

Characterization of phytochemical compounds (qualitative and quantitative) in the endophytic fungi the bark of the *Duwet* plant

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Article History

Received : May 08th, 2022

Revised : May 25th, 2022

Accepted : June 08th, 2022

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Abstract: This study aims to characterize the phytochemical compounds in the endophytic fungi the bark of the *Duwet* (*S. cumini* L.). *Duwet* skin samples were taken from various regions in East Java, including Batu City, Malang Regency, Pasuruan Regency, Lamongan Regency, Gresik Regency, and Situbondo Regency. The method used in this research is an experiment, namely the isolation of endophytic fungi, qualitative and quantitative phytochemical content tests. The results showed that there were variations in the content of endophytic fungi in the *duwet* bark from one location to another. All samples of endophytic fungi isolate from *Duwet* stem bark contained flavonoid compounds, alkaloids, terpenoids, phenolics and tannins. Phytochemical compounds of endophytic fungi in the bark are the result of symbiosis between endophytic fungi and their host plants. Endophytic fungi live in intracellular tissue, then absorb nutrients from their host plants, and include phytochemical compounds found in the bark of the *duwet* plant.

Keywords: characterization, endophytic fungi, phytochemical compounds, the bark, *duwet*

Introduction

All parts of *Duwet* plant (*Syzygium cumini* Linn) with other names *Syzygium jambolanum* Lam or *Eugenia cumini* Druce can be utilized for traditional medication by society (Machado et al., 2013); (Singh et al., 2019); (Sharma et al., 2013). One part of *duwet* plant which can be utilized for medication is its bark. Bark of adult *duwet* plant shows wide zone corks distinguished into upper and lower cork zones, forming rhytidoma; cork consisting of elongated tangential rectangular cells, several thick upper layers, stratification and reddish brown, having a group of 2-4 stone cells and fine elements from phloem; thin and colorless lower corks; with cambium; secondary phloem consisted of sieve tissue, and phloem vessels; parenchyma phloem with thin walls and in form of polyhedral; oval and elongated sclereid cells. Phloem vessel contains 1-4 reddish brown cells, there is calcium

oxalate crystal rosette, round to oval starch grains, with diameter 5-11 μ .

S. cumini bark contains *betulinic acid*, *beta sitosterol*, *friedelin*, *epi friedelanol* and *ester epi friodelanol (eugenin)*. The bark also contains *beta sitosterol D glucoside*, *kaempferol-3-o-glucoside*, *quercetin*, *myricetin*, *astragalin* and *gallic acid* (Rodrigues et al., 2015; Rajkumari et al., 2018; Yadav et al., 2011). There is a necessity to search for new ecological niches as potential sources of natural bioactive agent for medical, agricultural, and industrial application which are unprecedented, should be renewable, environment friendly, and accessible (Negri & Tabach, 2013; Yadav et al., 2014). The discovery of natural products has an essential role in the quest of new medicine, and as the most potential source to the discovery of new bioactive molecule. Natural product is chemical compounds originated from living organism. The most notable producer of natural products can be found in various organism groups including

plants, animals, marine macro-organisms, (sponges, coral reefs, and algae), and microorganisms (bacteria, actinomycetes, and fungi). The discovery of natural products involves isolation, structural elucidation, and developing biosynthetic pathway from secondary metabolites (Subedi *et al.*, 2014). More than 20,000 bioactive metabolites come from microbes (Iasnaia Maria de Carvalho *et al.*, 2017).

Fungi are one of the most crucial groups from eukaryotic organisms well-known for producing many new metabolites which are directly used as medicines or functioned as a main structure for synthetic modification (Palanichamy *et al.*, 2018; Balakumaran *et al.*, 2015; Tavares *et al.*, 2020). Explained that many fungi secondary metabolites with various chemical structures and their extensive biological activities reflected a high synthesis capability of fungi. There are 1,500 fungi metabolites reported possessing anti-tumor and antibiotics activities and some had been permitted as medicine. These include *micafungin*, anti-fungi metabolite from *Coleophoma empetri* (*mycophenolate*, a compound from *Penicillium brevicompactum*, used as prevention of kidney transplantation refusal; *rosuvastatin* from *Pecillium citrinum* and *P. brevicompactum* applied to treat *dislipidemia*; *cefditoren pivoxil*, antibiotic compound with wide spectrum originated from *Cephalosporium* sp; and *Illudin S.*, a sesquiterpenoid compound from *Omphalotus illudens*; which has anti-cancer activities (Rogerio *et al.*, 2010; Tavares *et al.*, 2020).

