International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304, ISSN(Online): 2455-9563
Vol.11, No.03, pp 198-205, 2018

Bacterial Endo-Symbiont Inhabiting *Durio zibethinus leaves* and their Antibacterial Potential

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Abstract : Drug resistance in bacteria has become a global concern and the search for new antibacterial agents is urgent and ongoing. Endophytes provide an abundant reservoir of bioactive metabolites for medicinal exploitation, and an increasing number of novel compounds are being isolated from endophytes. In the present study, endophyte was isolated from the leaves of *Durio zibethinus*. The selected endophyte was identified by 16s rRNA partial genome sequencing and investigated for their antimicrobial activity. The preliminary phytochemical test was conducted for the affirmation of phytoconstituents in the endophytic crude extract (DZLM). Antimicrobial activity was assessed against seven human pathogenic ATCC strains. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were recorded. The selected MIC dose was screened by Kirby-Bauer agar well diffusion method. The pre-screening of DZLM showed the presence of various phytoconstituents. DZLM exhibited the highest MIC and MBC of 250 µg/mL and 500 µg/mL respectively, against *Bacillus subtilis* and *Staphylococcus aureus*. At MIC of 250 µg/mL, DZLM portrayed significant inhibition zone against ATCC strains comparable to gentamicin. This study is the first report about the antimicrobial activity of endophyte residing in *Durio zibethinus* leaves able to produce bioactive agents with pharmaceutical potential and may provide a new lead in the pursuit of new biological sources of drug candidates

Keywords: *Durio zibethinus*, Endophytes, Preliminary screening, Quantitative analysis, Antibacterial.

International Journal of PharmTech Research, 2018,11(3): 198-205

DOI: http://dx.doi.org/10.20902/IJPTR.2018.11301

Introduction

The advancement of resistance by pathogenic bacteria to current medications or antibiotics is a significant issue confronted by global health community[1] and has turned into a global issue[2]. A few variables have led to this situation, for instance, pervasive and unseemly use of antimicrobial drugs, poor hygiene, incline rate of immunocompromised patients and deferral infection's diagnosis[3].

A wide range of microbial species, endophytes that colonize within the plant tissue symbiotically without causing evident harm[4]. Endophytic bacteria have a mutual relationship with the host and deliver compounds that promote the vegetative development, competitiveness followed by assurance against pathogens and herbivores[5]. Endophytes speak to a sizeable assorted quality of microbial adaptations that have developed in peculiar and unordinary conditions, making them an extraordinary wellspring of study and research for new medications for industrial, healthcare and agriculture fields[6,7]. These microorganisms are notable for delivering bioactive secondary metabolites, for instance, lactones, phenols, phenylpropanoids, lignans, isocoumarins, quinones, steroids, terpenoids and alkaloids[8].

Plants utilized as a part of the conventional drug have assumed a vital part in the look for new bioactive strains of endophytes, as it is conceivable that their desirable qualities are an after effect of the metabolites delivered by their endophytes[9,10]. Notwithstanding this potential, a collection of these plants stays to be examined in regards to their endophytic composition especially *Durio zibethinus murr*. Durian (*Durio zibethinus murr*) is known to be as King of fruits in Southeast Asia. Durian pulp has been accounted to have bioactive compounds including carotenoids, flavones, anthocyanins and flavonoids[11,12]. The hull, roots, fruit and leaves have been customarily used for various ailment medicines[13]. In numerous scientific reports, the ethanolic fruit peel extract had appeared to have hyperglycaemic impediment, and ethanolic rind extract had high antioxidants[14].

Materials and Methods

Chemicals

Gentamicin was purchased from Sigma-Aldrich Corporation, USA. All the chemicals used were of analytical grade.

Sample collection

The plant materials of *Durio zibethinus* were collected from Baling, 09100, Kedah. The plant materials, leaves were sampled for the study of endophytic bacterial communities. Healthy and mature plant which shows none visual disease symptoms was carefully picked for sampling. The plant materials were brought in the laboratory in sterile bags and processed within few hours after sampling. The herbarium voucher specimen accession number of *Durio zibethinus* is AIMST/FOP/08.

