IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 4, Issue 4 (Jul. – Aug. 2018), PP 21-30
www.iosrjournals.org

Molecular Identification of Endophytic Bacteria from The Stem's Bark of Moringa Plant and Their Antibacterial Activities

Nurul Ilmi[1], Dwi Soelistya Dyah Jekti[1,2], Lalu Zulkifli[1,2*]

[1](*Master Program of Science Education, University of Mataram, Indonesia)*
[2](*Study Program of Biology Education, University of Mataram, Indonesia)*
Corresponding author : Lalu Zulkifli

Abstract : *Moringa oliefera is one of the plants that is scientifically proven to contain secondary metabolites of flavonoids, alkaloids, phenols that can inhibit bacterial activity. Extraction of bioactive compounds from plants is considered inefficient because it requires a large biomass. The effective method is by utilizing endophytic bacteria associated with the plant. The aim of this research were to know the ability of endophytic bacterial isolate of bark of moringa plants in inhibiting the growth of gram-positive and gram-negative bacteria, and to characterize the biochemical and morphologycal properties of the isolated endophytic bacteria, and to determine species of endophytic bacterial isolate based on 16S rRNA. The stage of this research were endophytic bacterial isolation, antibacterial activity test, biochemical and morphological characterization, and molecular identification of the endophytic bacteria isolates based on 16S rRNA barcoding. Four isolates (KK1, KK2, KK3 and KK4) endophytic bacteria were isolated from the bark of moringa stems. Antibacterial activity test, showed that 4 endophytic bacterial isolates were able to inhibit the growth of B. cereus, S. aureus, E. coli and S. marcescen. Based on molecular identification with 16S rRNA, endophytic bacterial isolates of moringa plant stems was very closely related to B. cereus JL., and B. cereus strain ATCC 14579.*

Keywords – *antibacterial activity, endophytic bacteria, stem of moringa plant, 16S rRNA*

Date of Submission: 02-07-2018 Date of acceptance: 21-07-2018

I. Introduction

The world of traditional medicine has long used Moringa for the treatment of various diseases, including the recovery of liver disease and is often used to complement modern medicine in patients with chronic illness including AIDS and HIV. In general, parts of moringa plants, such as leaves, fruits, flowers and roots, have long been used for various purposes in daily life, such as food, medicine, dyes, animal feed and also wastewater purifier [1]. Moringa plant parts such as leaves, seeds, flowers, roots, and stem bark have been shown to be a source of antimicrobial ingredients in medicine [2].

The moringa plant contains antibacterial compounds such as, 4-(α-L-rhamnopyranosyloxy) benzyl isothiocyanate, peterygospermin, 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate [3], whereas in studies conducted by [4], the moringa leaf scouring suspension dissolved in water can be used as an antibacterial. In addition, the results of phytochemical screening showed that the bark of Moringa oleifera contains a class of steroid, flavonoid, alkaloid, phenol, and tannin compounds [5]. Utilization of bioactive compounds from a medicinal plant is generally done by extracting parts of the

plant. Utilization by direct extraction from the plant part is not effective, because if the medicinal plants are constantly taken to be extracted bioactive compounds then the availability of these plants in the environment will decrease. An efficient method for obtaining bioactive compounds is to isolate endophytic microbes capable of producing the number of bioactive compounds required, thus not necessarily extracting the bioactive compounds from their host plants [6].

It is likely that endophytic bacteria that settle in the plant tissue have the ability to synthesize the same antibacterial compounds as their host plants [7], so that endophytic bacteria may have an inhibitory effect on the growth of pathogenic bacteria. Endophytic bacteria are bacteria living in host tissue without causing symptoms [8]. Endophytic bacteria are known to produce antibacterial active compounds [9].

Several studies have shown that endophytic bacteria isolated from medicinal plants have the ability to inhibit the growth of pathogenic bacteria, for example endophytic bacteria isolated from red betel plants [10], Annona squamosa [11], cloves plants cloves [12].

Endophytic bacteria are bacteria living in host tissue without causing symptoms (Bhore and Sathisha 2010). Endophytic bacteria are known to produce antibacterial active compounds [9].