Plants can be functioned as hosts of many microorganisms recognized as endophytes. An endophyte is a microorganism (mostly fungi and bacteria) inhabiting host plant for all or several parts of its life cycle. It colonizes internal plant tissues under epidermis cell layers without causing obvious damage or *sympto-matic* infection on its host, living in intracellular tissues and seemed that it can penetrate living cells (Tavares *et al.*, 2020; Sharafeldin & Rizvi, 2015). An endophyte forms unobtrusive infections in healthy plant tissues or in almost all life cycle and its host's tissues emerge without symptoms, and is still asymptomatic for years and only becomes a parasite when its host is pressured (Kaur & Bansal, 2020; Palanichamy *et al.*, 2018).

Endophytic fungi are *polifiletik* ecological group of fungi which are various, mostly are *ascomycetes* and *anamorfik* fungi (H Kara, 2014); (Sharafeldin & Rizvi, 2015); (Rosannah, 2014). The symbiosis of endophytic fungi and host yields many bioactive metabolites (Kaur & Bansal, 2020). Many of endophytic fungi possess ability of synthesizing bioactive compounds which can be used as potential source of pharmacy. Endophytic fungi have been proved to be beneficial for the discovery of new medicine as suggested by chemical diversity of secondary metabolites. Many of endophytic fungi have yielded new anti-bacteria, anti-fungi, anti-viral, anti-inflammation, anti-tumor, and other compounds of alkaloid, steroid, flavonoid and terpenoid derivation and other structure types (Rashied *et al.*, 2022; Rogerio *et al.*, 2010); Prajapati *et al.*, 2021). Therefore, the existence of medicinal microorganism source will eliminate harvesting process and extract plants which are slowly growing and relatively rare, and price for the medicine will be cheaper and medicines will be quickly available through microbial (fungi or bacteria) multiplication (Palanichamy *et al.*, 2018).

Several criteria that should be considered in plant selection strategy, i.e.: First, plants from unique ecological environment niches and growing in special habitat especially those possessing distinguished biological survival ability (Palanichamy *et al.*, 2018; Mudiana & Ariyanti, 2020). Second, plants which have *ethnobotanical* history and are used for traditional medicines should be studied, because inhabiting endophyte can be a compound source that has bioactive compounds as possessed by those plants. Third, endemic plants, that have lives in a certain area with critical condition. Fourth, plants growing in area of great biodiversity also have endophytic potential with large variety too. Fifth, plants surrounded by pathogen infected plants, and do not show symptoms of infection which indicate that endophytic fungi have antimicrobial activities compared to other plants. Sixth, young plant tissues are more suitable for isolation of endophytic fungi than older tissues which frequently contain many additional fungi which make isolation of slow-growing fungi difficult.

Materials and Methods

Endophytic Fungi Isolation

Media PDA Making

10.73 g PDA media were dissolved in 275 ml distilled water, then heated until boiling then placed on petri dishes of 10 ml each and in test tubes of 5 ml each using micropipette.

Peptone Media Making

About 4.49 g peptone was dissolved in 450 ml distilled water, then heated until boiling. Then it was taken 9 ml each and placed on other test tubes.

Media Sterilization

Media which had been placed in petri dishes and test tubes were waited until a bit cool, then wrapped using brown papers and tied with threads and sterilized using autoclave in the temperature of 150 °C for 15 minutes. Then the media were cooled and wrapped again and kept in the refrigerator and the media were ready to be used after 3x24 hours or approximately 3 days of storage.

