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Antioxidants and Phytochemical Analysis of Endophytic Fungi Isolated from a Medicinal Plant *Catharanthus roseus*

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Abstract: Four endophytic fungi have been tested for antioxidant properties using different assays; DPPH radical-scavenging activity, ferric reducing antioxidant power (FRAP) and ferrous ion chelating activity (FCA). The test of polyphenolic content also has been done for both total phenolic content (TPC) and total flavonoid content (TFC). There was no result on the half maximal concentration (IC_{50}) for both DPPH and FCA assays for all fungi. However, through FRAP assays the results were ranged from 0.336±0.01 to 0.477±0.11 mmol Fe^{2+}/g extract where *N. sphaerica* had the highest result. This fungus also showed the highest results on TPC and TFC, which were 0.030±0.000 (mg GAE/g) and 0.038±0.001 (mg QE/g) respectively.

Keywords: Filamentous fungi, crude fungal extract, DPPH, TPC, TFC.

INTRODUCTION

Antioxidant is defined as a compound that can delay, inhibit or prevent the antioxidant of oxidizable materials through scavenging free radicals and diminishing oxidative stress [1]. This is supported by Halliwell *et al*. [2] from their report that antioxidant is any substances that present antitumour at low concentration compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate. The mechanism of this antioxidant compound is by associating with decreased DNA damage; diminished lipid peroxidation maintained immune functions and inhibited malignant transformation of cells [3]. Antioxidant properties can be determined by using a few methods such as DPPH scavenging activities, ferric reducing antioxidant power and ferrous chelating activities. However, DPPH scavenging activity is the most popular method to determine the antioxidant properties since it is a simple method [4].

DPPH is a stable and nitrogen – centred free radical which produces violet colour in ethanol solution [5] with a chemical compound of $C_{18}H_{12}N_5O_6$. According to Huang *et al.* [4], by being a stable free radical, the DPPH assay is used to evaluate the ability of antioxidant to scavenge free radicals. It gives reliable evidence concerning the antioxidant ability of the tested compounds to act as free radical scavengers or hydrogen donors. It is supported by Yamagushi *et al.* [6] when DPPH free radicals become paired with hydrogen from a free radical scavenging antioxidant, its purple color fades rapidly to yellow to form reduced DPPH-H. Hence, it was reduced to a yellow coloured produced with the addition of the fractions in a concentration [5].

Thus, the studies on antioxidant activities are important for pharmaceutical industries. Part of that, the major groups of phytochemicals that have been suggested as a natural source of antioxidant may contribute to the total antioxidant activity of plant material, including polyphenols, carotenoid and traditional antioxidant vitamins such as vitamin C and E [7]. Phenolic is a compound possessing one or more aromatic rings with one or more hydroxyl groups [1] and has been considered as a powerful antioxidant in vitro and proved to be more potent antioxidant than vitamin C, vitamin E and carotenoids [8] where natural phenolic can affect basic cell functions that related cancer development by many different mechanisms.

The mechanisms of phenolic compounds for antioxidant activities are neutralizing lipid free radicals and preventing decompositions of hydro peroxides into free radicals due to the presence of hydroxyl groups [5]. Thus, this study was about determination of antioxidant properties of endophytic fungi from *C. roseus* plant and phytochemical analysis of these endophytic fungi.

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MATERIALS AND METHODS

Samples preparation

All fungi strains used in this project were previously isolated and identified by Ayob and Simarani [9]. The crude fungal extract has been prepared [10] and divided into crude mycelia extract (CME) and crude supernatant extract (CSE). Briefly, the cell free filtrate was extracted with a dichloromethane (3x200 ml) and evaporated to dryness using rotary evaporator. The extracted sample was weighed to constitute the CSE. Meanwhile, in order to prepared the CME, the mycelia biomass has been freeze-dried and extracted twice with a mixture of dichloromethane:methanol (1:1, v/v) for 1 h.

