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Endophyte Extract From Nattle (*Urtica dioica* L.) Against Staphylococcus aureus and Escherichia coli

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Abstract. Endophytes are microorganisms that colonize the plant host tissue. Endophytic colonies are able to produce secondary metabolites in accordance with their host plants, endophytes have been found in every plant species studied to date and are recognized as a source of new natural compounds that have the potential to be exploited in medicine. This study aims to isolate and obtain endophytic fungi and to test secondary metabolites that have the potential as antibacterial against Staphylococcus aureus and Escherichia coli. Plant samples were taken around the village of Mamben, Wanasaba. The research method used is experimental laboratory. This research was conducted by isolating and identifying endophytic fungi from nettle stems. This investigation was resulted a type of endophytic fungal strain using Potato Dextrose Agar (PDA) media. The production of secondary metabolites of endophytic fungi was obtained by fermentation method using brown rice for a month then macerated for 3 days using methanol solvent and tested for secondary metabolites and antibacterial activity. The endophytic fungal extract of nettle stem contains alkaloids and flavonoids and is able to inhibit the growth of Staphylococcus aureus bacteria with the highest concentration of 60% with an inhibition zone of 18 mm. whereas, the extract of nettle stem endophytic fungus on Escherichia coli has not an inhibition zone.

Keywords : Endophytes, Nettle stem, Antibacterial

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Introduction

Currently, efforts in lead compound discovery have been commenced to utilize endophytic fungi lived in plant tissues such as leaves, twigs, small branches or roots [1]. Endophyte are microorganisms that are feasible to explore as natural product sources. they are sustainable strategies to produce diverse secondary metabolites and lead to discover natural drug derived from endophytic fungi [2]. Endophyte have been found in every plant species investigated presently, and have been recognized as potential sources of lead compound in organic synthesis [3].

Secondary metabolites are produced by plants as a defense mechanism against predators and pathogenic microorganisms [4]. The ability of endophytic fungi to produce secondary metabolites appears as a result of genetic interaction to their host plants to produce biological activities similar to secondary metabolites produced by their hosts [5]. Exploration of bioactive compounds is currently slowly diverted and tends to utilize microbes like endophyte as the producers of secondary metabolites which have several advantages such as fast growth, great survival in extreme environments and alteration of environmental conditions resulting in the production of various secondary metabolites. Nature plays an important role as the main supporting compounds in structural framework as a drug candidates [6], [7].

Generally, the activity of metabolites produced by an endophytic fungus can be predicted from the habitat of the host plant of the endophytic fungus. Endophytic fungal host plants that are resistant to pests and pathogenic microorganisms will tend to have secondary metabolites that have the potential to be studied due to biological and biochemical interactions [8]. Nettles are a plant living in fertile soil. they are naturally resistant to pests and diseases. In addition these plants have been carried a mystery of endophyte and their secondary metabolite accumulation due to resistant to pests [9].

The pharmacological activities of endophytic fungi are generally antibacterial and antifungal. In addition, they also play a role in the enzyme industry with various groups of compounds including peptides, polyketides, alkaloids, hybrid peptides and organic acids [10]. This study aimed to isolate and identify secondary metabolites such as alkaloids and flavonoids in fungal extract on nettle (*Urtica dioica* L.) stems and to test the endophytic extracts to the growth activity of Staphylococcus aureus and Escherichia coli bacteria. These secondary metabolites were used by plant-endophyte symbiosis as a resistance barrier to suvive in their environment. So there is a great chance of finding antibacterial candidates for these types of secondary metabolites [11].

Experimental

Media Preparation

Potato Dextrose Agar (PDA)

9.36 grams of PDA media, chloramphenicol 0.005 grams and distilled water 240 mL were stirred at room temperature. The solution was sterilized by autoclaving at 121 °C for 15 minutes. Then it was poured into a petri dish and stored in an incubator overnight [3].

