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Antioxidant Potential of Ethanol and Ethyl Acetat Extract of *Ganoderma* sp. Mycelium

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Abstract

Ganoderma sp. Banyumas 1 isolate that reffered as *Ganoderma* sp. is a new discovered isolate from Banyumas, Central Java, Indonesia expected to have a potential properties of antioxidant of medicinal mushroom. This study aimed to determine the antioxidant potential and the appropriate solvent for it's extracting from *Ganoderma* sp. This research result showed that ethyl acetate was able to extract as many as 15.57%, while etanol was only able to extract 3.87% active compounds from dried 28 days old *Ganoderma* sp. mycelium cultivated in the Mushroom Complete Medium (MCM). Extract of ethyl acetate (non-polar) extraction of mycelium of *Ganoderma* sp. had a potential character as an antioxidant source and performed a better result than from ethanolic (polar) extraction as shown in the IC50 value. Extract from ethyl acetate extraction had an average IC50 value smaller than from ethanolic extract (581.80 < 1285.67). Extract from ethyl acetate extraction resulted in a higher amount of phenol than that ethanolic extract 29.23 < 57.67. Inhibition percentage of both extracts at 65% was known to occur at concentration of 1000 ppm for ethyl acetate extract and 2000 ppm for ethanolic extract. An important finding was that ethyl acetate can be used as appropriate solvent for extracting antioxidant compound better than ethanolic. In conclusion, the mycelium extract of *Ganoderma* sp. extracted with ethyl acetate and ethanol as solvent is potential to be used as a source of natural antioxidants. This research result has benefit in developing potency of local resources as herbal resources.

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INTRODUCTION

Ganoderma sp. isolate is a local isolate from Banyumas, Central Java isolated by the researchers. Since a long time ago, it has been placed in the top rank of medicinal mushrooms and so called as "king of herbal medicine" (Jaelani, 2008). Modern pharmacological studies showed that *Ganoderma* sp. could be used in healing several diseases. Previous study by Senggoro (2005) was investigating the activity and bioactive compound from *Ganoderma* sp. mycelium grown on solid medium containing cellulose from palm fruit. Some mushrooms are known to contain medicinal compounds and so called as medicinal mushrooms e.g. *Ganoderma lucidum*. This mushroom is known to be potentially used as a medicament especially its basidiospores, mycelium and fruiting body.

According to Yue *et al.*, (2008), *G. lucidum* has been used as a traditional medicine in China for more than 2.000 years. Wachtel-Galor *et al.*, (2004) stated if *G. lucidum* could maintain the consumer's health. Analysis of *Ganoderma lucidum* by Stamets (2000) both qualitatively and quantitatively reported that *Ganoderma lucidum* contain different bioactive compounds which depend on its growth medium and conditions. Different growth media attracts the mushrooms to produce different amount of bioactive compounds (Stamets, 2000). The mycelium of *Ganoderma* sp. isolate is extracted using a maceration method applying ethanolic and ethyl acetate solvents. Solvents were chosen based on components of the samples.

Antioxidant activity can be identified using either polar solvents or non-polar solvents such as alkaloids and triterpenoids (Zazouli *et al.*, 2016). Information of source of antioxidant and its effective solvent is essential. However, studies of antioxidant compounds from fungi are still very limited, especially the extraction of antioxidant compounds from dry filtrate culture of *Ganoderma* sp. which isolated with different solvents. Recent research by Ekowati *et al.* (2017) studied different macro fungi which was *Pleurotus ostreatus* extract and its potential as anti-cervical cancer cells, not it's antioxidant potential. *Ganoderma* sp. originating from several different cultivation locations is thought to have different abilities to produce bioactive compounds as the result of biosynthetic processes. Its ability is either influenced by genes or affected by physical and chemical environment factors. Examining antioxidant ability may lead to the discovery of superior strains. It is necessary to assess the potency of each strain to produce bioactive compounds that can be used as an antioxidant. It is also important to examine the most appropriate solvent in extracting dried isolate of *Ganoderma* sp. Current research aimed to study potential of *Ganoderma* sp. B1 isolate in producing antioxidants and to determine the solvent with the highest amount of antioxidant. This research was expected to give some scientific information about active compounds of *Ganoderma* sp. isolate especially the antioxidants content and the possibility of the use of *Ganoderma* sp. isolate as an antioxidant source.

