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Antioxidant and antimicrobial activities of endophytic fungi isolated from *Sesbania grandiflora (L.) Pers.*

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Abstract

Twenty eight different endophytic fungi were isolated from *Sesbania grandiflora (L.) Pers.* These endophytic fungal extracts were prepared, using ethyl acetate and evaluated for their chemical constituents in vitro. The antibacterial activity of crude extract was done by using broth microdilution and exhibited significant antimicrobial activity against an array of plant pathogenic bacteria included Xanthomonas axonopodis pv. citri, Xanthomonas axonopodis pv. glycines, Xanthomonas campestris pv.campestris and Acidovorax avenae subsp. Avenae range from 125-2000 µg/mL. The total antioxidant capacity was evaluated by using 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) assay ranged from 2.10 x 10-3 to 4.84 x 108 µg /mg extract. The total phenolic content of the fungal extracts was determined by using Folin-Ciocalteu procedures which ranged from 0 to 1730.78 µg gallic acid/mg extract. This is the first report of Sesbania grandiflora (L.) Pers. producing endophytes which have antimicrobial and antioxidant activities. This investigation reveals that the metabolites produced by a variety of endophytic fungi from Sesbania grandiflora (L.) Pers. can be a potential source of novel natural antimicrobials and antioxidants agents.

Keywords: Antimicrobials, Antioxidant, DPPH, *Sesbania grandiflora (L.) Pers.*.

Introduction

Endophytic fungi, microorganism hidden within healthy host plant, were poorly investigated group among other microorganisms. They represent an abundant and dependable source of novel bioactive compounds with huge potential for exploitations in a wide variety of medicinal, agricultural and industrial areas [1]. Fungal endophytes residing within the plants not only mediate interactions between host plants and herbivores and pathogens, but can also control food-web structure by disrupting the transfer of energy from plants to upper trophic levels and produce metabolites similar to or with more activity than that of their respective hosts [2]. There are many reports and studies on the metabolites isolated from the endophytes They are good sources of novel secondary metabolic products having diverse structural groups and showing antibacterial, antifungal, anticancer, antiviral, antioxidant, insecticide, antidiabetic and immunosuppressive [3,4].

Agricultural plant diseases caused by plant pathogenic fungi and bacteria are one of the major economic damages in agriculture in the world, which cause harvest losses in crop production approximately 12% or even higher in developing countries [5]. Strategies to control plant diseases with classic method by using synthetic chemical compound may produce harmful side effects, such as serious environmental pollution and the development of multi- resistant strains. Thus, there is a continuing need for more effective and safer method, especially those with novel modes of action, and natural products play a key role in the search for such compounds [6,7]. Endophytes have been proven to be a rich and reliable source of biologically active and/or chemically novel compounds for exploitation in modern medicine, agriculture and industry attention for their ability to produce a variety of bioactive secondary metabolites which showed modest antimicrobial activity in recent years.

Reactive oxidant species (ROSs) plays an important role in creating oxidative stress, which could lead to cell injury and death. They also cause alteration of platelet functions [8]. These free radicals occur in the body during an imbalance between ROSs (Reactive Oxygen Species) and antioxidants. Antioxidants are thought to be highly profitable in the management of reactive oxygen species-mediated tissue impairments. To reduce the harm of ROSs to the human body, sufficient of exogenous antioxidants are required. The search for naturally occurring antioxidants especially of plant origin had increased greatly in the past decades [9,10]. The endophytic fungi are one of the potential sources of new natural bioactive products from their secondary metabolites. Many endophytic fungi have shown antimicrobial activity and antioxidant properties. Although a number of bio-pharmacological compounds from endophytes have been previously identified, the information related to their antioxidant activities is very scanty [11].

