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Biological Activities and Chemical Profiles of Selected Soil Fungi Isolated from Sirindhorn Peat Swamp Forest of Narathiwat, Thailand

(Aktiviti Biologi dan Profil Kimia Kulat Tanah Terpilih yang Dipencilkan dari Hutan Paya Gambut Sirindhorn di Narathiwat, Thailand)

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ABSTRACT

A total of 54 soil fungi were collected from Sirindhorn peat swamp forest in Narathiwat Province which is the last peat swamp forests in Thailand. They were extracted using four different solvents and 104 crude extracts were obtained. The bioactivity screening of these samples was done employing alpha-glucosidase inhibition, anti-inflammation using nitric oxide inhibition and cytotoxicity using cancer cell line (MCF-7). The results showed that SPSF224 crude extract had the best biological activities in the screening tests and was then selected for further study. The SPSF224 was identified as Penicillium maximae*. The crude extracts of* P. maximae *were determined for chemical profile, IC_{50} value of alpha-glucosidase inhibition, antioxidant and antimicrobial activities against* Staphylococcus aureus, Trichophyton mentagrophytes, *and* T. rubrum*. This was the first report of chemical investigation and biological activity evaluation of* P. maximae*.*

Keywords: Alpha-glucosidase inhibition; antidiabetic; anti-microbial; antioxidant; Penicillium maximae

ABSTRAK

Sebanyak 54 kulat tanah telah dikumpulkan daripada hutan paya gambut Sirindhorn di Wilayah Narathiwat yang merupakan hutan paya gambut terakhir di Thailand. Kesemuanya diekstrak menggunakan empat pelarut yang berbeza dan 104 ekstrak kasar diperoleh. Penyaringan bioaktiviti sampel ini dilakukan dengan menggunakan aktiviti perencatan alfa-glukosidase, anti-radang menggunakan perencatan nitrik oksida dan sitotoksisita menggunakan titisan sel kanser (MCF-7). Hasil kajian menunjukkan bahawa ekstrak kasar SPSF224 mempunyai aktiviti biologi terbaik dalam ujian saringan dan kemudian dipilih untuk kajian lebih lanjut. SPSF224 dikenal pasti sebagai Penicillium maximae*. Ekstrak kasar* P. maximae *ditentukan untuk profil kimia, nilai IC_{50} perencatan alfa-glukosidase, aktiviti antioksida dan antimikrob terhadap* Staphylococcus aureus, Trichophyton mentagrophytes *dan* T. rubrum*. Ini adalah laporan pertama penyelidikan kimia dan penilaian aktiviti biologi* P. maximae*.*

Kata kunci: Antidiabetik; antimikrob; antioksidan; Penicillium maximae*; perencatan alfa-glukosidase*

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterised by hyperglycemia due to the lack of insulin secretion, action or both. DM is a major non-communicable disease or NCDs in the world. The number of people with diabetes worldwide between the ages of 20 and 79 in 2017 was 425 million peoples and will be increased to 629 million by 2045 (WHO 2019). The Bureau of Non-Communicable Diseases, Ministry of Public Health, Thailand showed the statistics of diabetic patients per 100,000 population per year in Thailand in 2007 and 2015 were 500,347 and 802,017, respectively (Bureau of NCD 2019).

A defect in insulin production is the primary cause of rising blood sugar levels. Alpha-glucosidase enzyme is a key enzyme located at the brush border of the small intestine used for delaying blood glucose absorption which hydrolyses 1, 4 alpha-glycosidase bonds in the terminal position of the polysaccharide chain (Gao et al. 2008). The complex carbohydrates in the lumen of the small intestine have to be broken up by alpha-glucosidase before the resulting glucose molecules can be taken up and finally reach the bloodstream. Postprandial hyperglycemia can be improved by inhibition of small intestinal alpha-glucosidase activity (Hakamata et al. 2009). The drugs currently being used for hyperglycemia treatment by alpha-glucosidase inhibitory mechanism include miglitol (Kalra 2014), voglibose (Horii et al. 1986) and acarbose (Bischoff 1994) which have been initially isolated from microorganisms.

It is well known that long term DM is often associated with secondary complications due to over production of free radicals and malfunction of antioxidant process giving rise to increasing oxidative stress. From previous clinical studies, the results of antioxidant treatments showed that they could improve symptoms of diabetic polyneuropathy and increased glucose transport in skeletal muscle cells. The report also presented that the intake of antioxidant could reduce the risk of development on type 2 DM (Scott & King 2004).

Moreover, DM patients are susceptible to be infected with pathogens such as *Staphylococcus aureus*, *S. epidermidis, Mycobacterium tuberculosis,* and *Streptococcus pneumoniae* because their immune systems are weak (Casqueiro & Alves 2012). Therefore, combination treatment with antioxidant and antibacterial agents could help to improve DM treatment effectively (Fuchs et al. 2013). The antimicrobial effects of some of active ingredients of *Penicillium* species including griseofulvin, is one of antifungal antibiotics currently used for systematic fungal infection treatment which was isolated from *P. griseofulvum* (MacMillan 1954).

Fungi are found in a variety of habitats such as soil, associated with plants, water, associated with other fungi and ruminant guts (Dighton & White 2017). Secondary metabolites produced by fungi usually show inhibitory or toxicity effects on other organisms. Accordingly, fungal metabolites have been developed to be used in pharmaceuticals (Shwab & Keller 2008) due to their antibacterial, antifungal, anticancer (Zhao et al. 2013), anti-inflammatory (Deshmukh et al. 2009), antioxidant (Sadananda et al. 2014) and cytotoxic activities (Turbyville et al. 2006).

