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Isolation and Antibacterial Activity of Endophytic Bacteria Isolates from Gambir (*Uncaria gambir* (Hunter) Roxb.)

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Abstract : A study on isolation and antibacterial activity of endophytic bacteria of gambir (*Uncaria gambir* (Hunter) Roxb.), along with its antibacterial extract activity test on clinical pathogenic bacteria has been conducted. Isolation of gambir endophytic bacteria was done in nutrient agar incubated at ambient temperature for 48 hours. Samples of gambir leaves and root was used for source of endophytic bacteria. Bacterial isolate was propagated using Mueller Hinton Agar. Bacterial culture was extracted using methanol as solvent. To test methanol extract activity of bacterial isolates, concentration of 20, 40, 60, 80, and 100% (v/v) were used in paper disc. Pathogenic bacterial isolates such as *Escherichia coli*, *Streptococcus mutans*, and *Staphylococcus aureus* were used for antibacterial test. Inhibition zone formed around paper disc indicated antibacterial extract activity against the bacteria. Endophytic bacterial isolates of gambirwas identified using Vitek Compact 2.0®based on biochemical test. Isolates SO02 and SO03 showed relatively higher antibacterial activity, with inhibition zone of 17 and 30 mm, respectively. Methanol extract of SO02 showed to have higher activity of >10 mm at 100% of extract concentration, compared to that of SO03 which only showed <10 mm) against *E. coli*.High inhibition against *S.mutans* and *S.aureus* was obtained at of 60%. Chemical identification found that methanol extract of SO02 and SO03 showed to have flavonoid and tanning group. Bacterial identification showed that SO02 and SO03 were closely related to *Enterobacter cloacae* and *Gemella morbillorum* with similarity of 98 and 86%, respectively. However, accurate species of these two isolates should be determined.

Keywords : Antibacterial activity, *Enterobacter cloacae*, flavonoid, *Gemella morbillorum*, tannin, *Uncaria gambir*.

Introduction

Antibacterial is one of the characteristics of medicinal plants which acts as therapeutic agent towards plant and human diseases commonly used to treat various disease. Endophytic bacteria is not merely in medicinal plant producing beneficial biological compounds to cure disease, but it is also proven as rich source

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of new chemical and biological compound molecules. Research on endophytic bacteria from medicinal plant has been reported numerously, such as of *Styrax benzoin* L., *Chromolaena odorata*, *Glycyrrhiza uralensis*, and *Artocarpus altilis* (Parkinson) Fosberg[1,2,3,4]. Considering the most recent global concern regarding antibiotic resistance on pathogenic microorganism and prevalence of potent mutated strains, which causes concern, exploration to find novel natural products from endophytic bacteria has been done continuously. It is primarily applied to isolate endophytic bacteria from medicinal plants which has the potential in producing novel antibiotic type.

Indonesia is acknowledged as the main exporter of gambir in the world (80%) centered from North Sumaterato South Sumatera. Generally, gambir is utilized as material in tanning industry, textile dyes, component of betel/thomsoniae, and traditional medicine[5,6]. As traditional medicine, gambirhas been used as clinical and therapeutic medication. Laboratory scale research affirmed that gambir extract has secondary metabolite to biological activity. Several research have reported potential bioactive compound from gambir extract in vitro, among them are antihyperlipidemia, antioxidant, antiradical, immunomodulator, skin regeneration, cytotoxic, and antibacterial compounds[7,8,9,10,11]. Literature concerning gambir extract potential triggers researchers to make an effort in exploring further through exploiting and exploring bacteria associated with gambir plant. Endophytic bacteria is capable to form symbiosis with the inner tissue of their hosts and performs ecologically interaction without causing any infection symptom with the host of their habitat[12,13,14].

Microbial group that has been recognized having association with gambir plant is still limited to mold group. Endophytic fungi group has been successfully isolated and reported from various gambirparts, including leaf, stem, fruit, and root. There were 53 mold strains collected during previousresearch, in which the large group were from *Aspergillus, Cladosporium, Diaporthe, Fusarium, Penicillium, Pestaliotopsis, Phoma*and *Phomopsis*[15]. Study concerning endophytic bacteria bioprospecting associated with gambirhas not been conducted. This study was to know potential of endophytic bacterial isolates of gambir as a source of antimicrobial compound against pathogenic bacteria.

