BRAZILIAN JOURNAL OF MICROBIOLOGY

Clinical Microbiology

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

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ARTICLE INFO

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

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

SPD-10AV vp UV/vis detector. Chromatographic separation was achieved using methanol, acetic acid, and water in the ratio 18:2:80 (v/v) as the mobile phase under isocratic condition. The column was washed with methanol, equilibrated with the filtered and degassed mobile phase for 30 min, and 20 μL of 1 mg/mL standard mixture or 2 mg/mL chloroform fraction (CF) was injected. The sample or standard run was carried out for 1 h at 1 mL/min flow rate and the detection system was set at 280 nm.

Preparative HPLC analysis

Preparative HPLC analysis of the chloroform fraction (CF) was carried using Shimadzu (LC-8A) series HPLC system, DI diode array detector (SP-M10AVP), reverse phase C18 column (20 mm × 250 mm). Samples (2 mL, 30 mg/mL) were injected into the column repeatedly and the reference molecule, quercetin was used as a standard (1 mL, 1 mg/mL). An Isocratic elution method was employed using a mobile phase of methanol, acetic acid and water in the ratio 18:2:80. The flow rate was 10 mL/min and the absorption was measured at a wavelength of 280 and 320 nm. From the gradient elution, four major peaks were obtained at retention time 20.5, 30.1, 37.5 and 57.1 min and were collected, and labelled CHF1 through CHF4. The collected isolated fractions were dried by removing the solvent using rotavapour at 40 °C. The samples were kept in a vacuum desiccator for removal of traces of water and then stored at 4 °C until further analysis.

Antibacterial activity of four fractions obtained from preparatory HPLC

Twenty-five microliter of each of the four fractions CHF1-CHF4 (0.25 mg) obtained from preparative HPLC were loaded onto the sterile disc and placed on the microbial lawn and antibacterial activity was evaluated by measuring the zone of inhibition against the test organisms. Each organism was tested in triplicates.

Characterization of isolated active principle

Among the isolated fractions, CHF1 eluting at 20.5 min was again re-chromatographed under same condition to verify its purity and then characterized by spectroscopic methods as follows.

LC/MS

The analytical LC/MS experiment was performed in Waters Acquity UPLC system coupled with Synapt G2 Si HDMS mass spectrometer. Waters MassLynx and TargetLynx softwares were used for data acquisition and data processing respectively.

LC conditions

Two microlitre of 2 mg/mL CHF1 was injected into Acquity BEH C18 RP column (130 Å, 1.7 μm, 10 mm × 50 mm). Binary gradient containing mobile phase A (0.1% formic acid in water, v/v) and mobile phase B (acetonitrile) was used for chromatographic separation at 15,000 psi and 0.3 mL/min flow rate. The gradient used was as follows: 98% A and 2% B from 0 to 4 min, 2% A and 98% B from 4 to 6 min, and 98% A and 2% B from 6 to 8 min. Column and sample temperatures were maintained at 50 °C and 24 °C respectively; and the run time was set for 8 min.

MS conditions

Time of flight MS (TOF-MS) was used in negative electron spray ionization mode. Capillary, sampling cone and extraction cone voltages were set at 1.8 kV, 40 kV and 4 kV respectively, with a source temperature of 100 °C and pressure of 3.5 mbar. Nitrogen was used as the desolvation gas at 200 °C with a flow rate of 500 L/h and pressure of 1.4 mbar. Helium (damping gas, 180 mL/min, 2 mbar), Argon (trap gas, 2 mL/min, 7.6 mbar) and nitrogen (IMS gas, 90 mL/min, 2.5 mbar) were the gases used in Tri-wave. Collision induced dissociation was used as the fragmentation method at a trap collision energy of 4 eV. Ion mobility separation (IMS) was set on a linear ramp with IMS gas velocity ascent from 300 to 600 m/s, and wave height (V) ascent from 8 to 20. Transfer wave velocity was set at 247 m/s with a wave height of 0.2. TOF was operated between 50 and 1500 m/z with low mass resolution of 4.7 and high mass resolution of 15. Data was obtained in continuum mode with full scan acquisition time of 8 min, scan time of 1 s and interscan delay of 0.024 s.

Minimum inhibitory concentration of the active principle

Minimum inhibitory concentration of the active compound was determined by micro broth dilution technique as per CLSI guidelines. Briefly, the cell suspensions were prepared from bacterial cultures grown on Trypticose soya broth and was adjusted to $1–2 \times 10^5$ cells/mL as per McFarland's standards. The standard reference drug, chloramphenicol (4–246 μg/mL) and the bioactive molecule, 3,3′,4′-Trihydroxyflavone (16–1024 μg/mL) were prepared in Mueller-Hinton broth. Each individual drug concentrations (90 μL) were mixed with 10 μL inoculum in 96 well plate. Chloramphenicol was used as a positive control, while the broth without active compound served as negative control. The above treated bacterial plates were incubated at 37 °C and observed after 24–48 h and optical density was measured at 600 nm in Tecan plate reader. All the tests were conducted in triplicates.