Bark Samples Preparation

Duwet bark samples were taken from various areas in East Java, including city of Batu, regency of Malang, Pasuruan, Lamongan, Gresik and Situbondo. 1 g bark samples were washed with sterilized distilled water until clean, then the samples were pulverized using a mortar hammer until smooth and suspended. Sample suspensions were placed in a tube with 9 ml peptone in it, then were vortexed until forming precipitation and suspension (as diluent 10^{-1}) next it was carried out the staged dilution process as much as 6 times dilution until reaching concentration of 10^{-6} .

Sample Inoculation on Media

1 μ l of suspension from each dilution was taken and inoculated on PDA media in petri dishes, then was incubated in temperature of 25 °C then further observed on the 5th until 7th day after inoculation.

Observation

Observation was carried out from the 7th day on endophytic fungi culture growing on PDA media in petri dishes. Of all dilutions, it was

chosen endophytic fungi which grew dominantly.

Planting on Slanted Media

Selected endophytic fungi isolates were taken with an ose needle, then planted on slanted PDA media inside test tubes then the tubes were shut with cottons and kept in the temperature of 30 °C for further processes.

Phytochemical Test

Endophytic Fungi Supernatant Preparation

0.5 g endophytic fungi which had been separated from PDA media were dissolved with 2 ml of distilled water while pulverized with a mortar hammer. The solution then was centrifugated at speed of 6,000 rpm for 5 minutes. Supernatant was taken and diluted until 25 ml. Supernatant solution produced from the dilution was used in qualitative and quantitative testing processes.

Phytochemical Qualitative Determination

Flavonoid Qualitative

Flavonoid testing was conducted with methods by (Parwata, 2016); 5 ml of samples were placed in beaker glasses, then added 30 ml of distilled water and stirred using a magnetic stirrer for 2 hours. The solution was filtered using whatman no. 42 filter paper. Every 10 ml of the filtrate was added with 5ml of 1 M ammonia and 5 ml of concentrated sulfuric acid. Formation of yellow colour indicated the presence of flavonoid.

Alkaloid Qualitative

Alkaloid test used was the test presented by (Baud *et al.*, 2014); 5 ml of supernatant was added with 20 ml of 50% sulfuric acid in ethanol. Then the solution was heated until boiling for 2 minutes in waterbath. Then it was filtered using whatman no. 42 filter paper. The filtrate was made into alkaline by adding 5 ml of 28% ammonia solution in a separating funnel. Then 5 ml of chloroform solution was added, that formed solution with two separated phases, then the upper phase solution was taken. 2 ml of the upper phase solution obtained was added 0.5 ml of Dragendorffs' reagent (1.7 g of Bismuth subnitrate, 20 ml of glacial acetic acid, 80 ml of distilled water, and 100 ml of 50% KI solution in water were all mixed as stock solution. 10 ml of

stock solution was added with 20 ml of glacial acetic acid, then added with distilled water up to 100 ml as reagent which was ready to be used). The presence of orange sediment showed the presence of alkaloid.

Terpenoid Qualitative

Test used methods conducted by (Chigozie *et al.*, 2014); 5 ml of supernatant was placed in 100 ml beaker glass, then added 30 ml of chloroform. The solution was stirred using magnetic stirrer for 2 hours. Then it was moved into separated funnel. The solution was left until forming separated phases solution. The upper phase was taken, and every 5 ml of the upper phase was added 3 ml of concentrated sulfuric acid. The formation of reddish-brown color showed the presence of terpenoid.

Phenolic Qualitative

Phenolic test used methods conducted by (Lemino Singh & Bag, 2013); 1 ml of sample was placed into test tube, then added with 5 ml of distilled water and homogenized with a vortex. Then 10 drops of 10% Pb acetate solution was added. The presence of white sediment showed the presence of phenolic.

Tannin Qualitative

The test used (Chigozie *et al.*, 2014) methods. 1 ml of supernatant was put into test tubes filled with 5 ml of distilled water. The solution was homogenized with a vortex. Then it was heated in waterbath until boiling and left for 10 minutes. The solution was cooled, then filtered with whatman no. 42 filter paper. 5 ml of the filtrate was added with 3 drops of 0.1% FeCl₃ solution. The formation of green to dark blue color showed the presence of tannin.