Beef Peptone Agar (BPA)

Preparation of BPA agar media was carried out by weighing 9.36 grams of media powder into 240 mL of distilled water. The media is poured into a beaker then homogenized and heated until boiling. The solution was sterilized by autoclaving at 121 °C for 20 minutes. Then it was poured into a petri dish and stored in an incubator overnight to harden [3].

Isolation of Endophyte

The identified stem samples were approximately 1-2 cm in size and were sterilized in 70% ethanol for 1 minute, 5% sodium hypochlorite for 5 minutes and 70% ethanol for 30 seconds. The dry segments were cut in half and placed in PDA media. Covered with plastic wrap and incubated for 3-7 days. The fungal strains appearing in each medium were sub-cultured in new PDA media to obtain isolated pure fungal strains [3].

Fermentation of Endophyte

All fungi isolated in PDA media containing isolated fungi were cut into 2 mm square and inoculated with rice medium in Erlenmeyer. This medium contains 250 grams of raw rice in 250 mL of distilled water then filtered, sterilized and autoclaved at 121 °C for 20 minutes. In addition, Erlen-

meyer flasks containing all isolated fungal strains were covered with cotton and Wrap laul plastics were incubated for four weeks. Furthermore, the fermented mushrooms were extracted with methanol for three days. The methanol extract was added to the sample tube and thickened with a rotary evaporator [3].

Chromatography Test

The chemical profile was carried out by TLC analysis. Each dry extract from the first fermentation was added with a few drops of methanol and then shaken using a water bath. The eluent system for TLC analysis used chloroform and methanol in a ratio of 10: 1. Analytical visualization of the compound TLC was carried out by spraying with a 10% vanillin solution in sulfuric acid [7].

Phytochemical test

Alkaloids

The extract of nettle stem endophytic fungus was dissolved with 5 mL of 2 N hydrochloric acid. The solution obtained was then divided into 3 test tubes. The first tube was used as a blank, 3 drops of Dragendroff's reagent was added to the second tube, and 3 drops of Meyer's reagent were added to the third tube. The formation of a reddish brown precipitate in the second tube and a white to yellowish precipitate in the third tube indicated the presence of alkaloids [12].

Flavonoids

The test was carried out by taking 2 mL of endophytic mushroom extract each, then heating for 5 minutes. After heating, 0.1 grams of magnesium metal and 5 drops of concentrated hydrochloric acid were added. If the sample solution is yellow, orange to red, it is positive for flavonoids [12]

Antimicrobial Activity Test

The media used for the antibacterial test was BPA media. Each petri dish containing BPA media was divided into 8 regions and labeled to place positive control discs *(*1 grams of chloramphenicol (100% w/v), negative controls (Dimethylsulfoxide) and 6 treatments with concentrations of 10%, 20%, 30%, 40 respectively. %, 50%, 60%.

Pure cultures of S. aureus and E. coli bacteria suspended with a turbidity level of 0.5 Mc Farlan (Bacterial density 1.5 x 108). The pure culture was taken with sterile cotton and scratched in BPA media until the entire surface of the petri dish was covered [14].

Data analysis

Analysis of research data with endophytic fungi against Staphylococcus aureus and Escherichia coli bacteria by looking at the inhibition zone of the fungal extract, using the disc method. The diameter of the inhibition zone is categorized according to its inhibitory strength. The weak, medium and strong categories have inhibition zone diameters < 5 mm , 6-10 mm , 11-20 mm, respectively [14].

Results and Discussion

In this study, endophytic fungi were isolated from the stems of nettle (*Urtica dioica* L). Isolation was continued by testing the activity of secondary metabolites that have the potential to inhibit the growth of Staphylococcus aureus and Escherichia coli bacteria. Isolation of endophytic fungi aims to obtain fungal strains found in nettle stems, in this study a type of endophytic fungal strain from nettle stems was obtained after incubation for 7 days at 25 °C. In the study [15] the addition of chloramphenicol as an antibacterial and incubated for 2 days at 25 °C produced 2 types of isolates, whereas according to the study [16] with the same incubation temperature for 2 days without the addition of antibacterial obtained 3 isolates of endophytic fungi. Meanwhile in the study [17] which used a temperature of 30 ° C incubated for 4 days also obtained fewer isolates. This is due to the influence of the incubation temperature and the length of incubation time and the nutrients of the media used.