The aim of this study was to know the ability of endophytic bacteria isolates obtained from the bark of the moringa plants in inhibiting the growth of gram-positive bacteria (*B. cereus, S. aureus*) and Gram-negative bacteria (*E. coli, S. marcescen and P.mirabilis*), and (ii) to determine endophytic bacterial isolate species based on 16S rRNA.

II. Material Methods

2.1.Isolation of endophytic bacteria

The surface of the bark of the moringa plant in fresh condition was washed with running water and cut into ± 3 cm length, sterilized with 70% alcohol, 4% NaOCl, rinsed twice with aquades. Sterilized samples were cut 0.5 cm long and then grown in NA and TSA media, incubated at 32 ° C and observed daily until a bacterial colony appeared on the media. The endophytic bacteria colonies grown were separated from other colonies and re-planted on NA medium and incubated at 32 ° C for 24 hours. The growing bacterial isolates were then purified repeatedly to obtain pure isolates [13]

2.2. Antibacterial activity test of the endophytic bacteria

For the antibacterial activity test, two oses of endophytic bacterial isolate were inoculated into 10 ml of NB medium, incubated at 32^0C at a 48-hour incubator-shaker device, and centrifuged at 5000 g for 30 minutes to obtain supernatant from endophytic bacteria. Meanwhile, the refluxed pathogenic bacteria were diluted by mixing 1 ose of the suspension of pathogenic bacteria into a test tube containing 0.9% NaCl. Homogenized using a vortex and turbidity standardized with a concentration of 10^{-9} CFU. Inhibitory test was performed by the well method on MHA media, and used ciprofloxacin as a positive control and aquades as a negative control [14]

2.3.Biochemical and morphology characterization of the isolates

Some of the biochemical tests performed in this study include Triple Sugar Iron (TSI) test, Simmon citrat test, urea hydrolysis test, motility test, carbohydrate test (Glucose, Sucrose, Lactose, Maltose, Mannitol). Gram staining was also done to determine the cell morphology of the isolates.

2.4. Molecular identification of endophytic Bacteria

DNA extraction was performed by adding 1 ose of endophytic bacteria sample into 200 µl DNA ZOL buffer (Kit) and vortexed for 1 min, 100 µl of 95% ethanol added and allowed to stand for 5 min. The suspension was then centrifuged at 12,000 g for 4 min and washed 2 times with 200 µl of

75% ethanol and allowed to stand for 4 min. Centrifuged at 9000 g for 2 min, and dissolved in 40 µl aquades and stored at -200C. 16S rRNA gene amplification was performed using a universal primer 16S-rRNA, 63F primer (5'-CAG GCC CAC TAA GTC ATG CAA) and 1387R (5'-CGG CGG GGC CAA GTA WGT) [15]. The PCR reaction composition were 2x PCR Master Mix Solution 10 µl, 2 µl DNA template, 1 µl primary 63F, 1 µl Primer 1387R, and added aquades up to a total of 20 µl.

DNA amplification was performed using My Cycler (Bio Rad) tool. Pre-PCR conditions were set at 94 ° C for 10 min, followed by 38 PCR cycles consisting of denaturation at 94 ° C for 30 s, annealing at 55 ° C for 30 s and extension at 72 oC for 45 seconds. After 35 cycles, Post PCR was performed at 72oC for 10 minutes and at 20 ° C for 1 minute [16].

The PCR product was electrophoresed at 2% agarose gel at a voltage of 100 V and a current strength of 400 A for 30 minutes. The marker in use is 100 bp DNA Ladder (Invitrogen). The electrophoresis results were visualized under ultraviolet light and photographed using Doc Gel Doc (Bio Rad). The obtained PCR product is subsequently sequenced, the sequence data is edited using Clustal W in the MEGA 7 program and the results are compared with the existing sequences in GenBank by using the BLAST search facility on the NCBI website (http://www.ncbi.nlm.nih.gov) [16].