Materials and Methods

Sampling and Isolation of Endophytic Bacteria

Samples of gambir leaves and root was picked up in PahaeJulu, North Tapanuli, North Sumatera, Indonesia, and brought toLaboratory of Microbiology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara. Disinfection was performed on sample pieces by using EtOH 70% for one minute, NaOCl 2% for 6 minutes, and EtOH 70% for 30 seconds to remove the remaining NaOCl. Samples were washed using sterile distilled water,cutinto 1-2 cm size, and aseptically put onto nutrient agar (NA). Culture was incubated at ambient temperature for 48 hours. Subsequently, grown bacterial isolates was isolated using NA.

Morphological and Biochemical Characterization of Endophytic Bacteria

Endophytic bacterial isolate was characterized based on morphology of colony and cell shape using gram staining method.Simple biochemical test includingstarch hydrolysis test on Starch Agar medium, gelatin hydrolysis on semi-solid gelatin, citrate on Simmons' Citrate Agar(SCA), hydrogen sulfide on Triple Sugar Iron Agar (TSIA), motility on Sulfide Indole Motility (SIM), and catalase using $H_2O_2$3% was performed.

Clinical Pathogenic Bacterial Isolate Culture

Pathogenic bacterial isolates *Escherichia coli*, *Streptococcus mutans* and *Staphylococcus aureus* were provided from Microbiology Laboratory of Faculty of Medicine of Universitas Sumatera Utara.Bacterial cultures were incubated at 37°C for 24 hours in NA. Cultures were maintained on NA slants at 4 °C, and subsequently cultured in nutrient broth (NB) at 37°C prior to antimicrobial assay.

Antibacterial Assay of Endophytic Bacterial Isolates from Gambir

As much as 10 µL endophytic bacteria (≈10^8 CFU/mL) was put on blank disc of 0.6 cm of diameter. The disc was put on NA inoculated previously with clinical pathogenic bacterial isolates. Culture was incubated at ambient temperature for 2 days. Inhibition zoneformed around the disc was measured as antimicrobial activity**.**

Extraction of Antibacterial Compound from Potential Endophytic Bacteria

Secondary metabolite of endophytic bacterial isolate was extracted following method by Suryanto *et al.*[16].Endophytic bacterial suspension (≈10^8 CFU/mL) was inoculated using steriled cotton swab on surface of Mueller Hinton Agar (MHA) and incubated for 24-48 hours at ambient temperature. Culture was copped into small pieces using spatula and put into 250 ml flask containing 200 ml methanol. The mixture was macerated for 24 hours in the flask wrapped with aluminum. Macerate was filtered using steriled filter paper. Macerate was evaporated using rotary evaporator.

Assay of Endophytic Bacterial Methanol Extract on Clinical Pathogenic Bacterial Isolates

Method proposed by Suryanto *et al.*[17] was applied in conducting this test. Endophytic bacteria methanol extract was prepared in various concentration of 100, 80, 60, 40, and 20% (v/v) in dimethyl sulfoxide. As much as 10 µL gambir endophytic bacterial extract was dripped on blank disc and left dried. Clinical pathogenic bacterial isolate (≈108 CFU/mL) was inoculated using steriled cotton swab on NA.Paper disc containing bacterial extract was put on bacterial lawn. Culture was incubated at ambient temperature for two days. Inhibition zone around paper disc indicating antibacterial activity was measured as diameter of clear zone.

Identification of Gambir Endophytic Bacteria Using Vitek Compact 2.0®

Isolate identification was performed using Vitek Compact 2.0®an automatic identification system for bacteria.

Results and Discussion

Morphological and Biochemical Character of Endophytic Bacteria

Tenendophytic bacteria were isolated from leave and root of gambir. They showed different characteristics in terms of their colony shape, edge, elevation, and color as presented on **Table 1**. Eightand two isolates were obtained from gambir leaves and root respectively. This study was to show biochemical shape and characteristic variation of endophytic bacteria isolate from gambir. Endophytic bacteria isolate with different morphological characteristic was chosen for further study.

