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Antioxidant Effect of *Chlorella vulgaris* on Physiological Response of Rat Induced by Carbon Tetrachloride

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Abstract

Chlorella vulgaris is an algae with high nutrition content. Carbon tetrachloride (CCl_4) is a hepatotoxic chemical.The aims of this study were to determine the effect of C. vulgaris extract on the physiological response of liver such as MDA, SOD and GPx activity on rat after induced by CCl_4 exposure as well as to determine the effective dose of *C. vulgaris* extract as antioxidant that can neutralize CCl_4 exposure. This research was conducted experimentally with Completely Randomized Design that consists of 6 treatment and 5 times repetition. The doses of *C. vulgaris* extract used were 3, 4, and 5 mg per /100 g of rat'sbody weight (BW).The administration of C. vulgaris extract was performed within 30 days, while the CCl_4 (0.25 ml/100 g BW) was administered orally on the day 9, 12, 16, 19, 23, and 26. Parameters measured were levels of MDA, SOD and GPx of rat blood serum.The results showed that the administration of *C. vulgaris* extract can inhibit lipid peroxidation indicated by decrease in MDA activities and oxidative stress by increasing SOD and GPx activity. In conclusion, 5 mg/100 g BW of *C.vulgaris* extract is an effective dose to be uses as endogenous antioxidant to protect the liver cell from damage caused by CCl_4 exposure. The benefit of *C. vulgaris* as a supplement for antihepatotoxin in humans.

How to Cite

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INTRODUCTION

Chlorella vulgaris is a type of green algae that grows in fresh water. The whole plant is used to make nutritional supplement and medicine.*C. vulgaris*) is a good source of protein, fats, carbohydrates, fiber, chlorophyll, vitamins, and minerals (Kim et al., 2009; Baky and Baroty, 2013). Lidiane et al. (2018) stated that *C. vulgaris* can be used as a great source of functional food, nutraceuticals and food supplements. Frietas (2017) reported that *C. vulgaris* has vitamin B 12, vitamin C, iron mineral, 58% protein and poly unsaturated fatty acids. The cell wall of *C. vulgaris* must be broken down before people can digest it.

Astaxanthin is the main carotenoid pigment found in *C. vulgaris* equipped with two asymmetric carbon located at the 3 and 3′ position of the benzenoid rings on either end of the molecule, this molecule has been proposed as super vitamin E due to its natural antioxidant activities (Chonglong et al., 2014). The essential functions of carotenoid as antioxidant are to deactivate free radicals such as singlet molecular oxygen and Reactive Oxygen Species (ROS) i.e. anion superoxide ($O2^-$),hydroxyl radical (OH^-) and peroxyl radical.Reactivity of free radicals hold the potential of initiating oxidative stress, an imbalance between endogenous antioxidant and free radical, resulted in cell damage (Yadav et al., 2016).

The industrial solvent carbon tetrachloride (CCl_4) is a potential environmental hepatotoxin. Carbon tetra chloride included in aliphatic hydrocarbon,which is colorless, volatile, pungent smell like ether, solubility in low water and nonflammable. It is widely used in industry of refrigerant and fuel. This toxin can enter to human body via inhalation, ingestion and skin contact. Chronic intoxication by CCl_4 leads to liver damage, brain, lung and renal. Carbon tetra chloride is also used in laboratory experiment for inducing liver damage in treated animals. The function of liver is to detoxify toxic compound that enter the body. Carbon tetrachloride can be metabolized to free radical intermediates by cytochrome P450 in the hepatocytes; these free radical intermediates is trichloromethyl (CCl_3^-)free radical. The excessive amount of CCl_3^-can lead to lipid peroxidation chain reaction that causes hepatic cell damage. Lipid peroxidation affects the hepatocyte cell integrity, resulted in hepatocyte damage (Foaud et al., 2018; Kamble & Rao, 2018; Susatyo et al., 2018).