Statistical analyses

Statistical analysis was performed with Graph Pad Prism (Graph Pad Software, Inc.). One-way ANOVA followed by student's 't' test was used for testing potent fraction with other fractions. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ were considered statistically significant. Data are expressed as mean ± SEM.

Results

Species identification of the isolates

The clinical isolates used in this study were identified by 16S rDNA sequence analysis. Fig. 1 shows the single sharp 1500 bp amplified region of the 16S rDNA (Fig. 1A and B). The size of the amplified DNA was confirmed by using a standard marker DNA ladder. The partial 16S rDNA sequences thus obtained
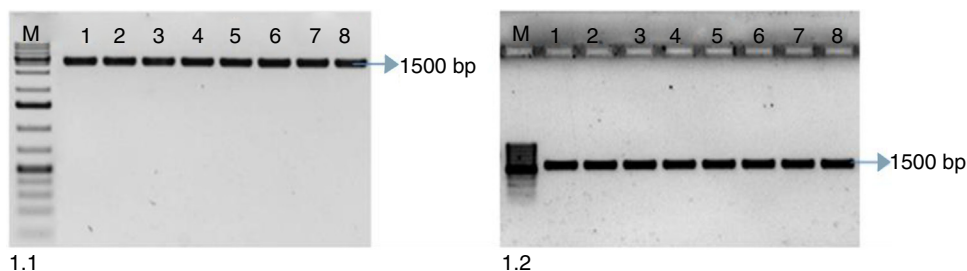
Fig. 1 – Clinical isolates identified by 16S rDNA analysis. (1.1 and 1.2): 1500 bp amplified region of the 16S rDNA of the bacterial isolates from diabetic wound and UTI, respectively.

were retrieved in FASTA format and subjected to BLAST search and deposited in Gen bank under the accession numbers as listed in (Table 1). In total, 16 organisms were identified with 8 organisms each from wound and urine samples.

Bioassay guided fractionation

Fractionation of methanol crude extract and phytochemical analysis

The total yield of crude methanol extract was found to be 5.25 g/100 g of the dry powder. Fractionation of the crude methanol extract (Fig. 2) by solvent–solvent partitioning gave four different fractions: hexane (yield = 47.23%), chloroform (yield = 24.57%), ethyl acetate (yield = 15.23%) and methanol–water (yield = 7.04%). Phytochemical analysis of these four fractions revealed the presence and absence of medicinally important phytoconstituents such as alkaloids, tannins, saponins, terpenoids, flavonoids, glycosides, phlobatannins and steroids. Chloroform fraction had alkaoids, tannins, saponins, flavonoids, glycosides and

steroids whereas, methanol–water fraction had alkaloids, tannins and glycosides. However, the hexane fraction and ethyl acetate fraction were rich in at least one of phlobatannins, terpenoids, glycosides, saponins and steroids.

Antibacterial activity

The four solvent fractions (HF, CF, EF and MF) were tested for their efficacy against the isolated MDROs. The antibacterial activity of the four fractions against a total of 16 isolates is shown in Fig. 3A and B. Chloroform fraction was found to be more potent when compared with other three fractions. It showed maximum activity against *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus epidermidis* and *Klebsiella pneumoniae* and less inhibition against *Staphylococcus haemolyticus* and *Pseudomonas aeruginosa*. Hexane fraction showed maximum activity against *S. haemolyticus* and *E. aerogenes* but a minimum effect was found on other organisms when compared to chloroform fraction. While, ethyl acetate fraction was less effective
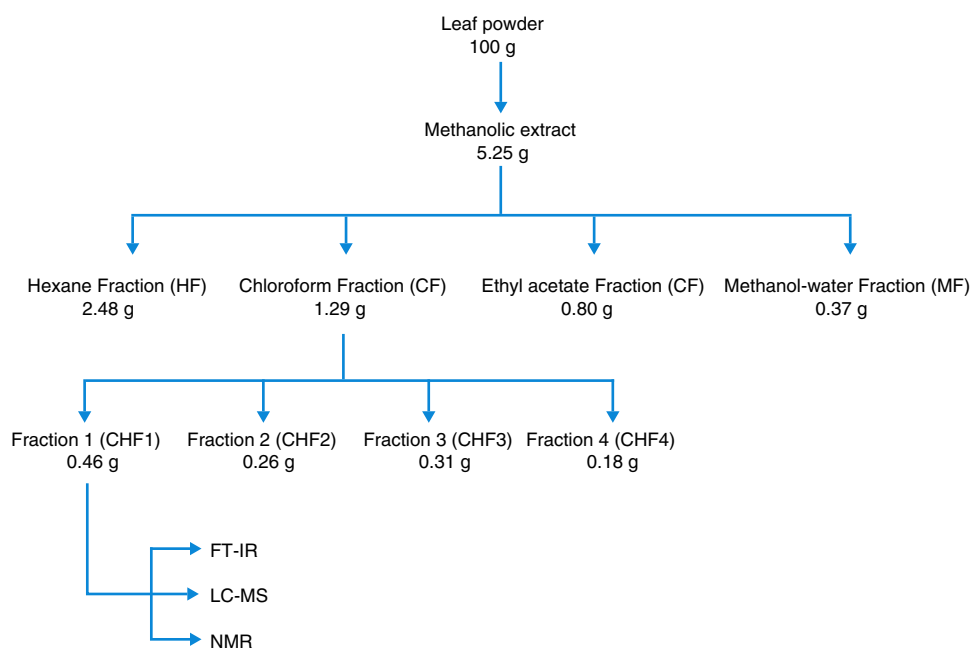

