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Antioxidant Effect of *Centella asiatica* Ethanolic Extract to Superoxide Dismutase (SOD) Level on *Cyprinus carpio* Liver

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KEYWORDS

Centella asiatica Linn;
Antioxidant;
DPPH;
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Abstract *Centella asiatica* is a herbal medicine has been used for many studies in some animals as antioxidant properties. This study was aimed to enhance the level of SOD in *Cyprinus carpio* after oral administration of C. *asiatica* extract. In the present study, phytochemical screening of bioactive constituents, antioxidant assay, and their effect on SOD in *C. carpio* liver were investigated. *C. asiatica* extract was obtained by maseration for 24 h using ethanol as a solvent. *C. asiatica* extract with 50, 100, 150 and 300 mg kg^{-1} body weight were administered every 3 days for two weeks. On the last day of experiment, fishes were killed and liver were removed from the body. The results showed that C. *asiatica* extract had some bioactive compounds such as flavonoid, alkaloid, terpenoid, tanin, saponin and DPPH scavenging activity with an IC_{50} value of 125 µg mL^{-1}. C. *asiatica* extract also enhanced the level of SOD in fish liver. The SOD level was significantly difference compared to the control group with P<005. These results indicate that the ethanolic extract of C. *asiatica* had a potential antioxidant properties by scavenging free radical and enhancing the SOD level as antioxidant deffense inside the body.

Introduction

Free radicals can caused damage to cells and tissues organs such as the liver, kidneys, and heart in both humans and animals. This damage can caused cell death and various degenerative diseases (Kregel and Zhang, 2006). Liver is a vital organ and had many important functions, including hepatotoxic metabolism and detoxification. This organ is the target of free radical concentration enhancement (Mohamed, 2009). Several factors including endogenous factors such as by-product of metabolic process or exposure that enters through respiratory can spread throughout the body. Environmental factors also trigerred free radicals such as cigarette smoke, pollutants, UV light, radiation and xenobiotics (Young and Woodside, 2001). Antioxidants can reduce and prevent cell damage by neutralizing free radicals before these radicals attacked cells so can prevent damage to lipid membrane, proteins, enzymes, carbohydrates, and DNA (Fang *et al.*, 2002). Nowadays, antioxidant properties from natural sources are believed to be safer for health than synthetic antioxidants. Some plants has been reported to have potential as a source of natural antioxidants, such as bran extract (Arab *et al.*, 2011), ethanol extract of serai leaves (Hasim *et al.*, 2015) and dragon fruit (Cho and

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Yong, 2011). Compounds that have antioxidant activities such as vitamins C and E, carotenoids (carotene and xanthophyll), and polyphenols (flavonoids, phenolic acids, lignans and stilbenes) can fulfill the antioxidant needs in the body (Oroian and Escriche, 2015).

The other plants also had a antioxidant properties is *Centella asiatica*. *C. asiatica* has been used for medicine in the recent years. This vine had purplish or pink white flowers. These flowers bloom during April to June. The fruit is rectangular and round. This plant develops in the shady, moist and wet such as paddy fields and river side (Singh *et al.*, 2010). *Centella asiatica* can be found throughout the tropics and sub-tropics areas, such as Southeast Asia, India, Sri Lanka, parts of China, Madagascar, South Africa, Southern United States, Mexico, Venezuela, and Columbia (Ravi *et al.*, 2008). C. *asiatica* contains some bioactive consituents, such as alkaloid, saponin, fenol, triterpenoid, and flavonoid. Phenol, triterpenoid, and flavonoid are known for their antioxidant properties (Chippada and Vangalapati, 2011). This plant also has triterpenes, such as asiaticoside, madecassoside, asiatic acid, and madecassic acid that has been known for their hepatoprotective effect (Zhao *et al.*, 2014). *C. asiatica* was reported to have free radicals scavenging activity (Hashim *et al.*, 2011) and anti-lipid peroxidative activity caused by free radicals (Katare and Ganachari, 2001). Oral administration of C. *asiatica* extract also been reported as an anti tumor by Babu and Paddikkala (1994). According to Upadhyay *et al.* (2002), *Centella asiatica* contains brahmicacid, isobrahmic acid, brahminoside and brahmoside. It has psychotropic properties, sedatives and anticonvulsants. It is also useful in dementia, mental and anxiety disorders and increased memory, concentration in children with disabilities. Furthermore, Wang *et al.* (2003) isolated pectin from *Centella asiatica* which showed immunostimulant activity. The methanol extract of *Centella Asiatica* also shows immunomodulatory effects (Jayathirtha and Mirsha, 2004). In previous study, C.*asiatica* water extract contained flavonoid (0.361 g / 100 g) showed free radical scavenging activity with IC_{50} value of 31.25 µg mL^{-1} (Pittella *et al.*, 2009).

