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Research Article
Endophytic Fungi from Surian (*Toona sinensis* Roem) and Antioxidant Potency from its Culture

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Abstract

This study aims to isolate and identify endophytic fungi from tissues of stem wood at different age of *Toona sinensis* and test the antioxidant activity produced from the culture of these fungi. Twenty five isolates were obtained and identified into 7 types of fungi classes; compromising of *Trichoderma* sp., *Aspergillus* sp., *Rhizopus* sp., *Cladosporium* sp., *Penicillium* sp., *Alternata* sp. and *Chepalosporium* sp. The fungus were then cultured into 100 mL of PDB medium, agitated at 150 rpm, at 28°C for 1 week. Cultures containing antioxidant compound were extracted using n-hexane and ethyl acetate and the antioxidant activity was determined by DPPH method. The ethyl acetate extracts of culture filtrate has higher antioxidant activity comparing than n hexane extracts. Among the 25 isolates, the antioxidant activity of n-hexane extract of culture *Penicillium* sp., number 1, isolated from 5 years surian branch had the greatest DPPH radical scavenger of 27.79% and the lowest one was 22.29% obtained from *Cladosporium* sp. and *Trichoderma* sp., number 8. Whereas antioxidants compound extracted by ethyl acetate of *Rhizopus* sp., number 2, from 5 months stem shoot had the greatest DPPH radical scavenger of 81.01% and the lowest one is from isolate *Aspergillus* sp., number 1 from white branch shoot of 5 months which had an activity of 68.72%. This result indicated that surian have many kind of endophytic fungus and some of are potential for producing antioxidant compound.

Key words: Endophytic fungi, identification, isolation, *Toona sinensis*, antioxidant

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

One of the alternative ways of obtaining antioxidants compounds is to extract it from endophytic fungi in plants. Several studies support this idea and show that many endophytic fungi produced antioxidant compound from their culture (Visalakchi and Muthumary, 2010; Pimentel *et al*., 2011; Sadananda *et al*., 2011; Zhao *et al*., 2014; Govindappa *et al*., 2011). Usually, many researchs of bioactive compound were carried out from the plant itself, so that the research on the activities of endophytic fungi on plants is still uncommon. This includes the minimum amount of research of endophytic fungi that has been done on Surian tree (*T. sinensis*), which is a wide-spread in Indonesia (Sun *et al*., 2013).

It is generally known that leaves of surian produced some bioactive compound that functioned as antioxidant such as gallic acid and its derivatives, gallotannins and flavonol glycosides (Cheng *et al*., 2009; Hseu *et al*., 2008), also pestasin and isopestasin that have molecular structure which resembles of flavonoid (Radji, 2005).

Endophytic fungi are defined as a type of fungi that can live and make a colony inside the tissue of plants, at a certain period of their life, without causing any harm in host that able to produce biological compound that similar or same as the host, as a result of genetic recombination with its host. Every high level plant may contain a number of endophytic fungus. Endophytes inhabit higher plants which are considered to be a reservoir of a novel metabolites that potential for medicinal, agricultural and or industrial exploitation (Strobel and Deasy, 2003; Rodriguez *et al*., 2009). Therefore, the research of isolation, identification continued by assaying antioxidant activities were carried out. The aims of this research were identification of the endophytic fungal of surian and knowing the potensity for producing antioxidant compound from the fungal culture.

MATERIALS AND METHODS

Materials: All endophytic fungus were isolated from stem of *T. sinensis* specimens collected in Rancakalong, Sumedang, West Java. Indonesia. All endophytic fungus were isolated from three varieties of age, like 5 months representing young age, 5 years and 7 years representing late young age as well as 10 years and 12 years representing middle age, represent different stages of surian life.