Fig. 2 – Flow chart of bioassay guided fractionation. Crude methanol extract was fractionated by solvent–solvent fractionation followed with separation using preparative HPLC to obtain sub-fractions. The potent fraction is characterized using FTIR, LC–MS and NMR spectral data.

Table 1 – List of drug resistant organisms used in the study.

Type of infection	Gram negative	Gram positive
Diabetic wound	*Pseudomonas aeruginosa* – KY069054 *Klebsiella pneumoniae* – KY069048 *Enterobacter aerogenes* – KY069049 *Escherichia coli* – KY069050	*Staphylococcus aureus* – KX611101 *Enterococcus faecalis* – KY069051 *Staphylococcus epidermidis* – KY069052 *Staphylococcus haemolyticus* – KY069053
Urinary tract infection	*Escherichia coli* – KX816955 *Klebsiella pneumoniae* – KY021157 *Pseudomonas aeruginosa* – KX816953 *Enterobacter aerogenes* – KX878983 *Proteus mirabilis* – KX878984	*Staphylococcus epidermidis* – KX816954 *Staphylococcus aureus* – KY021156 *Enterococcus faecalis* – KY021157

when compared with other two fractions and methanol–water fraction had the least activity against organisms tested from wound samples.

Interestingly, in case of UTI isolates, chloroform fraction showed maximum antibacterial activity. Maximum activity was observed against *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, *E. faecalis* and *E. coli*. Hexane fraction was effective against *E. aerogenes*, *Proteus mirabilis* and *S. epidermidis* but showed least

activity against *K. pneumoniae* and *E. coli*. Ethyl acetate fraction was found to be effective against *E. aerogenes* and *E. faecalis* but a moderate activity was observed for *P. aeruginosa*, *P. mirabilis* and *S. aureus*. However, the methanol-water fraction was not found to be effective against any organisms.

Since chloroform fraction showed potential antibacterial effect, it was sub-fractionated using preparative HPLC which resulted in four Sub-fractions (Fig. 4). The total percentage
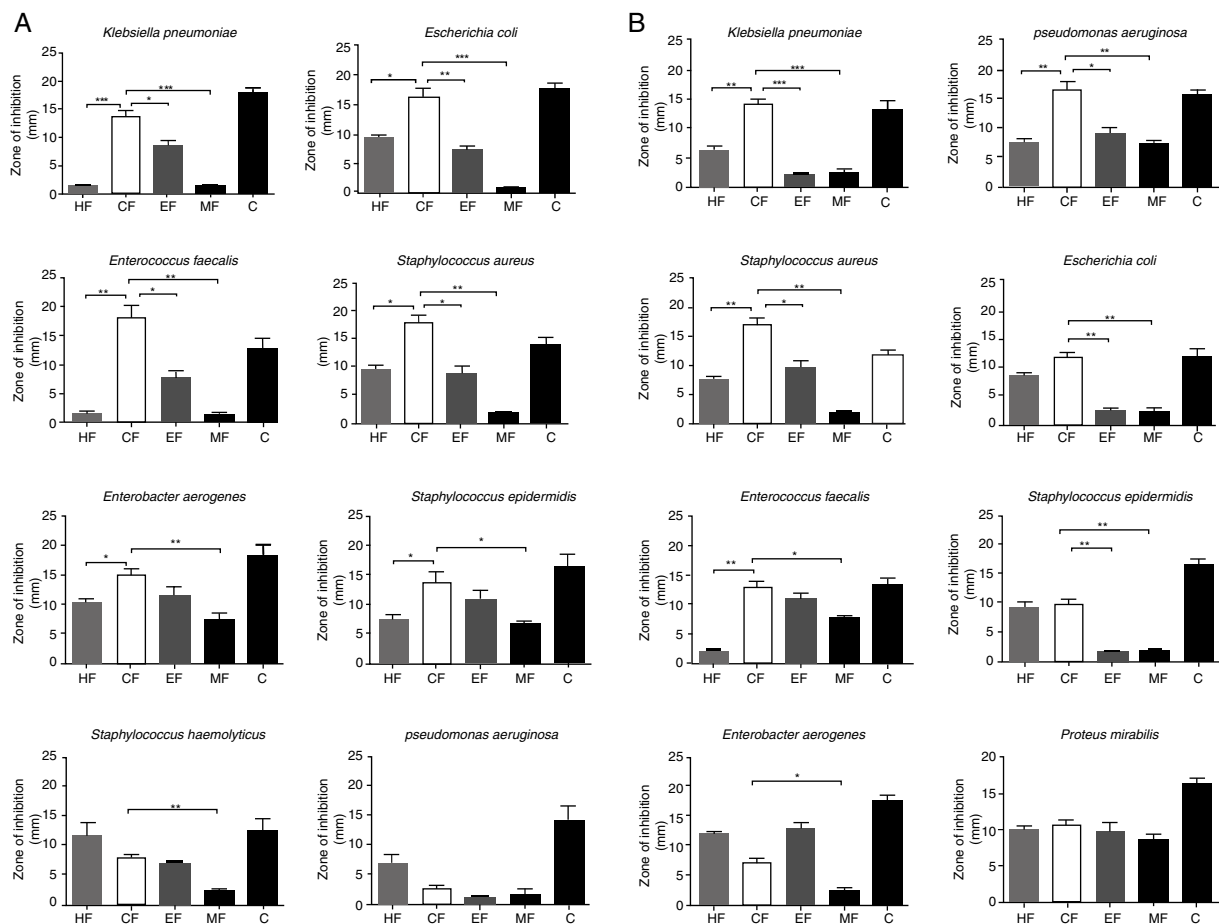

