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Potential Endophytic Microbes Selection for Antidiabetic Bioactive Compounds Production

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ABSTRACT

Endophytic microbes ability to produce bioactive compounds in common with its host plant is an opportunity to get source material antidiabetic drugs are natural, inexpensive and environmentally. With the aim of investigating the potential of endophytic fungi that have the potential to produce antidiabetic bioactive compounds , a total of 45 endophytic fungi were isolated from 6 species of Indonesian medicinal plants, , i.e., Mahoni (*Swietenia mahagoni* Jacq.), Sambiloto (*Andrographis paniculata* Ness.), Kumis kucing (*Orthosiphon spicatus* BBS), Mengkudu (*Morinda citrifolia* L.), Sirih merah (*Piper crocatum* L.) and Sirih hitam (*Piper ornatum* sp.) has been done. Fungi isolates were fermented in Potato Dextrose Broth medium for 14 days, then extracted with ethylacetate followed by Thin Layer Chromatography (TLC) test. Screening was conducted using α-glucosidase test. The results showed that there are 7 fungi isolates, i.e., A.Ap.3F (98.84 and 81.40%), A.Ap.4F (96.87 and 81.40%), B.Ap.1F (98.48 and 87.49%)., B.Os.1F (93.91 and 89.01%), A.Pc.1F (87.66 and 82.08%), B.Pc.1F (85.63 and 89.35%) and B.Pc.2F (83.51 and 87.57%) which gave inhibitory activity to α-glucosidase enzyme. These results demonstrate that 7 endophytic fungi has the potential to produce antidiabetic agents.

Key words: α-glucosidase, antidiabetic, endophytic fungi, Indonesian medicinal plant, potato dextrose broth, thin layer chromatography

INTRODUCTION

Endophytic fungi is one of potential natural resources for new antidiabetic compound sources. Endophytic microbes are bacteria including *Actinomycetes*, or fungi which spend part or whole of its lifespan inside intra- or intercellular tissue of its healthy host without giving any symptoms (Tan and Zou, 2001). Endophyte and its host can build symbiosis mutualism to latent phytopathogen relationship involving numerous secondary metabolites produced by both endophytes and its host. Endophytes increase adaptation capability of its host and host's indurance against disease caused by pathogen. Endophytes also have potency in medicinal, agricultural and industrial development (Strobel *et al*., 2002).

These groups of fungi are widely recognized as prolific sources of bioactive secondary metabolites that might represent useful leads for the development of new pharmaceutical bioagents.

Since more than 1.5×10^6 endophytic fungi are thought to thrive within the estimated 270,000 species of vascular plants, the prospects for additional discoveries of metabolites from these fungi are promising (Bhilabutra *et al*., 2007).

From practical point of view, drug production by endophytic microbes fermentation will give more profit since it will be fast, reproducible, unlimited and weather/season independent. Easy to increase microbes capability by genetic engineering and different cultivation condition, we can produce different products. The discovery of endophytic fungi with capability to produce the exactly same active compound produced by their host leads to a new approach in active compound production from natural product commercially. Production of useful compounds can be increased by endophytic fungi biotechnology for affording demands while keeping biodiversity and ecosystem sustainable (Onifade, 2007).

Endophytes have been investigated to be a rich source of novel biological active secondary metabolites (Zhang *et al*., 2006). Novel antibiotics, antimycotics, immunosuppressants and anticancer compounds are only a few examples of which have been found after the isolation and culturing of individual endophytes followed by purification and characterization of some of their natural products (Strobel *et al*., 2004). Many researches showed that some Indonesian medicinal plants have potencies as antidiabetic compound sources. Some of them are Mahoni (*Swietenia mahagoni* Jacq.), Sambiloto (*Andrographis paniculata* Ness.), Kumis kucing (*Orthosiphon spicatus* BBS), Mengkudu (*Morinda citrifolia* L.), Sirih merah (*Piper crocatum* L.) and Sirih hitam (*Piper ornatum* sp.). Many publications were reported the antidiabetic activity of these medicinal plants but information about their potential endophytic microbes with capability in producing antidiabetic active compounds are seldom (Arifin *et al*., 2006).