Sesbania grandiflora (L.) Pers is distributed widely through Tropical Asia including India, Indonesia, Myanmar, Philliphines and Thailand [12]. The chemical constituents found in this plant are galactommannan, linoleic acids, beta-Sitosteral, and carbohydrate [13]. It is not only a native economic tree, but also its bark, leaves and flower can be used in traditional medicine for treatment of cold, fever, stomach disorder, diarrhea and jaundice and as skin cleanser. Traditionally, the bark is used as astringent and used for the treatment of smallpox, ulcers in the mouth and alimentary canal, in bitter, in juvenile, infantile disorders of stomach, scabies [14]. The active compounds from the leaves are considered to be excellent sources of vitamin C, and calcium. Pectin and saponin are also reported that they can be found in the leave of this plant [15]. The leaves are used as aperients, diuretic, and tonic in form of poultice and they are applied to bruises. Recently, some reports showed that its leave presence of high amount of total phenols, flavonoids and ascorbic acid [16, 17]. However reports on the different endophytic fungi isolated from this plant are seldom seen. Further systematic investigation of endophytic fungi including their antimicrobial values and antioxidant properties has not been investigated as well. The present study was aimed to isolate and identify endophytic fungi and also evaluate for their antioxidant and antimicrobial activities against plant pathogen. The ecological information and new source of natural products with antimicrobial and antioxidant activities will be obtained from this study.

Experimental

Isolation of endophytic fungi

Healthy leaves and stem were collected from *Sesbania grandiflora* (L.) *Pers,* grown in the forest area of Nakhon Nayok Province, Thailand. The fresh-cut ends of plant samples were cut by sterile scissor, wrapped with Parafilm M (3M Co.Ltd.), and placed in zip-lock plastic-bags and stored less than 72 h prior to isolate of endophytic fungi. Samples were cleaned under running tap water for 5 min and then air-dried. Before surface sterilization, the cleaned stems were cut into pieces 5-cm long. Leaves and limb fragments were sterilized by immersion in 70% ethanol for 1 min, 5% sodium hypochlorite solution for 5 min and sterile distilled water for 1 min two times [18].(Petrini 1991). The surface-sterilized leaves and stems were cut into small pieces about 0.5 x 0.5 cm2 using a sterile blade and placed on sterile half strength potato dextrose agar plates. The plates were incubated at room temperature for 24 – 72 h. The hyphal tip of endophytic fungus growing out from the plant tissue was cut by a sterile pasture pipette and transferred to a sterile half strength potato dextrose agar plate. After incubation at room temperature for 7-14 days, colony morphology of each endophytic fungi was determined. Pure culture of endophytic fungi were obtained by several times subculture.

Preparation of endophytic fungi extracts

Twenty eight isolates of endophytic fungi that previously showed potential antimicrobial activities by screening method using agar diffusion assay and antagonistic assay (data not showed) were selected for cultivation in appropriate liquid medium under standstill condition. The fresh mycelium of different endophytes grown on Potato Dextrose agar (PDA) plates at 30 ºC for 3–6 days were cut into 1x1 cm. 6-8 pieces and inoculated into 3 of 250 mL erlenmeyer flasks containing sterile glucose yeast extract broth (containing 0.01% of peptone, 0.00%5 of NaCl, 0.003 % of yeast extract). These flasks were incubated at 30 ºC for 21 days. The biomass of each endophyte was separated by filtering through gauze cloth. The filtered liquid was then extracted with 300 mL ethyl acetate three times. The extract was evaporated to dryness using rotary evaporator and weighed to constitute the crude extract.

Preparation of bacterial culture

Four plant pathoginic bacteria, i.e., Xanthomonas axonopodis pv. citri, Xanthomonas axonopodis pv. glycines, Xanthomonas campestris pv.campestris and Acidovorax avenae subsp. avenae were kindly given from faculty of agricultural, Kasetsart University. Individual colonies isolated from 24 or 48 hrs culture plates of tested bacteria were suspended in 2 mL sterile 0.85% NaCl solution. The bacterial cell suspension was inoculated in Nutrient broth and mixed for 15 secs to ensure homogeneity. The turbidity of microbial suspension was diluted to match the turbidity of a 0.5 McFarland standard by spectrophotometry (OD = 0.08 - 0.1 at 625 nm) corresponding to 1x 10^8 CFU/mL and subsequently diluted to 1:100 with Nutrient broth to obtain the bacterial cell of 10^6 CFU/mL.