Methods

Isolation of endophytic fungi from surian plant: The isolation of endophytic fungi was performed as (Zeng *et al*., 2011). Briefly, after rinsing with distilled water, all samples were surface-disinfected and then sequentially washed with 75% ethanol (1 min), 2.5% sodium hypochlorite (15 min) and sterilized water (three times). The leaves were torn off the samples, then placed on Potato Dextrose Agar (PDA) medium supplemented with antibiotics (200 μg mL^{-1} chlorampenicol) and incubated at 28±1°C for about one week. Aliquots of 1.0 mL of the last wash were also inoculated in PDA to evaluate the effectiveness of the disinfection process. The mycelium originating from the tear of the sample was purified and cultured under the same conditions.

Cultivation and metabolite extraction of endophytic fungi: The test fungus from agar culture (3 mm at diameter) were grown in 250 mL erlenmeyer flasks containing 100 mL of PDB medium. The test fungus were inoculated and incubated for 1 week at shaker 150 rpm and 26±2°C. After incubation, the culture filtrate was filtered through Whatman paper No. 2 to remove mycelia and then extracted with one equal volumes of n-hexane and ethyl acetate solvent. The organic phase was collected and the solvent was then removed by evaporation under reduced pressure at 45°C using rotary vacuum evaporator. The dry solid residue was re-dissolved in methanol and the crude extract was evaluated for their antioxidant activity (Sadananda *et al*., 2011).

Physiological or phenotypic identification of endophytic fungi: Physiological or phenotypic identification of endophytic fungi was performed by observing the macroscopic as well as microscopic characteristics of the isolates and also by referring to taxonomic standards. Macroscopic observations were done to differentiate the isolates based on the characteristics of their colony, mycelium and hyphae. While, microscopic observations were performed to characterize hyphae and spore morphologies of the isolates (Ravindran *et al*., 2012).

Antioxidant activity (DPPH radical scavenging activity) test of endophytic fungi metabolites: The free radical scavenging activities of extracts were measured by using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH). Briefly, extract concentration of (0.1 mg mL^{-1}) endophytic crude extract dissolved in methanol (75 μL) was mixed with 250 μL of methanolic solution containing 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma) radicals obtained from fresh DPPH solution was prepared by mixing 24 mg of DPPH in 100 mL methanol and storing it at 20°C prior of use. 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. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging (\%)} = \frac{\text{A0-A1}}{\text{A0}} \times 100$$

where, A0 is the absorbance of the control reaction, A1 is absorbance in the presence of the sample.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi from *T. sinensis*: Isolation continued by identification of endophytic fungi from surian tree in several different level of age (5 month until 12 years old) resulted data that was displayed in Table 1. The total amount of endophytic fungi that was obtained from this research were 25 isolates from 7 genus.

Analysis of endophytic fungi isolate color and the color of its metabolites: There are two kinds of colour appeared from the isolate, one is from the secondary metabolite (state by symbol*) of the fungi and the other one is from the fungal itself (no symbol). The observation showed that some isolates had different colour of metabolite from the fungi mass, the other one is the metabolite has same color as the mass and the other one the metabolite is colorless. The components within the metabolites (including the antioxidants) were most likely to affect or gave a difference to the overall color of the metabolite (Sadananda *et al*., 2011).

Analysis of colony and microscopic observation of endophytic fungi: Figure 1 shows seven different genus microscopic data of that were obtained by wet preparation glasses and macroscopic data of fungal colonies on PDA plates. The identification of endophytic fungal were carried out using literature from the book "Laboratory Manual of Microbiology" (Cappuccino, 2008).

Observation on *Trichoderma* sp., that was growing on PDA plates showes that at the initial growth of the fungi, it produce white micellium that developed to green color during the maturation stage, but still covering by white missellium. After full development, the entire mycelium became green (Ismail and Tenrirawe, 2013). Microscopic analysis, showed that *Trichoderma* sp. isolates were single celled, had a well-defined branched conidiophore, did not form a bundle, had an ellipse-shaped conidia with a green-blue color and were in a small terminal group (Ismail and Tenrirawe, 2013).