METHODS

This research applied an experimental quantitative descriptive. Potential of antioxidant components obtained from *Ganoderma* sp. Banyumas 1 isolate determined using DPPH test, based on the observed color changes using spectrophotometer. It was assumed that the change in colour was due to the ability of mycelium extract of the mushroom in trapping the free radicals. Data of colour change were then analyzed by checking the IC50 value, followed by drawing a graph of percentage of the DPPH trapped by the mycelium. Total phenolic compounds, flavonoids, and terpenoid were also examined. In order to know the differences of those two groups, a statistical analysis was applied using an independent sample T-test. Two variables called dependent (antioxidant capacity) and independent (type of solvents) were noted as secondary parameters. Main parameter were total phenolic compounds and test of antioxidant capacity applying the DPPH. Data obtained were then drawn as curve of relations of antioxidant capacity contained with free radicals trapped in the extract. Supporting parameters were yields of mycelium extract of *Ganoderma* sp. and its flavonoids and terpenoids contents measurement.

Preparation of mycelium extract of *Ganoderma* sp.

Dried mycelia were macerated using ethanol at 1 : 9 ratio for 1 x 24 hours. Extraction and sieving were done in triplicate to get first, second and third levels of extract using Whatman paper no. 41. First and second level which contain ethanolic extracts were then mixed, sieved and placed in a vacum pump then being evaporated. Similar steps were done to the extract which contain ethyl acetate.

Test of Antioxidant Capacity Using a DPPH (Sheikh *et al.*, 2009), Total Phenolic Compounds (Matanjun *et al.,* 2008), Flavonoid Content

and Terpenoid Compounds. The test of antioxidant capacity using DPPH was done by dissolving solid extract in methanol at 125, 250, 500, 1000, and 2000 ppm concentrations. As many as 2ml of each extract concentration was mixed in 2ml DPPH 0,16 mM in methanol, vortexed for 1 minute and left for 30 minutes then measured for their absorbance in a spectrophotomer at 517 nm wavelength. Control was divided into two; negative control wth DPPH and methanol as much as 4 ml volume and positive control which is solution of negative control with addition of α tocopherol. Reduction in absorbance that represented increase ability of solution in trapping the free radical DPPH was calculated using formula applying a regression equation in precentage. Meanwhile, the test of total phenolic compounds was done by preparing a standard solution by weighing 0.025 g tannic acid in 25 ml methanol, then sequentially dissolved for the concentration of: 0 ppm, 25 ppm, 50 ppm, 75 ppm, 100 ppm, and 125 ppm. Two ml of each concentration was then placed in reaction flasks containing 5 ml free ionic water and 0.5 ml reagent Folin ciocalteau (10x). Solution was then vortexed and added with 1 ml Na_2CO_3 5% and re-vortexed. Absorbance was measured at 750 nm wavelength.

Data of samples analysis were taken from 1 ml of each concentration that was then added by 5 ml free ionic water and 0.5 ml Folin-ciocalteau (10 x dissolving). The tested solutions then were vortexed and left for 1 minute and added by 1ml Na_2CO_3 5% solution, vortexed and left for another 30 minutes in a dark room for absorbance measurement under 750 nm wavelength. Then the test of flavonoid content was done by adding 1 ml extract by some drops of strong H_2SO_4 to change its color. The present of flavonoid component was represented by color change into orange or cream. The test of terpenoid compounds was done by dissolving 1 ml extract in 10 ml chloroform and added with 10 drops of acetic acid anhydride and 3 drops of strong H_2SO_4 and left for several minutes. Positive reaction was represented by the change of solution's color into soft red.