Nettle stalks were incubated for 3 to 7 days at a temperature of 20-25 °C on PDA media to grow endophytic mycelium (see Figure 1). The media contains chloramphenicol which functions as an antibacterial. Furthermore, the growing fungi were purified or subcultured into other new PDA media. The purification was aimed to diferentiate endophytic colonies with different morphology to become single isolate. fungus was selected based on other morphological differences, but the

morphology of the endhopyte grew on this medium did not vary, only one type and tended to have the same color. The macroscopic and microscopic characteristics of the fungus obtained have the characteristics in Figure 2. The isolated fungus grew on PDA media by producing white isolates in the initial growth, and the isolates changed color to dark green at the day sixth of growth and the fungal hyphae texture was like cotton. while microscopic observations produced round conidia like ovoid, conidia consist of cells, round and grow in chains. The macroscopic and microscopic characteristics according to Figure 2 and based on the classification according to [18], it can be seen that the isolate belongs to the Moniliaceae family, genus Penicillium sp. In addition, the fibrous hyphae emerge in green color on it's growth media [19], the presence of strands of conidia clustered around phialide [20] and also according to [21] the morphology of penicillium showed criteria that resemble to this study such as colony surface color is yellowish green, radial of colony growth type, and smooth texture of colony surface.

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The purified endophyte were fermented on brown rice to investigate and evaluate to obtain a chemical metabolite profile of the fungus. Fermentation was carried out for 3-4 weeks. The growth rate of the fungus in this study was fast where the fungus covered all brown rice media within 3 weeks. During fermentation, the temperature used is equivalent to room temperature. Brown rice is used as a source of nutrition which has the advantage of being easy to obtain and easy to prepare. At the fermentation stage that must be considered in the process such as temperature, humidity and the possibility of contamination must be controlled. Environmental changes can affect the growth of fungi and the production of their metabolites. After 4 weeks, the printed media was soaked in methanol for 3 days and heated on a water bath.

