Bioprospecting of fruit extract and endophytic bacteria isolated from dewandaru (Eugenia uniflora L.) as antibacterial against colorectal bacteria

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Abstract. Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the fourth leading cause of cancer-related deaths in the world. The gut bacteria are an important player in the development of colorectal cancer. Dewandaru (Eugenia uniflora L.) have been used as a traditional medicine to treat various diseases. Many of antibacterial metabolites produced by the fruit and its endophytic bacteria. This study aimed to find Antibacterial activity of ethanolic extract of dewandaru fruit using Kirby - Bauer disk diffusion method and to determine Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) using Dilution Tube Method (DTM). Endophytic bacteria were isolated from dewandaru fruit by using sterilization treatment followed by serial dilution agar plate and streak technique. All the isolates and ethanolic extract with different concentration were evaluated for the antimicrobial activity against colorectal bacteria (Streptococcus bovis, Enterococcus faecalis, Escherichia coli and Salmonella enterica). The results showed resistant – strong inhibitory zone category of fruit extract and isolated endophytic bacteria. The isolates were identified as Bacillus cereus, Bacillus amyloliquefaciens, and Bacillus sp.

Keywords: Antibacterial, endophyte, extraction, rare fruit, Kirby-Bauer

1. Introduction
Pathogenic bacteria caused various illnesses and could also be carcinogenic agent. Bacteria participate in development of colorectal cancer using various mechanisms, such as proinflammatory and procarcinogenic pathways induction, production of free radical effect and genotoxins, and conversion of procarcinogenic factors into carcinogens [1].

One of the most common cancer case is colorectal cancer (CRC). Colorectal cancer is abnormal tissue growth in the inner lining of rectum or colon [2]. Global Burden of Cancer (Globocan) in 2018 stated the incidence of colorectal cancer in Indonesia was 12.8 per 100 thousand adult population with mortality rate 9.5 percent [3]. CRC is the third most common cancer in the United States, and the fourth leading cause of cancer-related deaths, with estimated number 133.000 new cases and 50.000 deaths every year [4]. There were 50,630 death cases caused by colorectal cancer in 2018 [5].

According to some researches there are numbers of carcinogenic bacteria species such as Bacteroides fragilis, Bifidobacterium longum, Clostridium clostridioforme, Enterococcus faecalis, Escherichia coli, Fusobacterium nucleatum, Helicobacter pylori, Ruminococcus obeum, Salmonella
enterica, and Streptococcus bovis. Those bacteria are usually present in large quantities in colon and rectum of colorectal cancer patients [6].

Infection and cancer have several related aspects such as induction of chronic swelling that can cause DNA damage, cell migration and proliferation through various mechanisms [7]. Every species of colorectal bacteria has special mechanism to induce cancer. S. bovis has cell wall antigens that can excessively induce the expression of cyclooxygenase-2 (COX-2) to cause cellular proliferation, angiogenesis, and inhibits apoptosis. Those activities show occurrence of cancer [8]. E. faecalis species can grow uncontrollably, then increase the possible mutations and change the final product of metabolism to become harmful [9].

DNA damage can also be induced by genotoxin. E. coli harbor the polyketide synthetase and encode a genotoxin named colibactin. Colibactin can break DNA double-strand and cause chromosomal instability in human cells [6]. It has been reported that AvrA, a protein released by S. enterica enhances epithelial proliferation and promoting tumorigenesis [10].

Many researches and development of alternative antibiotics have started utilizing various natural resources all around the world to resolve bacterial infection. Indonesia is known as mega-biodiversity country has many potential source of natural antibiotics. Dewandaru (Eugenia uniflora L.) is one of the rare fruits that grow in Indonesia and has lots of history of use as traditional medicines in several local areas [11]. Dewandaru fruit is considered to be effective in treating various diseases, such as antihypertensive and digestive disorders. In some regions, dewandaru is considered to have diuretic, hypotensive, anti-inflammatory, antidiarrheal, and antimicrobial compounds [12].

