Antagonistic Endophytic Fungi of *Hedychium coronarium* J. Koenig from Hutan Sibayak and Taman Hutan Raya, North Sumatra against *Staphylococcus aureus* ATCC® 29213™

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Abstract. Possible role of endophytic microbes has been receiving much attention from different aspects and all plants are believed to have an endophytic association. Some medicinal plants have been reported to harbor endophytic microbes capable of producing bioactive compounds. Endophytes may benefit plant host by secreting similar bioactive compounds leading to systemic plant defenses through synthesis of antimicrobial compounds. In this study, we reported that rhizome of *Hedychium coronarium* J. Koenig (a member of Zingiberaceae), native of Hutan Sibayak and Taman Hutan Raya, North Sumatra, is inhabited by various endophytic fungi morphotypes. Total of 28 fungal morphotypes were recovered from rhizome parts and differentiated based on morphological characters. Most fungal strains displayed antibacterial activities ranging from 0.00 (no activity) to 30 mm (very strong activity) against *Staphylococcus aureus* ATCC® 29213™. Based on this preliminary result, we may propose some antagonistic strains as candidates for further study regarding to the production of antibacterial compounds.

1. Introduction

Drug resistances are still considered as global health problems and issues that need to be investigated through several approaches and strategies. Intensive studies have revealed that plants constituted major bioactive metabolites in overcoming the clinical health issues. Unfortunately, the progressing resistance exhibited from infectious microorganisms, may lead the development of plant antibiotics to a deadlock situation. Therefore, an interesting and unique strategy may be applied through utilization of another rich sources of antibiotics, which are known as endophytes [1].

Endophytes are microorganisms that living inside a plant without causing any negative drawbacks yet promoting many advantages to plants. Wide range of metabolites may be explored through laboratory studies of endophytes, especially from endophytic fungi. Endophytic fungi have proven as major producers of antibiotics from associated plants with history of medicinal use or even plants living in unique habitats. Efforts to employ endophytic fungi as future producers of novel compounds or antibiotics are to be explored in this study [2].

Butterfly ginger, *Hedychium coronarium* J. Koenig, or *Gandasuli* (Sundanese, Indonesia), is a species member of Zingiberaceae originating from tropical Asia. The plants have been known as medicinal plants used in several ailments through traditional remedies in treating cold, headache, arthritis and injuries. Biological properties of its phytochemical compounds exhibit properties such as:
antioxidant, anti-inflammatory, antitumour, analgesic, hepatoprotective, anti-allergic, antihypertensive, antiasthmatic, anthelmintic, larvicidal, leishmanicidal and antimicrobial [3,4]. The rhizomes contain essential oils and organic compounds with potential antimicrobial properties.

Essential oils of fresh rhizomes of H.coronarium exhibit antimicrobial activities against Trichoderma sp. and Candida albicans while less susceptible to Bacillus subtilis and Pseudomonas aeruginosa [5]. Essential oils of H.coronarium cultivated in India displayed strong antibacterial activity against gram positive bacteria tested, i.e. B.cereus, B.subtilis and Staphylococcus aureus [6]. Methanolic and aqueous extracts of leaves and rhizomes of H.coronarium exhibit antibacterial activity against Staphylococcus aureus and S.epidermidis while less susceptible to Excherichia coli and Pseudomonas aeruginosa [7]. Variety of results have shown that rhizome of H.coronarium may be used as potential sources of antagonistic endophytic fungi against bacterial pathogens.

From our knowing, bioprospecting study of Hedychium species, especially H.coronarium and associated endophytes displaying antibacterial activities is still less reported. One study reported collection of endophytic fungi isolated from Hedychium acuminatum exposing strong antibacterial activities against S.aureus and B.subtilis [8]. In this preliminary study, we reported some potential endophytic fungal strains isolated from H.coronarium, collection of North Sumatera that displayed strong antibacterial activity against Staphylococcus aureus ATCC® 29213™.

2. Materials and Methods

2.1 Plant Material

The plant materials used in this study were specimens of fresh Hedychium coronarium rhizome collected randomly from Hutan Sibayak and Taman Hutan Raya, Deli Serdang regency, North Sumatera province, Indonesia. Rhizomes were stored in plastic bags and duplicates were authenticated by Herbarium Medanese, Universitas Sumatera Utara for identification purpose. In laboratory, rhizomes were cut into small pieces and used in isolation step.

2.2 Isolation of Endophytic Fungi

Isolation step performed in this study was based on standard surface-sterilization procedure [9]. Small pieces of H.coronarium rhizome were surface-sterilized by dipping into 75% ethanol for 2 min, 5.3% NaOCl for 5 min and 75% ethanol for 30 secs. The pieces were washed again with sterile distilled water to remove solutions. Samples were dried on Whatman filter paper and cut into another 1–2 cm smaller pieces. The pieces were placed on top of Potato Dextrose Agar (Oxoid™) supplemented with 0.1% chloramphenicol. Plates were incubated in ambient condition for 7 days for maximizing fungal growth from each pieces. Protruding hyphae was observed daily and small portion was taken using inoculation needle. Purely grown strains were differentiated based on their morphological characters.