III. Results and Discussion

3.1 Aantibacterial activity test of endophytic bacteria isolates

Four endophytic bacterial isolates were obtained from the bark of moringa plant, ie: KK1, KK2, KK3 and KK4. The endophytic bacteria isolates obtained were then tested for inhibitory power (antibacterial activity test). Screening of endophytic bacterial isolates capable of inhibiting the growth of pathogenic bacteria was performed using a pouring plate method. The results of the endophytic bacterial inhibition resistance test of moringa (KK) stem bark showed that all isolates had inhibitory effect on bacterial growth of *B. cereus, S. aureus, E. coli* and *S. marcescen*. KK3 has no inhibitory power to E. coli and on P. mirabilis bacteria. Meanwhile, all isolates have no inhibitory power against P. mirabilis. The antibacterial test results were demonstrated by the formation of a clear zone around the test bacterial colony (Fig. 1) and an antibacterial assay test that had no inhibitory absence of a clear zone formed (Fig. 2). Isolates of endophytic bacteria capable of producing clear zones are presented in Table 1.

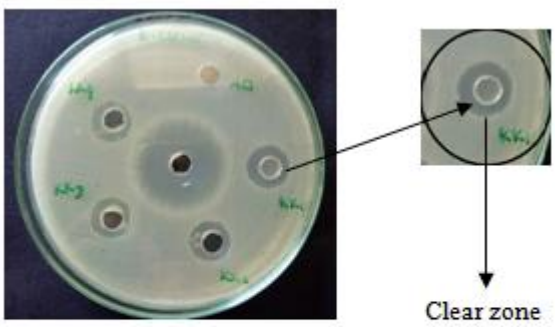

Figure 1. Results of Antibacterial activity test of endophytic bacterial isolated from the bark of stem of moringa plant (KK) to Bacillus cereus. Control (+) = ciprofloxacin and Control (-) = aquades)

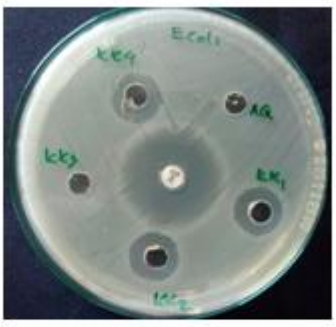

Figure 2. Results of antibacterial activity test of endophytic bone bacteria isolate from Moringa plant (KK) to E. coli. (Ket: control (+) = ciprofloxacin and control (-) = aquades)

The results of the endophytic bacterial (KK1, KK2, KK3, KK4) inhibition test showed that all isolates had inhibitory effect on bacterial growth of Basillus cereus, Staphylococcus aureus, Escherichia coli and Seratia marcescen. KK3 has no inhibitory power to E. coli and on P. mirabilis bacteria. Meanwhile, all isolates have no inhibitory effect against *P. mirabilis* (Table 1)

Tabel 1. Diameter of inhibition zone of endophytic bacteria isolate from stem of moringa plants against pathogenic bacteria

No	endophytic bacterial isolate	Average of inhibition zone diameter (mm)					
		KK1	KK2	KK3	KK4	Control (+)	Control (-)
1	*S.aureus*	13	14	14	14	38	-
2	*B. cereus*	12	12	13	13	28	-
3	*E. coli*	15	15	-	14	25	-
4	*Seratia marcescen*	10	13	12	16	22	-
5	*P.mirabilis*	-	-	-	-	34	-

Note : KK = isolates of endophytic bacteria from the stem of the moringa plant Control (+) = *ciprofloxacin*; Control (-) = aquades

The isolate of endophytic bacteria from the bark of moringa plant stem obtained was 4 isolates, all of which had inhibitory effect on the B. cereus, S. aureus, Seratia marcescen and E. coli test bacteria except KK3 had no inhibitory effect against E. coli and all isolates KK1, KK2, KK3 and KK4 do not have inhibitory power of P. mirabilis. The formation of clear zones around the isolate colonies of endophytic bacteria indicates the presence of inhibitory power. According [17], that the formation of clear areas around the colonies of bacterial isolates endofit indicate the presence of antibacterial compounds that are able to kill or inhibit the growth of pathogenic bacteria. Bacterial inhibitory test is a unit of method to determine the degree of susceptibility of bacteria to anti-bacterial substances or to know the pure compounds that have anti-bacterial activity [18]. Endophytic bacteria can inhibit the growth of test bacteria by inhibiting bacterial cell wall synthesis, disrupt bacterial metabolism, disrupt bacterial cell membrane permeability, inhibit bacterial protein synthesis and destroy bacterial nucleic acid synthesis [19].