Peat swamp forests are tropical moist forests and they create a thick layer of acid soil (Thawai 2004) with high relative biodiversity and humidity (Boonyuen et al. 2012). The microbes living in this area can survive in this harsh environment. The Sirindhorn Peat Swamp Forest Nature Research and Study Centre is located in one of the last remaining peat swamp forests in Thailand. This forest area is reported to spread over 224 square kilometers (ORDPB 2019). Preliminary study showed that various fungi such as *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp., and *Gongronella* sp. have been isolated from soil obtained from Sirindhorn peat swamp forest. Pharmacological activities of these fungi such as antifungal (Wang et al. 2012), cytotoxic, insecticide (Rukachaisirikul et al. 2013), antibacterial (Cazar et al. 2005), antioxidant (Trisuwan et al. 2011), antimalarial and anticancer (Trisuwan et al. 2014) have been reported. This study aimed to search for the active substances for antidiabetic activity from fungi isolated from the soil of Sirindhorn peat swamp forest by assessing alpha-glucosidase inhibitory efficacy. The effective compound or extract could serve as a lead substance for further development of anti-diabetic drug. Additionally, bioactivity screening of alpha-glucosidase inhibition, antiinflammation and cytotoxicity of the fungal crude extracts from Sirindhorn peat swamp forest are herewith reported.

Materials and Methods

Fungal materials

The 54 soil fungi were collected from Sirindhorn peat swamp forest, Narathiwat, Thailand. They were isolated by the researchers from Department of Microbiology, Faculty of Science, Prince of Songkla University, Songkhla, Thailand. The isolated fungi were cultivated and extracted using four different solvents to obtain 104 sample extracts which were then screened for bioactivity determination. The bioactivity screening performed on these samples were alpha-glucosidase inhibition, anti-inflammation (nitric oxide inhibition) and cytotoxicity using cancer cell line (MCF-7).

Determination of alpha-glucosidase inhibitory activity

This assay was modified from previously reported

protocol using colorimetric method in 96-well plate (Dej-adisai & Pitakbut 2015). Samples (2 mg/mL) were added to 50 µL of 10 mM phosphate buffer solution (pH7), which contained 2 mg/mL of bovine serum albumin and 0.2 mg/mL of sodium azide, and then incubated at 37 °C for 2 min with 50 µL of α-glucosidase enzyme (1 unit/mL). The result of α-glucosidase inhibition activity was processed using the following equations:

$$\text{Velocity} = \frac{\Delta \text{ Absorbance at 405 nm}}{\Delta \text{ Time}}$$

$$\% \text{ Inhibition} = \frac{\text{V control-V sample}}{\text{V control}} \times 100$$

DETERMINATION OF ANTI-INFLAMMATORY ACTIVITY

Inhibitory effect on nitric oxide (NO) production by the 104 fungal extracts (25 µg/mL) were carried out using RAW264.7 cells following Dej-adisai et al. (2018). NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. The reagent consisted of *N*-(1-naphthalenediamine) and sulfanilic acid. Under acidic conditions, sulfanilic acid (sulfanilamide) was converted by nitrite to a diazonium salt. *N*-(1-naphthalenediamine) was added into diazonium salt to form a colored azo dye (red-pink color). The optical density was measured with a 570 nm using microplate reader. L-nitroarginine (L-NA), indomethacin and caffeic acid phenethyl ester (CAPE) were used as positive controls.

DETERMINATION OF CYTOTOXIC ACTIVITY

Cytotoxic activity against human cancer cells for 104 fungal extract samples (25 µg/mL) were determined by performing sulforhodamine B (SRB) colorimetric assay. SRB assay was used for measuring the cellular protein content using two of the sulfonic groups to bind to basic amino acid in the cell under mild acid condition, which show pink color. Human cancer cells (human breast carcinoma cell line (MCF-7)) and a human normal cell (human gingival fibroblast cell line (HGF)) were used for cytotoxicity test. Camptothecin was used as a positive control. The experiment was tested in 96 well plates and detected using microplate reader at wavelength 492 nm (Dej-adisai et al. 2018).

FUNGAL IDENTIFICATION

Fifty-four soiled fungi were collected from Sirindhorn peat swamp forest in Narathiwat Province which is the last peat swamp forests in Thailand. They were extracted by 4 different solvents and obtained 104 crude extracts. The bioactivity screening of the 104 crude extracts showed that SPSF224 crude extract had the best biological activities. SPSF224 was selected for further species identification which was performed by Leibniz-Institut DSMZ-Deutsche Sammlungvon Mikroorganismen und Zellkulturen GmbH, Germany. The fungus was subjected to DNA extraction and sequencing of the rDNA ITS fragment (Schoch et al. 2012). Additionally, we amplified and sequenced the large subunit of rDNA (LSU) and partial calmodulin gene (Visagie et al. 2013). The fungus rDNA-ITS fragment showed two different operons and was therefore not useful for identification. Comparison of assembled calmodulin sequences was performed with GenBank, Myco ID and various databases as well as with the type species of *Penicillium maximae* (GenBank Accession: KC773821). *P. maximae* belongs to the section Sclerotiora within the genus *Penicillium* (Visagie et al. 2014).

FUNGAL FERMENTATION AND EXTRACTION OF SPSF224

SPSF224 was grown on potato dextrose agar (PDA) at 25 °C for five days. Five pieces of 0.5 × 0.5 cm^2 mycelial agar plugs were inoculated into 500 mL Erlenmeyer flasks containing 300 mL of PDA at room temperature for 21 days. The fungal culture (60 L) was filtered to separate into wet mycelia and the filtrate. The filtrated broth was transferred to a separatory funnel and the same volume of ethyl acetate was added each time. The organic layer was evaporated to dry under reduced pressure to provide broth ethyl acetate extract, BE (red-brown paste, 5.77 g). The fungal mycelium was soaked for 3 days in methanol. The MeOH layer was concentrated by an evaporator and added to 150 mL of distilled water. The mixture was then shaken with 500 mL of hexane. The aqueous layer was extracted three times with an equal volume of ethyl acetate and then evaporated to obtain cell hexane extract, CH (yellow-brown paste, 1.35 g), cell ethyl acetate extract, CE (red-brown paste, 2.47 g) and cell methanol extract, CM (black-brown paste, 3.59 g). Then these extracts from SPSF224 were further studied for chemical profiling and biological activities.