Antibacterial activity of Endophytic fungi extracts

The antimicrobial activities of the 28 endophytic fungi crude extracts were assayed against plant pathogenic bacteria. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts were determined by using the standard broth microdilution method and a modified resazurin microtiter plate assay as recommended by the NCCLS and CLSI methodology. The NCCLS, the CLSI M27 A2, the CLSI M38-A were intended for bacterial testing with a few modifications [19-21]. The serial two fold dilutions were made in a concentration ranged from 250 µg/mL to 4000 µg/mL. Fifty µL of Test sample solutions and 50 µL of bacterial suspension containing 1 10^6 cfu/mL were added into each well of the 96-well microplate. Each well of the negative control contained 50 µL of microbial suspension and 50 µL of 2% DMSO. Microtiter plates were incubated at 37ºC, 24 hrs. After incubation period 10 µl of the sterile resazurin indicator solution (0.15 mg/ml) was added to each well. The color change was then assessed visually. The blue or purple color (oxidized form) was recorded as positive. Any color changes from blue or purple to pink or colorless (reduced form) were recorded as negative. The lowest concentration at which the blue or purple color occurred was taken as the MIC value. All tests were carried out in triplicate.

The MBC were determined by inoculating 1 loop of sample from wells that showed no apparent growth from the MIC assays onto Muller Hinton agar (MHA) plates. The plates were incubated at 37ºC, 24 hrs. After incubation period, the plates were examined for growth or lack of growth for each dilution subculturing. No growth indicated that the endophytic fungi crude extract sample was bactericidal at that dilution. Growth indicated that the sample was bacteriostatic at that dilution. The lowest concentration showing no visible growth on agar subculture was taken as MBC value.

DPPH radical scavenging activity

The free radical scavenging activities of endophytic extracts were measured by using 2, 2-diphenyl-1-picryl-hydrazyl (DPPH). Briefly, 100 µl of the extract concentration of 312.5 –10,000 µg/mL prepared in ethanol was mixed with 100 µl of 0.2 mM. 2, 2-diphenyl-1-picryl-hydrazyl (DPPH, Sigma) prepared in methanol. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank [22]. Simultaneously, a control was prepared without sample extracts. The capability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = $[(A_0 - A_1/A_0) \times 100]$

Where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the sample. The extract concentration providing 50% inhibition (IC_{50}) was calculated by interpolation from linear regression analysis. IC_{50} value (µg /mL) is the effective concentration which DPPH radicals were scavenged by 50%.

Determination of total phenol compounds

Total phenolic compounds were determined using Folin-Ciocalteu's method [22]. Briefly, 20 µl of extract concentration of (312.5 – 10,000 g/ml) prepared in ethanol was mixed with 100 µl ml of Folin - Ciocalteu's reagent. Then 80 l of 2% aqueous sodium carbonate was added into the mixture. The mixture was incubated for 30 mins and the absorbance of the mixture was measured at 765 nm against reagent blank. The content of total phenol was calculated on the basis of the calibration curve of gallic acid and the results were expressed as g of gallic acid equivalents (GAEs) per mg of extract.

Results

A total of 28 isolated were obtained from the leaves, and stems of Sesbania grandiflora (L.) Pers. Among of them, 24 were isolated from stem and 4 from leave. Based on their morphological characteristics, isolated endophytic fungi were classified into six genera, i.e., Acremonium spp., Fusarium spp., Phaeoacremonium spp., Phomopsis spp., Paecilomyces spp., and *Cladosporium* spp. The majority of these isolated endophytic fungi belonged to the Acremonium spp. (42.86%), and Fusarium spp. (35.71%) (Table1). Moreover, it was noted that different isolates exhibited different strengths of antibacterial and antioxidant activities.