Dewandaru is an evergreen shrub or small multi-trunked tree, can reach 7 – 8 m height and has slender branches and a spreading growth habit. Leaves of dewandaru are simple, opposite, ovate to ovate-lanceolate with rounded base and short acuminate to sharp pointed apex, with 2-6 cm length and 1-3 cm width. The flowers occur on long stalks, borne in axillary inflorescences singly or in groups of two to four. The small flowers have four white, persistent sepals and four delicate, recurved, white petals. There are 50-60 prominent white stamens within the yellow anthers. The fruit is a 2-4 cm wide berry with six to ten ribs and is flattened at the poles. Fruit color turns from green to yellow then orange and finally to dark red when ripe [13].

Dewandaru contained phenols, tannins, saponins, glycosides, flavonoids, sterols, resins, balsams, alkaloids and essential oils [11]. Based on several studies it has been proven that metabolites obtained from parts of dewandaru plants such as leaves and seeds have anti-inflammatory, anti-cancer, and antibiotic activities against gram positive and negative bacteria [14].

Antibiotics can also be obtained from metabolites produced by microorganism. Medicinal plants were usually needed in large quantities to be extracted so their bioactive compounds can be collected, this method was certainly not efficient considering the availability of dewandaru fruit. The use of endophytic microbes could be a solution because of their ability to produce bioactive compounds that were similar to their original plants. Bioactive metabolites discovered from endophytes may be related to the independent development of these microorganisms. Alternatively, endophytes may have combined genetic information from their host plants, which stimulates their adaptability and enhances their defense mechanisms against pathogens and insects [15].

2. Methods
There are several methods used in this study. Dewandaru fruits (Eugenia uniflora L.) were collected in August 2018, at Padalarang, West Bandung, Indonesia. Bacterial strains used in this study were collected from Microbiology Laboratory Padjadjaran University, and PT. Biofarma Indonesia, they are Streptococcus bovis ATCC 33317, Escherichia coli ATCC 25922, Enterococcus faecalis, and Salmonella enterica ATCC 14028.

2.1. Extraction
Dewandaru fruit were extracted by maceration process using 2 litre ethanol 95% for 2 kg fruit flesh, stirred two times for 48 hours. The extract was concentrated by vacuum rotary evaporator at 45°C until
it became pasta form [16]. The fruit extract was then dissolved into various concentrations (80%, 40%, 20%, 10%, 5%, and 2.5%).

2.2. Isolation and secondary metabolite production of endophytic bacteria
Dewandaru fruit surface was sterilized using ethanol 70% for 1 minute, NaOCl 0.5% for 1 minute, and sterile aquadest. Fruit sample was mashed and dissolved as much as 1 gram into 9 ml NaCl repeatedly to get concentration $10^{-7}$ and $10^{-8}$. Dissolved sample from concentration $10^{-7}$ and $10^{-8}$ was inoculated using spread plate technique as much as 1 ml into Nutrient Agar (NA), then incubated for 48 hours. Forming different colonies were isolated into new medium and then incubated. Isolated endophytic bacteria was taken as much as 2 loops and inoculated into 9 ml Nutrient Broth (NB), then incubated for 48 hours. Endophytic bacteria was centrifuged with 5000 rpm for 15 minutes. The supernatant formed from endophytic bacteria culture was separated and pipetted into paper disc.

2.3. Identification of endophytic bacteria
There are three methods used for endophytic bacteria identification, consist of macroscopic observation, gram staining, and biochemical assay using Vitek 2-compact Biomerieux. In macroscopic observation the morphology of colonies were observed including size, shape, elevation, margin, and color. Bacterial cell was observed using Gram staining (1884) procedure.

2.4. Antibacterial activity in vitro assays
The suspension of bacterial assay was pipetted 0.1 ml and rubbed into the entire surface of Mueller Hinton Agar (MHA) using cotton swab. Disc paper which was already soaked with dewandaru fruit extract or endophytic bacteria metabolite were placed on the surface of medium using tweezers. All of inoculated medium then incubated for 24 hours at 37°C with three times replication. The diameter of the inhibition zone formed was measured using ruler and determined based on inhibition zone category. Ampicillin 0.05g/ml as positive control and aquadest as negative control were used in this study.