Phytochemical Quantitative Determination

Flavonoid Quantitative

Flavonoid quantitative determination followed the procedure presented by (Chigozie *et al.*, 2014); 50 ml of 80% methanol in distilled water was added to 10 ml of supernatant sample in beaker glass, after homogenized, the solution was closed and left for 24 hours in the room temperature. Formed supernatant was discarded and the residue was extracted using ethanol with the same volume three times. The solution then was filtered, the residue gathered on the filtered

paper was transferred to the porcelain cup, then dried and scaled. The weight of the residue was the weight of flavonoid. The flavonoid percentage was calculated by comparing flavonoid weight to the samples used.

Alkaloid Quantitative

Alkaloid quantitative determination was conducted according to (Parwata, 2016) method. 200 ml of 10% acetate in ethanol was added to 10 ml of supernatant sample in a beaker glass and left for 4 hours. The solution then was evaporated until ¼ part of it remained, then added 15 drops of ammonium hydroxide solution (1:1) in distilled water until forming sediment. The solution was left for 3 hours until forming perfect sediment. The supernatant fluid was discarded, while the sediment was washed with 20 ml of 0.1 M ammonium hydroxide, then filtered using whatman no. 42 filter paper of which weight had been known. The weight of the residue was the weight of alkaloid formed. The concentration was determined by comparing formed alkaloid weight to the used sample weight.

Terpenoid Quantitative

Terpenoid determination was based on methods conducted by (Indumathi *et al.*, 2014); 10 ml of supernatant sample was added by 90 ml of ethanol and stirred with magnetic stirred for 24 hours. The extract was then transferred to separating funnel. 10 ml of petroleum ether was added to the extract, so that a solution with two separated phases formed. Take the upper solution and put it in a beaker glass of which weight had been known. Petroleum ether solution was evaporated to dry, then the beaker was scaled. The weight difference of the beaker glass after drying and the weight of the initial beaker glass was the weight of terpenoid. Terpenoid percentage was determined by comparing the weight of terpenoid to the sample used.

Phenolic Quantitative

Phenolic quantitative determination was conducted based on method used by (Ahmad *et al.*, 2017; Bello *et al.*, 2018). 1 ml of supernatant sample was added with 4 ml of 35% Folic C solution in distilled water. The solution was homogenized with a vortex for 1 minute and left for 1 minute, then added 2 ml of 10% Na₂CO₃, hence the solution was homogenized with a

vortex for 1 minute and left for 2 hours in the room temperature. The green color was formed of which absorbance value was measured at 765 nm using a uv vis shimadzu uv-1800 spectrophotometer. Phenolic concentration was determined with the standard gallic acid curve.

Tannin Quantitative

Tannin quantitative determination was based on what conducted by (Chigozie et al., 2014); (Chi Chi, 2020). 50 g of sodium tungstate (Na_2WO_4) was dissolved in 37 ml of distilled water, Folin Denis reagent was made. Then 10 g of phosphomolybdic acid ($\text{H}_3\text{PMo}_{12}\text{O}_{40}$) and 25 ml of phosphoric acid (H_3PO_4) were added. After refluxing for 2 hours, the solution was cooled and diluted with distilled water to 500 ml.

10 ml of supernatant sample was placed in an Erlenmeyer flask, then added 100 ml of distilled water. The solution was boiled for 1 hour then filtered using whatman no. 42 filter paper. 10 ml of the filtrate sample was transferred into 100 ml

volumetric flask, then added 5 ml of Folin Denis reagent and 10 ml of saturated Na_2CO_3 solution then added distilled water until 100 ml. The solution was left at temperature of 25 °C for 30 minutes for color formation. Green colour formed was measured its absorbance value at 700 nm wavelength with a uv vis shimadzu uv-1800 spectrophotometer. Tannin concentration was determined with the standard gallic acid curve.