Fig. 3 – (A) Antibacterial activity of four extracts against wound isolates: HF, hexane fraction; CF, chloroform fraction; EF, ethyl-acetate fraction; MF, methanol–water fraction; C, chloramphenicol. CF showed maximum activity against *E. faecalis*, *S. aureus*, *E. coli*, *E. aerogenes*, *S. epidermidis* and *K. pneumoniae* and less inhibition against *S. haemolyticus* and *P. aeruginosa*. Data are represented as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, CF compared with HF, EF and MF. (B) Antibacterial activity of four extracts against UTI isolates: HF, hexane fraction; CF, chloroform fraction; EF, ethyl-acetate fraction; MF, methanol–water fraction; C, chloramphenicol. Chloroform fraction (CF) showed maximum activity against *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, *E. faecalis* and *E. coli* and less inhibition on *P. mirabilis*, *S. epidermidis* and *E. aerogenes*. Data are represented as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, CF compared with HF, EF and MF.
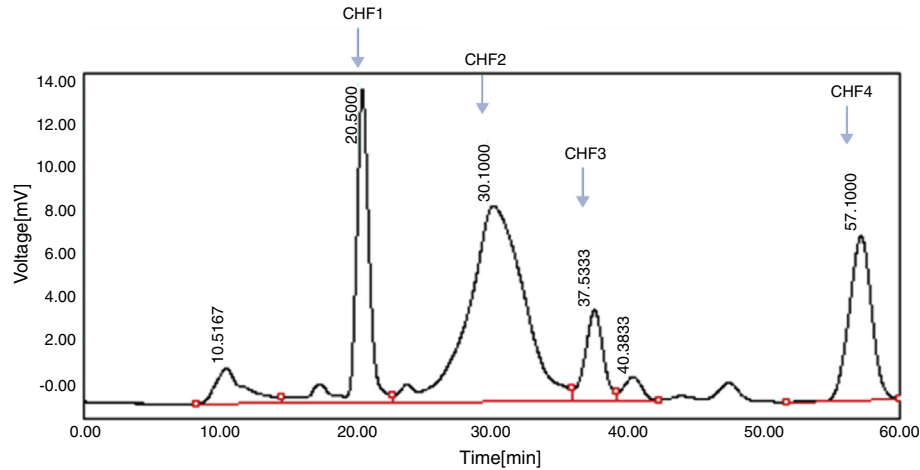
Fig. 4 – Sub-fractionation of chloroform fraction using preparative HPLC. Four different sub-fractions were obtained from chloroform fraction (CF) at a retention time of 20.5 min (chloroform HPLC fraction 1, CHF1), 30.1 min (CHF2), 37.53 min (CHF3) and 57.1 min (CHF4).
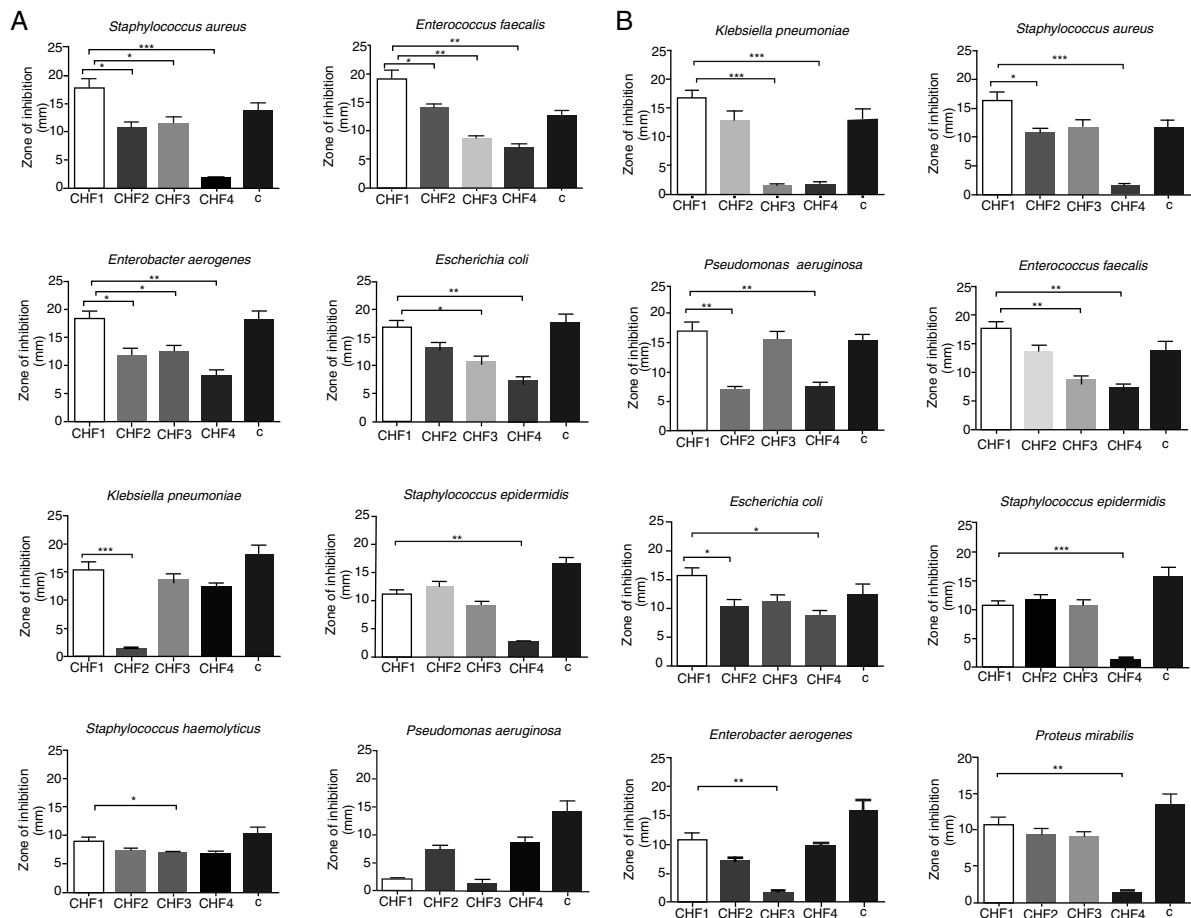