Antioxidant effect of C. *asiatica* can be replaced the antibiotic treatments for prevention of fish diseases. Animals have an antioxidant defense mechanism to neutralize ROS, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), as well as non-enzymatic antioxidants, glutathione (GSH) (Scandalios, 2005). Superoxide dismutase (SOD) is a primary antioxidant enzyme as preventive defense system in organism caused by free radicals (Valko *et al.*, 2007). By-product of metabolic process and exposure that enters the body can caused free radical formation. Free radicals damaged cellular macromolecules, such as carbohydrates, proteins, lipids and nucleic acids. Therefore, SOD can effectively eliminate free radical in various tissues and reduce the damage. SOD catalyzed the conversion of superoxide anions (O_2^-) to hydrogen peroxide (H_2O_2). Thus, the bioactive constituents of C. *asiatica* as secondary antioxidant expected to break the chain reaction of free radicals and can increase the level of SOD as a primary antioxidant.

Materials and methods

Preparation of Centella asiatica L. Extract

500 g simplicia of *Centella asiatica* L. produced by UPT of Materia Medica, Batu, Malang. *C. asiatica* L. extract was obtained by maseration for 24 h using 98% ethanol (5 L) as a solvent. Residues and filtrates were separated using buchner funnel. Residues were re-extracted with 5 L of 98% ethanol for 24 h. The filtrate obtained by rotary evaporator vacuum (50°C) until more concentrated. This

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procedure produced 35 g extract of C. *asiatica* L.

Phytochemical Screening of Centella asiatica L. Ethanolic Extract

Qualitative phytochemichal analysis of C. *asiatica* L. extract was done with standard method by Harborne (1998). Flavonoid, alkaloid, tanin, saponin and terpenoid were tested.

Radical Scavenging Assay of Centella asiatica L. Ethanolic Extract Using DPPH

DPPH (Merck, USA) assay was described by Killedar *et al*. (2013) and Aizad *et al.* (2016) with slight modification. DPPH concentration was 0.1 mM and sample concentration was 10, 50, 100, 150, and 200 µg mL^{-1}. Percentage inhibition was calculated as DPPH radical scavenging activity (%) = [(Abs control–Abs sample)/Abs control] X 100; where Abs control is absorbance of DPPH radical; Abs sample is absorbance of DPPH radical along with extract. The IC_{50} values of the extract was calculated using linier regression.

Experimental Animals and Treatments

The study was used 150 *Cyprinus carpio* (10-12 g). Fishes were randomly divided into 5 groups with 10 fishes in each aquarium (3 repetition). Group I: 0 mg kg^{-1} b/w C. *asiatica* extract. Group II: 50 mg kg^{-1} b/w C. *asiatica* extract. Group III: 100 mg kg^{-1} b/w C. *asiatica* extract. Group IV: 150 mg kg^{-1} b/w C. *asiatica* extract. Group V: 300 mg kg^{-1} b/w C. *asiatica* extract. All the extract were given orally every 3 days for 15 days.

Measurement of Superoxide Dismutase (SOD)

Liver was removed from the body in the last day of treatments. The level of SOD was determined according to the method of Durak *et al*, 1996 with slight modification. Sample was measured with Shimadzu Co. BioSpek UV-1601PC UV-Visible Spectrophotometry in 570 nm.

Statistical Analysis

Levene homogenity test was used for homogenity of the data. Then, it tested with One way analysis of variance (ANOVA) and Tukey HSD post hoc test. The data expressed as mean from SOD level (ng mL^{-1}) in the liver of C. *carpio* in-vivo treatments. This statistical analysis was performed using SPSS 16.0. The results are significant if $p<0.05$.

Results and discussion

Extraction and Phytochemical Screening of Centella asiatica L. Ethanolic Extract

Extraction of *Centella asiatica* used maseration method involve separation of biaoctive of plant tissues from inactive/inert components by using selective solvents. The solvents diffused into solid plant material during extraction. This process make the compounds with similar polarity are more soluble. This method is best suitable for use in case of the thermolabile drugs (Ncube *et al.*, 2008). The purpose of extraction procedure of medicinal plants is to eliminate the unwanted material and gain the important part with therapeutic property. The type of extraction, time, temperature, nature of solvent, solvent concentraion and polarity can affect the quantity and secondary metabolite extract (Tiwari *et al.*, 2011). Low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate are some properties of a good solvents in plants extractions (Eloff, 1998).

Based on the Table 1, analysis of *C. asiatica* L. ethanolic extract contains bioactive constituents such as flavonoid, alkaloid, terpenoid, tanin, and saponin. Based on the previous study, bioactive constituents on C. *asiatica* are mainly triterpenes compounds such as asiaticoside, madecassoside, asiatic acid, and madecassic acid (Shukla *et al.*, 1999).