The occurrence of lipid peroxidation indicated by an increase in Malondialdehyde as the end product of lipid peroxidation and a decrease in antioxidant activity super oxide dismutase (SOD) as well as glutathione peroxidase (GPx). The function of SOD is to catalyze the dismutation of superoxide anion to hydrogen peroxide (H_2O_2). This reaction is continued by GPx which catalyze H_2O_2 into water (H_2O) and oxygen (O_2) (Lobo et al., 2010; Mbata et al., 2018; Goodarzi et al., 2018). The free radical CCl_3^- causes lipid peroxidation and oxidative stress. Oxidative stress is the imbalance between free radicals and antioxidant enzymes resulted in hepatocyte damage and decrease in endogenous antioxidants level, such as Super oxide dismutase (SOD) and Glutathione Peroxidase (GPx). Administration of *C.vulgaris* as exogenous antioxidant can prevent the oxidative stress in liver.

Administration of *C. vulgaris* extract will increase SOD, GPx, glutathione, vitamin C, and catalase in rats exposed by CCl_4 (Peng et al,. (2009).In order to determine the potential activity of *C. vulgaris* as antioxidant, carbon tetra chloride (CCl_4)was used as xenobiotic agent. Administration of *C. vulgaris* extract to animal exposed by CCl_4 was expected to be neutralize the toxic effect of trichloromethyl free radical. The aims of this study were to determine the effect of *C. vulgaris* extract on the physiological response in rats' liver such as MDA, SOD and GPx activity on rat after induced by CCl_4 exposure as well as to determine the effective dose of *C. vulgaris* extract as antioxidant that can neutralize CCl_4 exposure.

METHODS

Research Location and Time

This research was conducted in Animal House, Laboratory of Animal Physiology, Laboratory of Ecotoxicology and Research Laboratory of Faculty of Medicine Universitas Jenderal Soedirman, Purwokerto.

Animals and Research Ethics

Thirty male white rat Wistar strain with 2-3 month of age and 150-200 g of weight from LPPT IV Universitas Gadjah Mada Yogyakarta was used in this study. Permission for using animal had been approved by Ethical Committee of Health Research Dr. Moewardi General Hospital, Faculty of Medicine Universitas Sebelas Maret number 461/V/HREC/2017.

Production of *Chlorella vulgaris* extract

Production of *C. vulgaris* extract was use maceration method (Cha et al., 2010). Dried *C.vulgaris* weighed as much as 50 g and was added

to 100 ml of 96% ethanol in beaker glass. This solution was mixed and left for 24 hours until it was sedimented. After 24 hours, the macerated *C. vulgaris* was taken and placed in clean beaker glass. Furthermore, the macerate it was re-macerated by using 96% of ethanol. Re-maceration was done three times in 24 hours, repeatedly until the color of the mixture faded. Obtained solution was evaporated using Vacuum Rotary Evaporator to remove solvent and to obtain thick *C. vulgaris* extract.

Experimental Design

This research was conducted experimentally with Completely Randomized Design consist of 6 treatments and 5 times repetition. The rats were separated into 6 groups namely, P1, P2, P3, P4, P5, P6. Before the administration of *C. vulgaris* extract, the rats in each group was acclimated within 10 day with pellet feed twice a day (on 7 AM and 4 PM). Furthermore, the feeding was continued routinely until 30 days of experiment with the same time administration.

In 30 days of experiment,*C. vulgaris* extract was administered to the rats in P4, P5, and P6 groups were given *C. vulgaris* extract twice a day (before meals) with dose of 3 mg/100 g BW, 4 mg/100 g BW, 5 mg/100 g BW, respectively, the gastric probe. Induction using of 0.25 ml/100 g BW of CCl_4 was conducted to P3, P4, P5, and P6 using gastric probe on day 9, 12, 16, 19, 23, and 26. P1 group as healthy control were given only physiological NaCl without *C. vulgaris* extract while P2 as standard control were given *C. vulgaris* extract and physiological NaCl. P1 and P2 group were free of CCl_4 induction.