The results of endophytic bacterial inhibition resistance test showed that endophytic bacterial isolates from the bark of moringa plant stem have strong antibacterial potency. The strongest inhibitory of 16 mm indicated by KK4 endophytic bacterial isolates against S. marsecent test bacteria and the weakest inhibitory was 12 mm in KK1 and KK2 endophytic bacterial isolates to B. cereus. The endophytic bacteria isolates obtained showed that endophytic bacteria were able to inhibit gram positive bacteria and gram negative bacteria. According to [20], the antibacterial activity of certain compounds (secondary metabolites or antibiotics) that can inhibit the growth of both gram-positive and gram-negative bacteria, is said to have a wide spectrum. Conversely an antibiotic that is only effective against certain classes of gram bacteria is said to be a narrow spectrum antibiotic.

3.2 Endophytic bacterial morphology and biochemical characteristics

The results of the morphological and biochemical tests are shown in Table 2. Gram staining results show that endophytic bacteria including Gram positive (KK2 and KK4) and Gram negative (KK1 and KK3), have basil and cocus cell form. endophytic bacteria in the form of bacilli can form spores. Spore-producing bacteria are more resistant to extreme environmental pressures because of their stopping cell metabolism or dormancy if present in a poor environment [21]. Gram positive bacteria have only a single plasma membrane surrounded by a thick cell wall of peptidoglycan [22]. Gram-negative bacteria have thin peptidoglycan cell walls located between the inner membrane and the outer membrane [23].

Table 2. Morphological and biochemical results of endophytic bacterial isolates of moringa stem's bark

No	**Test/ Isolat**	**KK1**	**KK2**	**KK3**	**KK4**
1	Gram	-	+	-	+
2	Cell shape	Bacillus	Coccus	Bacillus	Bacillus
3	Spore formation	-	-	-	+
4	TSI	+/+	-/-	+/+	-/-
5	Urea	-	-	+	-
6	Motilitas	+	-	-	+
7	Simon sitrat	+	+	+	+
8	Manitol	-	-	-	-
9	Maltosa	+	-	-	+
10	Laktosa	-	-	-	-
11	Sukrosa	-	-	-	-
12	Glukosa	+	-	-	+

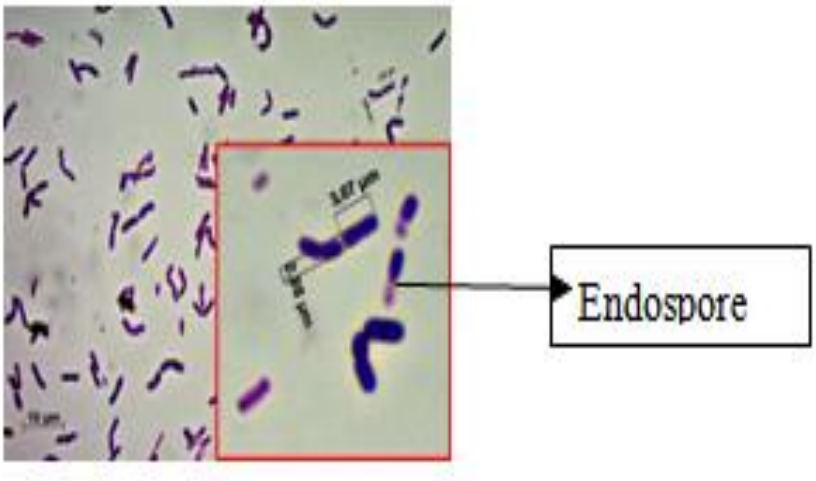

Figure 3. Isolate KK2 (Cell shape is bacillus and Gram-positive bacterium)