Table1.Morphological characteristic of endophytic bacterial isolates from gambir

Isolate Source	Isolate code	Colony Morphology			
		Shape	**Edge**	**Elevation**	**Color**
Leaf	SO01	Irregular	Undulate	Flat	White
	SO02	Circular	Entire	Convex	White
	SO03	Circular	Entire	Flat	White
	SO04	Filamentous	Filamentous	Flat	White
	SO05	Irregular	Entire	Flat	White
	SO06	Irregular	Curled	Umbonate	White
	SO07	Circular	Entire	Flat	Cream
	SO08	Irregular	Lobate	Flat	White
Root	SO09	Irregular	Undulate	Flat	White
	SO10	Circular	Entire	Flat	White

Further assay on simple biochemical characteristic ofbacterial isolates was showed varied in isolate characteristics (**Table 2**). This study was the first report regarding endophytic bacteria from gambir of North Sumatera. Leaf is part of the plant which has the most endophytic bacteria, in which total population reached 10^7 per cm^2. Previous research also reported that bacteria which usually colonizes in leaf forming aggregate or clump, making it possible for endophytic bacteria correlate to each other[18].These isolates were argued to have capability in tolerating stressor given by the plant during its stay on the tissue.

Table2.Biochemical characteristic of endophytic bacteria from gambir

Isolate	Gram	Cell Shape	Biochemical Reaction									
			Citrate	Gelatin	Motility	Amylase	Catalase	Glucose	Sucrose	Lactose	Precipitated	Rift
SO01	+	Basil	-	-	+	+	+	+	+	+	-	-
SO02	-	Basil	-	-	+	+	-	+	-	-	-	-
SO03	+	Coccus	-	-	-	-	-	+	+	+	-	-
SO04	-	Basil	-	-	+	+	+	+	+	+	-	-
SO05	+	Basil	-	-	+	+	+	+	+	+	-	+
SO06	+	Basil	+	-	+	-	+	+	+	+	-	+
SO07	-	Coccus	+	-	+	+	+	+	+	+	-	+
SO08	+	Coccus	+	-	+	-	-	+	+	+	-	+
SO09	-	Coccus	+	-	+	-	+	+	+	+		-
SO10	-	Coccus	+	-	+	+	-	+	+	+	-	-

Antibacterial Activity of Endophytic Bacteria

All gambir endophytic bacterial isolates showed to have antibacterialactivity (**Figure 1**) against three pathogenic bacteria of *S. mutans*, *S. aureus,* and*E. coli* to some extent (**Table3**). SO02 and SO03 isolates demonstrated to have higher antibacterial activity with inhibition zone of 17 and 30 mm. Endophytic microorganism, especially bacteria, have a role in host plant defense against pathogen attract through secretion of bioactive metabolite which has antimicrobial properties[19].

Figure 1. Result of antibacterial assay of SO02 against *S. aureus*

Table 3.Antibacterial activity of endophytic bacteria against pathogenic bacteria

Isolate	Inhibition Zone Diameter (Mean mm ± S.D)		
	S. mutans	***S. aureus***	***E. coli***
SO01	9.75 ± 0.35	8.25 ± 0.71	10.25 ± 1.41
SO02	11.25 ± 1.10	31.13 ± 7.95	8.25 ± 0.00
SO03	8.88 ± 0.88	17.38 ± 0.88	8.75 ± 2.47
SO04	9.25 ± 0.71	14.00 ± 1.41	9.63 ± 0.18
SO05	10.13 ± 0.88	7.38 ± 0.53	8.50 ± 0.71
SO06	8.38 ± 3.36	8.50 ± 0.71	9.75 ± 0.35
SO07	10.00 ± 1.06	9.88 ± 0.53	10.38 ± 0.18
SO08	10.13 ± 0.53	7.25 ± 0.35	7.63 ± 0.88
SO09	9.00 ± 1.06	7.63 ± 0.88	9.25 ± 0.35
SO10	9.63 ± 0.18	11.00 ± 1.41	7.63 ± 0.88

Leaf is part of the plant which ispotential as microbial source producing antimicrobial compound against pathogenic microbes. Several medicinal plants accumulate their bioactive compounds in leaf in which associative microbeswhich survives in the tissue contribute[20]. Das *et al.*[21]found that two bacterial isolates *Bacillus* and *Pseudomonas* from kunubuti leaves (*Hyptis suaevolens*:Lamiaceae)showed to be activeagainst *B. subtilis, S. aureus, E. coli,* and *Candida albicans*. Mohamad*et al.*[22]also reported that endophytic bacteria isolates from medicinal plant leaves of licorice (*Glycyrrhiza uralensis*: Fabaceae) were active against *S.aureus, B. cereus, E. coli* and *Salmonella enteriditis*.