Analisys Method

Data from several tests were then analyzed using SPSS statistical program while quantitative data were analyzed by an independent T-test. Qualitative data from both flavonoid, and terpenoid tests were described by comparing the data taken from baseline and after being treated or even between the treatments.

RESULTS AND DISCUSSION

Regenerated mycelium of *Ganoderma* sp. isolate were grown in an MCM (Mushroom Complete Medium) for 28 days on an orbital shaker and then weighed. As much as 12.79 g constant dry weight of 319.07 g fresh weight was prepared for ethanolic extract. Meanwhile, fresh weight for mycelium prepared for ethyl acetic extraction was 753.41 g and reached a constant weight at 28.83 g. Dried mycelium of each preparation was grinded and extracted by macerating in two different solvents to get the crude extract weight as well as its yield of mycelium extract. Weight of crude extract derived from ethanol solvent was 1.99 g with 16.69% of mycelium extract, while crude extract derived from ethyl acetate solvent was 0.79 g with 3.87% mycelium extract. A statistical analysis using an independent T test ($T_{calculated}$: 3.259 > T_{table}: 2.776) showed that the two solvents producing different amount of extract (Table 1). Percentage of yield of mycelium extract of ethanolic extraction was higher than that of ethyl acetic which might be because the component contained in the mycelium of *Ganoderma* sp. isolate have more polar characteristics than non-polar ones (Figure 1).

Septiana and Asnani (2013) stated that selection for solvent for extraction process have to be based on it's compounds polarity. Agarwal *et al.* (2012) reported that bioactive compounds in the mycelium of *Ganoderma lucidum* were polysaccharides (β-D-glucans, heteropolysaccharides and glycoprotein) which were dissolved in water- and triterpenoid. Gowrie *et al.* (2014) reported that *Ganoderma lucidum* contains alkaloids, carbohydrate, saponin, protein, amino acids, phytosterols, fats, triterpenoid, flavonoid, phenolic compounds and tannin which might be used as antiinflammation, antibacteria, anticancer and antioxidants. Padmasari *et al.* (2013) stated that saponin, flavonoid, oils, alcaloids, tannin and glycosides were dissolved in ethanol solvent.

Table 1. Fresh weight, dry weight, crude extract weight and yield of *Ganoderma* sp. mycelium extract

Extraction solvents	Weight (g)	Yield of Mycelium Extract (%)
Ethanol	1.99	16.69 a*
Ethyl Acetic	0.79	3.7 b*

*different letters showed a significant difference

Figure 1. Colony of *Ganoderma* sp. Banyumas 1 isolate on Potato Dextrose Agar, 15 days at room temperature (27-29°C), reverse white to brownish.

Phytochemistry

Phenolic compounds play a significant role in attacking free radicals. Kinsella et al, (1993) stated that this compound can be used as antioxidants due to its ability to attack free radicals and peroxides radicals leads to slowing down lipid oxidations. Flavonoid is one among those phenolic compounds contained in some plants and mushrooms. Flavonoids are the most common compound in plant's tissues and being produced as secondary metabolites (Redha, 2010). Pietta (2000) reviewed flavonoids as antioxidant and assesed the antioxidant capacity in relation with their chemical structures critically. Owing to the unique characteristics of flavonoids, they are likely to be radical scavengers, reducing agents, hydrogen donors, singlet oxygen quenchers and/or metal chelators to reduce the amount of free radcal in the body. In order to know the flavonoids content of the mycelium of *Ganoderma* sp. isolate, a further test in form of qualitative test was conducted (Table 2).

The table shows that mycelium of *Ganoderma* sp. isolate extracted by both ethanol and ethyl acetate contains flavonoids and terpenoids as represented by the color change. However, the change of the color noted in this study was categorized as weak because both solvents have a closed-fraction in polarity. Dielectric constant of ethanol is 24.5 and ethyl acetate is 6.0 which means that both solvents are able to extract both polar and non-polar compounds from its substrates. Buchari & Sulaeman (2003) stated that constant dielectric is one among those parameters used to judge the polarity level of a particular solvent, the higher dielectric constant means the more polarity.