The study was aimed to select potential endophytic fungi from 6 Indonesian medicinal plants which traditionally used as antidiabetic drug and proven to have potency as antidiabetic active compounds sources. Those 6 medicinal plants are Mahoni (*Swietenia mahagoni* Jacq.), Sambiloto (*Andrographis paniculata* Ness.), Kumis kucing (*Orthosiphon spicatus* BBS), Mengkudu (*Morinda citrifolia* L.), Sirih merah (*Piper crocatum* L.) and Sirih hitam (*Piper ornatum* sp.).

MATERIALS AND METHODS

Research material: Research materials are 6 species of well known Indonesian medicinal plants for antidiabetic, i.e., Mahoni (*Swietenia mahagoni* Jacq.), Sambiloto (*Andrographis paniculata* Ness.), Kumis kucing (*Orthosiphon spicatus* BBS), Mengkudu (*Morinda citrifolia* L.), Sirih merah (*Piper crocatum* L.) and Sirih hitam (*Piper ornatum* sp.) collected from Bogor and Bandung areas. This research was conducted during February-October 2010.

Isolation and purification of endophytic microbes: Plant's stem in 2 cm for length and 1 cm for diameter were washed by water followed by sterilization using ethanol 70% for 1 min and ethanol 75% for 30 sec. Stems were cut longitudinally into 2 pieces then were placed on petri disc filled with Corn Meal Malt Agar (CMMA) mixed with 0.05 mg mL^{-1} chloramphenicol (Theantana *et al*., 2007). Others were placed on Potato Dextrose Agar (PDA) mixed with 0.05 mg mL^{-1} chloramphenicol (Croizer *et al*., 2006). Those agars were incubated at 25°C for 3 days. Purification were done by transferring colony to fresh PDA followed by incubation at 25°C for few times until we got pure colony. Pure colony are kept on slanted PDA in the refrigerator (-80°C) for further treatment.

Production of endophytic microbes bioactive compounds: Production and fermentation of endophytic microbes chemical compounds were done by cutting 1 week old endophytic microbe colony in medium agar using cork borer tobe inoculum disc with diameter of 1.8 cm. Inoculum was inoculated into 250 Potato Dextrose Broth (PDB) medium in Erlenmeyer flask size 1000 mL and fermented for 14 days with agitation 150 rpm at room temperature (Onifade, 2007). Fermented fungi were extracted using ethylacetate and filtrated using Whatman filter paper number 41 until we got filtrate and biomass of fungi. Filtrate collected was evaporated using evaporator and were dried using oven at 40°C. Dried biomass was extracted again using ethylacetate to get the extract (Sunil *et al.*, 2009).

***In vitro* antidiabetic activity test of active compounds:** Screening was done to get isolates which can produce a chemical compound with inhibitory activity to α-glucosidase enzyme. In this screening, we were having test to all ethylacetate extracts of endophytic fungi (both filtrate and biomass extracts). As control we used quercetin, a well known flavonoid compound which have inhibitory activity to α-glucosidase enzyme. All samples and standard solution were measured at concentration of 50 ppm (Suarsana *et al.*, 2008).

In vitro antidiabetic activity test were done using α-glucosidase method (Saijyo *et al.*, 2008). Inhibition capability were calculated using formula:

$$\text{Inhibition}(\%)=\frac{C-S}{C}\times 100$$

where, S showed sample absorbance and C showed blank absorbance with quercetin solution as standard.

Thin layer chromatography test: Both filtrate and biomass ethylacetate extract of endophyte isolates were tested by thin layer chromatography analysis using eluent chloroform-methanol (5:1) and silica gel GF254 as solid phase. Spray reagent used is Serium sulphate.

RESULTS AND DISCUSSION

Isolation of endophytic fungi: Isolation and purification of endophytic fungi from Mahoni (*Swietenia mahagoni* Jacq.), Sambiloto (*Andrographis paniculata* Ness.), Kumis kucing (*Orthosiphon spicatus* BBS), Mengkudu (*Morinda citrifolia* L.), Sirih merah (*Piper crocatum* L.) and Sirih hitam (*Piper ornatum* sp.) gave results 45 fungi isolates. Complete results of isolation can be seen in Table 1.

