Isolation of endophytic bacteria on african leaves (*Vernonia amygdalina)* and its activity against *Escerichia coli* bacteria

Arfiandi[1], Neri Fadjria[1], Alfa Satria Nanda[1] and Mhd. Riza Marjoni[2]*
1. Department of Microbiology, Dwi Farma Academy of Pharmaceutical, Bukittinggi 26121, West Sumatera, INDONESIA
2. Department of Phytochemistry, Dwi Farma Academy of Pharmaceutical, Bukittinggi 26121, West Sumatera, INDONESIA
*mhdriza.marjoni@gmail.com

Abstract
The endophytic bacteria are beneficial microorganisms that interact with host plants without causing any damage or infection to them. Some studies state that certain endophytic bacteria can produce chemical compounds that have health effects, especially endophytic bacteria isolated from medicinal plants. African leave (Vernonia amygdalina) is a medicinal plant that has many health benefits and has long been used in the treatment of a variety of conditions.

This study aimed to do endophytic bacterial isolation and screening on Bitterleaves and test the activity of Escherichia coli. The number of endophytic bacteria that were isolated were 2 isolates: Nan-1 and Nan-2. Both isolates had potential activity (characterized by the formation of inhibition zones) against Escherichia coli bacteria. The formation of inhibition zones indicated the possibility of compounds having an antibacterial effect. The isolate that showed the largest inhibition zone was Nan-2, so it could be concluded that the isolate was the most potential isolate as an antibacterial compound.

Keywords: Antibacterial, endophytic bacteria, *Vernonia amygdalina*.

Introduction

Endophytic microbes are endosymbiotic microorganisms including bacteria, fungi and actinomycetes which spend all or part of their lives inside the living tissue of the host plant without causing adverse symptoms to the host plant itself[1].

The results of a number of research on endifitic microbes show that endophytic microbes can play a role in producing secondary metabolites which are bioactive metabolites and can function to kill pathogenic bacteria. Bioactive metabolites produced by endophytic microbes can be in the form of anti-microbial compounds that can inhibit the growth of other types of microbes; these compounds include anti-bacterial compounds, anti-fungal compounds, remodel enzymes, plant regulatory substances andanti-tumor compounds. The compounds contained by endophytic bacteria will be similar to those needed by the plants[2]. The presence of endophytic bacteria in plant tissue is known to stimulate the plant growth and act as a biological control agent. Its ability to penetrate into the plant's internal tissue can be caused by the presence of extracellular enzymes in the form of cellulase produced by these bacteria. After penetration, endophytic bacteria will colonize thereby inhibiting the growth of pathogenic bacteria through the mechanism of space and nutrition competition[3,4].

One of the plants that have bioactive compounds is African leaves (*Vernonia amygdalina*). This plant originates from the western continent of Africa, Nigeria. In West Sumatra, this plant is known as *Daun Afrika Selatan* or the South African leaf[5]. Based on the past studies, the African leaves plants contain the compounds of tannin vernonioside A1, A2, A3, A4, B1, B2, B3, flavonoids, luteolin, luteolin 7-O-β-glucoronicide, luteolin 7-O-β-glucoside, terpenes, coumarin, phenol acids, lignans, xanthons, anthrakuinones andedotides (peptides)[6].

Recent studies report that compounds such as alkaloids, terpenoids, flavonoids andsteroids can inhibit a number of microorganisms. This plant is also traditionally used to treat rheumatism, malaria, diarrhea, hypertension and gout. In fact, this plant is also widely used by the people as vegetables[7]. Based on the background problems, this study aims to isolate endophytic bacteria from the African leaves plant (*Vernonia amygdalina*) and test the antibacterial activity of the endophytic bacterial isolates against pathogenic *E. coli* bacteria using the paper disc method.

Material and Methods

Materials and apparatus: The materials used in this study are fresh African leaves (*Vernonia amygdalina*) obtained from the Bukittinggi area, *Escherichia coli* culture (collection from the Bacteriology Laboratory of the Faculty of Medicine of Andalas University), nutrient agar (NA) media, nutrient broth (NB) media, 5,2% sodium hypochlorite, 70% ethanol, distilled water, McFarland 0.5, safranin and tetracycline antibiotics. The tools used include Petri dishes, test tubes, centrifuges, shakers, autoclaves and incubators.