Table 1. Isolation of endophytes from *Sesbania grandiflora (L.) Pers* on PDA media

Types of endophytes	Number of isolated	Isolated from	
		Leave	Stem
Fusarium spp.	10	+	+
Phaeoacremonium spp	3	-	+
Acremonium spp.	12	+	+
Phomopsis spp.	1	-	+
Paecilomyces spp.	1	-	+
Cladosporium spp.	1	-	+

+ = presence, - = absence

In the present work, the antibacterial activities were conducted using microdilution method and a modified resazurin microtiter plate assay. The susceptibility testing of 28 endophytic fungi crude extract against 4 plant pathogenic bacteria including *Xanthomonas axonopodis* pv. *citri*, *Xanthomonas axonopodis* pv. *glycines*, *Xanthomonas campestris* pv. *campestris* and *Acidovorax avenae* subsp. *Avenae*. It was found that most of endophytic fungi crude extract can exhibited potent antibacterial activity which at least one of them can inhibit these bacteria (Table 2).

Among of 24 from 28 endophytic fungi extract presented significant anti microbial activity against *X. axonopodis* pv. *citri* (85.71%) with MIC and MBC value range from 250-2000 and 1000-2000 µg/mL respectively. The highest inhibition was showed by extract of isolated 23 and 38. While 21 (75%) extract were active against *X. axonopodis* pv. *glycines* with MIC and MBC value range from 250-2000 and 500-2000 µg/mL respectively. The highest inhibition was demonstrated by extract of isolated 23. Twenty three (82.14%) extract were highly active against *X. campestris* pv. *campestris* with MIC and MBC value range from 125-2000 and 2000 µg/mL respectively. The highest inhibition by extract of isolated 23. Twenty two (78.57%) of 28 endophytic fungi extract can inhibit *A. avenae* subsp. *avenae* with MIC and MBC value range from 125-2000 and 500-2000 µg/mL respectively. The highest inhibition was exhibited by extract of isolated 20 and 23 (Table 2).

Table 3 showed the difference in total antioxidant capacity (DPPH) and total phenolic content among selected endophytes. It was observed that DPPH is varied in all tested endophytic fungi. The crude extract of isolated 17 and 20 exhibited a good antioxidant activity with IC_{50} value of 7.50×10^{-3} and 2.10×10^{-3} µg/mL respectively, compared to the IC_{50} value of ascorbic acid which is 4.83 µg/mL. In addition, the total phenolic content, displayed a rather low activity with IC_{50} ranging from 0 to 1730.78 µg gallic acid/mg extract. A highest total phenolic content (TPC) was found in the extracts of isolated 43 with a value of 1730.78 µg gallic acid. The phenolic compounds in the endophytic fungi isolated from *Sesbania grandiflora(L.) Pers.*, may have contributed significantly to their antioxidant activity.

Table 2 Percentage of susceptibility testing and Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of crude extract from different isolates from *Sesbania grandiflora (L.) Pers*. determined by broth microdilution.

endophytic fungi crude extract	Tested plant pathogenic bacteria			
	Xag	Xac	Xcc	Aaa
Active endophytic fungi crude extract N = 28 (%)	24 (85.71%)	21 (75%)	23 (82.14%)	22 (78.57%)
MIC (µl/ml)	250-2000 (N= 24)	250-2000 (N= 21)	125-2000 (N= 23)	250-2000 (N= 22)
MBC (µl/ml)	1000-2000 (N= 10)	500-2000 (N= 10)	2000 (N=5)	500-2000 (N= 9)

(Xag: *Xanthomonas axonopodis* pv. *citri*, Xa*c: Xanthomonas axonopodis* pv. *glycines,* Xcc : *Xanthomonas campestris* pv. *campestris* and Aaa : *Acidovorax avenae* subsp. *avenae*)