FUNGAL PURIFICATION

The extract from SPSF224 BE (5.767 g) was loaded onto the quick column by dry-loading technique. Then, a gradient solvent system as a mobile phase was eluted through the column with a mixture of hexane, ethyl acetate, methanol and water from 100:0:0:0 to 0:50:40:10 to yield 19 subfractions F1 to F19. F12 (0.972 g) was further purified using classical column chromatographic technique. The separation was carried out using a mixture of chloroform and methanol from 100:0 to 50:50 to obtain 11 subfractions. F12-8 (0.121 g) was loaded on the surface of Sephadex® LH-20 column chromatography using methanol as eluent to produce 8 fractions. F12-8-8 (68 mg) was purified by Sephadex® LH-20 column chromatography. The same elution protocol was repeated giving 7 subfractions. Fraction F12-8-8-4 was isolated and determined as semi-purified PM1 (30.2 mg).

GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Gas Chromatography-Mass Spectrometer (GC-MS) (5977A Series GC/MSD System, Agilent Technologies, USA) was used to analyse the crude extracts. The samples were chromatographed on capillary columns, size 30 m × 250 µm × 0.25 µm. One microliter of extract was injected into an injector at 70 ºC and held for 3 min. The temperature was increased by 15 ºC/min until it reached 200 ºC without holding. It was then increased with a program rate of 3 ºC/min to 300 ºC and was held for 10 min. The identification of the components in the extract was carried out by comparison of their retention time and mass spectral fragmentation pattern with those stored in the computer library.

CHEMICAL SCREENING

The extracts from SPSF224 were further studied for its chemical profile by identification of the presence of tannin, alkaloids, steroid, triterpenoid, cardiac glycoside, saponin, anthraquinone, coumarin, and flavonoid. The chemical screening tests were performed according to previously reported methods (Farnsworth 1966; Harborne 1984; Trease & Evans 1983) with some modifications.

DETERMINATION OF TOTAL PHENOLIC CONTENT (TPC)

Total phenolic content of *P. maximae* extracts was estimated using the Folin-Ciocalteu colorimetric assay according to the method of Singleton and Rossi (1965) with some modifications. Stock solutions of samples and standard (gallic acid) at the concentration of 5 mg/mL were dissolved in absolute ethanol. Total phenolic contents were obtained from regression equation Y= 0.0101X + 0.0111 with R^2 value of 0.999 and expressed as mg/g gallic acid equivalent using the equation below:

$$\text{Total phenolic content (mg/g)} = \frac{c\ x\ DF\ x\ V}{W}$$

where C is the concentration of gallic acid (µg/mL); DF is the dilution factor; V is the volume of fungal extract (mL); and W is the weight of fungal extract (mg).

DETERMINATION OF TOTAL FLAVONOID CONTENT

The total flavonoid content of *P. maximae* extracts was determined by aluminum chloride colorimetric method as described by Chang et al. (2002) with some modifications. The sample (500 µL of 1 mg/mL) was mixed well with 1.5 mL of 95% ethanol, 100 µL of 10% $AlCl_3$, 100 µL of 1M CH_3COOK and 2.8 mL of distilled water. Five varying concentrations of quercetin were used in preparing the standard curve. Regression equation of the curve, Y=0.0033X+0.0072 with R^2 value of 0.999 was obtained. The equation was used to calculate the quercetin content in 1 g of sample (mg quercetin equivalence (QE)/g of sample).

DETERMINATION OF ANTIOXIDANT ACTIVITY

The radical scavenging activity of the various extracts of *P. maximae* were determined using 2,2-diphenyl-1-picrylhydrazil free radical scavenging assay. The samples (200 µg/mL) or 2 mL of 200 µg/mL quercetin were mixed well with 2 mL of 6×10^{-5} M DPPH. Ethanol was used as blank. The mixtures were incubated for 20 min in the dark. Measurement of absorbance at 520 nm was done using UV-vis spectrometers and percentage of antioxidant activity was calculated using the following equation:

$$\%\ \text{inhibition} = \left(\frac{ODcontrol - ODsample}{ODcontrol}\right) x\ 100$$

DETERMINATION OF ANTIMICROBIAL ACTIVITY

The preliminary screening of antimicrobial activity was carried out using the agar disc diffusion assay (Lorian 2005). *Staphylococcus aureus* (ATTC 25923), *S. epidermis* (TISTR 517), *Propionibacterium acnes* (DMTS 14916) and Methicillin-resistant *S. aureus* (MRSA 1350II06) were selected for Gram positive bacteria. *Escherichia*

coli (ATCC35218) and *Pseudomonas aeruginosa* (ATCC10145) were selected for Gram negative bacteria. *Candida albicans* (TISTR 5779) was selected for yeast and *Microsporum gypseum*, *Trichophyton rubrum,* and *T. mentagrophytes* were selected for fungi. Five fungal extracts of SPSF224 were dissolved in DMSO and diluted to achieve a concentration of 200 µg/mL and then 10 µL of samples were dropped on the sterile paper disc. All test plates were incubated for 24 h at 37 °C for bacteria, 24 to 48 h at 35 °C for yeast and 7 days at 30 °C for fungi. The appearance of zone of inhibition on the plates was measured in diameter (mm).