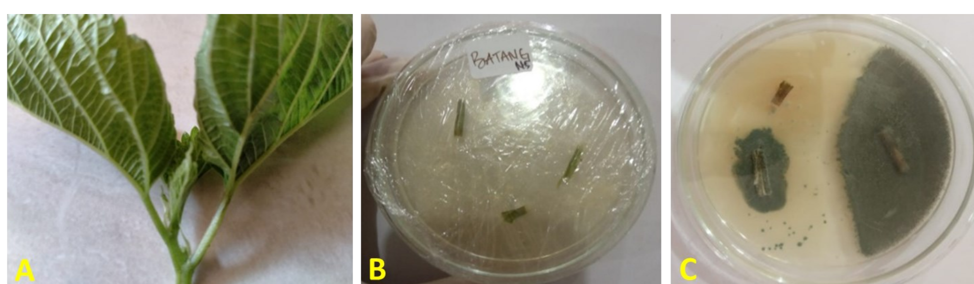

Figure 1. Nettle stalks: a) plant; b) before; c) after incubation

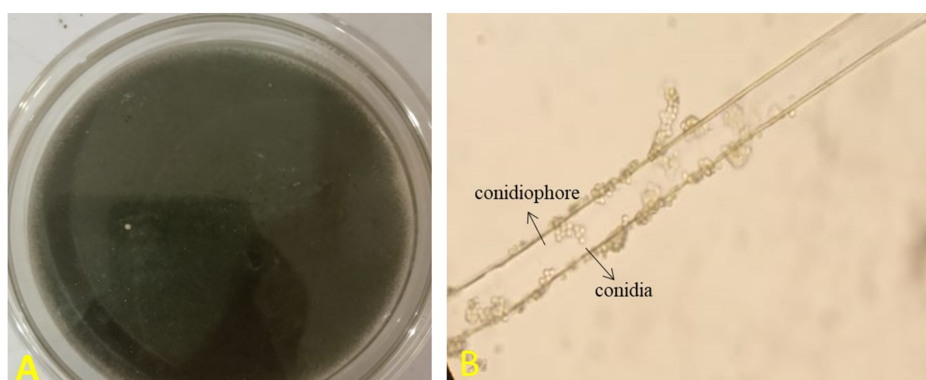

Figure 2. a) Endophyte sample; b) microscope magnification

Identification of the chemical content of secondary metabolites from the extract of the nettle stem endophytic fungus resulted in the content of alkaloids and flavonoids, which can be seen in Table 1.

The test resulted suggested that the endophytic mushroom extract of nettle stem contains secondary metabolite components, namely Alkaloids and Flavonoids (Figure 3). Positive reactions to alkaloids were the presence of a white precipitate using Meyer's reagent and a brownish precipitate using Dragendrof's reagent, and positive reactions for flavonoids indicated a change in color to yellow after the addition of magnesium and HCl. In alkaloids consist of nitrogen atom on the structural framework in cyclic system and contain various substituents such as amine groups, amides, phenols, and methoxy so that alkaloids are semipolar. In alkaloid identification with Mayer reagent, the nitrogen in alkaloids was predicted to react with metal ion of potassium (K^+) from potassium tetraiodomercurte (II) producing a complex of potassium-alkaloid precipitating [22]. While the Dragendorff's reagent was reacted to amine groups to form ammonium salt after prepared with acid due to base properties of amine groups. Then the ammonium salts react with potassium-tetraiodo-bismuthate to form salt complex give color alteration yellow to orange to red to brown depans on type of the alkaloids [23]. Identification of falvonoids was used Shinoda test. The strong acid hydrolyzed the glycoside-flavonoid to aglycone-flavonoid and the presence of yellow color was indicated the existence of the flavone or isoflavone groups [24].

Antibacterial activity investigated with Staphylococus aureus and Escherichia coli. Results of the study was found that there was a clear zone of endophytic extract from nettle stems against Staphylococcus aureus, whereas the extract showed negative activity against Escherichia coli. The distinction of Staphylococcus aureus and Escherichia coli located in composition and the structure of cell walls. In Escherichia coli as gram negative and Staphylococcus aureus as gram positive bacteria can affect the antibacterial activity of a chemical compound. Single layer of gram-positive bacterial cell walls contains 90% peptidoglycan layer or tend to polar wall cell surfaces with low lipid content (1-4%). membrane cell layers of gram negative bacteria have more complex cell walls consisting of three layers, namely the outer layer of lipoprotein, the middle layer of lipopolysaccharide and the inner layer of peptidogly-

Tabel 1. Result of Phytochemical Analysis

Chemical Component	Reagent	Observation
Alkaloids	Mayer	White precipitate
	Dragendroff	Orange precipitate
Flavonoids	Mg, HCl	Yellow