Preparation of blood serum

Blood collection was done through orbitalis vein of rat on day 31 after treatment. Furthermore, the blood was centrifuged with a speed of 4000 rpm. The blood serum was then separated from erythrocytes.

Measuring the parameters

Malondialdehyde (MDA) was detected by Thiobarbituric Reactive Substances (TBARS) method with spectrophotometer at 535 nm; SOD activity was detected by Randox SOD with spectrophotometer at 520 nm and GPx activity was detected by ELISA method with Elisa Reader at wavelength of 450 nm.

Data Analysis

Data obtained from the research were analyzed by using ANOVA and Advanced Tuckey Test.

RESULT AND DISCUSSION

Malondialdehyde (MDA) level

The results of MDA level in control and treatment groups can be seen in Figure 1. The data shows that on day 31 the positive control group (P3) has the highest MDA activity, and while P1 has the lowest MDA activity compared to the other treatments. MDA levels are widely used as indicators of lipid peroxidation and describe the amount of free radical that enter the body. MDA, one of several by-products of lipid peroxidation process, is a biomarker that provides an indication of lipid peroxidation activity (Kuyumucu & Aycan, 2018). Lipid peroxidation can be described generally as a process under which oxidants such as free radicals attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) that involve hydrogen abstraction from a carbon, with oxygen insertion resulting in lipid peroxyl radicals and hydroperoxides. (Antonio et al., 2014).

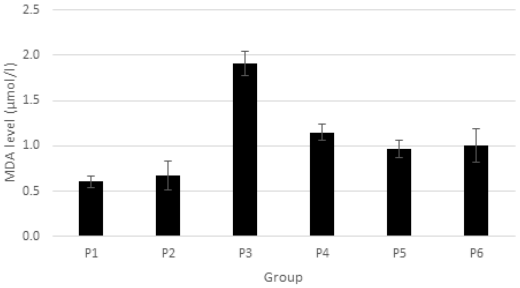

Figure 1. MDA level in control and treatment groups. Note: P1 (healthy control), P2 (standard control), P3 (positive control induced by CCl_4),P4 (treatment with *C. vulgaris* extract 3 mg/100 BW), P5 (treatment with *C. vulgaris* extract 4 mg/100 BW), P6 (treatment with *C. vulgaris* extract 5 mg/100 BW)

The mechanism of elevating MDA level because after rat's administered by CCl_4, it metabolized by cytochrom P-450 in the endoplasmic reticulum of liver then converted to CCL_3^- (Tricholoromethyl radical) which initiated lipid peroxidation reaction in phospholipid of the cell membran hepatocyte (Lobo et al., 2010). Tricholoromethyl radical bind covalently with protein, fat and DNA and $CCl_3O_2^-$ will accumulate in reticulum endoplasmic. Increase of CCl_3^- and

$CCl_3O_2^-$levels in hepatocyte initiate the lipid peroxidation reaction in phospholipid that composes the cell membran of hepatocyte, and lead to cell membrane dysfunction (Thangakrishnakumari et al., 2017). Free radical that comes from Fenton reaction will also increase. Fenton reaction is the formation of O_2^-, OH^- and Fe^{3+} from the non-enzymatic reaction of Fe^{2+} with H_2O_2. CCl_3^-free radical increase the Fenton reaction that produce O_2^-, OH^- several fold more compared to normal condition and resulted in oxidative stress in various cell especially hepatocyte.

Glycolipids, phospholipids (PLs), and cholesterol (Ch) are also well-known targets of damaging and potentially lethal peroxidative modification. Lipids can also be oxidized by enzymes like lipoxygenases, cyclooxygenases, and cytochrome P450. In response to membrane lipid peroxidation and according to specific cellular metabolic circumstances and repair capacities, the cells may promote cell survival or induce cell death. Early studies showed that a probable biochemical route for MDA metabolism involves its oxidation by mitochondrial aldehyde dehydrogenase followed by decarboxylation to produce acetaldehyde, which is oxidized by aldehyde dehydrogenase to acetate and further to CO_2 and H_2O. On the other hand, phosphoglucose isomerase is probably responsible for metabolizing cytoplasmic MDA to methylglyoxal (MG) and further to D-lactate by glyoxalase enzymes system with GSH as a cofactor (Yin et al., 2011; Pizzimenti et al., 2013).