Determination of polyphenolics content in the endophytic fungi

1. Total phenolic contents

Total phenol content (TPC) was measured by the Folin-Ciocalteu method [11]. Briefly, 20 µL of crude fungal extract (CFE) was mixed with 100 µL of Folin-Ciocalteu reagent (diluted 10-fold with distilled water) in a 96-well microplate and incubated for 5min before added with 75 µL of sodium carbonate solution (75 g/L). After 2 h incubated in darkness at room temperature, the absorbance was measured at 740 nm with a microplate reader (Tecan Sunrise, Austria). Tannic acid (100 – 1000 µM) was used as a standard for calibration and construction of a linear regression line and water was used as a blank. The total phenolic content was estimated as mg tannic acid equivalent (mg TAE)/g of dry extract.

2. Total flavonoid contents

Total flavonoid content (TFC) was measured according to [12, 13]. Briefly, 50 µL of crude fungal extract (CFE) were added with 70 µL of distilled water and 15 µL of sodium nitrite solution (5 %) in a 96-well microplate. The solutions were well mixed and incubated at room temperature for 5 min. Then, 15 µL of aluminium chloride solution (10 %) was added into the mixture and incubated for 6 min. Then, 100 µL of 1 M sodium hydroxide solution was added and the absorbance was measured at 510 nm with a microplate reader (Tecan Sunrise, Austria). The total flavonoid content (TFC) was estimated from quercetin (200 – 1000 µM) standard curve and the results were expressed as mg quercetin equivalent (mg QE)/g of dry extract.

Antioxidant activity assays DPPH radical scavenging activity

The free radical scavenging activity of crude fungal extract (CFE) was measured in terms of hydrogen donating ability by using DPPH radical according to Margithas *et al*. [14] and Ablat *et al.* [13]. Briefly, 40 µL of crude fungal extracts with different concentrations (0.05 – 2 mg/mL) were mixed with 200 µL of 50 µM DPPH solution in methanol. Then, the mixture was immediately shaken before incubated in the dark at room temperature for 15 min. The absorbance was measured at 517 nm with a micro plate reader (Tecan Sunrise, Austria). Butylated hydroxyanisole (BHA) with concentrations of 5 – 80 µg/mL was used as a standard while the control was ethanol. The percentage of inhibition activity of the extracts was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [(A_1 - A_2) \times 100] / A_1$$

Where ,

A_1 = Absorbance of control
A_2 = Absorbance of sample

The DPPH radical scavenging activity was estimated from the graph plotted against the percentage inhibition and compared with the standard in order to find the Half Maximal Inhibitory Concentration (IC_{50}).

Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) activities of crude fungal extracts were measured according to Muller et al. [11]. Briefly, 20 µL of crude fungal extract (CFE) in methanol were mixed with 200 µL of daily prepared ferric reducing antioxidant power (FRAP) reagent contained 5 mL of 10 mM tripyridyl triazine (TPTZ) in 40 mM hydrogen chloride (HCl), 5 mL of 20 mM iron (III) chloride ($FeCl_3$), and 50 mL of 0.3 M acetate buffer; pH 3.6 in 96-well microplate. The formation of the TPTZ-Fe^{2+} complex in the presence of antioxidant compounds in the extract was measured at 595 nm with a microplate reader (Tecan Sunrise, Austria) after incubating for 8 min. Ferrous sulphate ($FeSO_4$) solution (0.2 – 1 mm) was used for a standard calibration curve while the methanol was used as blank. The ferric reducing antioxidant power (FRAP) value was evaluated according to the linear regression between standard solutions and

absorbance at 595 nm and the results were estimated as mmol Fe^{2+}/g of dry extract from triplicated tests.

Metal chelating activity

Metal chelating activity or ferrous ion chelating activity (FCA) of the CFE was determined by measuring the formation of the ferrozine (Fe^{2+}) complex, according to the procedure described by Decker and Welch [15]. 10 µL of extracts with different concentrations (50 – 800 µg/mL) were mixed with 120 µL of distilled water and 10 µL of 2 mM iron (II) chloride ($FeCl_2$) in a 96-well microplate. 20 µL of 5 mM ferrozine was added to the mixture in order to initiate the reaction. The reaction mixture was incubated for 20 min at room temperature and was measured at 562 nm along with EDTA-Na_2 (5 – 80 µg/mL) as a standard metal chelator. 20 µL of distilled water was used as a blank while 100 µL of methanol was used as a control. The percentage of inhibition of ferrozine (Fe^{2+}) complex formation was calculated according to the following formula:

$$\text{Ferrous ion chelating activity (\%)} = [(A_1 - A_2) \times 100] / A_1$$

Where,

A_1 = Absorbance of control
A_2 = Absorbance of sample

The metal chelating activity was estimated from the graph plotted against the percentage inhibition and compared with the standard in order to find the Half Maximal Inhibitory Concentration (IC_{50}).