Isolation of Endophytic Bacteria

The plant materials were rinsed gently in running water to remove superficial injury and soil particles. After proper washing, the samples were cut into small pieces. The isolation of endophytes was done according to the method described by with slight modifications[15]. The surface sterilization was carried out by treating the plant material with 70% ethanol for 30 secs, followed by immersion in 95% ethanol for 10 secs and again in 5% sodium hypochlorite solution for 4 mins. Subsequently, the segments were rinsed four times with sterile distilled water. The samples were cut into at least 3 to 4 mm in diameter and 0.5 to 1 cm in length. In each petri plate, 5 to 6 segments were placed on the nutrient agar petri plates. The efficiency of the surface sterilization procedure was assessed by adding few drops of water from the last wash on the agar plate. The petri plates were incubated at 37^{o}C for 24 h to 36 h.

16S rRNA partial gene sequencing

The PCR amplification of 16S rRNA gene of the selected endophyte strains was done by using the forward primer (Bak ll W-F 5'- AGT TTG ATC MTG GCT CAG -3') and reverse primer (Bak-R 5'- GGA CTA CHA GGG GGG TAT CTA AT -3'). The PCR amplification was carried out in a thermocycler with the

following condition, initial denaturation at 95˚C for 4 mins, cycle denaturation at 9˚C for 30 secs, cycle annealing at 52˚C for 30 secs, cycle extension at 72oC for 30 secs, repeat cycle steps 30 more times, final extension 72˚C for 5 mins. The sequenced DNA data was BLAST analysed by NCBI database to identify the sequence similarity reported gene sequences in GenBank[16].

Bacterial Endophytic Crude Fraction Extraction

The isolated endophytic bacteria were grown as subcultures on nutrient agar petri plates for 24 h. The subculture was inoculated in an Erlenmeyer flask containing 1 L of nutrient broth and then incubated at 110 rpm on orbital shaker at 37oC for 3 to 4 days. After fermentation, the culture broth was added with brine solution and ethyl acetate solvent respectively, with the ratio of 1:3. The culture broth was then extracted with ethyl acetate in a separating funnel by shaking vigorously. The organic phase was filtered after which anhydrous sodium sulphate was added. The ethyl acetate solvent was evaporated by rotary evaporator. The extract was dried and concentrated then was weighed and stored at -4ºC[17].

Preliminary phytochemical screening

DZLM were used for preliminary screening of phytochemicals such as alkaloids, carbohydrates, phenols, amino acids, steroids, anthocyanins, proteins, flavonoids, saponins, mucilage, gums, glycosides and tannins using standard biochemical testing methods[18].

Anti-bacterial Activity

Standardization of Microbial Inoculum

The microorganisms used were four gram-positive bacteria (*Bacillus subtilis*- ATCC 11774, *Enterococcus faecalis*- ATCC 29212, *Staphylococcus aureus*- ATCC 29213, *Streptococcus pyogenes*- ATCC 19615) and three gram-negative bacteria (*Neisseria gonorrhoeae*- ATCC 43069, *Pseudomonas aeruginosa*- ATCC 10145, *Escherichia coli*- ATCC 10799). The strains were maintained on Mueller Hinton Agar at 36 ºC. The inoculums were adjusted to 0.5 McFarland[19].

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum inhibitory concentration (MIC) was determined using the test tube dilution method. 1 ml of Nutrient broth was added to sterile test tubes. 1 ml of 1 mg/ml test solution was transferred in one tube and serially diluted ranging from 31.25 µg/ml to 500 µg/ml. The following test tube, 0.1ml of inoculum was inoculated, and the tubes were incubated at 37ºC for 24 h. The growth of microorganism was monitored using the turbidity of the tubes. MIC was determined with the lowest concentration of extract that suppresses bacterial growth which was determined by the absence of turbidity in the broth[20].