Research Methods for Isolation of Endophytic Bacteria: Fresh plant samples were cleaned under running water and then cut into 1-3 cm lengths. The sample pieces were immersed in 70% ethanol for 1 minute and 5.25% sodium hypochlorite solution for 5 minutes and then washed with 70% ethanol three times. The sample pieces were sliced in a separate manner and then planted in nutrient agar (NA)

media that contained nystatin. The media containing the sample were incubated at room temperature in a dark state and observed every day until there was colony growth. If within 24 hours there had been no microbial growth around the plant sample, surface sterilization was said to be successful.

The growing endophytic bacteria were purified one by one and cultivated in order to tilt. Pure endophytic bacterial isolates were morphologically identified based on the colony's color, edge shape, elevation, consistency and growth rate[8].

Screening to obtain potential endophytic bacterial isolates: Endophytic bacterial isolates from tilted agar were regenerated into NA media while the test bacteria were regenerated into 5 mL nutrient broth media (NB) and then incubated for 24 hours at 28-30°C. 0.4 mL of bacterial liquid culture of the test was put into 80 mL of NA media at ± 40°C. Then 20 mL was poured into a sterile Petri dish and left until it solidified. Endophytic bacterial isolates to be tested were inoculated into media containing pathogens and then incubated for 24 - 48 hours. The inhibitation zones formed were observed and compared with positive control (Tetracycline 30 µg). Then the diameter of the inhibition zones was measured and endophytic bacterial isolates that positively showed inhibition zones were referred to as potential isolates[8].

Results and Discussion

The common part of the plant that can be an entry point for endophytic bacteria is the root, but the parts that are exposed to direct air such as flowers, stems, leaves (through stomata) and cotyledons can also be an entry point for endophytic bacteria. Endophytic bacteria that have entered the plant can grow only at a certain point or spread throughout the plant. These microorganisms can live in vascular vessels or in the intercellular space of roots, stems, leaves and fruit[9-11]. This is consistent with the statement of Bacon and Hinton[11] who stated that the number of endophytic bacteria in plants cannot be determined with certainty, but these bacteria can be detected by isolating the agar media.

The agar media used to isolate endophytic bacteria in this study were agar nutrient media (NA). This was a rich media consisting of yeast extract, peptone, NaCl and agar. Endophytic bacteria can live on NA media due to the complex nature of the media and most likely the media had a composition similar to conditions in plants.

The endophytic bacteria started to grow on the Bitterleaves (*Vernonia amygdalina*) after pieces of the plant parts were inoculated on NA media for ± 48 hours (2 days). This statement is supported by Zinniel et al,[9] Simarmata et al[10] and Jalgaonwala et al[13] who stated that the selected time of at least 2 days for incubation aims to ensure that the growing bacteria are endophytic bacteria, instead of contaminant bacteria.[12,13]

Endophytic bacterial colonies that were successfully isolated from the Bitterleaves (*Vernonia amygdalina*) showed diversity, both in terms of color, shape and speed of growth. This is in accordance with Bhore and Sathisha[14] who stated that endophytic bacteria in one host plant generally consist of several genera and species. The diversity of endophytic bacteria in a plant is also influenced by the plant growth conditions, especially the soil. In some cases, plants of the same type or species have endophytic bacteria which are not always the same. In some plants there are specific endophytic bacteria that inhabit them.

The results of the this research found two endophytic bacterial isolates called Nan-1 isolates which had cream-colored characteristic and Nan-2 isolate which had a yellowish color. Furthermore, for microscopic observations, a gram staining testing was carried out. In this test the two isolates were a group of gram-positive bacteria (Table 1).

Table 1
Results of macroscopic bacterial observations

Morphological characteristics (bacterial color and gram staining)	Isolate Code
The colony has a cream color and a purple gram color	I Nan-1 Isolate
The colony has a deep yellow color and a purple gram color	II Nan-2 isolate

Gram-positive bacteria were known to maintain violet crystalline dye and the bacteria appear purple while gram-negative bacteria would lose violet crystalline dye when washed with alcohol andbacteria would absorb safranin dye so that it turned red[15].

The potential resulting from the isolation of endophytic bacteria on the Bitterleaves (*Vernonia amygdalina*) against *E. coli* bacteria can be seen based on the clear zone that is around the disk paper (Table 2). The formation of clear areas around the colony of endophytic bacterial isolates indicates the possibility of antibacterial compounds capable of killing or at least inhibiting the growth of pathogenic bacteria. This is confirmed by Simarmata et al[10] and Kumala and Siswanto[16] who conducted a similar test with endophytic bacteria from Indonesian medicinal plants.