Statistical analysis

All data were analysed by using analysis of variance (IBM SPSS Statistics, Version 20; IBM Corp., Armonk, New York) to determine the significant studies on antioxidant and polyphenolics compounds. The regression among variables was showed in surface plot graph.

RESULTS AND DISCUSSION

Total phenolic contents (TPC) of the crude fungal extract (CFE) were tested using Folin-Ciocalteu's reagent and expressed as Gallic acid equivalent (y = 0.0042x + 0.0196, r2 = 0.9996). While total flavonoid contents (TFC) were measured based on the equivalent of quercetin (y = 0.0676x + 0.0142, r2 = 0.9997). The results showed that crude supernatant extract (CSE) of *N. spherica* had a higher value of both total phenolic contents (TPC) and total flavonoid contents (TFC), which were 0.030 mg GAE/g and 0.038 ± 0.001 QE/g respectively (Table 1). The lowest total phenolic contents (TPC) and total flavonoid contents (TFC) were crude mycelia extract (CME) of *N. sphaerica* (0.023 mg GAE/g) and CFE of *C. gloeosporioides* (0.022 ± 0.001 QE/g) respectively.

According to Huang *et al.* [16], main natural products of secondary metabolites in plant and fungi were phenolic compounds where phenol and flavonoid compounds have been reported to possess different bioactivities. The antioxidant activity of crude fungal extracts was determined by using a methanol solution of DPPH reagent, ferric reducing antioxidant power (FRAP) and metal chelating activity (FCA). The percentage of DPPH radical scavenging activity ranged from 9.03 % to 17.15 % and 0.26 % to 9.03 % of mycelia and supernatant samples respectively (Table 2). However, there was no data for half maximal inhibitory concentration (IC_{50}) due to low scavenging activity.

The ferric reducing antioxidant power assay was measured based on the reaction between Fe^{3+}-TPTZ and antioxidant potentials to form Fe^{2+}-TPTZ in the fungi extract. The highest results showed that crude supernatant extract (CSE) of *N. sphaerica* produced 0.477±0.11 mmol Fe^{2+}/g of extract while the lowest result was crude mycelia extract (CME) of *C. gloeosporioides* produced 0.336±0.01 mmol Fe^{2+}/g of fungi extract (Table 3).

The metal chelating (FCA) activity was measured based on the chelation of ferrous ion with ferrozine and formed ferrous-ferrozine complex. The fungi extract were prepared in different concentrations (50-800 µg/mL) (Table 4 and Figure 1). Through ferric reducing antioxidant assay, it showed that crude supernatant extract (CSE) of *N. sphaerica* has the highest result 0.477±0.11 mmol Fe^{2+}/g of extract while crude mycelia extract (CME) of *C. gloeosporioides* has the lowest result 0.336±0.01 mmol Fe^{2+}/g of extract (Table 5).

Table 1. The polyphenolics content of crude fungal extracts

Samples	TPC (mg GAE/g)	TFC (mg QE/g)
CG (CME)	0.024±0.001^{f}	0.022±0.002^{f}
CG (CSE)	0.028±0.002^{c}	0.022±0.001^{f}
MP (CME)	0.024±0.001^{f}	0.034±0.001^{b}
MP (CSE)	0.027±0.001^{d}	0.023±0.001^{e}
NS (CME)	0.023±0.000^{g}	0.025±0.002^{d}
NS (CSE)	0.030±0.000^{a}	0.038±0.001^{a}
FS (CME)	0.025±0.001^{e}	0.029±0.003^{c}
FS (CSE)	0.029±0.001^{b}	0.023±0.001^{e}