3. Results

3.1. Dewandaru fruit extract antibacterial assay
The inhibition zones produced by dewandaru fruit extract are vary in very weak to strong range categories. Dewandaru extract inhibited S. bovis growth with inhibition zone diameter 6.7 - 22 mm. The biggest inhibition zone was 22 mm which was in strong category. Inhibition zones produced by the extract were bigger than inhibition zone produced by positive control ampicillin started from concentration 10%. Inhibition zones diameter in average produced by dewandaru fruit extract were 7 - 26.7 mm against E. faecalis, 7 – 19.3 mm against E. coli, and 7 – 12 mm against S.enterica. The results of antibacterial assay for both dewandaru fruit extract and ampicillin showed greater antibacterial activity against gram-positive bacteria than gram-negative bacteria, while negative control did not produce inhibition zone to the colorectal bacteria. Gram-positive bacteria is more sensitive to antibacterial compounds than gram-negative bacteria, this caused by differences in cell walls structure of two types of bacteria. The result of dewandaru fruit extract antibacterial assay against colorectal bacteria can be seen in table 1.

Gram-negative bacteria have more complex structure than gram-positive bacteria. The cell wall of gram-negative bacteria are constructed with lipoprotein, peptidoglycan and lipopolysaccharide. Gram-positive bacteria has simpler cell wall structure, that causes antibacterial compounds to enter the bacterial cell more easily [17]. Thus the bacterial inhibition of dewandaru fruit extract is stronger against gram-positive bacteria. Dewandaru bacterial assay against all the test bacteria showed that the higher concentration tested, the bigger inhibition zone produced. The test result can be seen in figure 1 – 5.
Table 1. Antibacterial activity of dewandaru fruit (*Eugenia uniflora* L.) extract against colorectal bacteria.

| Bacterial Assays | Extract Concentration (%) | Inhibition Zone Diameter (mm) | Average (mm) | Category |
|------------------|---------------------------|------------------------------|--------------|----------|
|                  |                           | P 1  | P 2  | P 3  |          |        |
| *Streptococcus bovis* |                           | 2.5  | 7    | 7    | -       | 6.7     | very weak |
|                   |                           | 5    | 9    | 8    | 9       | 8.7     | very weak |
|                   |                           | 10   | 12   | 11   | 11      | 11.3    | weak      |
|                   |                           | 20   | 19   | 18   | 18      | 18.3    | medium    |
|                   |                           | 40   | 20   | 19   | 19      | 19.3    | medium    |
|                   |                           | 80   | 23   | 21   | 22      | 22      | strong    |
| control +        |                           |      |      | 10   |          |          | weak      |
| control -        |                           |      |      | -    |          | -        |           |
| *Enterococcus faecalis* |                           | 2.5  | -    | -    | -       | -       | -         |
|                   |                           | 5    | 7    | 7    | 7       | 7       | very weak |
|                   |                           | 10   | 9    | 9    | 10      | 9.3     | very weak |
|                   |                           | 20   | 13   | 14   | 14      | 13.7    | weak      |
|                   |                           | 40   | 18   | 19   | 19      | 18.7    | medium    |
|                   |                           | 80   | 28   | 26   | 26      | 26.7    | strong    |
| control +        |                           |      |      | 38   |          |          | strong    |
| control -        |                           |      |      | -    |          | -        |           |
| *Escherichia coli* |                           | 2.5  | -    | -    | -       | -       | -         |
|                   |                           | 5    | 7    | 7    | 7       | 7       | very weak |
|                   |                           | 10   | 8    | 10   | 9       | 9       | very weak |
|                   |                           | 20   | 12   | 14   | 14      | 13.3    | weak      |
|                   |                           | 40   | 14   | 16   | 16      | 15.3    | medium    |
|                   |                           | 80   | 18   | 20   | 20      | 19.3    | medium    |
| control +        |                           |      |      | 25   |          |          | strong    |
| control -        |                           |      |      | -    |          | -        |           |
| *Salmonella enterica* |                         | 2.5  | -    | -    | -       | -       | -         |
|                   |                           | 5    | -    | -    | -       | -       | -         |
|                   |                           | 10   | -    | -    | -       | -       | -         |
|                   |                           | 20   | 7    | 7    | 7       | 7       | very weak |
|                   |                           | 40   | 11   | 10   | 10      | 10.3    | weak      |
|                   |                           | 80   | 13   | 12   | 11      | 12      | weak      |
| control +        |                           |      |      | 28   |          |          | strong    |
| control -        |                           |      |      | -    |          | -        |           |