Table 2
Average inhibition zone of endophytic bacterial isolates against *E. coli* bacteria by caliper method

Isolate	*E. coli*
Sterile Discs	-
Tetracycline	1.34 cm
Nan-1 Isolate	6.7 mm
Nan-2 isolate	7.3 mm

The antibacterial potential of endophytic bacterial isolates on Bitterleaves (*Vernonia amygdalina*) against *E. coli*

bacteria can be seen from the clear zone formed around the disk paper.

The samples used in this study were Bitterleaves (*Vernonia amygdalina*) obtained in the city of Bukittinggi. From the research, two pure isolates of endophytic bacteria were found on the Bitterleaves. The endophytic bacteria were selected based on color, shape of the colony's edge and gram staining.

This study obtained two isolates with different types of bacterial colonies that were coded Nan-1 isolates and Nan-2 isolates. The diversity of endophytic bacteria in plants was also determined by soil conditions.[14] The diversity occurs depending on the growing environment of the plants.

The results of the study showed that there were two endophytic bacterial isolates, namely cream-colored Nan-1 isolate and yellowish-colored Nan-2 isolate. Furthermore, for microscopic observations, a gram staining test was carried out with both isolates being gram-positive.

Gram-positive bacteria were known to maintain violet crystalline dye and the bacteria appear purple while gram-negative bacteria would lose violet crystalline dye when washed with alcohol andbacteria would absorb safranin dye so that it turned red.[15]

The potential resulting from the isolation of endophytic bacteria on the Bitterleaves (*Vernonia amygdalina*) against *E. coli* bacteria can be seen based on the clear zone that is around the disk paper.