The mechanism toxicity of CCl_3^- as a free radical of CCl_4 depends on oxygen availability (anaerobic or aerobic conditions). In anaerobic conditions, the dimerization process change CCl_3^- to hexachloroethane which can directly bind to lipids, microsomal proteins and heme as a part of CYP450. In aerobic condition, CCl_3^- which is trapped in oxygen bonds can form trichloromethyl radicals.This process break down CCl_3^- into phosphagen ($COCl_2$) and electrophilic forms of chlorine. CCl_3^- and $COCl_2$ are the main initiators of lipid peroxidation (Manibusan *et al*., 2010). This process resulted infragmentation of phospholipid membranes of hepatocyte and the formation of MDA as the end product of lipid peroxidation, so that, the MDA level in P3 increase. Lipid peroxidation process followed by the occurrence of hepar liver damage.

After treatment with *C. vulgaris* extract on day 31, MDA activity of P4, P5 and P6 decrease due to the carotenoid (a derivate of polyphenolic compound) content of *C. vulgaris* donated electron H^+ to CCL_3^- so it became neutral. This result is in accordance with study by Lidiane et al., (2018) that found that polyphenolic compound of *C. vulgaris* can protect cell from the damage caused by free radicals. *C.vulgaris* also contains vitamin E which canbreak the chain of lipid peroxidation so that it can prevent cell damage. *C. vulgaris* is also rich in vitamin C which has function to recycle the production of vitamin E (Peng, et al., 2009; Buono et al., 2014).

The results of statistical analysis showed very significant differences between positive control(CCl_4 exposure) and treatment (*C. vulgaris* extract) groups ($p<0.05$). Tukey test result showed that there were no significant difference among P4, P5 and P6. It can be concluded that all treatments have the same effect to reduce MDA activity, although the MDA activity is still higher than P1 (healthy control) and P2 (standard control). Normal value of MDA < 1 mmol/l.

Super Oxide Dismutase (SOD) Activity

The results of SOD activity in control and treatment groups can be seen in Figure 2.

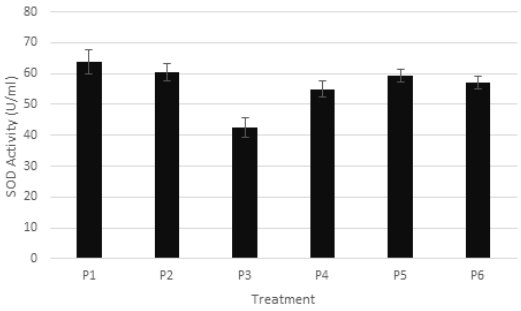

Figure 2. SOD activity in control and treatment groups. Note: P1 (healthy control), P2 (standard control), P3 (positive control induced by CCl_4), P4 (treatment with *C. vulgaris* extract 3 mg/100 BW, P5 (treatment with *C. vulgaris* extract 4 mg/100 BW), P6 (treatment with *C. vulgaris* extract 5 mg/100 BW)

The data of day 31show that the positive control group (P3) has lowest SOD activity while the SOD activity of P1, P2, P5 are higher than other groups. Super oxide dismutase is present in essentially in every cell in the body represented by a group of metalloenzymes with various prosthetic groups. It appears in three forms: Cu-Zn SOD:in the cytoplasm; Mn-SOD:in the mitochondrion and Cu-SOD: as an extracellular SOD. This is the first line to protect cells from the injurious effects of free radical especially anion superoxide (O_2^-) (Yadav, *et al*, 2016; Ighodaro &Akinloye, 2017). At the time CCl_3^- enter the liver, it will follow by an increase of Fenton reaction which produce Reactive Oxygen Species

(ROS) such as O_2^-, SOD becomes active and try to convert it into hydrogen peroxide (H_2O_2) and oxygen (O_2). However, there are a lot of O_2^- in liver so that SOD failed to neutralize H_2O_2 and the level SOD in serum decreases.