Based on the lable there are 11 isolates of *Trichoderma* sp., most of which were isolated from surian tree at the age of 10 year. *Aspergillus* sp., was found on isolate F2 white from surian branch of 5 months (*Aspergillus* sp., No. 1) and F2 light green from surian branch of 5 years (*Aspergillus* sp., No. 2). The colony of *Aspergillus* sp., was characterized with an initial color of white, then it turned a blue green color and this was followed by a black or brown color, indicating mature culture. Based on microscopic observations, single-celled spores (conidia) could be seen forming a chain. The end of the sterigma could be seen developing from the terminal ball of the conidiophore. Vesicles and a long conidiophore were also observed emerging from the septate mycelium.

Moreover, the fungi *Rhizopus* sp., was found on four of the isolates dominating the early years of a surian tree. Colony observations of *Rhizopus* sp., showed a white biomass with an unclear texture similar to that of cotton wool. From microscopic viewings, oval-shaped spores could be seen being either brown or colorless. The mycelium was not septate, but formed straight sporangiophores which had black sporangiums at the ends, each containing columella. The hyphae had a structure similar to roots, which was also known as rhizoids.

The fungi *Cladosporium* sp., were present in four of the isolates ranging from early to middle ages of surian tree. Colony observations of *Cladosporium* sp., showed that these fungi had small green-black colored colonies that were stacked together and had a powdery texture. From the

Table 1: Endophytic fungus obtained from *T. sinensis* in different age

Age	Tissue	Name and type of fungi	Name of fungal isolates
5 month	Branch	F1 green	*Trichoderma* sp., No.1
		F2 white	*Aspergillus* sp., No.1
		F3 yellow	*Rhizopus* sp., No.1
		F4 yellow-green	*Cladosporium* sp., No. 1
5 month	Stem	F1 green	*Rhizopus* sp., No 2
		F2 yellow	*Rhizopus* sp., No. 3
		F3 red*	*Rhizopus* sp., No. 4*
5 month	Seed	F1 white*	*Cladosporium* sp., No 2*
5 years	Stem	F1 green	*Trichoderma* sp., No.2
		F2 black	*Cladosporium* sp., No. 3
5 years	Branch	F1 yellow*	*Penicillium* sp., No. 1*
		F2 light green	*Aspergillus* sp., No.2
7 years	Stem	F1 green	*Penicillium* sp., No. 2
		F2 white	*Trichoderma* sp., No. 3
10 years	Stem	F1 green	*Trichoderma* sp., No. 4
		F2 white	*Trichoderma* sp., No. 5
		F3 red*	*Trichoderma* sp., No. 6*
		F4 yellow	*Trichoderma* sp., No. 7
		F5 light green	*Trichoderma* sp., No. 8
		F6 light yellow	*Trichoderma* sp., No. 9
		F7 brown*	*Trichoderma* sp., No. 10*
12 years	Stem	F1 green	*Trichoderma* sp., No. 11
		F2 yellow*	*Alternata* sp., No. 1*
		F3 red*	*Chepalosporium* sp., No.1*
		F4 white*	*Cladosporium* sp., No.4*