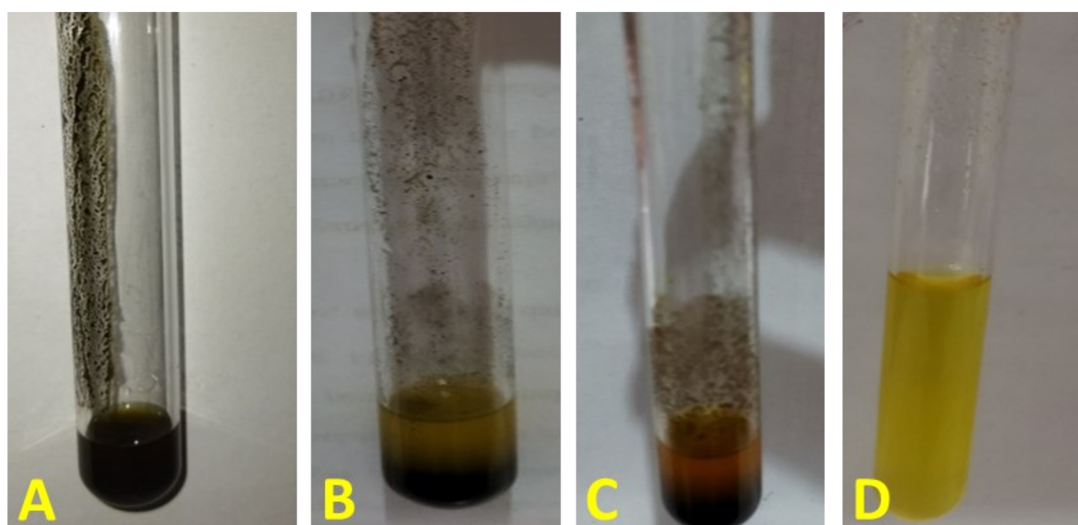

Figure 3. Phytochemical Test of Endophytic Extract: a) blanko; and in reagent : b) Meyer; c) Dragendrof; d) Mg, HCl/Flavonoid

can with lipid content reaching 11-12% [25].

Alkaloids and flavonoids are polar compouds that have amine group of alkaloids as polar compounds, and flavonoids have glycosidic groups and hydroxyl groups that act as polar functional groups. So that these compunds is easier to penetrate the polar peptidogly can layer of Staphylococcus aureus compared to Escherichia coli with three layer cell membranes of the non-polar lipid layer [26]. In addition, Escherichia coli contains more lipids, less peptidoglycan and have an outer bilayer membrane to inhibit compounds that penetrate to inner cells [27]. These circumstances induce endophytic extract can not provide antibacterial activity against Escherichia coli bacteria.

The results of the inhibition of endophytic fungus extract against Staphylococcus Aureus shown in Table 2 show that the negative control group Dimethyl-sulfoxide (DMSO) did not have inhibition, which was indicated by the average value of the clear zone of 0 mm. The clear zone started to form at 10% extract concentration with an average value of 3.3 mm. At 20% extract concentration, the clear zone decreased with an average value of 2.93 mm. At 30% extract concentration the clear zone increased with an average value of 13.73 mm. At 40% extract concentration, it increased with an average value of 14.76. At a concentration of 50% it increased again with an average value of 16. At a concentration of 60% the clear zone increased further and became the largest clear zone with an average values of 18 mm. The positive control group (chloramphenicol) produced the largest clear zone of all the treatment group with an average value of 26.33 mm. Compare with commercial antimicrobial, the 60% of extract still lower activity. This circumstance caused by the endophytic extract used in test were crude extract, different with pure compound in commercial antimicrobial.

Based on the calculation of the diameter of the inhibition zone, all the endophytic fungal extracts were classified as strong because they were > 10 mm and some were classified as very strong > 20 mm [28]. Alkaloids and flavonoids have activity against Staphylococcus aureus by damage the cell membrane of bacteria [29]. Moreover the alkaloids breakdown the bacteria growth by four mechanisms of action ; interfering cell division, inhibiting respiratory and enzyme activation, membrane disruption, and Affecting virulence genes [30].

Conclusion

*A*n endophytic fungus that has been isolated from nettle stem (*Urtica dioica* L.) is suspected to be Penicillium sp and contains alkaloid and flavonoids. The metabolite activity of the endophytic fungus isolates from nettle stems had the potential to produce antibacterial compounds against Staphylococcus aureus while Escherichia coli did not have antibacterial activity.

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Concentrations (%)	Inhibition zone (mm) replication			Average (mm)	Category
	I	II	III		
10%	3,1	3,5	3,3	3,3	weak
20%	3	3	2,8	2,93	weak
30%	15	14,2	12	13,73	medium
40%	15,3	14	15	14,76	medium
50%	17	14	17	16	strong
60%	11	21	22	18	strong
Chloramphenicol (100%)	29	26	24	26,33	very strong
Negative Control (DMSO)	0	0	0	0	none

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