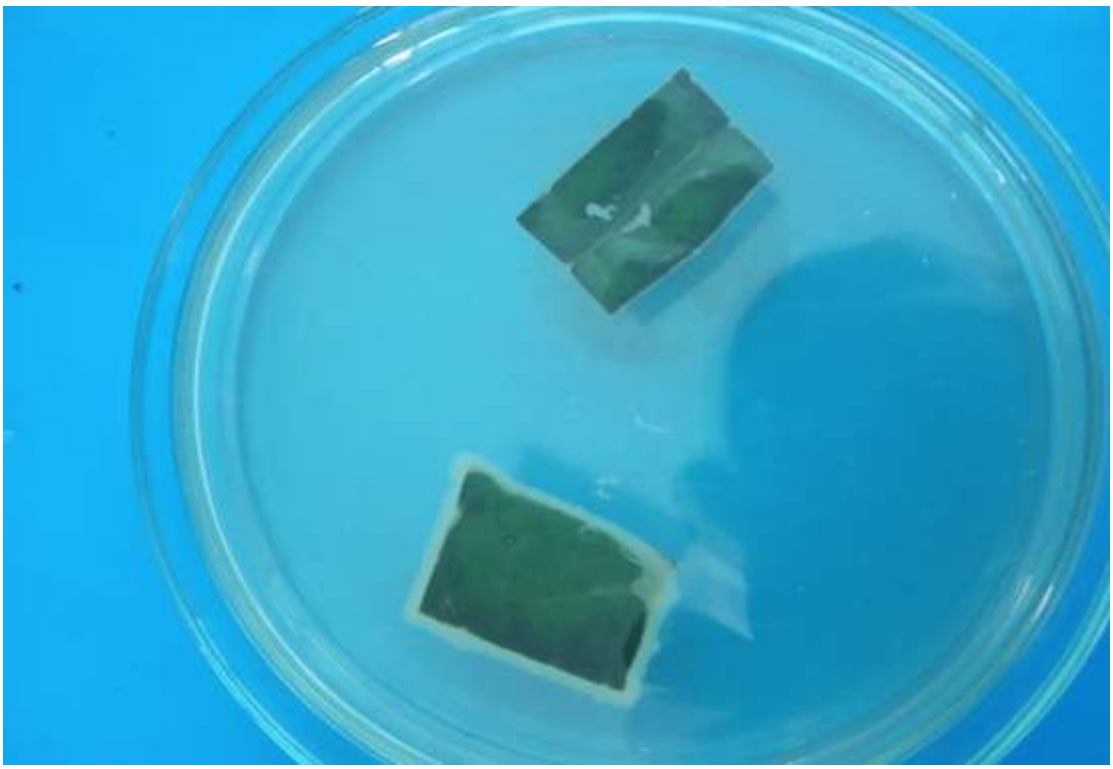

Figure 1: Isolation of Endophytic Bacteria

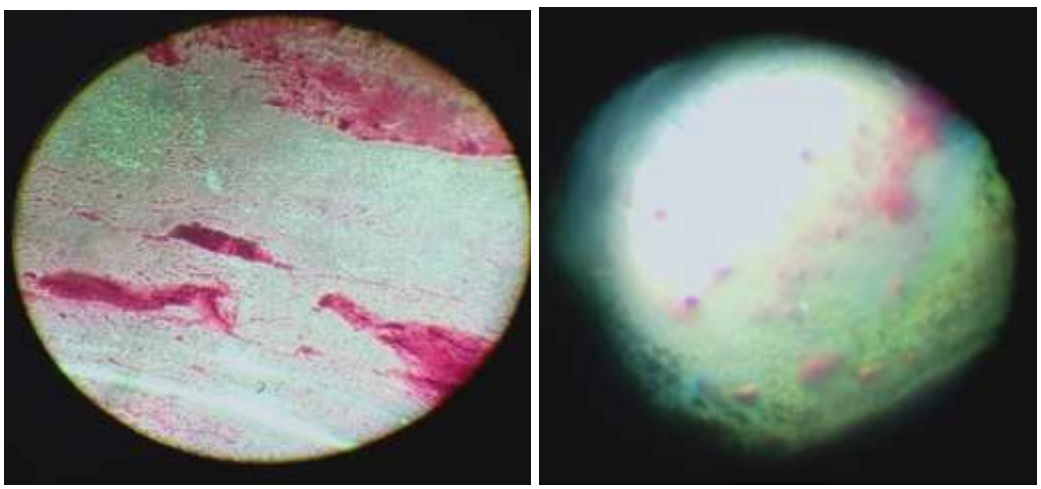

Figure 2: Isolates 1 and 2

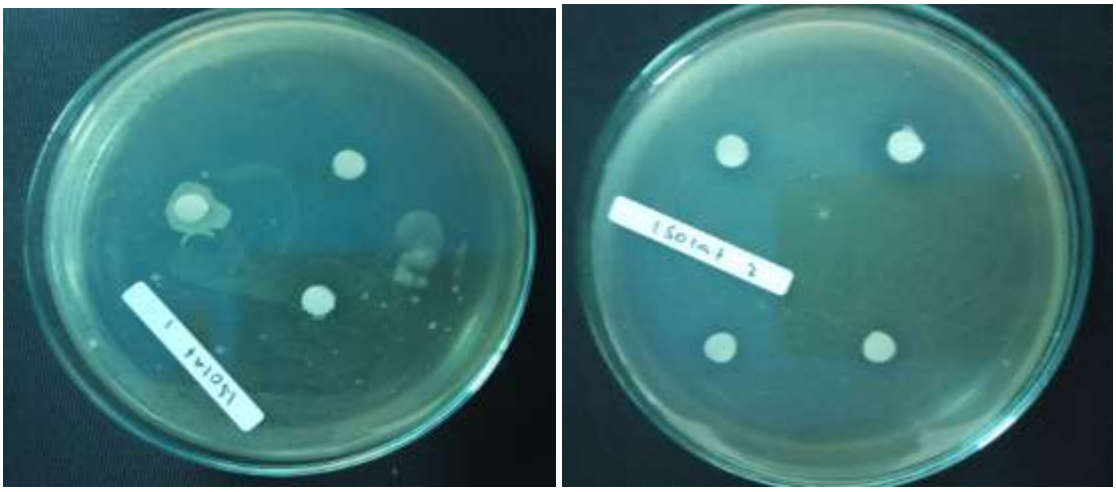

Figure 3: Inhibition zones of isolate 1 and 2

The results of the study showed that the two endophytic bacterial isolates had an antibacterial ability; Nan-1 isolates had inhibition zone diameters of 6.7 mm against *E. coli* bacteria and Nan-2 isolates had inhibition zones of 7.3 mm against *E. coli* bacteria.

The difference in diameter of the inhibition zone produced was probably due to differences in the structure and composition of the cell wall of each gram bacteria. The formation of a clear zone indicated the ability of endophytic bacterial isolates to produce secondary metabolites in the form of antibiotic compounds that could inhibit bacterial growth.[17]

Based on the results of research on isolation of endophytic bacteria on Bitterleaves for testing against E. coli bacteria, it can be seen that both isolates (Nan-1 and Nan-2) can inhibit the growth of *E. coli* bacteria. This is marked by the formation of a clear zone. Judging from the gram staining, the two isolates were gram-positive because both of them can maintain the violet crystal dye and the bacteria appear purple.