Values presented are the means ± SD, (n = 3). Values within the same column and having different superscript letters are significantly different ($p < 0.05$). CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica*, FS: *F. solani*, TPC: total phenolic contents, TFC: total flavonoid contents, CME: crude mycelia extract and CSE: crude supernatant extract

Table 2. The DPPH radical- scavenging activity of crude fungal extracts

Concentration (µg/mL)	DPPH radical – scavenging activity (%)									
	C. gloeosporioides		*M. phaseolina*		*N. sphaerica*		*F. solani*		VB	VC
	CME	CSE	CME	CSE	CME	CSE	CME	CSE		
50	20.73± 0.15^{b}	8.94± 0.77^{f}	26.49± 0.15^{a}	3.97± 0.26^{b}	9.52± 0.69^{e}	0.26± 0.02^{h}	10.75± 0.90^{d}	4.46± 0.38^{g}	17.57± 0.83^{c}	10.17± 0.86^{d}
100	4.99± 0.42^{d}	9.37± 0.80^{b}	16.98± 0.74^{a}	9.44± 0.74^{b}	9.03± 0.65^{b}	0.26± 0.02^{e}	9.86± 0.83^{b}	8.42± 0.72^{c}	9.73± 0.78^{b}	8.47± 0.71^{c}
150	9.19± 0.75^{f}	7.76± 0.67^{h}	20.82± 0.20^{b}	10.12± 0.79^{e}	16.80± 0.64^{d}	9.03± 0.40^{f}	10.14± 0.85^{e}	8.29± 0.71^{g}	24.89± 0.39^{a}	18.76± 0.81^{c}
200	15.75± 0.67^{e}	7.84± 0.67^{h}	19.22± 0.84^{c}	8.91± 0.69^{g}	17.15± 0.64^{d}	0.26± 0.02^{i}	9.73± 0.81^{f}	7.68± 0.66^{h}	29.05± 0.23^{a}	24.06± 0.42^{b}
250	8.83± 0.72^{g}	13.39± 0.59^{d}	25.53± 0.08^{a}	11.21± 0.45^{e}	10.29± 0.76^{f}	0.26± 0.02^{h}	15.85± 0.71^{g}	8.16± 0.70^{h}	24.98± 0.36^{b}	24.22± 0.44^{b}

Values presented are the means ± SD, (n = 3). Values within the same row and having different superscript letters are significantly different ($p < 0.05$).CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica*, FS: *F. solani*, VB: Vinblastine, VC: Vincristine, CME: crude mycelia extract, CSE: crude supernatant extract and BHA: Butylatedhydroxyanisole

Table 3. Ferric reducing antioxidant power (FRAP) of crude fungal extracts

Samples	Ferric reducing antioxidant power (mmol Fe^{2+}/g extract)
CG (CME)	0.336±0.01^{g}
CG (CSE)	0.400±0.04^{d}
MP (CME)	0.357±0.01^{e}
MP (CSE)	0.423±0.04^{c}
NS (CME)	0.347±0.01^{f}
NS (CSE)	0.477±0.11^{a}
FS (CME)	0.340±0.01^{f}
FS (CSE)	0.437±0.11^{b}
VB	0.103±0.01^{h}
VC	0.097±0.01^{i}
$FeSO_4$	0.595±0.02^{a}

Values presented are the means ± SD, (n = 3). Values within the same raw and having different superscript letters are significantly different ($p < 0.05$). CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica*, FS: *F. solani*, VB: Vinblastine, VC: Vincristine, CME: crude mycelia extract, CSE: crude supernatant extract and $FeSO_4$: Ferrous sulphate