Table 2. Phytochemistry test of mycelium of *Ganoderma* sp. isolate

Qualitative test			Remarks
Extract	Ethanol	Ethyl acetate	
Flavonoid	+	+	Orange or cream
Terpenoid	+	+	Pink

Remarks: - : absent, + : weak, ++ : strong, +++ : very strong

Qualitative test of this study showed that type of solvent did not affect flavonoid and terpenoid compounds which extracted. This might be because both compounds have a specific structure which has both polar and non-polar character at almost balance amount. Terpenoids for example contain both polar and non-polar characters in which the amount of non-polar is higher than that of polar ones. The total extract resulted from non-polar solvent tend to be more soluble than polar solvent. Terpenoid which was dissolved in polar solvent might be in form of globule with a polar character in its outer part (Septiana & Asnani, 2012). Ganoderic acid is a bioactive compound which derived from lanosterol of *Ganoderma lucidum* fruiting body and was reported to have pharmacologic activity in form of triterpenoids (Trigos & Medellin, 2011). Figure 2 shows the structure of flavonoid and ganoderic acid:

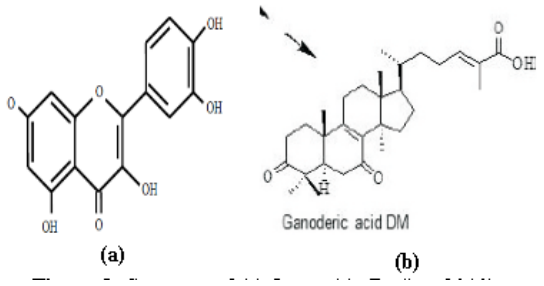

Figure 2. Structure of (a) flavonoid (Redha, 2010); (b) ganoderic acid (Trigos and Medellin, 2011).

Total Phenol

Analysis of total phenol was done as *Ciocalteau* method by measuring total phenolic compound quantitatively. Table 3 shows the analysis results of total phenolic compounds of mycelium of *Ganoderma* sp. mycelium extracted with ethanol and ethyl acetate.

Table 3. Results of total phenolic compounds contain in mycelium of *Ganoderma* sp. mycelium analysis by ethanol and ethyl acetate.

Total phenol (mg/g)	
Ethanolic extraction	29.23 a
Ethyl acetate extraction	57.67 a

*different letters show a significant difference.

A statistical analysis applying independent sample T test showed if average value of total phenolic compound resulted from both solvents did not significantly different ($T_{calculation}$ = -12.960 < T_{table} = 2.776). The result showed that ethyl acetate extraction produces 57 mg/g phenol -27.77 mg/g higher amount of total phenolic compound- rather than ethanolic extraction which produces 29.23 mg/g phenol. Mushrooms are known to produce phenolic compounds as secondary metabolites in form of polyketides, terpenes and steroid, and also ascorbic acids, flavone, beta carotene and lycopene which can be used to count total phenolic compound of the mushrooms (Phunita & Rajasekaran, 2014).

Phenol is a chemical compound characterized by its aromatic ring with one or more hydroxyl groups. Phenol which found in the food might be divided into two namely simple phenol and folic acids (P-cresol, 3-ethyl phenol, 3.4-diethyl phenol, hydroxyquinone, vanillin, and gallic acid), derivate of hydroxy cinnamic acid (p-coumarate, caffeic, phenolic acid and chlorogenic acid) and flavonoids (catechin, proanthocyanin, anti-cyanidin, flavone, flavanol and glycosides). Phenolic compounds are able to slowing down the rate of lipid oxidation by donating hydrogen atom to free radicals (Widiyanti, 2006).