3.3 Molecular identification of 16S rRNA Results

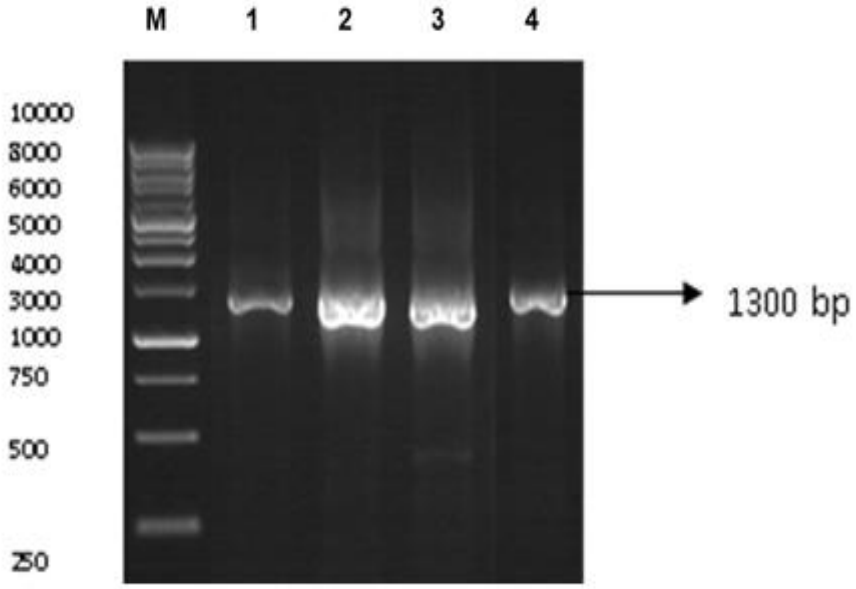

Figure 4. Electrophoresis results from PCR the amplification of 16S rRNA of the endophytic bacterial isolates. The DNA band size is ± 1300 bp. M (DNA marker), 1 (KK1), 2 (KK2), 3 (KK3), 4 (KK4).

The DNA sequence of 16S rRNA were edited using Clustal W in the MEGA 7 program and the results were compared with the existing sequence of 16S rRNA from other organisms in GenBank databases in the NCBI website. The result of this sequencing comparison is then visualized in phylogenetic tree form using MEGA 7 program which can show the kinship of sample isolate with the obtained sequences from other organisms.. Phylogenetics is important for describing taxonomic classification of an organism based on their evolutionary history [24]. According to [25], the phylogenetic tree is a logical approach to showing the evolutionary relationship between organisms.

Table 3. Genetic distance of endophytic bacteria isolate with refernces bacteria derived from GenBank based on 16 rRNA.

No	endophytic bacteria isolate	Reference bacteria	Genetic distance
1	KK1	*Pantoea anthophila strain LMG2558*	0,084
		Klebsiella sp KSC 16S	0,099
2	KK2	*Staphylococus pasteuri*	0,042
		Staphylococus sp BAB 5902	0,051
		Staphylococus epidermidis	0,048
3	KK3	*Burkholderia cepacia strain NBRC 14074*	0,015
4	KK4	*Bacillus cereus strain ATCC 14579*	0,003
		Bacillus cereus JL	0,003
		Bacillus mycoides B38V	0,006
		Bacillus cereus TMPSB-M20	0,009