Results and Discussion

Endophytic fungi isolation

Endophytic fungi isolation was carried out on six Duwet plant (*S. cumini*, L.) bark samples obtained from various areas including city of Batu, regency of Malang, Pasuruan, Lamongan, Gresik, and Situbondo. Result of Duwet bark endophytic fungi isolation from the six areas can be seen on figure 1.

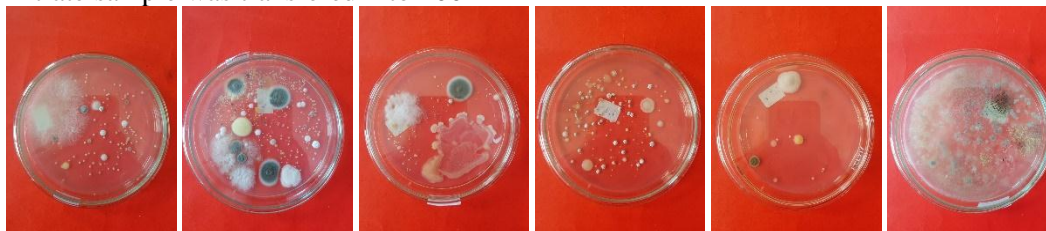


Figure 1. The *Duwet* Bark endophytic fungi isolation

Figure 1. indicated that *Duwet* bark endophytic fungi varied from one location to another. *Duwet* bark endophytic fungi from city of Batu were greenish white, round colonies with irregular edge, the elevation formed like a crater forming hyphae threads which were quite long and greenish white in colour. *Duwet* bark endophytic fungi taken from Malang regency, the fungi were green, round colonies form with white edge, the elevation rose and formed a circle, hyphae threads were thin and fine. *Duwet* bark endophytic fungi obtained from Pasuruan regency, the fungi were white, round form with irregular edge, rising elevation and covered with white hyphae threads. *Duwet* bark endophytic fungi from Lamongan regency, the fungi were white, round colonies form with regular rising edge, colonies size were smaller and denser, with elevation forming like a crater. *Duwet* bark

endophytic fungi taken from Gresik regency, the fungi were white, colonies formed round with flat and thinned edge. They had curved elevation with fine white hyphae threads (Chi Chi, 2020); (Wijayanti & Setiawan, 2018). *Duwet* bark endophytic fungi obtained from Situbondo regency, the fungi were blackish green, irregular colonies form with spreading edge, rising elevation and uneven (Bello et al., 2018); (Wijayanti & Setiawan, 2018); (Matule, 2018).

Phytochemical Qualitative Determination

Result of phytochemical determination was carried out qualitatively and quantitatively on *Duwet* (*Syzygium cumini* L) bark endophytic fungi isolate. Phytochemical determination carried out included flavonoid, alkaloid, terpenoid, phenolic and tannin group. Qualitative determination aimed to find out the presence of phytochemical compounds from certain groups.

The results of qualitative tests on phytochemical compounds of flavonoids, alkaloids, terpenoids,

phenolics, and tannins can be seen on Figure 2.

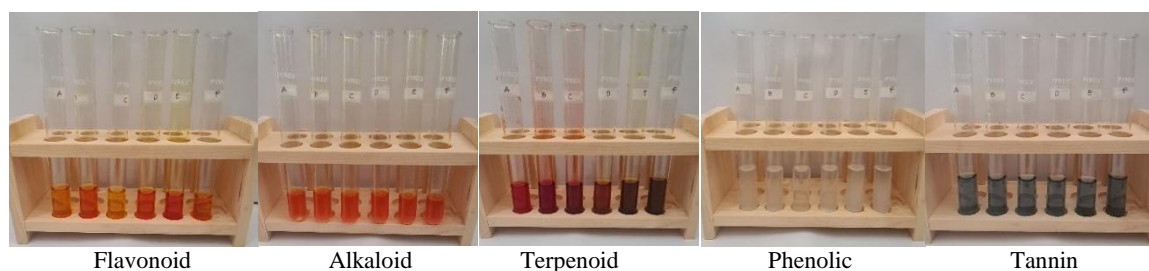


Figure 2. Qualitative test of phytochemical compounds on the endophytic fungi of *Duwet* Bark

The results of qualitative phytochemical test showed that all samples of the endophytic fungi isolate from the bark of *Duwet* contained flavonoids, alkaloids, terpenoids, phenolics and tannins. This is indicated by the reaction results indicating the presence of the five classes of phytochemical compounds being tested.