Results and Discussion

Bioactivity determination of fungal extracts

A total of 104 crude extract samples from 54 fungi collected from Sirindhorn peat swamp forest were determined for their biological activities as anti-alpha-glucosidase, anti-inflammatory and cytotoxic (Table 1). Thirty-eight samples showed anti-alpha-glucosidase activity of over 90% inhibition. Only two samples showed potential effects of anti-inflammatory and cytotoxic activities at 25 µg/mL which SPSF224BE and SPSF224CE. Hence, SPSF224 was selected for further studies.

TABLE 1.Bioactivity determination of 104 fungal extracts

No.	Extract	Anti-alpha-glucosidase activity (% inhibition)	Anti-inflammation (% inhibition)		Cytotoxicity on MCF-7 (% inhibition)
			Nitric oxide inhibition	Cytotoxic effect	
1.	SPSF001BE	43.95±3.65	35.39±3.57	-2.13±3.78	18.88±5.36
2.	SPSF002BE	27.80±3.73	43.63±2.78	0.94±1.30	59.42±4.08
3	SPSF003BE	99.71±0.29	37.73±2.22	1.26±4.89	11.24±5.36
4.	SPSF004BE	63.98±3.62	32.22±4.55	0.46±3.48	9.90±5.97
5.	SPSF005BE	18.19±4.00	108.64±2.04	85.23±1.71	99.32±0.22
6.	SPSF006BE	50.07±4.29	56.57±3.43	-2.94±2.90	38.63±2.57
7.	SPSF007BE	100.20±0.24	26.24±2.80	2.69±3.39	39.10±1.30
8.	SPSF008BE	99.55±0.08	23.42±3.15	1.12±1.04	11.78±5.60
9.	SPSF009BE	99.75±0.27	33.71±2.37	3.56±1.95	-3.90±5.39
10.	SPSF010BE	7.18±5.33	22.95±1.21	3.86±0.92	66.00±4.00
11.	SPSF001BE	43.95±3.65	35.39±3.57	-2.13±3.78	18.88±5.36
12.	SPSF012BE	100.56±2.86	51.01±4.30	2.24±2.13	0.85±4.99
13.	SPSF013BE	70.74±2.30	30.45±3.31	1.08±2.93	85.14±2.70
14.	SPSF028BE	99.71±0.29	123.29±2.13	87.81±0.76	92.18±0.15
15.	SPSF029BE	83.63±1.89	123.37±2.04	78.21±0.78	97.10±0.18
16.	SPSF030BE	68.62±7.05*	47.42±3.37	10.07±1.76	10.31±2.43
17.	SPSF031BE	98.55±2.69	35.87±1.76	11.89±1.02	34.92±2.62
18.	SPSF032BE	98.05±0.63	21.25±0.86	11.99±1.75	12.00±4.68
19.	SPSF033BE	99.50±0.09	124.72± 2.27	76.14±1.16	87.49±1.15
20.	SPSF034BE	91.26±0.65	28.02±2.88	14.27±2.50	4.43±1.57
21.	SPSF035BE	99.27±0.34	35.93±3.06	14.53±2.47	7.98±0.22
22.	SPSF037BE	97.61±2.82**	19.86±2.06	22.80±2.69	74.12±1.64
23.	SPSF038BE	36.72±1.52	29.12±2.91	15.58±1.68	3.40±3.60

24.	SPSF039BE	76.91±0.55	31.88±2.13	15.02±3.12	5.48±1.90
25.	SPSF040BE	96.72±0.82	36.10±4.11	18.17±0.53	5.27±2.84
26.	SPSF042BE	66.24±4.05	91.71±5.17	11.45±2.89	64.38±4.08
27.	SPSF043BE	96.87±0.34	81.32±1.14	7.58±2.08	24.63±1.65
28.	SPSF044BE	61.11±0.51	101.08±1.85	47.26±2.26	75.90±0.76
29.	SPSF045BE	82.56±0.96	47.38±2.42	14.09±3.07	19.99±2.34
30.	SPSF046BE	82.54±2.91	36.26±2.46	17.26±0.64	2.76±4.27
31.	SPSF047BE	91.51±0.68	67.22±1.75	15.86±2.28	17.17±2.44
32.	SPSF048CE	88.92±1.42	45.87±2.66	15.37±1.10	1.48±1.96
33.	SPSF049BW	54.69±3.32	100.73±2.93	16.54±0.58	73.93±0.94
34.	SPSF051BW	91.44±2.23	95.85±3.58	47.76±2.91	95.54±3.12
35.	SPSF206CM	17.98±2.89	29.19±3.30	20.58±2.08	10.60±1.28
36.	SPSF206BE	79.43±1.19	66.72±1.47	12.77±2.51	47.12±1.08
37.	SPSF208BE	98.31±1.19	49.56±1.84	3.63±3.35	30.30±3.79
38.	SPSF208CE	56.45± 2.57	87.78±4.80	47.18±0.96	69.91±2.07
39.	SPSF208CH	69.61±0.79	53.48±0.87	8.58±5.15	31.94±1.39
40.	SPSF209CE	60.34±1.41	56.99±0.99	4.97±5.9	25.51±1.83
41.	SPSF209BE	84.76±2.89	30.29±5.10	14.50±2.98	19.36±1.96
42.	SPSF209BW	27.98±3.35	23.05±2.55	15.63±2.04	3.10±2.20
43.	SPSF211CE	81.43±3.26	43.32±5.68	17.42±2.90	7.63±1.68
44.	SPSF211BE	101.26±1.73	-26.87±3.20	11.987±4.55	9.84±3.43
45.	SPSF213BE	64.55±0.98	32.73±2.66	11.35±1.45	1.12±2.28
46.	SPSF219BE	98.29±3.64	72.42±1.29	8.92±1.74	61.34±0.95
47.	SPSF221CE	27.00±2.80	69.44±2.22	54.79±1.63	86.15±1.03
48.	SPSF221CH	18.90±1.20	78.33±1.48	59.95±3.02	89.21±1.18
49.	SPSF221BE	89.86±4.55	81.60±1.59	41.67±1.99	34.61±1.29
50.	SPSF224BE	104.83±3.40	76.13±2.25	15.84±1.85	46.84±2.28
51.	SPSF224CE	100.00±00	91.07±2.32	22.83±1.79	76.62±1.39
52.	SPSF225CE	51.23±1.28	52.13±4.25	16.59±3.59	53.98±0.97
53.	SPSF225BE	88.02±1.71	-2.74±2.57	6.72±2.72	19.33±1.50
54.	SPSF226BE	86.91±1.94	55.37±1.36	18.10±1.13	40.49±2.47
55.	SPSF226CE	54.70±4.90	55.82±2.54	5.38±2.55	28.49±1.82
56.	SPSF226CH	29.86±3.83	47.69±1.43	16.99±1.99	30.81±1.64
57.	SPSF227BE	57.93±1.11	59.60±2.51	9.87±3.11	35.95±2.81
58.	SPSF227CE	18.55±0.16	79.97±1.55	3.58±0.07	43.53±1.70
59.	SPSF227BW	91.04±0.19	21.39±3.31	17.16±2.36	0.29±1.10
60.	SPSF234CE	100.32±0.36	17.71±2.50	18.94±2.47	33.28±1.01
61.	SPSF234BE	99.97±1.33	7.83±2.73	13..57±3.93	21.58±3.44
62.	SPSF234BW	74.81±2.17	21.58±3.09	18.51±2.54	-5.90±1.40
63.	SPSF236BE	61.00±2.34	45.59±1.35	16.54±5.70	27.39±1.34
64.	SPSF236CE	52.05±2.73	55.46±4.41	15.75±2.32	31.16±2.54
65.	SPSF238CE	54.05±1.68	48.16±1.83	22.35±2.05	51.68±1.10
66.	SPSF240BE	93.63±2.19	37.60±0.75	3.44±2.64	14.99±2.80
67.	SPSF240CE	62.57±3.85	68.24±1.09	7.45±3.44	60.05±2.44