These endophytic isolates were extracted with ethylacetate and produced 45 sample of ethylacetate filtrate and 45 sample of ethylacetate biomass.

Screening of endophytic fungi isolates using α-glucosidase test: α-glucosidase is an enzyme in intestine which can hydrolyze carbohydrate into simple sugar (glucose). Compound which can inhibit this enzyme activity will be very potential to be an antidiabetic drug since it can decrease blood sugar level by slowing the absorption of carbohydrate *post-prandial*. The results of the screening were summarized in Table 2.

In this test, we compared absorbancy of standard with samples. DMSO solution was used as blank solution while quercetin as standard. Absorbancy value of DMSO solution 0.1183 and absorbancy value of quercetin solution 0.1007. So, Inhibition percentage of standard 14.88%.

Table 1: Endophytic fungi isolated from some indonesian antidiabetic plants

		Ethyl acetate extracts weight	
Plants	Fungi isolates	Filtrate (g)	Biomass (g)
Mahoni (*Swietenia mahagoni* Jacq.)	A.Sm.1F	0.0887	0.1007
Mahoni (*Swietenia mahagoni* Jacq.)	A.Sm.2F	0.1598	0.1023
Mahoni (*Swietenia mahagoni* Jacq.)	A.Sm.3F	0.0886	0.0587
Mahoni (*Swietenia mahagoni* Jacq.)	B.Sm.1F	0.0830	0.1052
Mahoni (*Swietenia mahagoni* Jacq.)	B.Sm.2F	0.0922	0.0906
Mahoni (*Swietenia mahagoni* Jacq.)	B.Sm.3F	0.0584	0.0848
Mahoni (*Swietenia mahagoni* Jacq.)	B.Sm.4F	0.0907	0.1869
Sambiloto (*Andrographis paniculata* Ness.)	A.Ap.1F	0.1300	0.0711
Sambiloto (*Andrographis paniculata* Ness.)	A.Ap.2F	0.0675	0.0027
Sambiloto (*Andrographis paniculata* Ness.)	A.Ap.3F	0.0919	0.0605
Sambiloto (*Andrographis paniculata* Ness.)	A.Ap.4F	0.0750	0.0598
Sambiloto (*Andrographis paniculata* Ness.)	B.Ap.1F	0.0649	0.0.557
Sambiloto (*Andrographis paniculata* Ness.)	B.Ap.2F	0.0683	0.1104
Sambiloto (*Andrographis paniculata* Ness.)	B.Ap.3F	0.1022	0.0602
Sambiloto (*Andrographis paniculata* Ness.)	B.Ap.4F	0.0905	0.0681
Sambiloto (*Andrographis paniculata* Ness.)	B.Ap.5F	0.0967	0.0634
Kumis kucing (*Orthosiphon spicatus* BBS)	A.Os.1F	0.0901	0.1442
Kumis kucing (*Orthosiphon spicatus* BBS)	A.Os.2F	0.0661	0.0683
Kumis kucing (*Orthosiphon spicatus* BBS)	A.Os.3F	0.0902	0.1137
Kumis kucing (*Orthosiphon spicatus* BBS)	A.Os.4F	0.0536	0.2108
Kumis kucing (*Orthosiphon spicatus* BBS)	B.Os.1F	0.0814	0.0939
Kumis kucing (*Orthosiphon spicatus* BBS)	B.Os.2F	0.0626	0.0834
Kumis kucing (*Orthosiphon spicatus* BBS)	B.Os.3F	0.0563	0.0847
Kumis kucing (*Orthosiphon spicatus* BBS)	B.Os.4F	0.0865	0.0882
Mengkudu (*Morinda citrifolia* L.)	A.Mc.1F	0.0697	0.0557
Mengkudu (*Morinda citrifolia* L.)	A.Mc.2F	0.0588	0.1326
Mengkudu (*Morinda citrifolia* L.)	B.Mc.1F	0.0619	0.0603
Mengkudu (*Morinda citrifolia* L.)	B.Mc.2F	0.0584	0.0646
Mengkudu (*Morinda citrifolia* L.)	B.Mc.3F	0.0688	0.0538
Sirih merah (*Piper crocatum* L.)	A.Pc.1F	0.0551	0.0524
Sirih merah (*Piper crocatum* L.)	A.Pc.2F	0.0791	0.0747
Sirih merah (*Piper crocatum* L.)	B.Pc.1F	0.0639	0.1593
Sirih merah (*Piper crocatum* L.)	B.Pc.2F	0.0734	0.0423
Sirih merah (*Piper crocatum* L.)	B.Pc.3F	0.0727	0.1488
Sirih merah (*Piper crocatum* L.)	B.Pc.4F	0.0563	0.1391
Sirih merah (*Piper crocatum* L.)	B.Pc.5F	0.0665	0.0517
Sirih hitam (*Piper* sp.)	A.Ps.1F	0.0133	0.0100
Sirih hitam (*Piper* sp.)	A.Ps.2F	0.0089	0.0085
Sirih hitam (*Piper* sp.)	A.Ps.3F	0.0120	0.0879
Sirih hitam (*Piper* sp.)	A.Ps.4F	0.0064	0.0143
Sirih hitam (*Piper* sp.)	A.Ps.5F	0.0045	0.0184
Sirih hitam (*Piper* sp.)	B.Ps.1F	0.0113	0.0226
Sirih hitam (*Piper* sp.)	B.Ps.2F	0.0065	0.0115
Sirih hitam (*Piper* sp.)	B.Ps.3F	0.0148	0.0119
Sirih hitam (*Piper* sp.)	B.Ps.4F	0.0063	0.0157