Current study results were parallel to those resulted by Mau *et al.* (2002) who reported that fruiting body of several medical mushrooms contained total phenolic compounds as follows: *Coriolus versicolor* (23.28 mg/g), *G. lucidum* (47.25 mg/g), *G. tsugae* (51.28 mg/g), and *G. applanatum* (55.96 mg/g). Applying different solvent to extract bioactive compound of *Schizophyllum commune,* reported if extraction using ethyl acetate produced higher amount of total phenol than extracted with dichloromethanol or water which are known as polar solvents. Phenolic compound contains in mycelium of *Ganoderma* tend to be dissolved in non-polar solvents. It might be because its phenolic compound, in structure, it does not belong to simple phenol. Phenol structure varies from simple phenolic acid with on 1 ring), biphenyl and flavonoids which have 2 or more phenolic rings (Vattem *et al.*, 2005). The simple phenol contains in Ganoderma are *catechol* dan *hydroquinon* (Castellano *et al.*, 2012).

Capacity of trapping DPPH radicals

DPPH has widely used as a component to test chemical compound characterized by their ability of trapping free radicals or as hydrogen atom donor. Electron contained in DPPH radicals could be absorbed maximumly under 517 nm wavelenght by producing violet color (Septiana and Asnani, 2013). Antioxidant compound would donate proton to the DPPH radicals to slowing down its ability in absorbing the light and change the color from violet to yellow (Kalyoncu et al, 2010). Average percentage of *Ganoderma* sp. mycelium extract ability on DPPH radicals blocking (Table 4).

Table 4. Blockage of the DPPH radicals by mycelium extracted by ethanol and ethyl acetate

Concentration	Ethyl acetate (%)	Ethanol (%)
125	22.60	8.32
250	42.68	16.56
500	61.09	30.75
1000	65.02	52.60
2000	63.55	66.14

It can be concluded that extract which derived from ethyl acetate extraction produce better blocking activity than ethanolic extraction. At 125 ppm extract of ethyl acetate shows a blocking score of 22.60% in compared with 8.32% due to the present of extract from ethanolic extraction. Both extract, however, show a gradual increase in blocking when concentration is also increased and keep increasing up to a certain concentration (Figure 3).

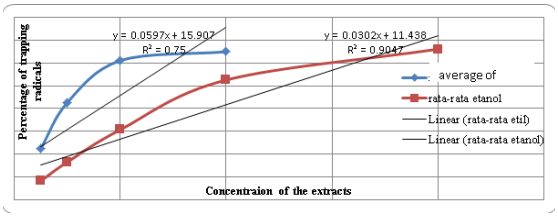

Figure 3. Graph of blocking percentage of the DPPH radicals by both extracts

Figure 3. shows if blocking percentage DPPH radicals by ethanol extract was significantly increased and reached its maximum level at 66.14% at concentration of 2.000 ppm. On the other hand, the highest blocking level of ethyl acetate was 65.02% reached at the concentration of 1.000 ppm but at a higher concentration (2000 ppm) its blocking ability was gradually decrease. Tocopherol which was treated as a positive control reached a constant increase in blocking activity. Dewi and Murtini (2007) added that antioxidant at higher concentration caused a higher activity but at a particular level its antioxidant activity would decrease. Extract of ethyl acetate extraction showed a better activity in blocking DPPH radicals than extract from ethanol extraction (Table 5).

Table 5. Analysis of extract of ethanol and ethyl acetate extraction of *Ganoderma* sp. isolate on blocking DPPH radicals

Average value of IC_{50} (ppm)	Extract from ethyl acetate extraction	Extract from ethanol extraction	control (α tocopherol)
	581.80 a	1285.67 a	1.37×10^{-5}

*different letters show significant differences

It might be then concluded if (H0) was accepted which means that mycelium of *Ganoderma* sp. isolate has potential character to be used as source of natural antioxidant.

A statistical analysis applying an independent sample T-test show that the average level of IC50 of both solvents did not significantly different ($T_{calculation} = 1.94 < T_{table} = 2.77$). The average level of IC50 of ethyl acetate extraction was still higher (581.80 ppm) or (0.58 mg/ml) while the extract from ethanolic extraction had 1285.67 ppm or 1.28 mg/ml. α tocopherol which was used as a positive control in blocking the DPPH radicals, showed the highest score on blocking the DPPH radicals with average of 1.3×10^{-5} ppm, but this level is still lower than that one shown by extract of mycelium of *Ganoderma* sp. isolate extracted by both ethanol and ethyl acetate. It might because of varies mechanisms of antioxidants in blocking the DPPH radicals. The DPPH method is a methodology which represent the number of antioxidant by releasing its hydrogen atoms to the DPPH in order to stabilized it.