68.	SPSF240CH	77.42±4.69	84.38±2.40	16.16±2.69	67.47±1.55
69.	SPSF241BE	85.43±0.48	65.92±1.54	11.48±4.19	22.65±2.12
70.	SPSF244CE	44.84±1.14	88.40±2.66	22.00±5.72	74.00±0.80
71.	SPSF244BE	51.29±1.87	27.26±2.09	7.28±0.22	16.22±3.81
72.	SPSF248CH	82.27±2.24	41.81±189	0.01±5.91	19.57±3.80
73.	SPSF248CE	69.61±0.79	95.63±2.32	-4.28±5.95	61.16±1.78
74.	SPSF248BE	86.91±1.94	103.34±1.94	35.60±0.45	81.46±1.08
75.	SPSF250BE	103.70±2.01	56.98±4.65	55.96±0.61	3.42±1.73
76.	SPSF304BE	84.80±0.74	82.98±1.31	8.60±3.55	59.79±4.21
77.	SPSF304CH	100.00±00	92.93±2.39	17.96±2.27	69.50±1.85
78.	SPSF304BW	52.97±1.23	51.44±3.15	20.26±1.69	6.56±3.73
79.	SPSF310BE	119.00±2.91	40.05±2.46	-24.76±6.66	15.08±2.77
80.	SPSF310CE	67.96±2.27	71.33±3.08	-7.60±7.98	20.42±1.30
81.	SPSF310BW	84.37±2.21	62.61±1.34	20.82±3.09	11.83±4.56
82.	SPSF312BE	85.73±1.09	85.23±2.13	15.40±2.22	86.36±0.88
83.	SPSF312CE	79.36±1.75	80.29±5.31	26.00±2.61	64.01±2.36
84.	SPSF318BE	53.42±1.51	53.16±1.44	2.73±2.20	21.58±2.80
85.	SPSF318CE	53.04±1.44	84.00±2.30	18.05±1.50	84.27±1.10
86.	SPSF325BE	100.00±00	25.83±1.72	4.16±1.89	70.96±4.20
87.	SPSF325CE	100.00±00	25.98±3.36	17.27±2.27	34.52±2.06
88.	SPSF325CH	100.00±00	31.76±6.15	22.57±1.83	17.53±3.26
89.	SPSF328BE	100.00±00	38.77±2.39	4.96±3.75	31.69±4.20
90.	SPSF329BE	86.27±3.19	27.53±2.42	13.96±3.06	-2.60±1.63
91.	SPSF329CE	100.00±00	-0.92±2.54	20.46±5.41	78.68±1.47
92.	SPSF329CH	100.00±00	10.69±1.47	35.15±4.35	55.29±2.21
93.	SPSF330BE	55.29±2.57	61.32±1.49	16.57±1.97	36.87±4.10
94.	SPSF330CE	98.49±1.23	37.64±1.56	11.59±1.39	15.18±1.16
95.	SPSF339BE	94.35±0.69	105.03±0.62	57.78±2.05	85.23±0.23
96.	SPSF339CE	96.87±0.60	98.44±0.57	16.25±3.11	24.43±2.10
97.	SPSF339BW	51.24±0.25	70.23±1.10	3.53±2.59	78.80±1.50
98.	SPSF347BE	77.16±2.57	39.60±3.54	16.07±1.66	24.57±3.57
99.	SPSF358BW	83.21±3.35	23.88±2.73	10.81±1.03	-2.36±1.44
100.	SPSF358BE	59.77±3.95	9.41±1.05	9.31±0.86	10.20±1.39
101.	SPSF358CE	98.90±3.72	28.65±1.01	5.22±4.23	16.98±2.24
102.	SPSF360BE	32.08±0.15	105.04±0.89	72.32±0.71	97.33±0.10
103.	SPSF360CH	13.12±4.26	105.22±1.07	89.32±0.29	98.90±0.20
104.	SPSF360BW	64.63±1.57	30.96±1.57	27.93±1.43	6.74±1.61
	Acarbose	86.87±0.66	-	-	-
	Camptothecin				94.01±0.17
	Indomethacin		61.08±0.68	25.42±2.99	