Table 3 Antioxidant capacity and total phenolic content of the endophytic fungi isolated from *Sesbania grandiflora (L.)* Pers

crude extract No.	DPPH (IC_{50}) (µg Vit C /mg)*	Total phenolic content of ethylacetate extract (µg gallic acid/ g extract)
2	$6.61x10^3$	240.79
3	$5.40 x10^3$	43.75
4	$1.10 x10^4$	488.69
9	$4.84 x10^8$	0
13	$3.70 x10^4$	224.37
14	$1.10x10^4$	976.37
17	$7.50 x10^{-3}$	1047.78
20	$2.10 x10^{-3}$	798.21
22	$7.50 x10^5$	0
23	$1.40 x10^4$	221.08
25	$3.80 x10^3$	312.48
27	$4.70 x10^3$	383.88
28	$1.40 x10^5$	545.36
30	$5.70x10^5$	138.99
37	$2.00 x10^6$	0
38	$1.60 x10^4$	0
39	$5.10x10^3$	79.88
43	$1.20 x10^3$	1730.81
48	$1.90 x10^4$	115.18
49	$5.50 x10^3$	113.27
50	$1.10 x10^4$	0
51	$7.60 x10^3$	170.19
52	$1.30 x10^8$	467.37
53	$1.40 x10^4$	81.52
54	$2.90 x10^5$	44.58
59	$2.90 x10^8$	0
63	$5.50 x10^4$	258.03
69	$2.20x10^4$	180.86
	Vitamin C control = 4.38	

*DPPH assay: IC50 > 250 µg /mL, inactive; >100 –250 µg /mL, weakly active; >50–100 µg /mL, moderately active; 10–50 µg /mL, strongly active;< 10 µg /mL, very strongly active.

This is the first report on the antibacterial against plant pathogenic bacteria and antioxidant activity of endophytic fungi isolated from *Sesbania grandiflora (L.) Pers.* Among all the isolated tested, 17, 20, 23, 38, and 43 possessed the most remarkable *in vitro* antibacterial and antioxidant activities as determined by different assays mentioned above. In order to facilitate their development into a novel source of natural antibacterial and antioxidants, further isolation, purification and characterization of the active compound constituents in ethyl acetate extracts are underway.

Discussion

Endophytic fungi, a potential source of medicinal compounds, have been attracted more attention in the recent years. It is reported that special eco-environmental microorganisms may produce special activated metabolites [23]. According to this notion, we isolated endophytic fungi from *Sesbania grandiflora (L.) Pers*., which known as traditional medicinal plant, to search for novel natural antibacterial and antioxidant activities.

Acremonium spp. and *Fusarium* spp. were dominant genera in *Sesbania grandiflora (L.) Pers*., which was also demonstrated in other plants [24,25]. There is great deal of reports about *Fusarium* spp. as plant pathogenic fungi. However, *Fusarium* spp. could not cause apparent disease to their host. Therefore, the effect of endophytic *Fusarium* spp. during the developmental stage of plant need to be further studied.

Antibacterial activities of plant endophytic fungi to plant pathogenic bacteria have been reported by a few groups [26]. In the present work, the antibacterial activities were conducted using microdilution method and a modified resazurin microtiter plate assay. The susceptibility testing of 28 endophytic fungi crude extract showed that most of them exhibited potent antibacterial activity against 4 plant pathogenic bacteria which at least one of them can inhibit these bacteria. However, the degrees of susceptibility of endophytes were greatly different depending upon isolates,

suggesting that several substances participated in antimicrobial activity.

The endophytic fungi extract presented significant antimicrobial activity against plant pathogenic bacteria. Among of them, *X. axonopodis* pv. *citri* showed the highest inhibition by endophytic fungi, followed by *X. campestris* pv. *Campestris*, *A. avenae* subsp. *Avenae*, and *X. axonopodis* pv. *glycines* respectively. The degree of inhibitory activities against plant pathogenic bacteria showed with MIC and MBC range from 125-2000 µg/mL.