Fig. 5 – (A) Antibacterial activity against diabetic wound isolates (HPLC fraction): CHF1, chloroform HPLC fraction 1; CHF2, chloroform HPLC fraction 2; CHF3, chloroform HPLC fraction 3; CHF4, chloroform HPLC fraction 4; C, chloramphenicol. Maximum activity was offered by CHF1 against *E. faecalis*, *E. aerogenes*, *S. aureus*, *E. coli*, and *K. pneumoniae* and less inhibition on *S. epidermidis* and *S. haemolyticus*. Data are represented as mean ± SD. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, CHF1 compared with CHF2, CHF3 and CHF4. (B) Antibacterial activity against UTI isolates in diabetic patients (HPLC fraction): CHF1 showed maximum inhibitory activity against *P. aeruginosa*, *E. faecalis*, *S. aureus*, *K. pneumonaie*, and *E. coli* and minimum inhibition against *E. aerogenes*, *P. mirabilis* and *S. epidermidis*. Data are represented as mean ± SD. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, CHF1 compared with CHF2, CHF3 and CHF4.

recovery of these sub-fractions; CHF1, CHF2, CHF3, and CHF4 were 35.65, 20.15, 24.03 and 13.95% respectively.

Sub-fractionation of chloroform fraction

Among the four sub-fractions screened for potential antibacterial activity against organisms isolated from diabetic wound (Fig. 5A), CHF1 showed maximum activity against *E. coli, K. pneumoniae* and *E. aerogenes* among gram negative isolates and also for *E. faecalis* and *S. aureus* among gram positive isolates. A moderate inhibition was observed for *S. epidermidis* and *S. haemolyticus*. CHF2 was effective against *E. faecalis, E. aerogenes* and *E. coli*. However, CHF3 and CHF4 were less effective when compared with CHF 1 and CHF2.