Values are mean±standard deviation of three replication (n=3); *0.5 mg/mL; **1 mg/mL; P= Positive control

PRELIMINARY QUALITATIVE CHEMICAL SCREENING OF *P. maximae* EXTRACTS

The qualitative metabolite analysis of *P. maximae* extracts was obtained as shown in Table 2. The results showed that tannin and alkaloid were present in all the crude extracts while cardiac glycoside, steroid, saponin, and terpenoid were absent. Flavonoid was found in cell ethyl acetate extract (CE), broth ethyl acetate extract (BE), and cell hexane extract (CH). Coumarin and anthraquinone were absent in cell ethyl acetate extract (CE).

This result showed that *P. maximae* produced various types of secondary metabolites. Moreover, the data were in accordance with previous reports that alkaloids, tannins, flavonoids, coumarins and anthraquinones had been found in *Penicillium* genus. Alkaloids were found in *P. aurantiogriseum*, *P. terlikowskii,* and *P. citrinum,* respectively (Kalinina et al. 2018; Lai et al. 2013; Waring et al. 1987). Tannins were found in *P. frequentans* (Bhardwaj et al. 2015). Flavonoids, coumarins and anthraquinones were obtained from *P. setosum* (George et al. 2019), *P. oxalicum* (Wang et al. 2014), and *P. chrysogenum* (Brunati et al. 2009).

TABLE 2. Chemical screening of *P. maximae*

Chemicals	Extracts*				
	CE	BE	BW	CM	CH
Tannin	/	/	/	/	/
Alkaloid	/	/	/	/	/
Cardiac glycoside	X	X	X	X	X
Steroid	X	X	X	X	X
Saponin	X	X	X	X	X
Flavonoid	/	/	X	X	/
Triterpenoid	X	X	X	X	X
Coumarin	/	X	X	X	X
Anthraquinone	/	X	X	X	X

/ = Present, X = Absent

*CE: cell ethyl acetate extract; BE: broth ethyl acetate extract; BW: broth water extract; CM: cell methanol extract; CH: cell hexane extract

SECONDARY QUANTITATIVE CHEMICAL SCREENING OF *P. maximae* EXTRACTS

DETERMINATION OF TOTAL PHENOLIC CONTENT

The total phenolic content of *P. maximae* extracts were shown in Table 3. Broth water extract (BW) and cell methanol extract (CM) showed low amounts of total phenolic content at 1.83±0.41 and 0.97±0.20 mg GAE/g dry weight of sample, respectively. Meanwhile, cell ethyl acetate extract (CE) contained the highest amount of phenolic content up to 8.89±0.40 mg GAE/g dry weight of sample.

DETERMINATION OF TOTAL PHENOLIC CONTENT

Total flavonoid content of *P. maximae* extracts quantified using the aluminum chloride colorimetric method showed that the total flavonoid content was highest in CE (7.73 mg QE/g dry w.), while flavonoid was absent in BW and CM (Table 3). The maximum phenolic and flavonoid

content were found in CE at 8.89±0.40 mg GAE/g and 7.73 mg QE/g, respectively. The results suggested that ethyl acetate was the most suitable solvent to isolate *P. maximae* secondary metabolites. This may be due to the low polarity property of ethyl acetate which selectively extracts low molecular weight phenol and flavonoid (Bhardwaj et al. 2015). Phenolic and flavonoid are major secondary metabolites of fungi and have been associated with antioxidative action in biological systems (Devi et al. 2012). The presence of phenolic and flavonoid compounds has been reported in *Penicillium* species including *P. chrysogenum* (Sikandar et al. 2020), *P. granulatum* (Chandra & Arora 2012), and *P. fumiculosum* (Jakovljević et al. 2014). Therefore, the phenolic and flavonoid compounds found in this study possibly possess antioxidant activity.

TABLE 3. Total phenolic and flavonoid contents of *P. maximae* extracts

Extracts*	Total phenolic content (mg GAE/g)	SD	Total flavonoid content (mg QE/g)	SD
CE	8.89	0.40	7.73	0.19
BE	6.12	0.20	2.24	0.15
BW	1.83	0.41	n/d**	n/d**
CM	0.97	0.20	n/d**	n/d**
CH	5.33	0.40	1.36	0.18

*CE: cell ethyl acetate extract; BE: broth ethyl acetate extract; BW: broth water extract; CM: cell methanol extract; CH: cell hexane extract

CHEMICAL PROFILING OF CELL HEXANE EXTRACT OF *P. maximae*

From this investigation, CH of *P. maximae* showed the highest activity of alpha-glucosidase inhibition. Thus, CH was selected for further analysis. The GC-MS chromatogram showed three dominant peaks at retention times of 15.9212, 26.6168, and 37.8967 min, respectively (Figure 1; Table 4). These peaks represented the *n*-Hexadecanoic acid ($C_{16}H_{32}O_2$), 2,6-dimethyl-N-(2-methyl-alpha-phenylbenzyl) aniline ($C_{22}H_{23}N$) and 3Beta-acetoxy-6-nitroandrost-5-en-17-one ($C_{21}H_{29}NO_5$), respectively. From the previous report (Artanti et al. 2012), *n*-Hexadecanoic acid had a low activity of alpha-glucosidase inhibition. Therefore, alpha-glucosidase inhibition potency of CH might be from 2,6-dimethyl-N-(2-methyl-alpha-phenylbenzyl) aniline ($C_{22}H_{23}N$) and 3Beta-acetoxy-6-nitroandrost-5-en-17-one ($C_{21}H_{29}NO_5$). However, the anti-alpha-glucosidase efficacy of these two compounds should be further studied to determine whether the anti-alpha-glucosidase capacity of CH possibly was due to the effect of each individual constituent or the synergistic action of the components in the extract.