The minimum bactericidal concentration (MBC) was determined by comparing the number of viable bacteria or inoculum with the initial number. Clear tubes from MIC study were diluted further and spread onto a nutrient agar plate and incubated for 24 h at 37°C. Colony forming unit (CFU) was recorded. The lowest concentration of extract that kills at least 99.99% of initial bacteria number is identified as MBC[21].

Agar Well Diffusion Method

The screening for anti-bacterial activity was conducted using agar well diffusion assay[22]. The selected bacterial inoculum was spread evenly onto a fresh Muller Hilton agar plate with the help of sterile cotton swab. 4 wells sized 12 mm in diameter are made into the agar plate above, and 50 µL of the extract was added to each well. These plates were incubated for 24 h at 36ºC ± 1ºC, under aerobic conditions. After the incubation period, bacterial growth was observed, and the diameter of inhibition zones was measured in mm. The referred antibiotic, Gentamicin was used as positive control and 10% DMSO as negative control[23]. The tests were carried out in triplicates.

Results and Discussion

Bacterial endophyte dwells within different plant tissues are generally unexplored as well are proclaimed to have a wellspring of novel natural products to be used in pharmaceutical, industrial and agricultural. The importance of endophytes has portrayed in protecting the host plant against insects and ailments[24]. In this study, endophytes were isolated from *Durio zibethinus* leaves. The surface disinfection of the excised tissue was done to ensure the expulsion of epiphytes. The tissue immersion in both ethanol and sodium hypochlorite has shown significant method in different studies to isolate the endophytes[25]. The small segments of the plant tissue, i.e. leave under aseptic or sterile conditions were transferred to the isolation media. The endophytes were selected and sub-cultured on Lysogeny broth (LB) agar and characterized by16S rRNA partial gene sequencing.

The genome sequence data obtained from 16S rRNA partial gene sequencing was used in the identification of bacterial endophyte. The 16S rRNA sequences nucleotide blast analysis reveals the identities of the sample based on hits analysis from mega blast (highly similar sequences) output. Based on the 16S rRNA sequence nucleotide blast analysis, the closest hit was treated as the identity of the respective endophytes sample. Based on the results obtained through 16S rRNA sequence nucleotide blast analysis, the endophyte identified as *Cronobacter sakazakii*. The 16S rRNA gene fragments nucleotide sequences have been submitted to the GenBank/DDBJ/EMBL under the accession number of MF615204.

The preliminary qualitative phytochemical analysis as appeared in Table No.1, DZLM has various phytoconstituents such as phenol, carbohydrate, alkaloid, flavonoid, steroid, mucilage and glycoside.

Table No.1: Phytochemical analysis of DZLM

Phytochemical Constituents	DZLM
Alkaloid	+
Flavonoid	+
Saponin	-
Steroid	+
Gums	-
Mucilage	+
Glycoside	+
Tannin	-
Protein	-
Amino acids	-
Carbohydrate	+
Reducing sugar	-
Monosaccharides	-
Starch	-
Anthocyanin	-
Phenol	+

Note: (+) = Present and (-) = Absent

The antimicrobial assay of DZLM was tested against human pathogenic ATCC bacterial strains. The results are depicted in Table No.2, DZLM exhibited the highest MIC and MBC of 250 µg/mL and 500 µg/mL against *Escherichia coli.*

Table No.2: The MIC and MBC of DZLM

Tested Microorganisms	DZLM	
	MIC µg/mL	MBC µg/mL
Bacillus subtilis	125	500
Enterococcus faecalis	250	1000
Staphylococcus aureus	125	500
Streptococcus pyogenes	250	500
Neisseria gonorrhoeae	250	500
Pseudomonas aeruginosa	250	1000
Escherichia coli	250	500

At MIC of 250 µg/mL, as depicted in Table No.3, DZLM portrayed significant inhibition zone against *S. aureus*, 10.3±0.58 mm and *B. subtilis*, 10.0±0.58 mm compared to gentamicin 14.7±1.00 mm and 14.0±1.01 mm, respectively.