Table 4. Metal chelating activity (FCA) of crude fungal extracts

Samples	Metal chelating activity (%)				
	Concentration (µg/mL)				
	50	100	200	400	800
CG (CME)	20.48±0.87^{h}	17.92±0.63^{h}	27.55±0.78^{f}	18.01±0.64^{g}	16.53±0.50^{i}
CG (CSE)	38.86±0.05^{d}	37.85±0.14^{c}	39.16±0.01^{b}	37.07±0.06^{c}	37.78±0.13^{c}
MP (CME)	20.76±0.50^{h}	18.65±0.73^{g}	12.33±0.13^{i}	18.47±0.72^{g}	21.51±0.55^{h}
MP (CSE)	24.44±0.59^{a}	18.96±0.70^{g}	18.33±0.64^{g}	18.67±0.67^{g}	23.61±0.58^{g}
NS (CME)	37.25±0.10^{e}	30.80±0.44^{d}	32.16±0.21^{d}	28.87±0.63^{e}	27.08±0.75^{e}
NS (CSE)	34.42±0.22^{f}	23.34±0.66^{e}	30.64±0.36^{e}	29.58±0.29^{d}	26.66±0.78^{f}
FS (CME)	39.72±0.15^{c}	30.59±0.75^{d}	38.31±0.12^{c}	29.06±0.88^{d}	32.44±0.50^{d}
FS (CSE)	24.41±0.66^{g}	19.06±0.79^{f}	15.78±0.28^{h}	25.79±0.49^{f}	26.26±0.47^{f}
VB	95.97±0.03^{a}	94.38±0.03^{b}	93.18±0.05^{a}	91.02±0.04^{b}	89.55±0.02^{b}
VC	97.50±0.03^{b}	95.27±0.00^{a}	93.91±0.01^{a}	92.52±0.05^{a}	92.41±0.05^{a}
EDTA-Na_2	98.29±0.11^{a}	93.71±0.05^{c}	88.11±0.15^{b}	83.31±0.09^{c}	77.59±0.02^{c}

Values presented are the means ± SD, (n = 3). Values within the same row and having different superscript letters are significantly different ($p < 0.05$). CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica*, FS: *F. solani*, VB: Vinblastine, VC: Vincristine, CME: crude mycelia extract and CSE: crude supernatant extract

Table 5: Antioxidant and phytochemical analysis of crude fungal extracts

Samples	DPPH (IC_{50} µg/mL)	FRAP (mmol Fe^{2+}/g extract)	FCA (IC_{50} µg/mL)	TPC (mg GAE/g)	TFC (mg QE/g)
CG (CME)	ND	0.336±0.01^{g}	ND	0.024±0.001^{f}	0.022±0.002^{f}
CG (CSE)	ND	0.400±0.04^{d}	ND	0.028±0.002^{c}	0.022±0.001^{f}
MP (CME)	ND	0.357±0.01^{e}	ND	0.024±0.001^{f}	0.034±0.001^{b}
MP (CSE)	ND	0.423±0.04^{c}	ND	0.027±0.001^{d}	0.023±0.001^{e}
NS (CME)	ND	0.347±0.01^{f}	ND	0.023±0.000^{g}	0.025±0.002^{d}
NS (CSE)	ND	0.477±0.11^{a}	ND	0.030±0.000^{a}	0.038±0.001^{a}
FS (CME)	ND	0.340±0.01^{f}	ND	0.025±0.001^{e}	0.029±0.003^{c}
FS (CSE)	ND	0.437±0.11^{b}	ND	0.029±0.001^{b}	0.023±0.001^{e}
VB	ND	0.103±0.01^{h}	22.00^{a}	-	-
VC	ND	0.097±0.01^{i}	22.00^{a}	-	-

Data are mean ± SD (n = 3). Values within the same row and having different superscript letters are significantly different ($p > 0.05$). DPPH: diphenyl-1-picrylhydrazyl, FRAP: ferric reducing antioxidant power, FCA: ferrous ion chelating activity, TPC: total phenolic content, TFC: total flavonoid content, ND: not detected, CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica*, FS: *F. solani*, VB: Vinblastine, VC: Vincristine, CME: crude mycelia extract and CSE: crude supernatant extract.

Table 6. The ANOVA of antioxidant and polyphenolic content on endophytic fungi from *C. roseus*

	Regression			
	Sum of Squares	**Mean Square**	**F**	**Sig.**
Fungi	0.000	0.000	0.011	0.919
Fungi*CFE	0.006	0.003	0.089	0.915
Fungi*CFE*Test	0.532	0.177	18.637	0.000

	Correlation			
	Sum of Squares	**Pearson Correlation**	**Sig. (1-tailed)**	**Sig. (2-tailed)**
Fungi	0.102	0.022	0.459	0.919
CFE	0.186	0.089	0.339	0.679
Test	-2.901	-0.853	0.000**	0.000**

Fungi (CG, MP, NS, FS); CFE (CME, CSE); Test (Antioxidant, TPC, TFC)
**correlation was significant at the 0.01 levels.