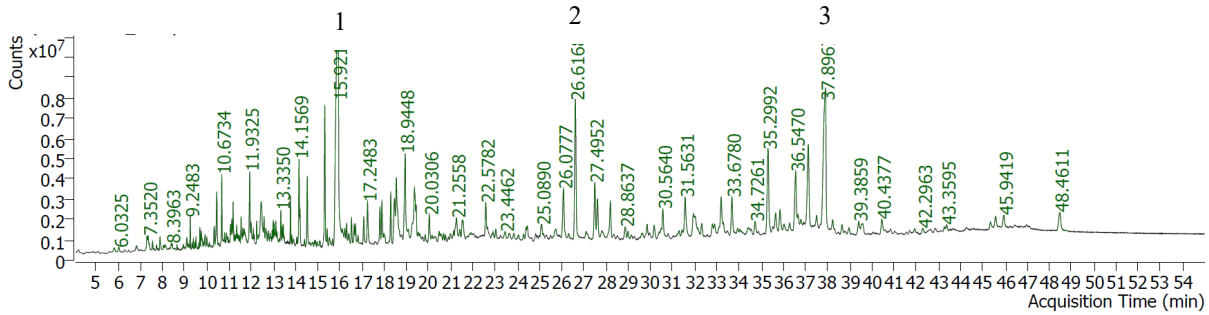

FIGURE 1. GC-MS chromatogram of cell hexane extract of *P. maximae*

TABLE 4. Dominant peaks from GC-MS chromatogram of cell hexane extract of *P. maximae*

No.	Component RT	Compound name	CAS#	Formula	Component Area	Match factor
1	15.9212	n-Hexadecanoic acid	57-10-3	$C_{16}H_{32}O_2$	99116607.0	96.4
2	26.6168	2,6 dimethyl-N-(2 methyl-alpha.- phenylbenzyl)aniline	119971-00-5	$C_{22}H_{23}N$	31006746.8	81.4
3	37.8967	3Beta-acetoxy-6-nitroandrost-5-en-17-one	31559-86-1	$C_{21}H_{29}NO_5$	54667640.7	71.4

CHEMICAL PROFILING OF SEMI-PURIFIED PM1

GC-MS analysis of semi-purified PM1 was shown in Figure 2. The chromatogram illustrated two dominant peaks at retention times (RT) of 14.1728 and 12.3793 min, respectively (Table 5). The highest peak (RT 14.1728) represented 3-(1-methylbutyl)-1,2,4-cyclopentanetrione ($C_{10}H_{14}O_3$) and the other (RT 12.3793) represented suberic dihydrazide ($C_8H_{18}N_4O_2$). Their alpha-glucosidase inhibitory efficacies have not been reported yet. To the best of our knowledge, the exact active substances of semi-purified PM1 on anti-alpha-glucosidase are still undefined. Thus, anti-alpha-glucosidase efficacy of these two ingredients should be further evaluated.

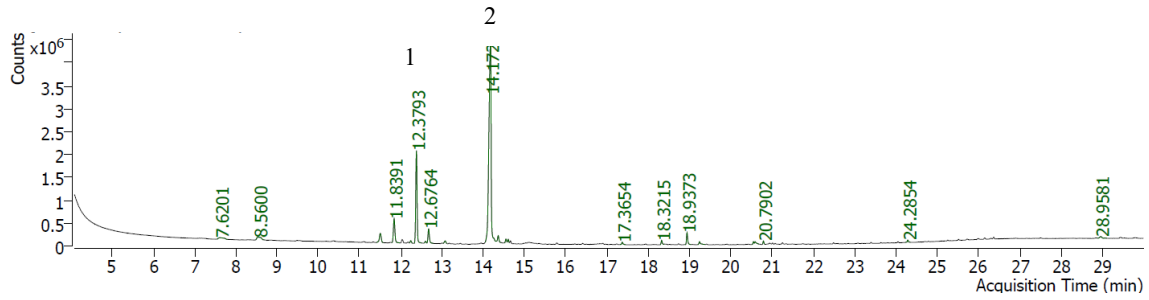

FIGURE 2. GC-MS chromatogram of semi-purified PM1

TABLE 5. Dominant peaks from GC-MS chromatogram of semi-purified PM1

No.	Component RT	Compound name	CAS#	Formula	Component area	Match factor
1	12.3793	Suberic dihydrazide	20247-84-1	$C_8H_{18}N_4O_2$	4511271	68.4
2	14.1728	3-(1-methylbutyl)-1,2,4-cyclopentanetrione	54644-19-8	$C_{10}H_{14}O_3$	16922005	82.3

BIOACTIVITIES OF *P. maximae*

ALPHA-GLUCOSIDASE INHIBITORY ACTIVITY

P. maximae extracts were determined for alpha-glucosidase inhibition and the half maximal inhibitory concentration (IC_{50}). The results showed that CH was the most active extract for alpha-glucosidase inhibition with IC_{50} of 490 µg/mL whereas acarbose as standard drug showed the IC_{50} value at 205 µg/mL (Table 6). The comparison of alpha-glucosidase inhibition from all SPSF224 extracts, CH, CE, and BE showed that their alpha-glucosidase inhibitory activities were higher than the others. Previous studies have reported that phenolic and flavonoid compounds could act as alpha-glucosidase inhibitors and regulators of hyperglycemia (Montefusco-Pereira et al. 2013). Therefore, phenolic and flavonoid compounds present in CH, CE, and BE could be the chemical groups in these extracts that exhibited the alpha-glucosidase inhibition.