Winarsi (2007) stated that vitamin E or α tocopherol is an hydrogen ion donor which able to change peroxyl radicals to become active tocopherol radicals. In this way, α tocopherol is able to disrupt the fatty acids chain. The main difference between positive control and extract was mainly due to the extract content of *Ganoderma* sp. mycelium that might have a different mechanism to block free radicals. Delouee and Urooj (2007) explained that a particular compound can be functioned as antioxidant by donating its proton to the free radicals, chelating metals, slowing down lipid peroxidation or blocking a single oxygen.

This study result was similar to previous result by Gowrie *et al.* (2014) about extraction of *Ganoderma lucidum* fruiting body using chloroform, ethanol and methanol. Percentage on absorption of the DPPH radicals by extract from chloroform extraction was 89.71%, ethanol 64.72% and methanol 74.05%. This result mean that the extract derived from extraction using non-polar solvents had a higher absorption on the DPPH radicals than those of polar solvents. Who had applied water, ethyl acetate, and dichloromethane to extract *Schizophyllum commune* found that the IC50 value of those solvents were as follow: ethyl acetate (0.219 mg/ml) < dichloromethane (0.641 mg/ml) < water (0.674 mg/ml). The IC50 value of extract from ethyl extraction, which is non-polar, was the lowest in compared with either dichloromethane or water. IC50 value is a particular number to represent the concentration of a particular extract (ppm) in blocking oxidation process by 50%. The smaller IC50 value means the higher antioxidant activity, and specifically it is grouped as follow: very strong antioxidant (less than 50 ppm), strong (50-100 ppm), mediocre (100-150 ppm) and weak (151-200 ppm) (Zuhra *et al.*, 2008). Ling *et al.* (2013) stated that the higher total phenolic compound then the higher ability in blocking free radicals.

Current study showed that the IC50 value of the extract from ethanolic extraction had a correlation with total phenol present in the extract which is represented by a regression correlation value of R2 = 0.840. However, IC50 value of extract from ethyl acetate extraction did not correlate with its total number of phenol in where the regression correlation is only R2 = 0.394. It is indicated that other compound that has ability as phenol was present, thus IC50 value of ethyl acetate extract was reduced. Trigos and Medellin (2011) stated if antioxidant activity of *Ganoderma lucidum* is infected by bioactive compounds contained in its mycelium, spores, or fruiting body namely triterpenoid and polysaccharides. There are more than 140 triterpenoid in *Ganoderma lucidum* with more than 200 polysaccharides. Frac-

tion of non-polar extract of *Ganoderma lucidum* contains more than 130 isolated triterpenoids, some of lanosterol derivate and showed a pharmacological activity and as antioxidant or anticancer, like ganoderic acids, ganoderiols, ganolucidic acids, lucidones and lucidenic acids. This result show that mycelium extract of *Ganoderma* sp. from ethyl acetate extraction had better antioxidant activity than from ethanolic extraction, due to its compound that non polar and had a better performance in blocking DPPH radicals. This research result has benefit in developing potency of local resources as herbal resources.

CONCLUSION

Based on the result and discussion before, it can be concluded that mycelium extract of Ganoderma sp. is having potential of antioxidant as shown by average precentage on blocking DPPH free radicals of both solvents that reached 65%. The extract from ethyl acetate extraction (non-polar) of *Ganoderma* sp. mycelium isolate had a potential character as antioxidant source better than the extract from ethanolic extraction as shown in the IC50 value of both types of extracts. Extract from ethyl acetate extraction had an average IC50 value smaller than ethanolic extract 581.80 < 1285.67. Extract from ethyl acetate extraction resulted in higher amount of phenol than that ethanolic extract 29.23 < 57.67.

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