Antimicrobial activities of compounds biosynthesized by plant endophytes have been demonstrated by many reports. Compound of endophytic fungi isolated from Hydrastis canadensis showed antimicrobial activities against many Gram positive bacteria, Gram negative bacteria, fungi and parasites [27]. Tejesvi et al. reported that endophytic species isolated from four medicinal plants in India have not only antibacterial activity but also antioxidant, and antihypertensive activities [28].

To explore the effects of the endophytic fungi isolated from *Sesbania grandiflora(L.) Pers.*, extract on *in vitro* antioxidant activity, the DPPH scavenging rate was studied. The hydroxyl radical is one of the most reactive free radicals, which can induce severe damage to biomolecules [29]. Antioxidants are compounds that inhibit or delay the oxidation process by preventing the initiation or propagation of oxidizing chain reactions. DPPH radical is a relatively stable free radical and has been widely used to evaluate the antioxidant activities of various biological samples. This method is based on the reduction of the stable 2, 2- diphenyl-1-picryl-hydrazyl radical (DPPH) in the presence of a radical scavenger or hydrogen donors due to the formation of nonradical form of DPPH-H [30]. Ascorbic acid was chosen as the standard antioxidant for this experiment. The DPPH radical contains an old electron, which is accountable for the absorbance at 517 nm and also for a visible deep purple color. DPPH is decolorized when it accepts an electron donated by an antioxidant compound, which can be quantitatively measured from the changes in absorbance.

In the present study, there are some different in total antioxidant capacity (DPPH) and total phenolic content among selected endophytes. It was observed that DPPH is varied in all tested endophytic fungi. In addition, the total phenolic content displayed a rather low activity with IC_{50} ranging from 0 to 1730.78 µg gallic acid/mg extract. However, negative results do not mean that bioactive constituents are absent or these endophytic fungi are not effective antioxidant activity. It may contain other active chemical components that produce a definite other physiological action. The endophytic fungi isolated from *Sesbania grandiflora(L.) Pers* which showed the highest phenolic compounds can be further subjected to isolate the therapeutic antioxidant compounds activity and evaluate their pharmacologically.

There is some previous research on the antioxidant activity of endophytic fungi from other medicinal plants. For example, Harper *et al.* obtained two antioxidants, pestacin and isopestacin, from the endophytic fungi *Pestalotiopsis microspora* [31]. Phongpaichit *et al.* reported that 22.5% of the extracts from endophytic fungi and garcinia plants exhibited remarkable antioxidant activities [32]. The results of our study are similar to those in previous reports and indicate that endophytic fungi may serve as a potential source of antioxidants. The data presented in the study demonstrated that endophytic fungus have phenolic content and show excellent activity of against DPPH radicals. Therefore, they could be a source of natural antioxidants. In addition, the characteristics of phytochemicals having antioxidants property should be further studied.

In conclusion, the results of the present study reveal that the ethyl acetate extracts of *Sesbania grandiflora (L.) Pers*. has broad ranges of antibacterial activities and could be good potential sources for screening programs of bioactive natural products. The antioxidant activities demonstrated clearly that these extract contains a number of antioxidant compounds which can effectively scavenge DPPH under in vitro condition. It's suggesting that multiple mechanisms are involved. This study indicated that the endophytic fungi extracts from *Sesbania grandiflora (L.) Pers*. are the good sources of natural anti plant pathogenic bacterial and antioxidants which might be useful in treating the diseases associated with oxidative stress and plant disease control activity.

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Author's contribution

All authors contributed extensively to the work presented in this paper. Pannapa Powthong designed, performed experiments, analysed data and wrote the paper; Pattra Suntornthiticharoen performed experiments and analysed data; Acharawan Thongmee, supervised the project, gave technical support, conceptual advice and revised the manuscript; All authors discussed the results and implications and commented on the manuscript at all stages.

Conflict of Interest

This paper has no conflict of interest.

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