2.3 Antagonism Assay of Endophytic Fungi

Representative clinical pathogen used in this study was Staphylococcus aureus subsp. aureus Rosenbech ATCC® 29213™, a gram positive bacteria which is susceptible to methicillin and other antibiotics. Antagonism assay were performed based on agar plug method in dual culture plate assay with modification of technical plating technique [10]. Direct colony suspensions from pathogenic strain were made by swabbing colonies into sterile physiological saline solution (0.95% NaCl) to obtain OD_{600}=0.5. One mL of cell suspensions were mixed with 15 mL molten PDA (45°C) medium, supplemented with 1% (w/v) yeast extracts for bacteria. Ratio of cell suspension with molten agar may be adjusted as 1 : 15 as this will give good and compact lawns. Molten agar medium were then plated to obtain bacterial lawns. Three plugs of aerial mycelium from each fungal endophytes were placed on top of medium and were counted as replicates. Illustration of dual culture plate assay in this study is shown in Figure 1. Plates were incubated for 2 days in ambient condition. Clear zones around mycelial plugs indicating antagonisms were measured using standard caliper in millimetre unit (mm).
3. Results and Discussion

_Hedychium coronarium_ J. Koenig was one of twenty-three species of Zingiberaceae that can be found in Hutan Sibayak. Hutan Sibayak may be considered as natural forest area that constitute for the most diverse Zingiberaceae species in North Sumatera [11]. Yet, we still sampled _H. coronarium_ from Taman Hutan Raya as comparison (Figure 1).

![Figure 1](image1.png)

Figure 1. Illustration of modified dual culture assay using three agar plugs of fungal colonies as replicates.

Our study found 28 (twenty-eight) fungal isolates from rhizomes of _H. coronarium_ sampled from representative tropical forest in North Sumatera. Each isolates were differentiated based on their morphological characters and were given a code. The results of antagonism assay against _S. aureus_ ATCC® 29213™ are presented in Table 1. Majority of isolates exhibiting antibacterial activity were mostly from Taman Hutan Raya (93%, 15 out of 16) than Hutan Sibayak (58.3%, 7 out of 12). Strong antibacterial activities were observed also from Taman Hutan Raya strains producing >30 mm diameter of inhibition zones (Figure 2).

![Figure 2](image2.png)

Figure 2. Samples of _Hedychium coronarium_ J. Koenig from Hutan Sibayak (A) and Taman Hutan Raya (B), Note the white-colored inflorescences protruding from the terminal pseudostem.
Table 1. Antagonism result of endophytic fungi against *S. aureus* ATCC® 29213™

| Locality       | Isolate Code | Diameter of Inhibition Zone (mm) |
|---------------|--------------|---------------------------------|
| Hutan Sibayak | JRD 1A       | 24.65                           |
|               | JRD 2A       | -                               |
|               | JRD 2B       | 8.46                            |
|               | JRD 2C       | 19.55                           |
|               | JRD 2D       | 23.61                           |
|               | JRD 3A       | 23.85                           |
|               | JRE 1A       | -                               |
|               | JRE 1B       | 9.41                            |
|               | JRE 2A       | -                               |
|               | JRE 2B       | -                               |
|               | JRE 4A       | 18.23                           |
|               | JRE 4B       | -                               |
| Taman Hutan Raya | S8A1     | 16.43                           |
|               | S8A2         | -                               |
|               | S8A3         | 28.73                           |
|               | S8B1         | 27.83                           |
|               | S8B2         | 36.10                           |
|               | S8B4         | 32.80                           |
|               | S8D1         | 28.80                           |
|               | S8D2         | 21.00                           |
|               | S8D4         | 0.00                            |
|               | S8E1         | 16.10                           |
|               | S8E4         | 35.33                           |
|               | S8E2         | 36.10                           |
|               | S8F3         | 25.73                           |
|               | S8F4         | 33.73                           |
|               | S8G1         | 33.13                           |
|               | S8G2         | 29.33                           |

By our understanding, this is the first report on evaluating antagonistic fungal endophytes isolated from rhizomes of *H. coronarium* in North Sumatera. Previous study in revealing number of culturable fungal endophytes from *H. coronarium* and *H. flavescens* has been reported in India with evaluation solely on their extracellular enzymes performance. Both species showed higher isolation effort on fungal endophytes from petiole and leaves rather than rhizomes [7]. The different occurrence of fungal endophytes based on locality indicated that habitat characteristics may affect the existence of certain fungal endophytic species in same plants. The phenomenon is termed as site-specific differences of same host in harboring endophytes [12]. In contrary, another study also revealed that there might be no consistent pattern of endophytes community similarity associated with variety, region, cultivation practice, and seasonal time and tissue type [13]. Since we only differentiated fungal isolates morphologically, molecular evidences in future study will give a better results in identifying endophytic fungal isolates from *H. coronarium* to support an ecological study.