We found that, the results obtained from chloroform fraction against microorganism of diabetic wound were similar to that of UTI samples (Fig. 5B). Consistently, CHF1 showed maximum inhibitory activity for *P. aeruginosa, E. faecalis, S. aureus, K. pneumonaie,* and *E. coli*. While, minimum inhibitory effect was observed for *E. aerogenes, P. mirabilis* and *S. epidermidis*. CHF2 was effective against *E. faecalis, K. pneumoniae* and *S. aureus* and a moderate zone of inhibition was observed for *P. mirabilis, S. epidermidis* and *E. coli* but the activity was less when compared to CHF1. Chloroform HPLC fraction (CHF3) was found to be more effective against Gram negative isolates than Gram positive isolates. Chloroform HPLC fraction 4 (CHF4) had least antibacterial effect when compared with other three sub-fractions. It was further subjected to check the purity of the CHF1 through analytical HPLC (Fig. 6A) and the structure of the active compound in CHF1 was identified as 3,3′,4′-Trihydroxyflavone (Fig. 6B) based on the NMR and other spectral data (Fig. 7).

Minimal inhibitory concentration of 3,3′,4′-Trihydroxyflavone

The active compound, 3,3′,4′-Trihydroxyflavone was significantly effective against diabetic wound isolates compared to UTI isolates. It showed an MIC value of 32 μg/mL against *E. faecalis* and *S. aureus*, and 64 and 128 μg/mL *K. pneumoniae, E. aerogenes* and *E. coli*, respectively. While, the concentration of reference antibiotic for the corresponding organisms ranged between 8 and 32 μg/mL. However, we found the MIC value against *S. epidermidis* and *S. haemolyticus* to be 1024 μg/mL. In comparison to pathogens from wound samples, the MIC value of 3,3′,4′-Trihydroxyflavone against *K. pneumoniae, S. aureus, E. faecalis* and *E. coli* was also found to be 64 and 128 μg/mL, respectively. The MIC of the reference antibiotic for these isolates ranged between 4 and 32 μg/mL. However, the MIC of *P. aeruginosa* was found to be 32 μg/mL, and appeared to be relatively greater than that of reference antibiotic.

Discussion

Opportunistic organisms take advantage of low resistance of the diabetic host to infection. The commensals of the body become pathogenic in conditions such as diabetic wound and UTI. Diabetics have severe neutropenia and impaired humoral as well as cellular immunity which may be the
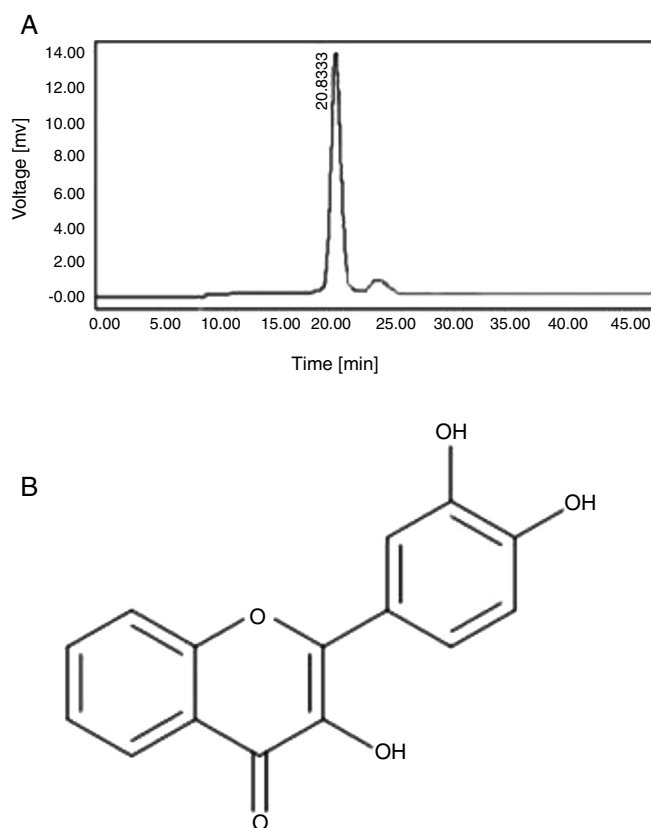
A