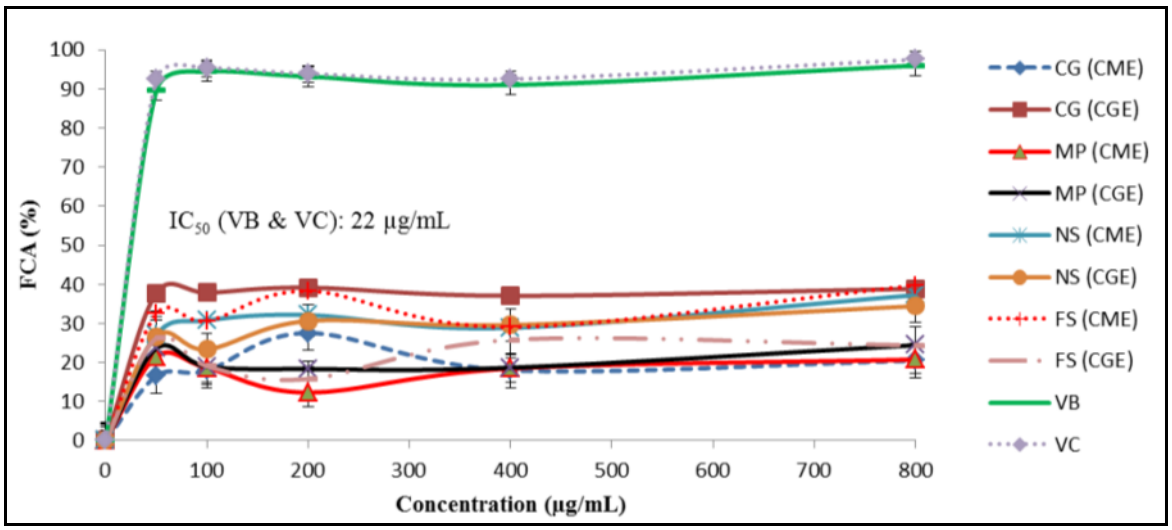

Figure 1. The antioxidant assay of crude fungal extracts from *C. roseus* plant by using ferrous ion chelating activity (FCA) at 562 nm. CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica*, FS: *F. solani*, VB: Vinblastine, VC: Vincristine, CME: crude mycelia extract and CSE: crude supernatant extract

This analysis also revealed that crude supernatant extract (CSE) of *N. sphaerica* has the highest properties for both antioxidant and polyphenolic; total phenolic content (TPC) and total flavonoid content (TFC). According to Ablat et al. [13], the extract containing the high value of total phenolic content and total flavonoid content showed the highest reducing power activity. The ferric reducing ability correlated well with total phenolic content (r2 = 0.9996), total flavonoid content (r2 = 0.997) contents in the fraction and this was supported by Velioglr et al. [17] from their report that the antioxidant activity of plant material is very well correlated with the content of phenolic compounds. The most significant studies on antioxidant and polyphenolics compounds were determined using an analysis of variance (ANOVA). Table 6 showed the regression of fungi*crude fungal extract*test was significantly different. Besides, the correlation among test was also significant at the 0.01 level.

CONCLUSION

N. sphaerica isolated from a medicinal plant *C. roseus* has potential as an antioxidant agent due to the significant result on both antioxidant and polyphenolic properties compared to the other species. Since there was no result on IC_{50} of DPPH radical – scavenging activity and IC_{50} of ferrous ion chelating activity, the data on ferric reducing antioxidant power was used as a comparison to a polyphenolic property. From the analysis, all CSE showed the highest result on the antioxidant properties through ferric reducing antioxidant power

(FRAP) assay compared to crude mycelia extract (CME) of each endophytic fungi and this also applied to the total phenolic content (TPC) analysis. Thus, as a summary, the crude supernatant extract (CSE) samples were more productive on antioxidant and polyphenolic assays. Meanwhile, *N. sphaerica* has a great potential as the antioxidant agents and also could produce the polyphenolic compounds.

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