Notation A is refers to endophytic fungi isolates planted on CMM (Corn Meal Medium), Notation B is refers to endophytic fungi isolates planted on PDA (Potato Dextrose Agar)

Table 2: The results of the screening of endophytic fungi isolates using α-glucosidase test

		Absorbans		α-glucosidase (%)*	
Plants	Fungi isolates	Filtrate	Biomass	Filtrate (g)	Biomass (g)
Mahoni (*Swietenia mahagoni* Jacq.)	A.Sm.1F	0.0995	0.0225	15.89	80.98
Mahoni (*Swietenia mahagoni* Jacq.)	A.Sm.2F	0.0327	0.0092	72.59	92.22
Mahoni (*Swietenia mahagoni* Jacq.)	A.Sm.3F	0.0224	0.0244	81.87	79.37
Mahoni (*Swietenia mahagoni* Jacq.)	B.Sm.1F	0.0443	0.0018	63.40	98.84
Mahoni (*Swietenia mahagoni* Jacq.)	B.Sm.2F	0.0407	0.0441	65.50	62.72
Mahoni (*Swietenia mahagoni* Jacq.)	B.Sm.3F	0.0072	0.0574	93.91	51.48
Mahoni (*Swietenia mahagoni* Jacq.)	B.Sm.4F	0.0148	0.0300	87.48	74.64
Sambiloto (*Andrographis paniculata* Ness.)	A.Ap.1F	0.0250	0.0225	78.87	80.98
Sambiloto (*Andrographis paniculata* Ness.)	A.Ap.2F	0.0934	0.0817	20.29	30.94
Sambiloto (*Andrographis paniculata* Ness.)	A.Ap.3F	0.0220	0.0018	81.40	98.84
Sambiloto (*Andrographis paniculata* Ness.)	A.Ap.4F	0.0220	0.0037	81.40	96.87
Sambiloto (*Andrographis paniculata* Ness.)	B.Ap.1F	0.0148	0.0018	87.49	98.48
Sambiloto (*Andrographis paniculata* Ness.)	B.Ap.2F	0.0301	0.0092	74.56	92.22
Sambiloto (*Andrographis paniculata* Ness.)	B.Ap.3F	0.0767	0.0206	35.15	82.59
Sambiloto (*Andrographis paniculata* Ness.)	B.Ap.4F	0.0817	0.0111	30.94	90.62
Sambiloto (*Andrographis paniculata* Ness.)	B.Ap.5F	0.0301	0.0206	74.56	82.59
Kumis kucing (*Orthosiphon spicatus* BBS)	A.Os.1F	0.0694	0.0380	41.34	67.87
Kumis kucing (*Orthosiphon spicatus* BBS)	A.Os.2F	0.1154	0.0354	2.45	70.08
Kumis kucing (*Orthosiphon spicatus* BBS)	A.Os.3F	0.0742	0.0275	37.28	76.75
Kumis kucing (*Orthosiphon spicatus* BBS)	A.Os.4F	0.0817	0.0354	30.94	70.08
Kumis kucing (*Orthosiphon spicatus* BBS)	B.Os.1F	0.0072	0.0130	93.91	89.01
Kumis kucing (*Orthosiphon spicatus* BBS)	B.Os.2F	0.0048	0.0244	95.94	79.37
Kumis kucing (*Orthosiphon spicatus* BBS)	B.Os.3F	0.0195	0.0342	83.51	71.09