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It has been reported that asiaticoside (Mustafa *et al.*, 2010) and flavonoid (Korkina and Afanasev, 1997) had wound healing effect due to induction of antioxidant level. Flavonoid are natural antioxidant from plants. It has been reported that flavonoid are responsible for the radical scavenging effects because their hydroxyls contain (Hanasaki *et al.*, 1994). Some flavonoid can scavenge superoxides, and other flavonoid can scavenge peroxynitrite. Peroxynitrite is a highly reactive oxygen-derived radical (DeGroot, 1994). The previous study says that flavones and cathechins are the most powerful flavonoids against ROS (Cui *et al.*, 2006).

Table 1. Phytochemical Screening of Ethanolic Extract of *Centella asiatica* L.

No	Bioactive Compounds	Description
1	Flavonoid	+ (Positif)
2	Alkaloid	+ (Positif)
3	Terpenoid	+ (Positif)
4	Tannin	+ (Positif)
5	Saponin	+ (Positif)

Alkaloid also showed a strong radical scavenger power. Their cytotoxic, antimicrobial, and anti HIV activities are related to their radical scavenging activity (Gu *et al.*, 2008). Further, tannin showed their antioxidants activity from their acceleration of lipid peroxidation (Hagerman *et al.*, 1998). Tannin, or polymeric polyphenolic are more potent antioxidant than simple monomeric phenolics. It is because their high molecular weight and the proximity of many aromatic rings and hydroxyl groups (Chan *et al.*, 2014). Furthermore, previous study claimed that saponins are contribute to the antioxidant activity on defatted kenaf seed meal extract. The purification of saponin significally enhance the primary antioxidant activity (Atanassova *et al.*, 2011). Therefore, thus bioactive compounds may potentially used as dietary antioxidants ingredient.

Radical Scavenging Assay of Centella asiatica L. Ethanolic Extract Using DPPH

Antioxidant activity of C. *asiatica* ethanolic extract determined by radical scavenging assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH is a free radical with absorption 517 nm. The measurement of radical scavenging is taken by absorption decrease (Chan *et al.*, 2014). DPPH is a stable free radical with deep violet colour. The solution of DPPH will loss their violet colour if mixed with a substance that donated a hydrogen atom (Kedare and Singh, 2011). DPPH is a easy, rapid, and inexpensive method to measure overall antioxidant capacity (Prakash, 2001). This method also used to quantify antioxidants in complex biological systems, for solid or liquid samples (Sendra *et al.*, 2006). DPPH method only used in aqueous and nonpolar organic solvents. This method can examined hydrophilic and lipophilic antioxidants (Prior *et al.*, 2005). DPPH assay has been used for examine antioxidant properties of wheat grain and bran, vegetables, herbs, and flours in several different solvent systems including ethanol, aqueous acetone, methanol, aqueous alcohol and benzene (Yu, 2001; Parry *et al.*, 2005).

C. *asiatica* extract as antioxidant donated protons to DPPH. Mixed colour between DPPH solution and ethanolic extract of C. *asiatica* produced a yellow greenish colour. Their solution colour is more yellow if the extract concentration is higher. Based on Table 2, ethanolic extract of C. *asiatica* in 10, 50, 100, 150, and 200 µg mL^{-1} had persentage of inhibition 32.29, 40.62, 47.52, 56.12, and 57.42% respectively. The more higher concentration of the sample, the more higher percentage of the inhibition. Further, a regression curve was made between

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concentration (x) and percentage of inhibition (y), so the equation of the regression is y = 0.1337x + 32.668 with IC_{50}=125.87 µg mL^{-1}. It is showed that *C. asiatica* ethanolic extract classified as moderately active. According to Molyneux (2004), antioxidant properties based on IC_{50} values can be divided into several groups. IC50 values <50 µg mL^{-1} were classified as very strong, 50-100 µg mL^{-1} classified as strong, 100-150 µg mL^{-1} classified as moderate, and 150-200 µg mL^{-1} classified as weak.

Table 2. Percentage of Radical Scavenging in Ethanolic Extract of *Centella asiatica* L.

No	C. *asiatica* (µg mL^{-1})	Absorbansi	Inhibition *(%)*
1	Control	0.768	-
2	10	0.520	32.292
3	50	0.456	40.625
4	100	0.403	47.526
5	150	0.337	56.120
6	200	0.327	57.422

The present study showed different result from the previous study, who claimed that IC_{50} value of C. *asiatica* ethanol extract is 35.6±1.3 µg ml^{-1} (Sugunabai *et al.*, 2015). Other study had similar result for IC_{50} aqueous extract of C. *asiatica* is 30 µg ml^{-1} (Kundu *et al.*, 2015). Previous studies by Andarwulan *et al*, (2015) declared C. *asiatica* ethanolic extract in different ecotype also had different value of IC_{50}. Their result revealed that the higher phenolic content in the extract coincided with higher antioxidant activity. Some factors contributed to variations of antioxidant and nutrient are climate difference, geographical, yearly, soil conditions, and pesticide usage.