After the administration of *C. vulgaris* extract, SOD activity of P4,P5,P6 increased even the P5 SOD level almost equal to P1 (healthy control) and P2 (standard control). *C.vulgaris* extract consists of carotenoid derivate of polyphenolics compound will neutralize O_2^- by giving H^+, so that the SOD can convert O_2^- into H_2O_2.Super oxide dismutase needs minerals such as Cu and Zn that *C. vulgaris*have. This is indicated by an increase SOD in serum of treated animals.

Carotenoids constitute a ubiquitous group of isoprenoid pigments. They are very efficient physical quenchers of singlet oxygen and scavengers of other reactive oxygen species. Carotenoids can also act as chemical quenchers undergoing irreversible oxygenation.The antioxidant potential of carotenoids is of particular significance to human health, due to the fact that losing antioxidant-reactive oxygen species balance results in "oxidative stress", a critical factor of the pathogenic processes of various chronic disorders. Data coming from epidemiological studies and clinical trials strongly support the observation that adequate carotenoid supplementation may significantly reduce the risk of several disorders mediated by reactive oxygen species (Fiedor& Burda., 2014).

The results of statistical analysis showed very significant differences among positive control and treatment groups ($p<0.05$).Tukey test result showed that there were no significant difference among P4, P5 and P6. *C.vulgaris* at dose of 4 mg/100g BW has the highest SOD activity compared to *C. vulgaris* at doseof 3 mg/100 g BW and 5 mg/100 g BW. This data indicates that *C. vulgaris* can be used as antioxidant to inhibit and neutralize the negative effect of CCl_4 exposure.

Glutathione Peroxidase (GPx) Activity

The results of GPx activity in control and treatment groups can be seen in Figure 3. The data shows that on day 31, the positive control group (P3) has lowest GPx activity and P1 has highest GPx activity compare to other groups. Glutathione peroxidase is a major and ubiquitously expressed antioxidant enzyme present in the cytosol and mitochondria. It is involved in the detoxification of hydrogen and lipid peroxides and acts as a peroxy nitrite reductase (Kuyumucu & Aycan, 2018; Wang et al., 2018).

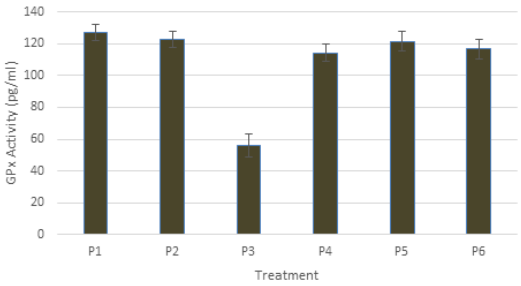

Figure 3. GPx activity in control and treatment groups. Note : P1 (healthy control), P2 (standard control), P3 (positive control induced by CCl_4), P4 (treatment with *C. vulgaris* extract 3 mg/100 BW, P5 (treatment with *C. vulgaris* extract 4 mg/100 BW), P6 (treatment with *C. vulgaris* extract 5 mg/100 BW)

Glutathion peroxidase activity is as important as endogenous antioxidant that protects cells from the dangerous effect of hydrogen peroxide and its derivates.H_2O_2 is normally produced endogenously in a number of cellular compartments, including the mitochondria, the endoplasmic reticulum, peroxisomes, and at the plasma membrane, and can play divergent roles as a second messenger or a pathological toxin. H_2O_2 is one of the major members of reactive oxygen species (ROS) and plays essential roles as a beneficial signaling agent or a toxic hazard in physiological and pathological processes (Ighodaro et al., 2017).