Antibacterial Activity of Methanol Extract Endophytic Bacteria from *Gambir*

It was shown that methanol extract of SO02 and SO03 inhibited clinical pathogenic bacterial isolates such as *E. coli, S. mutans, and S. aureus* to some extent (**Figure 2** and **Table 4**). Antibacterial activity test showed that SO02 inhibited more all clinical bacterial isolates tested, while SO03 seemed to be effective to *S. aureus*. This study showed that methanol extract of SO02 and SO03 might be potential to be applied as antibiotic compound against clinical pathogenic bacteria such as *E. coli, S. mutans,* and *S. aureus.*

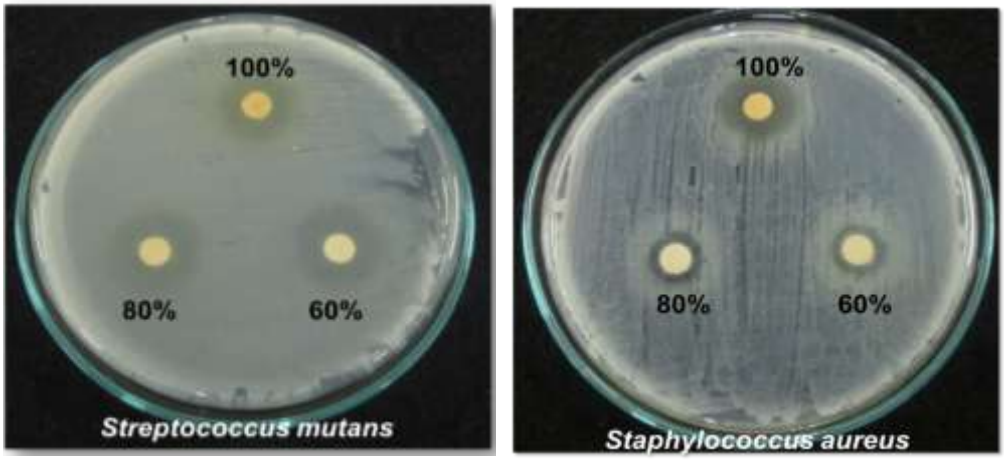

Figure2.Antibacterial activity from methanol extract of SO02 against pathogenicbacteria

Table4.Antibacterial activity of bacterial isolate methanol extract of gambiragainst clinical pathogenic bacteria

Isolate	Concentration	Diameter of Inhibition Zone Diameterin mm		
		S. mutans	***S. aureus***	***E. coli***
SO02	20%	8.55 ± 0.85	7.05 ± 0.28	7.90 ± 0.07
	40%	10.00 ± 0.78	7.50 ± 0.21	8.20 ± 0.21
	60%	14.55 ± 0.28	9.55 ± 0.42	8.75 ± 0.57
	80%	15.60 ± 1.63	10.20 ± 0.35	9.70 ± 0.35
	100%	11.20 ± 1.06	11.55 ± 1.27	13.60 ± 0.35
SO03	20%	7.60 ± 0.21	0.00 ± 0.00	6.68 ± 0.11
	40%	8.25 ± 1.13	7.00 ± 0.07	6.65 ± 0.28
	60%	8.40 ± 0.64	7.10 ± 0.35	7.40 ± 0.35
	80%	7.15 ± 0.42	6.25 ± 0.28	7.70 ± 0.21
	100%	7.10 ± 2.33	6.10 ± 0.35	8.83 ± 0.18

Identification of SO02 and SO03 Isolates Based on Vitek Compact 2.0®

Endopytic bacterial isolates, SO02 and SO03 were identified as *Enterobacter cloacae* (98%) (Table 6) and *Gemella morbillorum* (86%) (Table 7).based on biochemical characteristic against using Vitek Compact 2.0®.