*Isolates that displayed colored metabolites

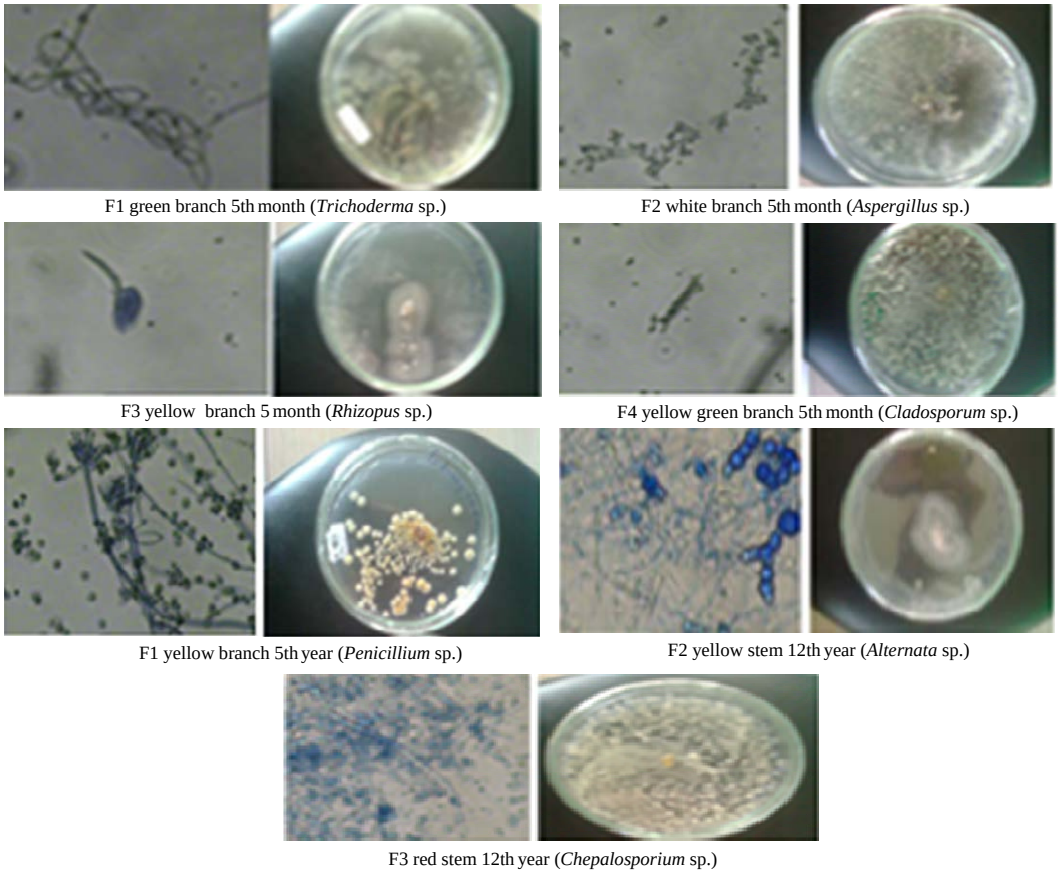

Fig. 1: Morphological observations of fungi isolates on agar plates (petri dish) as well as microscopic observations on wet preparation glasses with a zoom of 20×10 on a computer microscope, F1: Fungi No. 1, Right: Fungi colony in petri dish in PDA medium, Left: Microscope picture

microscopic observations, *Cladosporium* sp., had spores (conidia) at the ends of conidiophore complexes, which emerged from the brown-colored septate mycelium.

Furthermore, the fungi *Penicillium* sp., was found on isolate F1 yellow from surian branch of 5 years (*Penicillium* sp., No. 1) and F1 green from surian stem of 7 years (*Penicillium* sp., No. 2). These isolates were characterized with green or blue-green colored colonies. Macroscopic observations showed that *Penicillium* sp., had single-celled spores that formed a chain at the end the sterigma, which had emerged from the metula of the conidiophore. The conidiophore itself arose from the septate mycelium.

The fungi *Alternata* sp., was only found on one isolate, F2 yellow from surian branch of 12 years (*Alternata* sp., No. 1). This isolate produced greyish-green or black colonies, with grey edges that quickly occupied the Petri dish. Based on microscopic observations, pear-shaped multicellular spores were seen adhering to single conidiophores, emerging from the septate mycelium.

Lastly, the isolate F3 red from surian branch of 12 years was identified as *Chepalosporium* sp., No 1. This fungus was characterized with a moist, compact colony, similar to that of cotton wool. The aerial hyphae was either grey or red in color. From microscopic observations, single celled spores (conidia) with a shape of a cone or an ellipse were seen in groups at the end of the conidiophore. A slimy substance was also seen on the conidiophore which branched upright from the septate mycelium.

Endophytic fungi are distinctly distributed throughout the organs and tissues of plants and are associated with various plant structures, such as leaves, branches, stems, roots, shoots.

This research was different from the research that found two endophytes (*Aureobasidium* sp. and *Rhodotorula* sp.) were detected from our *T. sinensis* from leaves of *T. sinensis* in (Korea) (Sun *et al*., 2013).