Explanation: >20 mm: strong; 15-20 mm: medium; 10-15 mm: weak; <10 mm: very weak

3.2. Identification of endophytic bacteria

There were four isolates of endophytic bacteria isolated from dewandaru (*Eugenia uniflora* L.) and coded as DEB 01-04. The morphological characteristics of endophytic bacteria can be seen in table 2.

Biochemical assay using Vitek 2-compact Biomerieux managed to identify two of four isolates to the species level. Isolate DEB 01 was identified with compatibility 87% as *Bacillus cereus*. Isolate DEB 02 and isolate DEB 03 were not identified to the species level, so they will be called as *Bacillus sp.* 1 and *Bacillus sp.* 2. Isolate DEB 04 was identified with compatibility 93% as *Bacillus amyloliquefaciens*. All of endophytic bacteria isolated from dewandaru were identified as *Bacillus*. Genus *Bacillus* is commonly found in plants, this bacteria acts as biocontrol agent and growth stimulant. *Bacillus* has ability to inhibit the growth of certain pathogens [18]. The result of macroscopic and microscopic observation can be seen in figure 5 – 6.
Figure 1. Inhibition zone of dewandaru fruit extract against *Streptococcus bovis* (a) Concentrations 10%, 5%, and 2.5%; (b) Concentrations 80%, 40%, and 20%; (c) Positive and negative control.

Figure 2. Inhibition zone of dewandaru fruit extract against *Enterococcus faecalis* (a) Concentrations 10%, 5%, and 2.5%; (b) Concentrations 80%, 40%, and 20%; (c) Positive and negative control.

Figure 3. Inhibition zone of dewandaru fruit extract against *Escherichia coli* (a) Concentrations 10%, 5%, and 2.5%; (b) Concentrations 80%, 40%, and 20%; (c) Positive and negative control.

Figure 4. Inhibition zone of dewandaru fruit extract against *Salmonella enterica* (a) Concentrations 10%, 5%, and 2.5%; (b) Concentrations 80%, 40%, and 20%; (c) Positive and negative control.
Table 2. Macroscopic and microscopic observation of endophytic bacteria isolated from dewandaru fruit (*Eugenia uniflora* L.).

| Endophytic Bacteria | Macroscopic | Microscopic |
|---------------------|-------------|-------------|
|                     | Shape       | Elevation  | Margin  | Color       | Mucus | Cell  | Type of Gram |
| DEB 01              | circular    | raised     | entire  | white      | -     | bacil | positive     |
| DEB 02              | circular    | flat       | undulate| clear white| -     | bacil | positive     |
| DEB 03              | irregular   | flat       | lobate  | white      | -     | bacil | positive     |
| DEB 04              | circular    | raised     | entire  | pale yellow| -     | bacil | positive     |

3.3. Endophytic bacteria isolated from dewandaru antibacterial assay

Antibacterial activities produced by endophytic bacteria were in very weak to strong range category. Isolate *Bacillus* sp. 2 produced inhibition zone against all bacterial assay, meanwhile *Bacillus* sp. 1 produced inhibition zone against *E. faecalis* and *S. enterica* only. Inhibition zones diameter in average produced by isolate *Bacillus* sp. 1 were 20.3 mm against *S. bovis*, 10.3 against *E. faecalis*, 12.7 mm against *E. coli*, and 9 mm against *S. enterica*. Isolate *Bacillus* sp. 1 produced inhibition zone against *E. faecalis* 8.3 mm in diameter average and inhibition zone against *S. enterica* 7.7 mm in diameter average. The result of dewandaru endophytic bacteria antibacterial assay against colorectal bacteria showed in table 3.