Phytochemical Quantitative Determination

Quantitative determination aimed to find out concentration of phytochemical compounds from certain groups. Result of qualitative and quantitative determination of phytochemical compounds on *Duwet* bark endophytic fungi can be seen on table 1.

Table 1. Summary of qualitative and quantitative determination result of endophytic fungi isolate supernatant

Sample	Penentuan	Flavonoid	Alkaloid	Terpenoid	Phenolic	Tannin
<i>City of Batu (A)</i>	Qualitative	+	+	+	+	+
	Quantitative (µg/ml)	1208,47	63,16	187,44	3217,67	437,55
<i>Malang Regency (B)</i>	Qualitative	+	+	+	+	+
	Quantitative (µg/ml)	1375,83	74,69	203,45	3026,86	396,46
<i>Pasuruan Regency (C)</i>	Qualitative	+	+	+	+	+
	Quantitative (µg/ml)	1185,69	54,26	275,35	3319,24	487,32
<i>Lamongan Regency (D)</i>	Qualitative	+	+	+	+	+
	Quantitative (µg/ml)	1453,75	87,28	308,59	3857,49	463,18
<i>Gresik Regency (E)</i>	Qualitative	+	+	+	+	+
	Quantitative (µg/ml)	1409,76	89,11	341,21	3417,46	442,65
<i>Situbondo Regency (F)</i>	Qualitative	+	+	+	+	+
	Quantitative (µg/ml)	1327,44	79,45	318,47	3395,83	459,74

*Description: +, indicated positive result.

Table 1 indicated that all endophytic fungi isolates showed the presence of phytochemical compounds of flavonoid, alkaloid, terpenoid, phenolic, and tannin. Quantitative determination presented that on all endophytic fungi isolates, phenolic group was the compound with the highest concentration, followed by flavonoid, tannin, terpenoid, and alkaloid. The endophytic fungi isolate which had the highest flavonoid concentrations was the D endophytic fungi isolate, obtained from Lamongan regency. The

isolate which had the highest alkaloid concentration was the E endophytic fungi isolate, taken from Gresik regency. The isolate which had the highest terpenoid was the E endophytic fungi isolate, taken from Gresik regency. The endophytic fungi isolate with the highest phenolic concentration is the D endophytic fungi isolate, obtained from Lamongan regency. The endophytic fungi isolate with the highest tannin concentration was the C endophytic fungi isolate, taken from Pasuruan regency.

The presence of phytochemical compounds of endophytic fungi in the bark is symbiosis result between endophytic fungi and their host plants. Endophytic fungi live on intracellular tissues, then absorb nutrition from the host plants, including phytochemical compounds contained in the *Duwet* plant bark (Bello *et al.*, 2018); (Wijayanti & Setiawan, 2018). Therefore, endophytic fungi isolates obtained have potentials to be developed for gaining secondary metabolites which was beneficial for medication. Deeper exploration steps of secondary metabolites in the obtained endophytic fungi are needed.

Conclusion

The endophytic fungi isolation technique of *Duwet* (*S. cumini* L.) bark with PDA media was able to produce good endophytic fungi isolates. Endophytic fungi isolates taken from barks of *Duwet* plant from six different areas in East Java showed the existence of phytochemical compounds of flavonoid, alkaloid, terpenoid, phenolic, and tannin. Deeper exploration of secondary metabolites contained in endophytic fungi and identification of obtained endophytic fungi isolates need to be conducted as further steps for this study.

Acknowledgment

Appreciation was conveyed to P2M IKIP Budi Utomo Malang as the funder of the university's internal research grant. And the providers of research facilities are the Microbiology Laboratory of State University of Malang and the Chemistry Laboratory of Muhammadiyah Malang University.

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