Research in medicinal plant *Kadsura angustifolia* resulted the abundance, richness and species composition of endophytic asemblages were significantly dependent on

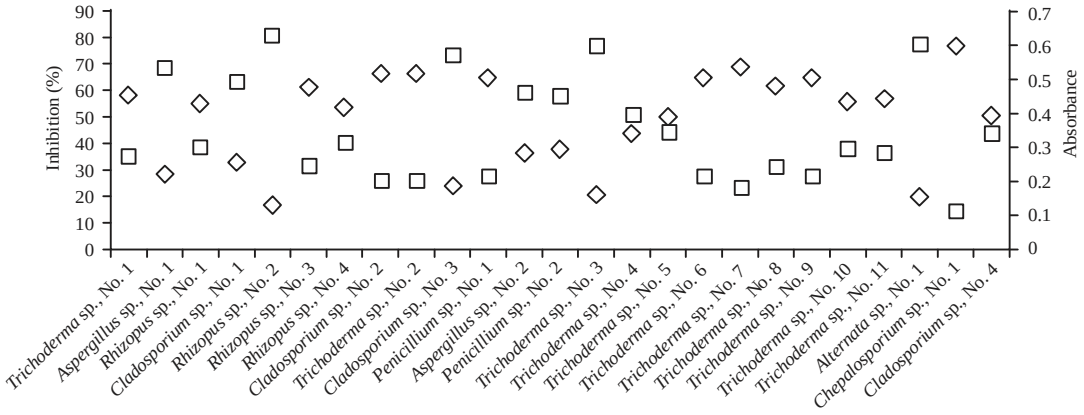

Fig. 2: Antioxidant activity of n-hexane extract culture of endophytic fungi from *T. sinensis* in different age

Table 2: Five isolates having the highest antioxidant activity (extracted with n-hexane)

Code	Sample fungi	Tissues	Name of isolate	Inhibition (%)
K	F1 yellow	Branch of 5 years	*Penicillium* sp., No. 1	27.79
Y	F4 white	Stem of 12 years	*Cladosporium* sp., No. 4	23.91
U	F7 brown	Stem of 10 years	*Trichoderma* sp., No. 10	23.48
X	F3 red	Stem of 12 years	*Chepalosporium* sp., No. 1	23.29
D	F4 yellow green	Branch of 5 months (Shoot)	*Cladosporium* sp., No. 1	22.29
S	F5 light green	Stem of 10 years	*Trichoderma* sp., No. 8	22.29

the tissue and the sampling site. From 134 morphospecies selected, 39 showed remarkable biocatalytic activity and were further identified as species belonging to the genera Colletotrichum, Eupenicillium, Fusarium, Hypoxylon, Penicillium, Phomopsis, Trametes, Trichoderma, Umbelopsis, Verticillium and Xylaria (Huang *et al*., 2015).

Research on Glycine max obtained that fungi *Ampelomyces* sp., *Cladosporium cladosporioides, Colletotrichum gloeosporioides, Diaporthe helianthi, Guignardia mangiferae* and *Phoma* sp., were more frequently isolated from the leaves, whereas the fungi *Fusarium oxysporum, Fusarium solani* and *Fusarium* sp. were prevalent in the roots. Greater richness was found in the leave but dominant index was higher in root (Fernandes *et al*., 2015).

A total of 97 endophytic fungal isolates were obtained from roots (35), stems (49) and leaves (13) of *B. napus*. *Alternaria alternata* is the dominant species accounting for 12.4% of the isolates. Several fungal genera including Acremonium, Alternaria, Aspergillus, Chaetomium, Epicoccum, Fusarium, Nigrospora, Penicillium and Rhizoctonia were isolated in this study (Zhang *et al*., 2014). Research of endophytic fungi in *Oficus indica* resulted that Cladosporium, the most frequent species in this study, with a frequency of 36.42% (Bezerra *et al*., 2012).

Antioxidant activity test: Antioxidant activity test was carried out for crude extract of endophytic fungi culture using n-hexane and ethyl acetate. Two different solvent (n-hexane and ethyl acetate) were used in this research to investigate which solvent were effective for extracting bioactive compounds from the endophytic culture of surian.