Conclusion

Endophytic bacteria isolated from Bitterleaves (*Vernonia amygdalina*) produce two endophytic bacterial isolates, namely Nan-1 and Nan-2. Gram staining in both isolates shows that isolates are gram positive. Both isolates obtained from the African leaves plant (*Vernonia amygdalina*) have antimicrobial ability.

Acknowledgement

The authors fully acknowledge Directur of Dwi Farma Academy of Pharmaceutical for the funds which made this important study viable and effective. This study was also supported by grants from the Dwi Farma Bukittinggi Pharmacy Academy Research Institute (No. 351.104047.08.2019).

References

1. Strobel G.A., Hess W.M., Ford E., Sidhu R.S. and Yang X., Taxol from fungal endophytes and the issue of biodiversity, *J. Ind. Microbiol. Biotechnol.*, **17**, 423-432 **(1996)**

2. Kumala S., Shanny F. and Wahyudi P., investigate endophytic microbes that live in the *Cassia fistula* L (Trengguli) plant and their ability to produce bioactive metabolite as anti microbial agents, *J. Farm. Indones.*, **3(2)**, 97-102 **(2006)**

3. Munif A., Djatnika I. and Widodo, Physiological Characters and Antibiosis Activity of Gramineous Crops Rhizobacteria, Againts Fusarium and Banana Growth Promoting, *J. Hortik.*, **17(2)**, 150-160 **(2007)**

4. Kulkarni K.D., Raghuwanshi K.S., Naik R.M., Borkar S.G. and Chimote V.P., Antifungal activity of camptothecin extracted from Mappia foetida against disease causing pathogens in pomegranate (Punica granatum L.), *J. Pure Appl. Microbiol.*, **9(1)**, 329-334 **(2015)**

5. Sembiring I., The Inotropic And Chronotropic Effect of African leaves (*Vernonia amygdalina Delile*) Ethanol Extract on isolated rat heart, Faculty of pharmacy north sumatera University **(2013)**

6. Ijeh I.I.and Ejike C.E.C.C., Current perspectives on the medicinal potentials of Vernonia amygdalina Del, *Journal of Medicinal Plants Research*, **5(7)**, 1051-1061 **(2011)**

7. Akah P.A., Alemji J.A., Salawu O.A., Okoye T.C. and Offiah N.V., Effects of Vernonia amygdalina on Biochemical and Hematological Parameters in Diabetic Rats, *Asian J. Med. Sci.*, **1(3)**, 108-113 **(2009)**

8. Powthong P., Jantrapanukorn B., Thongmee A. and Suntornthiticharoen P., Screening of antimicrobial activities of the endophytic fungi isolated from Sesbania grandiflora (L.) Pers., *J. Agric. Sci. Technol.*, **15(7)**, 1513-1522 **(2013)**

9. Zinniel D.K. et al, Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants, *Appl. Environ. Microbiol.*, **68(5)**, 2198-2208 **(2002)**

10. Simarmata R., Lekatompessy S. and Sukiman H., Endofitic microbial Isolation of Gynura procumbens AND ITS potential analysis as an antimicroba, *J. Biol. Res.*, **13**, 85-90 **(2007)**

11. Bacon C.W. and Hinton D.M., Bacterial endophytes: The

endophytic niche, its occupants andits utility, in Plant-Associated Bacteria **(2006)**

12.. Rana S.K, Pundir R.K. and Kaur A., Bioprospecting potential of foliar endophytic fungi associated with commonly used Indian medicinal plants, *J. Pure Appl. Microbiol.*, **10(1)**, 173-181 **(2016)**

13. Jalgaonwala R.E., Mohite B.V. and Mahajan R.T., Evaluation of Endophytes for their Antimicrobial activity from Indigenous Medicinal Plants belonging to North Maharashtra region India, *Int. J. Pharm. Biomed. Res.*, **1(5)**, 136-141 **(2010)**

14. Bhore S.J. and Sathisha G., 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 **(2010)**

15. M.J. and Pelczar E.C.S.C., Dasar-Dasar Mikrobiologi 2, in Elements of Microbiology **(1988)**

16. Kumala S. and Siswanto E.B., Isolation and Screening of Endophytic Microbes from Morinda citrifolia and their Ability to Produce Anti-Microbial Substances, *Microbiol. Indones.*, **1(3)**, 145-148 **(2007)**

17. Purwantini I., Wahyono Mustofa and Asmah R., Isolation of endophytic fungi from Artemisia annua, L and identification of their antimicrobial compound using bioautography method, *Int. J. Pharm. Pharm. Sci.*, **7(12)**, 95-99 **(2015)**.

(Received 28th December 2019, accepted 12th March 2020)