Rhizome of *Hedychium coronarium* J. Koenig have been reported to contain natural compounds such as: Diterpenes, Sesquiterpenes, Diarylheptanoids, Phenolics, Fatty acids and Steroids group [4]. Most of them are isolated in the form of essential oils that exhibit antimicrobial activity against gram negative bacteria and yeast [14]. Natural compounds in plants are known to be supported by endophytic microorganisms, especially fungal endophytes [1,2]. We assume that some of the isolated fungal endophytes may synthesize either intermediates or even similar bioactive compounds with *H. coronarium* through axenic fermentation. In the next study, we will try to evaluate their production of antimicrobial compounds in liquid fermentation broth or extraction of mycelium biomass. Further characterization of compound is also needed to answer our assumption or possibility of finding novel compounds.
Figure 3. Number of antagonistic fungal isolates against *S.aureus* ATCC® 29213™ grouped by inhibition zones-based (IZ) categorization of antibacterial potency: Very Strong (++++) IZ > 30 mm, Strong (+++) 21 ≥ IZ ≥ 30 mm, Mild (+) 11 ≥ IZ ≥ 20 mm, Weak (+) 1 ≥ IZ ≥ 10 mm, None IZ = 0 mm

Identified fungal endophytes from relatives of *Hedychium* has been reported in *Hedychium acuminatum* Roscoe. The study found 8 fungal endophyte species such as: *Fusarium solani*, *F. semitectum*, *Colletotrichum alienum*, *C.aotearoa*, *C.ti*, *C.coccodes*, *C.gloeosporoides*, and *Aspergillus parasiticus*. Antagonistic activity was evaluated by testing their cell-free fermentation broth against microbial pathogens using well diffusion agar method. Only two species were reported to exhibit considerable antagonism against *B.subtilis* and *S.aureus*. Although we only tested the fungal endophytes against sensitive strain, *S.aureus* ATCC® 29213™; potential candidates will be tested against drug-resistant strains in future study.

4. Conclusions

In this study, we successfully isolated 28 species of fungal endophytes from *Hedychium coronarium* rhizome with locality from Hutan Sibayak and Taman Hutan Raya, North Sumatera. Through antagonism assay using dual culture plate assay, we obtained 21 (twenty-one) antagonistic fungal endophytes. Most fungal endophytes (9 out of 12) showed strong antagonism against *Staphylococcus aureus* ATCC® 29213™ displaying Inhibition Zone (IZ) > 30 mm.

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References

[1] Strobel G A 2003 Endophytes as sources of bioactive products *Microbes Infect.* 5 535–44
[2] Strobel G and Daisy B 2003 Bioprospecting for Microbial Endophytes and Their Natural
Products Microbiol. Mol. Biol. Rev. 67 491–502

[3] Lim T K 2014 Edible medicinal and non medicinal plants: Volume 8, flowers Edible Med. Non Med. Plants Vol. 8. Flowers 1–1024

[4] Chan E W C and Wong S K 2015 Phytochemistry and pharmacology of ornamental gingers, Hedychium coronarium and Alpinia purpurata; a review J. Integr. Med. 13 368–79

[5] Joy B, Rajan A and Abraham E 2007 Antimicrobial Activity and Chemical Composition of Essential Oil from Hedychium coronarium Phyther. Res. 21 439–43

[6] Ho J C 2011 Antimicrobial, mosquito larvicidal and antioxidant properties of the leaf and rhizome of Hedychium coronarium J. Chinese Chem. Soc. 58 563–7

[7] Uzma F, Konappa N M and Chowdappa S 2016 Diversity and extracellular enzyme activities of fungal endophytes isolated from medicinal plants of Western Ghats, Karnataka Egypt. J. Basic Appl. Sci. 3 335–42

[8] Hastuti U S, Rahmawati D, Sari R Y, Fitri R D and Al Asna P M 2018 Antimicrobial activity of endophytic fungi isolated from a medicinal plant, Hedychium acuminatum Roscoe AIP Conference Proceedings vol 050002 (Japan: AIP Publishing) p 050002

[9] Yurnaliza, Aryantha I N P, Esyanti R R and Susanto A 2014 Antagonistic activity assessment of fungal endophytes from Oil palm tissues against Ganoderma boninense Pat Plant Pathol. J. 13 257–67

[10] Balouiri M, Sadiki M and Ibnoussa S K 2016 Methods for in vitro evaluating antimicrobial activity: A review J. Pharm. Anal. 6 71–9

[11] Siregar E S and Pasaribu N 2008 Inventarisasi Jenis-Jenis Zingiberaceae Di Hutan Sibayak Sumatera Utara J. Penelit. MIPA 2 22–4

[12] Gange A C, Dey S, Currie A F and Sutton B C 2007 Site- and species-specific differences in endophyte occurrence in two herbaceous plants J. Ecol. 95 614–22

[13] Ek-Ramos M J, Zhou W, Valencia C U, Antwi J B, Kalns L L, Morgan G D, Kerns D L and Sword G A 2013 Spatial and Temporal Variation in Fungal Endophyte Communities Isolated from Cultivated Cotton (Gossypium hirsutum) PLoS One 8

[14] Sabulal B, George V, Dan M and Pradeep N S 2007 Chemical Composition and Antimicrobial Activities of the Essential Oils from the Rhizomes of Four Hedychium Species from South India J. Essent. Oil Res. 19 93–7