Antioxidant activity test (n-hexane extracted): Based of the antioxidant assay that was carried out for the n-hexane extract from the culture of endophytic fungus isolated from surian tree, prosentase of DPPH radical inhibition can be seen at Fig. 2.

The graph above shows the result of the antioxidant test of the 25 fungi isolates extracted with n-hexane. Based on the graph, the five isolates having the highest antioxidant activity are listed below.

Furthermore, based on Fig. 2 a correlation could be seen in that as the absorbance decreases, the percent (%) of inhibition increases. A decrease in the absorbance is caused by the activity of antioxidant that scavenge the radicals of DPPH. The free radical DPPH (1, 1-diphenyl-2-picrylhidrazyl) is a purple-colored which can be converted into 1, 1-diphenyl-2-picrylhidrazine in the presence of antioxidants and will alternatively cause the change of the color from purple to a reddish purple and even yellow, depending on the capability of the antioxidants (Apak *et al*., 2007). From the results on Fig. 2 and Table 2, it can be resolved that isolate F1 yellow from Surian branch shoot of 5 months (*Penicillium* sp., No. 1) produced antioxidant compound with the maximum inhibition towards the free radicals. This was indicated by a

color change of DPPH, from purple to a reddish purple. The change in DPPH color signified that the antioxidants produced by *Penicillium* sp., No. 1 were able to obstruct free radical activities of DPPH (Ohtani *et al*., 2000). Fungi isolates that had the second, third, fourth and fifth best antioxidant activity were isolate F4 white from surian stem of 12 years (*Cladosporium* sp., No. 4), isolate F7 brown from surian stem of 10 years (*Trichoderma* sp., No. 10), isolate F3 red from surian stem of 12 years (*Chepalosporium* sp., No. 1), as well as F4 yellow green from surian branch shoot of 5 months (*Cladosporium* sp., No. 1) and F5 light green from surian stem of 10 years (*Trichoderma* sp., No. 8), respectively. The antioxidant activity of *Penicillium* sp., No. 1 and other fungi isolates extracted with n-hexane were all below 30%. This could have been caused by the low specificity of n-hexane, to bind and extract antioxidants compounds present in the fungi isolates. Nevertheless, other solvents could be used such as ethyl acetate which has been known to effectively extract antioxidants from endophytic fungi (Sadananda *et al*., 2011).

Antioxidant activity test (extracted with ethylacetate): Ethylacetate extract of endophytic culture isolated from surian tree resulted inhibition of DPPH radical , that can be seen in Fig. 3.

The graph above shows the result of the antioxidant test of the 25 fungi isolates extracted with ethylacetate. Based on the graph, the five isolates having the highest antioxidant activity are listed below.

Based on the results in Fig. 3 and Table 3, it can be concluded that isolate F1 green from surian branch shoot of 5 months (*Rhizopus* sp., No. 2) produced the highest amount of antioxidant and displayed the maximum inhibition towards the free radicals. This was indicated by a color change of DPPH, from purple to yellow. Fungi isolates that had the second, third, fourth and fifth best antioxidant activity were isolate F2 yellow from surian stem shoot of 12 years (*Alternata* sp., No. 1), isolate F2 white from surian stem of 7 years (*Trichoderma* sp., No. 3), isolate F2 black from Surian stem of 5 years (*Cladosporium* sp., No. 3) and isolate F2 white from Surian branch shoot of 5 months (*Aspergillus* sp., No. 1), respectively. The antioxidant activity of *Rhizopus* sp., No 2 and the other fungi isolates extracted with ethyl acetate were all above 30%. This indicated that ethyl-acetate was a better solvent, in that more antioxidants from the fungi were able to be extracted (Sadananda *et al*., 2011). The test results of this research indicate that the use of endophytic fungi as a source of antioxidants can be beneficial in the food and medical industry.