Chlorella vulgaris contains glutathione that can increase the GPx activity. Glutathione peroxidase utilizes glutathione as an electron donor to reduce ROS. Glutathione itself is not an enzyme, but merely a substrate for the antioxidant reaction catalyzed by glutathione peroxidase. In the human body, reduced glutathione disulfide is regenerated by the action of a second enzyme, glutathione reductase (Lobo et al., 2010).

Glutathione also serves as an antioxidant by donating electrons to maintain the activity of other antioxidant compounds such as vitamin C and vitamin E. Like catalase, glutathione peroxidase is a tetrameric protein but with selenium at the catalytic site. High levels of glutathione peroxidase have been shown to protect against oxidative stress in both in vitro cellular and in vivo animal studies. Dietary intake of polyphenols has been shown to increase the expression of antioxidant enzymes including SOD, catalase and glutathione peroxidase (Goodarzi et al., 2018). It is believed that these phytochemicals induce expression of antioxidant enzymes through the

nuclear factor-erythroid-2-related factor 2 pathway (Nrf2) (Mameghani, et al., 2014)

Hydrogen peroxide will be converted into water (H_2O) and oxygen (O_2) catalyzed by GPx. However, if O_2^- and H_2O_2 formation is excessive, GPx are not able to neutralize H_2O_2 and convert it into H_2O and O_2 so that GPx in P3 is decreased. Carotenoid of *C. vulgaris* administered to rat as an exogenous antioxidant that can inhibit oxidative stress and indicated by increasing of GPx activity. Antioxidants neutralize free radicals by giving one of its electrons (H^+) to the free radicals and transform them into non-radical forms. The addition of antioxidants can inhibit the rate of increase in the number of peroxide radicals (Ding & Lin, 2007). Research of Yun et al., (2011) indicated that administration of *C. vulgaris* on Sprawgue Dawley rat which induced by lead acetate can increase GPx activity in brain up to 63%. Astaxanthin of carotenoid is stronger up to several-fold than vitamin E as radical scavenger and has a potential effect to break the chain of lipid peroxidation (Buono et al., 2014).

According to Ding et al., 2007, GPx especially GPx-4 is widely expressed in normal tissues and is likely to play a role in protecting a variety of normal and neoplastic cells from lipid peroxidation. Among the primary cellular enzymes that reduce hydrogen peroxides, GPx-4 is known to have a broad range of cellular substrates but it is the only glutathione peroxidase to directly act on phospholipid hydroperoxides (Lobo et al., 2014). This fact implies that it has a unique involvement in the protection of cells against damage induced by lipid peroxidation.

In vitro and in vivo studies imply that antioxidant nutrients and related bioactive compounds from fruits and vegetables can protect us from oxidative stress. Antioxidants such as *C. vulgaris* as dietary supplements may prevent some ROS-induced damage in conditions of elevated oxidative stress during elevated environmental oxidant exposure or at weaken endogenous oxidative stress responses of an aged organism (Rahman, 2017).

The statistical analysis showsvery significant differences among positive control and treatment control positive and treatment groups ($p<0.05$).Tukey test result showed that GPx activity of P5 is equivalent compared to P1 and P2 and there are no significant differences GPxactivity among P4,P5 and P6. These results indicate that*C. vulgaris* extract with dose of 3,4 and5 mg/100g BW have the same effect to increase GPx activity.

The result of this study has benefit in developing *C. vulgaris* as herbal resource to protect hepatic cells damage seen from the physiological response of the liver caused by CCl_4 exposure. The increase in antioxidant enzymes activity (SOD and GPx) as well as decrease in MDA are indicated that *C. vulgaris* can protect the liver damage for CCl_4 exposure as a model of hepatotoxic chemical.

CONCLUSION

The administration of *C.vulgaris*extract can protect white rat from oxidative stress induced due to the administration of CCl_4. This result was indicated by the decrease in MDA activity as well as an increase in SOD and GPx activity. The most effective dose of *C.vulgaris* extract as exogenous antioxidant was 5 mg/100gBW.

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