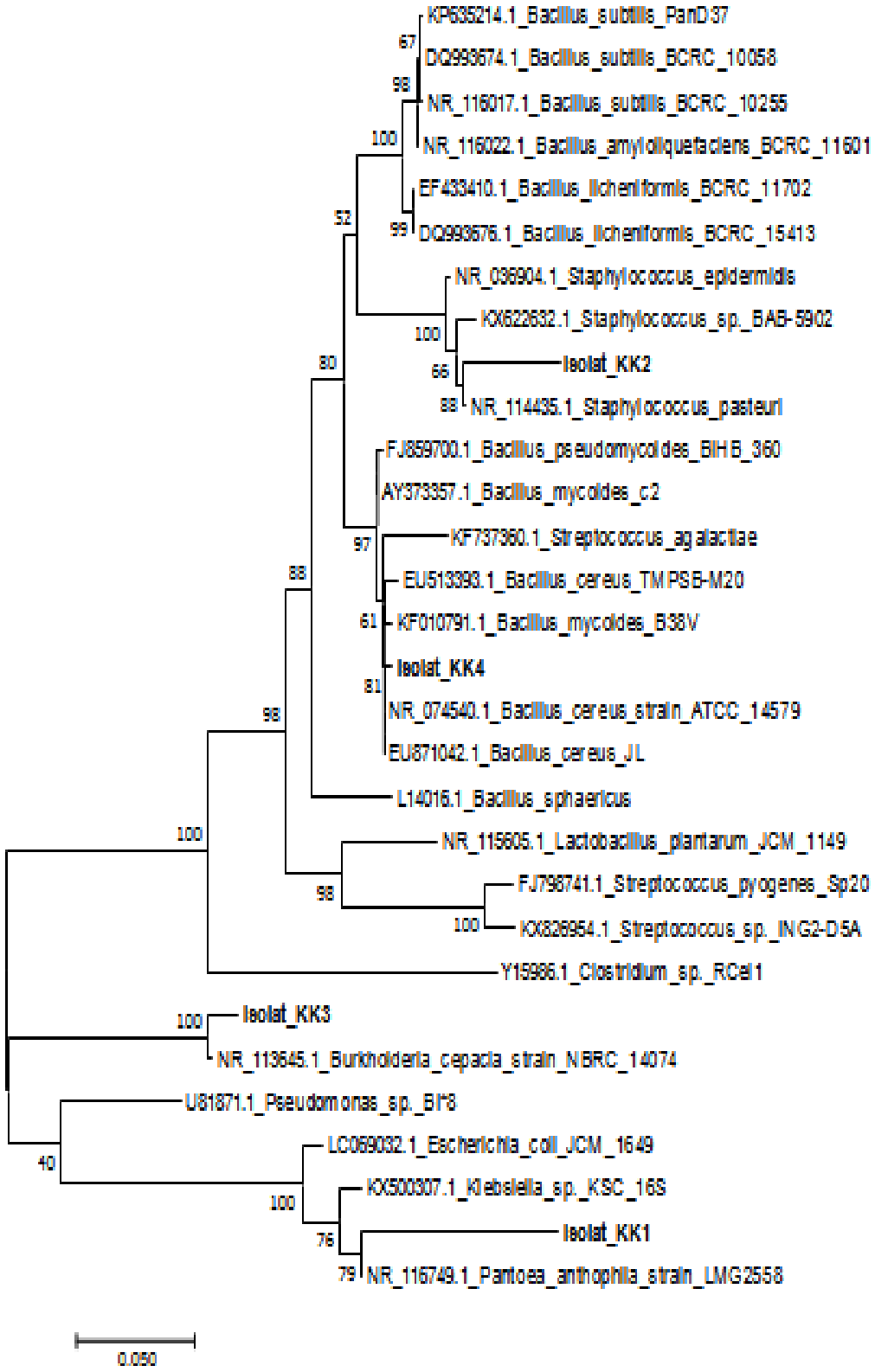

Figure 5. Phylogenetic tree showing the position of endophytic bacterial isolates of moringa stem bark (KK1, KK2,KK3 dan KK4) compared with others bacteria based on 16S rRNA gene sequences.