Endophytic fungus *Aspergillus* sp., from *Trigonella foenum-graecum* seeds demonstrated the highest both total phenolic content and antioxidant activity for 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay (Khiralla *et al*., 2015). The the methanol endophytic extracts of *Tabebuia argentea* contain more antioxidant activities than the chloroform extract. Phytochemical analysis revealed the presence of tannins, flavonoids, steroids, alkaloids, phenols

Table 3: The five isolates having the highest antioxidant activity (ethylacetate extract)

Code	Sample fungi	Tissues	Name of isolate	Inhibition (%)
E	F1 green	Stem of 5 months (Shoot)	*Rhizopus* sp., No. 2	81.01
W	F2 yellow	Stem of 12 years	*Alternata* sp., No. 1	77.57
N	F2 white	Stem of 7 years	*Trichoderma* sp., No. 3	77.04
J	F2 black	Stem of 5 years	*Cladosporium* sp., No. 3	73.60
B	F2 white	Branch of 5 months (Shoot)	*Aspergillus* sp., No. 1	68.72

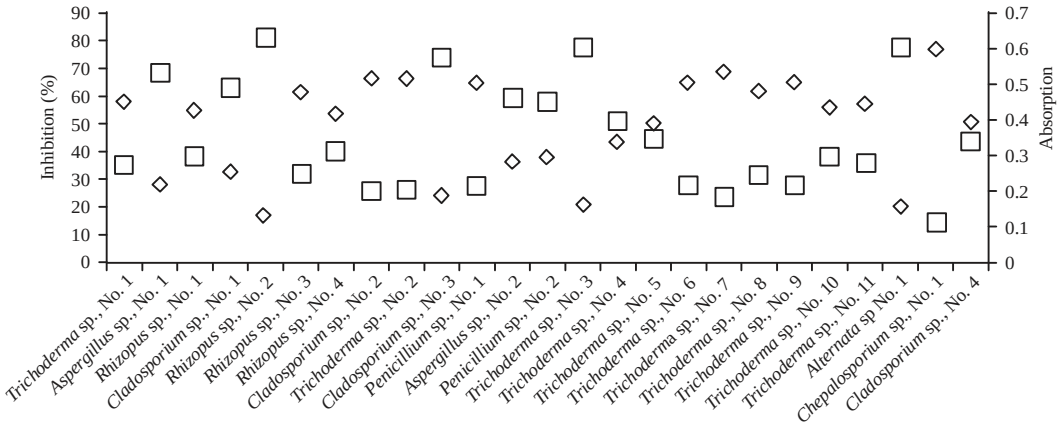

Fig. 3: Antioxidant aactivity of ethylacetate extract culture of endophytic fungi from *T. sinensis* in different age

and proteins from different solvents extracts of different endophytes.endophytes *Aspergillus niger* and *Penicillium* sp., are an important source of phenol compounds, which are a good source of antioxidant activity (Govindappa *et al*., 2013). The ethanol extracts of culture Endophytic fungi from *Crotalaria pallida*, which belongs to the Fabaceae family (Subfamily Faboideae), the members of which are herbs, shrubs and trees found in both temperate and tropical areas *Aspergillus niger* and *Fusarium oxysporum* showed potent antioxidant activity against ABTS, FRAP and DPPH radicals (Govindappa *et al*., 2011).

CONCLUSION

Based on this research, it can be concluded that, the diversity of endophytic fungi observed in *T. sinensis* were quite high. Twenty seven isolates from 7 genus such as Trichoderma, Aspergillus, Rhizopus, Cladosporium, Alternata. and Chepalosporium were dispersed in every part of the organ of surian in every age of the plant. *Trichoderma* sp. is the most frequent isolate that was found in 10 years old stem and another organ like branch and stem in 5 month, 5, 7 and 12 years old. Isolation and screening of endophytic fungi from *T. sinensis* resulted some fungus which could produce metabolites containing compounds with potent antioxidant capacity as it can be used as a potential source of natural antioxidant and bioactive compounds. Extraction endophytic culture with ethyl acetate resulted high antioxidant activity that was showed by DPPH radical scavenging by 81.01% by *Rhizopus* sp., although n-hexane extraction only resulted inhibition of 27.79% B.

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