and UTI were performed as follows: briefly, one hundred wound and urine samples each were collected from diabetic patients with random blood glucose levels >140 mg/dl and HbA1c >7.5 percent adopting aseptic conditions. The isolates were cultured on various selective media and identified by biochemical methods.[9] Further, the isolates were cultured on Mueller-Hinton medium, and tested against known antibiotics (Hi Media Laboratories, Pvt Ltd., Mumbai). Penicillin-G, oxacillin, erythromycin, linezolid, co-trimoxazole, vancomycin, cefotaxime, ciprofloxacin, tetracycline, imipenem, amoxyclav, ampicillin, gentamycin, cefuroxime, levofloxacin, norfloxacin, doxycycline HCl, amikacin chloramphenicol, cefoxitin, amphotericin-B, and clotrimazole. The organisms that offered resistance to three or more classes of antibiotics were selected and identified by molecular sequencing.

Molecular identification of the isolates

Bacteria
Genomic DNA was isolated by employing the conventional phenol-chloroform method.[10] Amplification of 16S rDNA was performed using the universal primers 27f (5′-AGAGTTTGATCCTGGCTCA-3′) and 1492r (5′-TACGGCTACCTTGTTACGACTT-3′).[11] The optimal cycling conditions were the following: 95 °C for 5 min; 35 cycles of 94 °C for 15 s, 56 °C for 30 s, and 72 °C for 90 s; and a final extension at 72 °C for 7 min. Each PCR mixture of 50 μL contained 1× Taq buffer (Fermentas, USA), 200 μM each dNTPs, 1.5 mM $MgCl_2$, 0.4 μM of primers, template DNA (100–250 ng) and 1.25 U of Taq polymerase (Fermentas, USA). The PCR products were loaded in 1.5% agarose gel in 1× TAE (Tris acetate EDTA) buffer and were allowed to run at 50 V for 45 min. The gel was stained with ethidium bromide bath (10 μg/mL) and the gel image was captured in a gel doc. DNA sequencing was carried out at Amnion Biosciences Pvt. Ltd., Bangalore.

Collection of plant material

J. wynaadensis was collected in the month of August 2014 from Western Ghats of Karnataka, India and authenticated by a taxonomist. A voucher specimen of this plant material is deposited at the herbarium library, JSS college of Pharmacology, Mysore, India (Accession No.: Jsscp-Pcog-16).

Extract preparation

The leaves were separated from the plant material, washed thoroughly under running tap water and shade-dried on sterile blotters. The ground material (100 g) was soaked in 500 mL of methanol and was placed on a water bath in a sealed container for proper steam effect at 50 °C for 4 h with frequent stirring. The extract was filtered through Whattman filter paper and the filtrate was evaporated to dryness under reduced pressure using a rotavapor (Buchi, Rotavapour R-3, Switzerland).

Bioassay guided fractionation

Fractionation of crude methanol extract
The methanol extract was weighed and reconstituted in methanol: water 1:1 (v/v), subjected for solvent–solvent partitioning with sequential addition of hexane, chloroform and ethyl acetate in the ratio 1:1 (v/v), the extraction was repeated thrice. The solvent fractions were concentrated to dryness in rotavapor to afford four solvent fractions: hexane (HF), chloroform (CF), ethyl acetate (EF) and methanol–water (MF).

Antibacterial activity of four solvent fractions
The pathogens isolated from diabetic wound and UTI were inoculated into 10 mL of sterile nutrient broth and incubated at 37 °C for 24 h. The cultures were aseptically swabbed on sterile Mueller-Hinton agar plates using sterile cotton swab. Twenty-five microliter of each of the four fractions (0.5 mg) was loaded onto the sterile disc placed on the microbial lawn and antibacterial activity was evaluated by measuring the zone of inhibition against the test organisms.

Analytical HPLC analysis
Analytical RPHPLC was carried out in a Shimadzu HPLC system using a C18 column (100 Å, 5 μm, 4.6 mm × 250 mm) containing LC-10AT vp pump, SCL-10A vp system controller, and

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