B

Fig. 6 – (A) HPLC fraction of CHF1; A single peak showing at RT 20.5 min. (B) Structure of 3,3′,4′-Trihydroxyflavone; IR: 3336 cm⁻¹ OH stretch, 1588 cm⁻¹, 1471 cm⁻¹ aromatic ring stretching, 1416 cm⁻¹ C=O stretch, 1195 cm⁻¹ C–O–C stretch, 1007 cm⁻¹, 781 cm⁻¹, 637 cm⁻¹ aromatic CH stretch,. ¹H NMR (400 MHz, DMSO-d_6, ppm): δ 5.35 (s 2 protons, phenolic OH)δ 6.87–8.07 (m, 7H, phenyl rings), 9.24–9.51 (s, 1H, OH proton). ¹³C NMR (100 MHz, DMSO-d_6, ppm): δ 115.73, 116.0, 118.57, 120.44, 121.75, 122.74, 124.79, 125.1, 133.77, 138.29, 145.52, 146.57, 148.06, 154.81, 172.9. LC–MS m/z 272 (M⁺).

major contributing factor to the cause of infection.[12] Although opportunistic fungi are rare compared with bacteria, they can be the cause of life threatening infections in immune compromised individuals. Thus, the major infective organisms may depend on several factors such as age, gender and severity of infection.

We identified eight different bacterial isolates in wound and UTI of patients with diabetes. Polymicrobial infections were also observed with as many as five isolates in a single individual. While, *S. aureus* was the most common organisms in wound; *E. coli* was most common in UTI. Gram negative bacteria were predominant in both wound and UTI when compared to Gram positive bacteria. This observation is in agreement with studies reported from several investigators.[13–16] The presence of *E. coli* as the most predominant pathogen in diabetic UTI is supported by several studies.[17–20] The major mechanism of drug resistance in UTI among patients with diabetes is the ESBL production by the infecting organisms. In our study, 30.8% of *E. coli* and 44.4% of *K. pnemoniae* were ESBL producers. This was
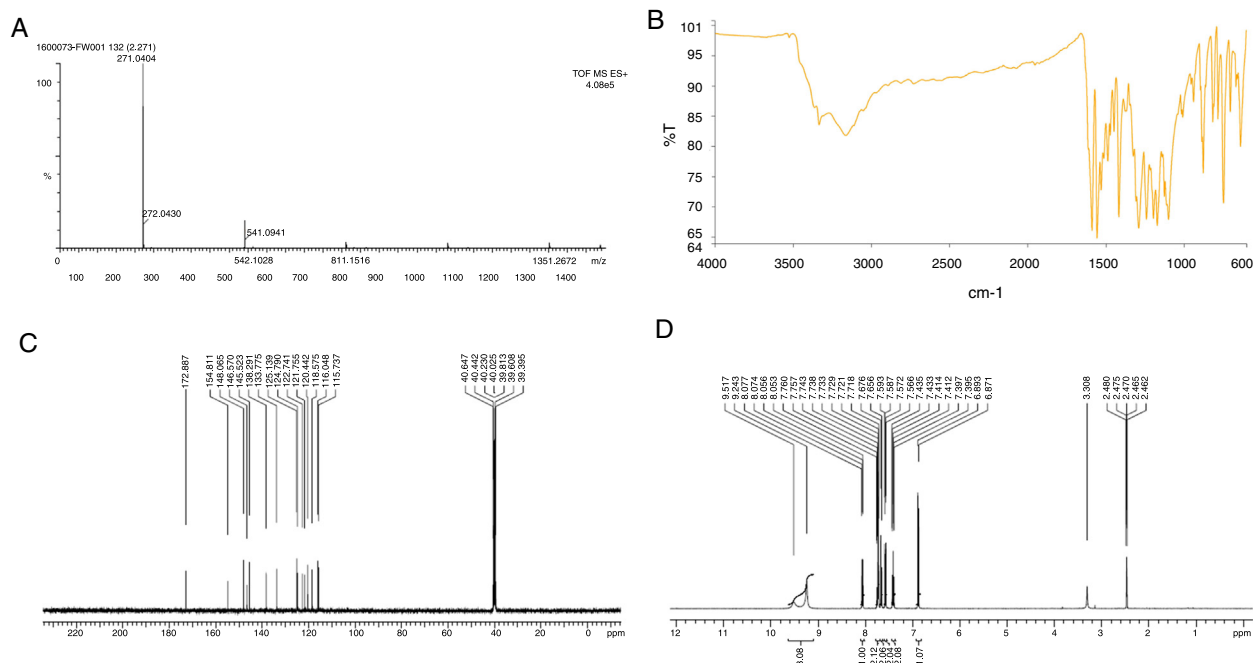
Fig. 7 – Spectral data of 3,3′,4′-Trihydroxyflavone. (A) LC–MS, (B) FTIR, (C) C NMR and (D) H NMR of chloroform HPLC fraction 1.

higher than 26.6% reported by Khurana.[21] The present study also confirms the colonization of MDRO notably by MRSA predominance with over 22% of *S. aureus* being methicillin resistant. This observation agrees with the study of Caputo,[22] who reported 20% MRSA in diabetic wound infections. Inhibition zone among associated bacteria are the great interest to search for antibacterial substances. Isolation and screening for secondary metabolite-producing bacteria have been strongly investigated. There are numerous reports on the antimicrobial activity of crude plant extracts and its bioactive compounds.[23]