Effect of Centella asiatica L. Extract on Hepatic Level of Superoxide Dismutase (SOD)

The first detoxification enzyme and the most powerful antioxidan in cells is called superoxide dismutase (SOD). This enzyme act as first line defense system in organismm against reactive oxygen species (ROS). SOD activity depends on metal cofactor such as iron (Fe), zinc Zn) copper (Cu) and manganese (Mn). This metalloenzyme catalyze superoxide anion ($O2^-$) to hydrogen peroxide (H2O2) and molecular oxygen (O2). Fe-SOD commonly found in prokaryotes and chloroplasts of some plants, while Mn-SOD present in prokaryotes and mitochondria of eukaryotes. The last is Cu/Zn-SOD which is major in eukaryotes, basically in cytosol but also found in chloroplasts and peroxisomes (Gill and Tuteja, 2010). SOD is necessary to cellular health, protecting body cells from excessive oxygen radicals and free radicals, also the other harmful agents that promote cell death.

The main organ for detoxification is liver. Thus, xenobiotic such as drugs and toxins can increase the damage of hepatic cells. This liver damage caused by hepatotoxic chemicals and the role of oxidative stress (Malhi and Gores 2008). Most of all organism possess antioxidant defense and repair systems. However, these systems are not enough to conquer the entire damage. Hence, therapeutic strategy such as supplementation of dietary antioxidant is promising in reinforcement the antioxidant defense and repair systems. Inhibition of oxidation process will be a course to prevent a liver damage (Kepekci *et al.*, 2013). The purpose of SOD assay in C. *carpio* liver is to determine the difference in SOD level after C. *asiatica* extract administration. It has been suggested that SOD supplementation will protect the immune system and significantly reduce chances of disease. Present study showed, that the level of SOD in the control groups was lower than the treated groups. The level of SOD in control group was 1,383 ng ml^{-1} , while in dose 50, 100, 150, 300 mg kg^{-1} body weight *Centella asiatica* was 1.405, 1.600, 1.663, 1.726 ng ml^{-1}

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respectively (Table 3). These results indicate that C. *asiatica* extract enhance the level of SOD in C. *carpio* liver. The SOD level was significantly difference compared to the control group with P<0.05. Treatment with 300 mg kg^{-1} C. *asiatica* had most significant result.

Table 3. SOD level in *Cyprinus carpio* L. Liver after Treatment with *Centella asiatica* L. Ethanolic Extract

No	Treatments (mg kg^{-1} b w^{-1})	Mean SOD level (ng ml^{-1})
1	Control	1.383
2	50	1.405
3	100	1.600
4	150	1.663
5	300	1.726

Kumar and Gupta (2002) demonstrated that C. *asiatica* extract with aqueous solvent had two effect in brain rats, specifically improving the learning and memory, also had an antioxidant property by decreasing lipid peroxidation and enhancing endogenous antioxidant enzymes. The other study by Choi *et al.* (2016) demonstrated that extract *Centella asiatica* increased the levels of SOD in the liver tissues. In the previous study, C. *asiatica* also had a cardioprotective effect in rats. This effect may involve the mechanism of prevention from lipid peroxidation and maintenance of antioxidant enzymes, such as SOD as well as scavenging of free radicals (Kumar *et al.*, 2015). Hussin *et al.* (2007) also showed an increased level of SOD significantly after administration of C. *asiatica.* Antioxidant components in C. *asiatica* such as phenolic compounds may have a role as inhibitor and chain breaking activity of free radicals production. Also, treatment with 100, and 200 mg kg^{-1} of *C. asiatica* by Sivakumar *et al*. (2018) showed a significant increase in the level of SOD due to the ability of the bioactive compounds to scavenge ROS. Those studies showed that administration of C. *asiatica* increased an antioxidant enzyme such as superoxide dismutase (SOD). Thus, can scavenge and neutralized the free radical.

Conclusions and suggestion

Centella asiatica ethanolic extract contains some bioactive compounds such as, flavonoid, alkaloid, terpenoid, tannin, and saponin. Those compounds exhibited antioxidant effect represented in their radical scavenging activity using DPPH with IC_{50} 125.87 µg mL^{-1}. The SOD level on the *Cyprinus carpio* liver significantly increased after administration of C. *asiatica* extract with P-value <0.05. Treatment with 300 mg kg^{-1} C. *asiatica* had most significant result. This study suggest that C. *asiatica* had an antioxidant effect by increase antioxidant mechanism such as SOD in liver tissues.

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