Table No.3: The inhibition zone of DZLM

Microorganisms	Zone of inhibition (mm, 250 µg/mL) ± SD	
	DZLM	Gentamicin
S. pyogenes	7.8±0.58	10.6±0.58
N. gonorrhoea	6.4±1.00	14.3±1.15
S. aureus	10.3±0.58	14.0±1.00
E. faecalis	7.3±1.15	14.3±0.58
B. subtilis	10.0±0.58	14.0±1.01
P. aeruginosa	8.6±1.00	12.3±1.15
E. coli	8.0±0.58	14.7±1.15

The antimicrobial activity of the crude ethyl acetate extract of bacterial endophyte was studied with the purpose of discovering novel bioactive compounds for therapeutic purpose. Many discoveries have exhibited that endophytic extract has antimicrobial properties[26] and recently considered as next novel source for gaining new bioactive compounds in drug discovery[27–29]. This outcome correlated with the discoveries of different reports where they revealed the endophytic antimicrobial activity[30]. Thusly, 16 out of 203 endophytes depicted antimicrobial action with a broad range against both gram-negative and positive pathogenic strains[31].

The antimicrobial study of DZLM was done by the MIC, MBC, and agar well diffusion method against Gram-positive and Gram-negative human pathogenic ATCC microbial strains. In both MIC and MBC, a serial dilution was carried out to identify the least concentration of extract to display antimicrobial properties. The least concentration of extract that hinders the microbial growth, likewise demonstrated no turbidity in media was known as MIC. Although MBC, the least extract concentration that bactericidal (100%). The agar well diffusion was assayed to test the effectiveness of sample against tested microbial strains in a grown culture by measuring the inhibition zone diameter[32].

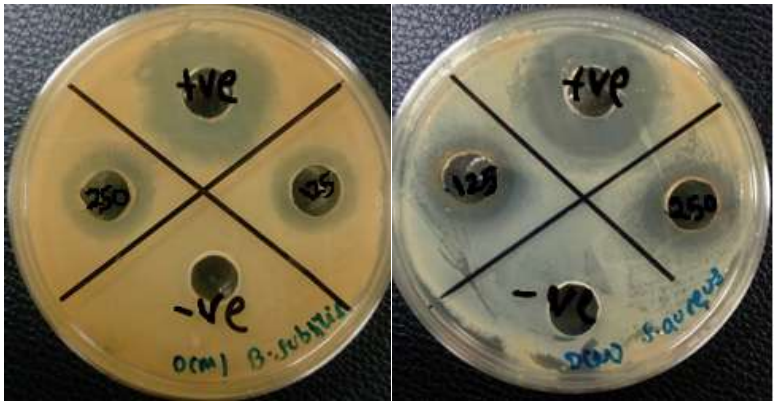

Figure 1: (a) *S. aureus* with a zone of inhibition (10.3 ± 0.58 mm); (b) *B. subtilis* with a zone of inhibition (10.0 ± 0.58 mm)

Conclusion

The present investigation presumes that the existences of bioactive compound in the crude extract of isolated endophyte, *Cronobacter sakazakii* from *Durio zibethinus* leaves has displayed antimicrobial action against various human pathogenic ATCC strains. It justifies saying that the *Durio zibethinus* which harbour the endophytes of the present study have been primarily used in folklore medicine. The endophytes harbours within the host plant hold remarkable potential in producing anti-microbial bioactive compounds. Although fractionation, purification and structural elucidation of the active compounds in the endophytic crude extract will reveal the compounds complexity responsible for its antimicrobial action.

Acknowledgements

Authors are thankful to AIMST University, Malaysia and Qassim University, Saudi Arabia for their support and facilities provided.

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