In the present study, we have shown the potential of *J. wynaadensis* as a source of natural antimicrobials. The chloroform fraction obtained through solvent–solvent fractionation showed strong antimicrobial activity against most of the tested drug resistant organisms. While the other fractions showed moderate antimicrobial activity. This is likely due to the presence of bioactive molecule in chloroform fraction. The antibacterial activity of the methanolic extract of *J. wynaadensis* against *K. pneumoniae* has also been reported by Ponnamma and Manjunath.[24] In a recent study reported by Subbu,[25] the methanol extract of *Tragia involucrata* was found to be effective against *E. aerogenes*, *P. aeruginosa*, *S. aureus*, *Proteus vulgaris* and *E. coli* of which were isolated from diabetic foot ulcers and UTI, which is in agreement with our results.

We performed further fractionation of the chloroform fraction to obtain four sub-fractions and upon screening for antibacterial properties, we found CHF1 to be highly potent when compared with other three sub-fractions (CHF2, CHF3 and CHF4). This led us to know that CHF1 indeed possesses the bioactive molecule responsible for its potent effects. The maximum inhibitory activity against *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *E. faecalis* could be due to a common mechanism of action. However, the minimum inhibitory effect

of all the sub-fractions observed against *P. mirabilis*, *E. aerogenes* and *S. haemolyticus* suggests that these pathogens might adopt a different kind of resistant pattern in order to multiply in great number. Identifying such resistant pattern will provide new insights to combat such MDROs. Therefore, we subjected CHF1 for its purity through HPLC and found >95% pure. Once the purity was confirmed, characterization and structure elucidation of the isolated molecule was carried out by using different spectroscopic techniques: ^{1}H (400 MHz) and ^{13}C NMR (100 MHz). Comparison of the ^{1}H and ^{13}C NMR along with the FTIR spectrum indicated the presence of hydroxyl group and presence of hydrogen and carbon atoms. The identified compound, 3,3′,4′-Trihydroxyflavone is a flavonoid. The antimicrobial activity of flavonoids has been reported earlier.[26] We speculate that the antimicrobial activity observed in this study is due to the presence of flavonoid. A flavonoid rich plant extracts from different species have been reported to possess antibacterial activity.[27,28] Several flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have been shown to possess potent antibacterial activity.[29] Antibacterial flavonoids may have various cell targets, instead of one particular site of activity. One of their molecular activity is to form complex with proteins through nonspecific strengths such as hydrogen bonding and hydrophobic impacts, and also by covalent bond formation. Hence, their method of antimicrobial activity might be identified with their capacity to inactivate microbial adhesins, compounds and cell envelope transport proteins.[30,31] The antimicrobial activity of 3,3′,4′-Trihydroxyflavone might be due to one or more of the mechanisms of action as mentioned above.

The minimal inhibitory concentration (MIC) of 3,3′,4′-Trihydroxyflavone, the isolated compound from *J. wynaadensis* extract against the tested bacteria are promising. According

to Rios and Recio,[32] MIC value ranging less than 1 mg/mL for crude extracts or 0.1 mg/mL for isolated compounds should be considered effective and also proposed that bioactive compounds that show activity in the range between 0.1 mg/mL (extract) and 0.01 mg/mL (isolated compound) would be of very interesting. While, Gibbons[33] suggests that isolated phytochemicals should have MIC values <1 mg/mL. The MIC of this compound ranging from 32 to 1024 μg/mL suggests that the 3,3′,4′-Trihydroxyflavone hold a lot of promise since the organisms tested were multi drug resistant. And hence, can be derivatized to increase its specificity and potency. The 3,3′,4′-Trihydroxyflavone structure can serve as a useful template for the synthesis of novel anti-microbial agents.

From our study and from other studies, it is evident that emergence of drug resistance is becoming a major obstacle to effective treatment of infections in diabetic individuals. The lack of effective and safe antibiotics to treat serious bacterial infections and lack of new antibiotic drug development by pharma industry is alarming. Consequently, botanicals as well as complementary and alternative medicine are seriously considered to address the issue of emerging drug resistance. The use of traditional medicinal plants for primary health care has steadily increased worldwide. Plants are a rich source of phytochemicals that could be used as antimicrobials and developed as new drugs.

Conclusion

Prevention and cure of infectious diseases using phytochemicals especially flavonoids are well known. The present study shows that 3,3′,4′-Trihydroxyflavone isolated from *J. wynaadensis* is an interesting biopharmaceutical agent against the multi drug resistant organisms isolated from diabetic wound and UTI infections. It 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.

Conflicts of interest

The authors declare no conflicts of interest.

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