Based on genetic distance and phylogenetic trees, endophytic bacterial isolates obtained from moringa plants are included in the genus of Bacillus and Cocus. Isolate bacterial endophytic bark of moringa plant (KK1 isolate) has kinship with Pantoea anthophila strain LMG2558, Klebsiella sp KSC 16S. While isolate KK2 had a kinship with Staphylococus pasteuri, KK3 isolate had a kinship with Burkholderia cepacia strain NBRC 14074, and isolate KK4 had the closest relative to B. cereus strain ATCC 14579 and B. cereus JL (Fig.5). Inhibitory test results showed that KK1, KK2, KK3 and KK4 isolates have inhibitory effect on S. aureus, B. cereus, E. coli and S. marcescen bacteria, and all isolates have no inhibitory effect on Proteus mirabilis growth. Ability of endophytic bacterial isolates from moringa stem bark to inhibit the growth of clinical isolates due to the presence of metabolite compounds possessed by endophytic bacterial isolates. This is in accordance with the [26], that secondary metabolite compounds produced by bacteria endofit can be used as a source of bioactive compounds that can inhibit the growth of other microbes. The results of this research showed that endophytic bacteria isolated from moringa plant have the antimicrobial activity which could be utilized in the future an an alternative source of antimicrobe compounds.

IV. Conclusion

Four isolates of endophytic bacteria were isolated from the bark of moringa. Based on the research result, isolate KK1, KK2, KK3 and KK4 able to inhibit the growth of pathogenic bacteria *S. aureus, B. cereus, E. coli* and *S. marcescen*. Endophytic bacteria isolated from the bark of moringa stem has the closest genetic relationship with *Bacillus cereus JL*. and *Bacillus cereus* strain ATCC 14579.

Acknowledgements

We would like to thank the staff members of Biomedical Research Unit of RSUD Mataram, Indonesia.

References

[1]. Krisnadi, A.D. 2015. *Moringa Super Nutrisi.* www.moringaina.com

[2]. Bukar, A., Uba, A., Oyeyi, T.I. 2010. Antimicrobial Profil of Moringa oleifera Lam. Extracts Against Some Food-Borne Microorganism. *Bajero Journalof Pure and Applied Sciences*. Vol. 3(1) : 43-48.

[3]. Fahey, J. W. 2005. *Moringa oleifera*: A Review of the Medical Evidence for its Nutritional, Therapeutic, and Prophylactic Properties. Part I. (http://www.malunggay-propagation.com/Jed_Fahey_text_GB.pdf)

[4]. Meitzer LS, Martin LP. 2000. Effectivenes of a Moringa Seed Ekstract in Treating a Skin Infection. Amaran th to Zai Holes. ECHO. USA.

[5]. Ikalinus,R., Sri Kayati, W., Ni Luh Eka S.,2015. Skrining Fitokimia Ekstrak Etanol Kulit Batang Moringa (*Moringa oleifera*). Indonesia Medicus Veterinus 4(1) : 71-79

[6]. Simarmata R, Lekatompessy S, Sukiman H. 2007. Isolasi mikroba endofitik dari tanaman obat sambung nyawa (Gymura procumbens) dan analisis potensinya sebagai antimikroba.

[7]. Kusumawati DE, Fachriyan HP, Maria B. 2014. Aktivitas antibakteri isolate bakteri endofit dari tanaman miana (*Coleus scutellariodes* L. Benth.) terhadap *Staphylococcus aureus* dan *Escherichia coli*. *Journal Current Biochemistry*. 1(1): 45-50.

[8]. Bhore SJ., Sathisha, G. 2010. Screening of endophytic colonizing bacteria for cytokinin-like compounds: crude cell-free broth of endophytic colonizing bacteria is unsuitable in cucumber cotyledon bioassay. *World J. Agric. Sci. 6 (4): 345-352*

[9]. Castillo UF., Strobel GA., Ford EJ. Munumbicins, wide-spectrum antibiotics produced by Streptomyces NRRL30562, endophytic on Kennedia nigriscans. Microbiology. 2002; (148):2675–85. Dalam: Ryan RP., Germaine K, Franks A, Ryan DJ, Dowling DN. Bacterial endophytes: recent developments and applications Mini Review. FEMS Microbiol Lett. 2008; (278):1–9.

[10]. Amrullah, L.W. Z. Jekti, DSD. Zulkifli, L. 2018. Isolation and Molecular Identification of Endophytic bacteria from Red Betel Root (Piper crocatum Ruiz & Pav) as a Producer of Anti-Bacterial Compounds. IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB) 4 (1), 27-32.