Table6.Identification of SO02 using Vitek Compact 2.0®

Well	Test	Results	Well	Test	Results
2	Ala-Phe-Pro-Arylamidase	-	33	Saccharose/Sucrose	+
3	Adonitol	+	34	D-Tagatose	-
4	L-Pyrrolydonyl-Arylamidase	-	35	D-Trehalose	+
5	L-arabitol	-	36	Citrat (sodium)	+
7	D-cellobiose	+	37	Malonate	+
9	Beta Galactosidase	+	39	5- Keto Gluconate	-
10	H_2S Production	-	40	L-Lactate alkalinisation	+
11	Beta-N-Acetyl-Glucosaminidase	+	41	Alpha Glucosidase	-
12	Glutamyl Arylamidase pNA	-	42	Succinate alkalinisation	+
13	D-Glucose	+	43	Beta-N-acetyl-Galactosaminidase	+
14	Gamma-Glutamyl-Transferase	+	44	Alpha Galactosidase	+
15	Glucose Fermentation	+	45	Phosphatase	-
17	Beta-Glucosidase	-	46	Glycine Arylamidase	+
18	D-maltose	+	47	Ornithine Decarboxylase	+
19	D-Manitol	+	48	Lysine Decarboxylase	-
20	D-Mannose	+	53	L-Histidine assimilation	-
21	Beta-Xylosidase	+	56	Coumarate	-
22	Beta-Alanine arylamidase pNA	-	57	Beta-Glucoronidase	-
23	L-Proline Arylamidase	+	58	O/129 resis. [Comp.Vibrio]	+
26	Lipase	-	59	Glu-Gly-Arg-Arylamidase	-
27	Palatinose	+	61	L-Malate assimilation	-
29	Tyrosine Arylamidase	+	62	Ellman	-
31	Urease	-	64	L-Lactate assimilation	-
32	D-sorbitol	+			

A study performed by Gonzalez *et al.*[23], affirmed *E. cloacae* could infect plant. In addition to being recognized as clinical and plant pathogen, several strains have been successfully isolated from healthy plants with the potential as triggering bacteria of plant growth, biocontrol agent against disease and able to live as endophytic[24,25].

Table7.Identification of SO03 using Vitek Compact 2.0®

Well	Test	Results	Well	Test	Results
2	D-Amygdalin	-	32	Polymixin B Resistance	-
4	Phosphatidylinositol Phospholipase C	-	37	D-Galactose	-
5	D-Xylose	-	38	D-Ribose	+
8	Arginine Dihydrolase 1	(+)	39	L-lactateAlkalinization	-
9	Beta-Galactosidase	-	42	Lactose	-
11	Alpha-Glucosidase	+	44	N-Acetyl-D-Glucosamine	+
13	Ala-Phe-ProArylamidase	+	45	D-Maltose	(+)

14	Cyclodextrin	-	46	Bacitracin Resistance	-
15	L-Aspartate Arylamidase	-	47	Novobiocin Resistance	-
16	Beta Galactopyranosidase	-	50	Growthin 6.5% NaCl	-
17	Alpha-Mannosidase	-	52	D-Mannitol	-
19	Phosphatase	(-)	53	D-Mannose	+
20	LeucineArylamidase	+	54	Methyl-B-D-Glucopyranoside	-
23	L-ProlineArylamidase	-	56	Pullulan	-
24	Beta Glucuronidase	-	57	D-Raffinose	-
25	Alpha-Galactosidase	-	58	O/129 res.[Comp.Vibrio]	+
26	L-Pyrrolydonyl-Arylamidase	+	59	Salicin	-
27	Beta-Glucuronidase	-	60	Saccharose/Sucrose	-
28	Alanine Arylamidase	-	62	D-Trehalose	+
29	Tyrosine Arylamidase	-	63	Arginine Dihydrolase-2	-
30	D-Sorbitol	-	64	Optochin Resistance	+
31	Urease	-			

SO03 was identified as *Gemella morbilliorum*a Gram positive bacteria, coccus, facultative anaerobe, non-motile,anddoes not form spores. *G. morbilliorum* has never been reported as endophytic bacteria at least from gambir. Other species, which is *G. was* reported as endophytic on *Solanum trilobatum*. Other study reported that *Gemella* sp.was isolated from heavy metal polluted soil in Bangladesh[26,27].

Conclusions

SO02 cell was gram-negative, while SO03 was gram-positive bacteria. These two isolates found from gambir showed to have antibacterial properties against clinical pathogenic bacteria of*E. coli*, *S. mutans*, and *S. aureus*. Their methanol cell extract showed to have antibacterial activity as well. Identification of endophytic bacterial isolates from gambir showed thatSO02 andSO03 was identified as *Enterobacter cloacae* and *Gemella morbillorum*, respectively with similarityof98 and 86%.

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