Kumis kucing (*Orthosiphon spicatus* BBS)	B.Os.4F	0.0121	0.0263	89.77	77.77
Mengkudu (*Morinda citrifolia* L.)	A.Mc.1F	0.0550	0.0817	53.51	30.94
Mengkudu (*Morinda citrifolia* L.)	A.Mc.2F	0.0121	0.0817	89.87	30.94
Mengkudu (*Morinda citrifolia* L.)	B.Mc.1F	0.0301	0.1047	74.56	11.50
Mengkudu (*Morinda citrifolia* L.)	B.Mc.2F	0.0598	0.0412	49.45	65.17
Mengkudu (*Morinda citrifolia* L.)	B.Mc.3F	0.0121	0.0322	89.77	72.78
Sirih merah (*Piper crocatum* L.)	A.Pc.1F	0.0146	0.0212	87.66	82.08
Sirih merah (*Piper crocatum* L.)	A.Pc.2F	0.0842	0.0367	28.83	68.98
Sirih merah (*Piper crocatum* L.)	B.Pc.1F	0.0170	0.0126	85.63	89.35
Sirih merah (*Piper crocatum* L.)	B.Pc.2F	0.0195	0.0147	83.51	87.57
Sirih merah (*Piper crocatum* L.)	B.Pc.3F	0.0121	0.0943	89.77	20.29
Sirih merah (*Piper crocatum* L.)	B.Pc.4F	0.0121	0.0385	89.77	67.46
Sirih merah (*Piper crocatum* L.)	B.Pc.5F	0.0195	0.0344	83.51	70.92
Sirih hitam (*Piper* sp.)	A.Ps.1F	0.0527	0.0234	55.45	80.22
Sirih hitam (*Piper* sp.)	A.Ps.2F	0.0842	0.0344	28.83	70.92
Sirih hitam (*Piper* sp.)	A.Ps.3F	0.0300	0.0892	74.64	24.60
Sirih hitam (*Piper* sp.)	A.Ps.4F	0.0504	0.0574	57.40	51.48
Sirih hitam (*Piper* sp.)	A.Ps.5F	0.0344	0.0344	70.92	70.92
Sirih hitam (*Piper* sp.)	B.Ps.1F	0.0435	0.0550	62.23	53.51
Sirih hitam (*Piper* sp.)	B.Ps.2F	0.0344	0.0598	70.92	49.45
Sirih hitam (*Piper* sp.)	B.Ps.3F	0.0598	0.0458	49.45	61.28
Sirih hitam (*Piper* sp.)	B.Ps.4F	0.0621	0.0278	47.51	76.50

Absorbancy of DMSO solution 0.118, absorbancy of quercetin solution 0.1007:

$$*\text{Inhibition percentage of sample}(\%) = \frac{\text{MSO} - \text{Sampel}}{\text{DMSO}} \times 100(\%)$$

Highest inhibition percentage against α-glucosidase enzyme was showed by biomass extract of fungus B.Sm.1F from Mahoni (*Swietenia mahagoni* Jacq.) plant which gave 98.84% inhibition while its filtrate extract only gave moderate inhibition percentage at 63.40%. On the contrary, fungus B.Sm.3F gave 93.91% inhibition percentage from its filtrate extract while its biomass extract only gave 51.48% inhibition. The chosen potential fungus is expected to have high inhibition percentage from both biomass and filtrate extracts.