TABLE 6. The alpha-glucosidase inhibitory activity of *P. maximae* extracts

Extracts/Compound	Anti-alpha-glucosidase activity (% inhibition)	IC_{50} (µg/mL)
Broth ethyl acetate (BE)	97.12±1.01	1008
Broth water (BW)	45.63±0.21	-
Cell ethyl acetate (CE)	118.59±2.71	1080
Cell hexane (CH)	104.03±0.42	490
Cell methanol (CM)	27.77±0.11	-
Semi-purified PM1	91.83±1.59	-*
Acarbose [p]	87.66±0.21	205

Data were expressed as mean±SD
[p] = Positive control
* = insufficient amount

ANTIOXIDANT ACTIVITY

Antioxidant activities of *P. maximae* extracts were determined by the DPPH radical scavenging activity. The highest activity of antioxidant was found in CE. However, its ability was less than a standard quercetin (Table 7). Huang et al. (2005) reported that phenolic compounds were the major antioxidant constituents of the endophytes. In addition, previous report showed the correlation between the phenolic and flavonoid contents with antioxidant activity (Shweta et al. 2010). In this study, CE exerted highest DPPH radical scavenging activity. Hence, it was possibly due to the highest level of phenolic and flavonoid contents in the extract.

TABLE 7. Inhibition of DPPH radical scavenging activity for *P. maximae* extracts

Samples*	% inhibition at 200 µg/mL**	SD
CE	31.25	5.89
BE	28.40	2.39
BW	2.66	0.33
CM	1.47	0.26
CH	11.69	2.14
quercetin	93.44	1.54

*CE: cell ethyl acetate extract; BE: broth ethyl acetate extract; BW: broth water extract; CM: cell methanol extract; CH: cell hexane extract.
**Values were represented with mean±standard deviation of three replications (n=3)

ANTIMICROBIAL ACTIVITY

Crude extracts of *P. maximae* were tested for antimicrobial activity using the disc diffusion method. Broth ethyl acetate extract (BE) showed inhibition zone against *S. aureus*, *T. mentagrophytes,* and *T. rubrum* at 8.1±0.1, 6.4±0.5, and 11.2±2.6 mm, respectively. Cell methanol extract (CM) also exhibited inhibition against *T. rubrum* at 12.6±3.5 mm. However, all extracts did not inhibit the Gram-negative bacteria, *E. coli*, *P. aeruginosa,* and yeast (*C. albicans*) (Table 8). This study was consistent with previous report that the extracts from *Penicillium* species also have the antibacterial potency on Gram positive bacteria including griseofulvin from *P. brasilianum* (Tang et al. 2015) and scleroderolide from *Penicillium* sp. FO-5637 (Tomoda et al. 1998). The result suggested that the extracts were potent in killing Gram-positive bacteria. Nevertheless, the extracts from other *Penicillium* species such as *P. Janthinellum* (Do Rosário Marinho et al. 2005) and *P. herquei* (Marinho et al. 2013) possessed bactericidal activity on Gram negative bacteria.

TABLE 8. Anti-microbial activity of *P. maximae*

Organisms	Zone of inhibition (Diameter, mm)					
	Standard drug*	CE**	BE**	BW**	CM**	CH**
P. aeruginosa	32.7±0.7	0	0	0	0	0
E. coli	23.1±0.1	0	0	0	0	0
S. aureus	22.0±1.8	0	8.1±0.1	0	0	0
S. epidermis	30.6±0.1	0	0	0	0	0
MRSA	21.3±1.0	0	0	0	0	0
P. acnes	25.4±1.1	0	0	0	0	0
C. albicans	13.5±0.4	0	0	0	0	0
T. mentagrophytes	42.4±1.5	0	6.4±0.5	0	0	0
T. rubrum	50.3±2.1	0	11.2±2.6	0	12.6 ±3.5	0
M. gypseum	27.0±1.0	0	0	0	0	0

* Standard drugs:
Norfloxacin : *P. aeruginosa*, *E. coli* 10 µg/disc
Oxacillin : *S. aureus*, *S. epidermidis* and *P. acnes* 1 µg/disc
Vancomycin : MRSA 30 µg/disc
Amphotericin B : *C. albicans* 25 µg/disc
** Concentration at 200 mg/mL
CE: cell ethyl acetate extract; BE: broth ethyl acetate extract; BW: broth water extract; CM: cell methanol extract; CH: cell hexane extract

CONCLUSION

Fifty-four soil fungi were collected from Sirindhorn peat swamp forest in Narathiwat Province, Thailand and 104 crude extracts were obtained from them. SPSF224 was selected for further study because of the potential effects on biological activities. SPSF224 was identified by molecular method as *P. maximae*. From phytochemical investigation, its crude extracts contained a variety of

secondary metabolites including tannins, alkaloids, flavonoids, coumarins and anthraquinones. *P. maximae* extracts also showed high potential effect on alpha-glucosidase inhibition and moderate potential effect on antimicrobial, anti-inflammation, and antioxidant activities. This study was the first report of chemical investigation and biological activities of the isolated fungus, *P. maximae*. Hence, this information could be used as database for further study of the lead compound for antidiabetic drug from fungi in the future.

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