Table 3. Antibacterial activity of endophytic bacteria isolated from dewandaru fruit (*Eugenia uniflora* L.) against colorectal bacteria.

| Bacterial Assays | Endophytic Bacteria | Inhibition Zone Diameter (mm) | Average (mm) | Category  |
|------------------|---------------------|--------------------------------|--------------|-----------|
|                  |                     | P 1 | P 2 | P 3       |            |           |
| *Streptococcus bovis* | DEB 1               | -   | -   | -        | -         | -         |
|                   | DEB 2               | -   | -   | -        | -         | -         |
|                   | DEB 3               | 22  | 19  | 20       | 20.3      | strong    |
|                   | DEB 4               | -   | -   | -        | -         | -         |
|                   | DEB 5               | -   | -   | -        | -         | -         |
| *Enterococcus faecalis* | DEB 01             | -   | -   | -        | -         | -         |
|                   | DEB 02             | 9   | 8   | 8        | 8.3       | very weak |
|                   | DEB 03             | 10  | 10  | 11       | 10.3      | weak      |
|                   | DEB 04             | -   | -   | -        | -         | -         |
|                   | DEB 05             | -   | -   | -        | -         | -         |
| *Escherichia coli* | DEB 01             | -   | -   | -        | -         | -         |
|                   | DEB 02             | -   | -   | -        | -         | -         |
|                   | DEB 03             | 14  | 12  | 12       | 12.7      | weak      |
|                   | DEB 04             | -   | -   | -        | -         | -         |
|                   | DEB 05             | -   | -   | -        | -         | -         |
| *Salmonella enterica* | DEB 01             | -   | -   | -        | -         | -         |
|                   | DEB 02             | 8   | 8   | 7        | 7.7       | very weak |
|                   | DEB 03             | 10  | 9   | 8        | 9         | very weak |
|                   | DEB 04             | -   | -   | -        | -         | -         |
|                   | DEB 05             | -   | -   | -        | -         | -         |

Explanation: >20 mm: strong; 15-20 mm: medium; 10-15 mm: weak; <10 mm: very weak

Endophytic bacteria inhibition zone was caused by specific secondary metabolite named bacteriocin. Bacteriocin is ribosomal synthesized peptide produced by bacteria, that can inhibit or kill
other bacteria, but will not harm the bacteria themselves because of their specific immunity. Bacteriocins is one of the weapons against pathogenic microorganisms due to the specific characteristics of large diversity of structure and function, natural resource, and being stable when exposed to heat. Specific bacteriocin produced by genus Bacillus is mersacidin. Mersacidin is a linear or globular peptide modified after translation containing lanthionine, β-methyl lanthionine and amino acids. B. amyloliquefaciens has two specific bacteriocins, they are amylohsin and difficidin [19]. The result of endophytic bacteria antibacterial assay can be seen in figure 7–8.

![Figure 5](image1.png) (a) DEB 01, (b) DEB 02, (c) DEB 03, (d) DEB 04.

![Figure 6](image2.png) (a) DEB 01, (b) DEB 02, (c) DEB 03, (d) DEB 04.

![Figure 7](image3.png) (a) DEB 02 against Enterococcus faecalis, (b) DEB 02 against Salmonella enterica.
Figure 8. Inhibition zone of endophytic bacteria isolated from dewandaru fruit (a) DEB 03 against Streptococcus bovis, (b) DEB 03 against Enterococcus faecalis, (c) DEB 03 against Escherichia coli, (d) DEB 03 against Salmonella enterica.

4. Conclusion

Fruit extract and endophytic bacteria isolated from dewandaru (Eugenia uniflora L.) showed various antibacterial activity from very weak to strong range categories. Dewandaru fruit extract has stronger antibacterial activity compared to endophytic bacteria against all bacterial assay. There were four identified endophytic bacteria isolated from dewandaru. The most potential endophytic bacteria is isolate Bacillus sp. 2.

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