From 9 endophytic fungi obtained from Sambiloto (*Andrographis paniculata* Ness.) there are 3 fungi which have high inhibition percentage both from biomass and filtrate extracts. They are A.Ap.3F (98.84 and 81.40%), A.Ap.4F (96.87 and 81.40%) and B.Ap.1F (98.48 and 87.49%).

Endophytic fungus B.Os.1F from Kumis kucing (*Orthosiphon spicatus* BBS) gave highest inhibition percentage compared to 7 other fungi obtained from this plant. It gave 93.91% from filtrate extract and 89.01% from biomass extract.

Most endophytic fungi obtained from Mengkudu (*Morinda citrifolia* L.) have moderate inhibition percentage. From 5 fungi, there are 2 potential fungi, i.e., A.Mc.2F with 89.77% inhibition for its filtrate extract and 30.94% for biomass extract and B.Mc.3F (89.77 and 72.78%).

From 7 endophytic fungi obtained from Sirih Merah (*Piper crocatum* L.) there are 3 fungi which have high inhibition percentage both from biomass and filtrate extracts. They are A.Pc.1F (87.66 and 82.08%), B.Pc.1F (85.63 and 89.35%) and B.Pc.2F (83.51 and 87.57%).

Most endophytic fungi obtained from Sirih Hitam (*Piper ornatum* L.) have low to moderate inhibition percentage (28-80%). From 9 fungi, only fungus A.Ps.1F which gave quite high inhibition percentage. Its biomass extract gave 80.22% inhibition while filtrate extract gave 55.45%.

Suarsana *et al.* (2008) obtained antidiabetic activity of quercetin standard using acarbose at concentration 50 ppm at 15.33%. In this research, antidiabetic activity of quercetin standard was 14.88%.

Screening to endophytic fungi from 6 Indonesian medicinal plants using α-glucosidase method resulted in 7 potential endophytic fungi with high inhibition percentage both from their filtrate and biomass extracts. They are A.Ap.3F, A.Ap.4F and B.Ap.1F from Sambiloto (*Andrographis paniculata* Ness.), B.Os.1F from Kumis Kucing (*Orthosiphon spicatus* BBS), A.Pc.1F, B.Pc.1F and B.Pc.2F from Sirih Merah (*Piper crocatum* L). Among those 7 potential fungi, A.Ap.1F has highest inhibition percentage. In a study conducted by Suarsana and his friends obtained value of tempeh extract on the inhibition of α-glucosidase enzyme of 11.89% (Suarsana *et al.*, 2008). Several compounds that can inhibit the enzyme α-glucosidase has been obtained by Saijyo *et al.* (2008) in the research by using ethanol from plant extracts bergenia ligulata, such as compound 1 inhibited 57.5% of α-glucosidase activity at a concentration of 0.25 mM and the ID_{50} (50% inhibition dose) value was 0.1 mM and Compound 5 inhibited 90.4% of α-glucosidase activity at a concentration of 0.25 mM and the ID_{50} (50% inhibition dose) value was 0.05 mM. In this study, 7 potential endophytic fungi provides inhibition values ranging between 80 to 98%.

CONCLUSION

Isolation and purification of endophytic fungi from 6 Indonesian medicinal plants obtained 45 fungi isolates. Mahoni (*Swietenia mahagoni* Jacq.) gave 7 isolates, Sambiloto (*Andrographis paniculata* Ness.) 9 isolates, Kumis kucing (*Orthosiphon spicatus* BBS) 8 isolates, Mengkudu (*Morinda citrifolia* L.) 5 isolates, Sirih merah (*Piper crocatum* L.) 7 isolates and Sirih hitam (*Piper ornatum* sp.) gave 9 isolates.

Screening procedure using Thin Layer Chromatography (TLC) test and α-glucosidase test resulted in 7 potential endophytic fungi with antidiabetic activity both from their filtrate and biomass extracts. They are A.Ap.3F, A.Ap.4F and B.Ap.1F from Sambiloto (*Andrographis paniculata* Ness.), B.Os.1F from Kumis Kucing (*Orthosiphon spicatus* BBS), A.Pc.1F, B.Pc.1F and B.Pc.2F from Sirih Merah (*Piper crocatum* L.).

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