[11]. Zulkifli, L., Jekti, DSD, Bahri, S. 2017. Isolasi, Karakterisasi Dan Identifikasi Bakteri Endofit Kulit Batang Srikaya (Annona Squamosa) Dan Potensinya Sebagai Antibakteri. Jurnal . Penelitian Pendidikan IPA 4 (1) : 21-29

[12]. Sepriana,C., Jekti, DSD. Zulkifli, L. 2017. Bakteri Endofit Kulit Batang Tanaman Cengkeh (Syzygium Aromaticum L.) Dan Kemampuannya Sebagai Antibakteri. Jurnal Penelitian Pendidikan IPA 3 (2) : 52-59

[13]. Desriani, Purwanto UMS, Bintang M, Rivai A, Lisdiyanti P (2014) Isolasi dan karakterisasi bakteri endofit dari tanaman binahong dan katepeng china. *Jurnal Kesehatan andalas 3(2):89-93*

[14]. Prayoga, E. 2013. Perbandingan Efek Ekstrak Daun Sirih Hijau (Piper Betle L) Dengan Metode Difusi Disk Dan Sumuran Terhadap Pertumbuhan Bakteri Staphylococcus Aureus. Fakultas Kedokteran dan Ilmu Kesehatan Universitas Islam Negeri Syarif Hidayatullah. Jakarta

[15]. Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J. & Wade, W. G. (1998). Design and evaluation of useful bacterium-speci®c PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol* 64, 795±799.

[16]. Resti Z, Habazar T, Putra DP, Nasrun. 2013. Skrining Dan Identifikasi Isolat Bakteri Endofit Untuk Mengendalikan Penyakit Hawar Daun Bakteri Pada Bawang Merah.J. HPT Tropika. ISSN 1411-7525.Vol. 13, No. 2: 167 –178.

[17]. Purwanto, UMS., Fachriyan, H., Maria B., 2014 Isolasi Bakteri Endofit dari Tanaman Sirih Hijau (*Piper betle* L.) dan Potensinya sebagai Penghasil Senyawa Antibakteri. Current Biochemistry Volume 1 (1): 51 – 57.

[18]. Hastowo,S. 2002. Mikrobiologi. Rajawali Pers: Jakarta.

[19]. Brooks, G.F., Janet, S.B., Stephen, A.M., 2007. Mikrobiologi Kedokteran. Edisi 23. Alih bahasa Hartono et al. EGC. Jakarta.

[20]. Tortora GJ, Berdell RF, Christine LC. 2001. Microbiology: An Introduction 7th ed. Benjamin Cummingis. New York.

[21]. Jenson I, dan Moir CJ (2003) *Bacillus cereus* and other *Bacillus* species. Ch 14 In: Hocking AD (ed) Foodborne microorganisms of public health significance. 6th ed, Australian Institute of Food Science and Technology (NSW Branch), Sydney, p. 445–478.

[22]. Madigan M. T., J. Martinko, J. Parker. 2003, Brock Biology of Microorganisms, 10th ed., Pearson Education, Inc., New York.

[23]. Cooper, G.M. & R.E. Hausman. 2007. The Cell: A Molecular Approach. 4th ed. Sinauer Associates, Inc., Sunderland.

[24]. Mount, D.W. 2001. Phylogenetic prediction. In: Bioinformatic, Sequence and Genome Analysis. Cold Spring Harbor laboratory. New York Press pp. 237 – 280.

[25]. Schmidt, H. 2003. *Phylogenetic Trees from Large Datasets. Inaugural-Dissertation*, Dusseldorf University. ////

[26]. Sudibyo. R.S. 2002. Metabolit Sekunder : Manfaat dan Perkembangannya dalam Dunia Farmasi. Yogyakarta: UGM Press

IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB) is UGC approved Journal with Sl. No. 4033, Journal no. 44202.

Nurul Ilmi "Molecular Identification OF Eentophytic Bbacteria FROM The Stem's Bark OF Morange Plant AND Their Aantibacterial Activities." IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB) 4.4 (2018): 21-30.