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Antioxidant Effect of *Clorella vulgaris* on Wistar Rat Kidney Induced by CCl_4: A Histopathological Review

✉Priyo Susatyo, Achmad Akbar Rifanda, Sorta Basar Ida Simanjuntak, Titi Chasanah

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Faculty of Biology, Universitas Jenderal Soedirman, Indonesia

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Abstract

Kidney is very susceptible to damage by toxicological compounds such as carbon tetrachloride (CCL). CCL produce free radicals, which cause lipid peroxidation and kidney damage and free radical release, which can be prevented by the administration of exogenous antioxidants, such as *Chlorella vulgaris*. The aim of study was to determine an effect of antioxidant of *C. vulgaris* on the histopathological features of Wistar rat kidney which is induced by CCL. Experimental study with *completely randomized design*. The variable was histopathology features of the kidneys. The doses of *C. vulgaris* extract were 3 mg, 4 mg, and 5 mg per 100 grams of rat body weight (BW). The administration of *C. vulgaris* extract was performed within 30 days, while the CCl_4 induction (0.25 ml/100 g BW) was administered orally on the day 9, 12, 16, 19, 23, and 26. Parameters were histopathology features of renal damage, proportion of tubular cell damage, and Bowman's space diameter. The results showed the administration of *C. vulgaris* extract was able to reduce the impact of damage caused by CCl_4 ($p<0.05$). This was supported by histologic observations, which was showing a decrease of picnotic and vacuolated cells, normal brush border, and decrease of Bowman's space. In conclusion, 5 mg/100 g BW of *C. vulgaris* extract can effectively protect the kidney from damage caused by CCL. The results of this study strongly support further research on immunostimulant content test *C. vulgaris* and determine the efficient dose for representative mammals animals also in humans.

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✉ Correspondence Author:
Jl. HR Boenyamin 708, Grendeng, Purwokerto, Banyumas, Jawa Tengah 53122
E-mail: p_susatyo@yahoo.com

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INTRODUCTION

Kidney has an important role in filtering metabolic waste products and toxic compounds on blood. This role involves tubular structure and counter-current on medulla, which play role in 25% of circulated blood reabsorption within the body (Weiner *et al.*, 2015). The high percentage of filtered blood makes this organ vulnerable toward damage due to toxic compound (nephrotoxic) within the blood.

Generally, nephrotoxic compound causes damage on kidney and reduces its function, such as excretion of metabolic waste products, synthesis of erythropoietin, maintain electrolyte balance, and maintain fluid balance (David *et al.*, 2012).

One of the nephrotoxic compound is carbon tetrachloride (CCl_4). Its toxicity is come from free radical from its metabolism by cytochrome P450 enzyme. Free radicals are atoms or molecules that have one or more unpaired electrons. This causes them are chemically reactive, which can cause chemical changes and damage on various components of living cells. In the human body, free radicals are considered to play a role in the occurrence of some diseases, such as aging (Fitmawati *et al.*, 2017). This compound will be metabolized into trichloromethyl (CCl_3^-) (Barsha *et al.*, 2017). Trichloromethyl reacts to oxygen molecule and forms trichloromethyl peroxy (CCl_3COO^-), which is a free radical (Fouw, 2007). This radical causes lipid peroxidation of phospholipids in cellular membrane and kidney cells damage (Antonio *et al*, 2014).

Impact of damage caused by free radicals can be reduced using antioxidant as protective agents. These agents are produced within the body continuously in the form of endogenous antioxidant (Hsu *et al.*, 2013). However, increased free radicals within the body can reduce effectivity of endogenous antioxidants as protective agents. Therefore, exogenous antioxidant is needed. Several example of exogenous antioxidants are phenolic and carotenoid compound within microalgae *Clorella vulgaris* (Goiris, 2012).

A wide variety of natural sources are under investigation to evaluate their possible use for new functional ingredient formulation. Some records attested the traditional and ancient use of wild harvested microalgae as human food but their cultivation for different purposes started about 40 years ago. The most popular species are Arthrospira (traditional name, Spirulina), *Chlorella spp*., Dunaliella spp. and Haematococcus spp (Tang & Suter, 2011). Microalgae provide a bewildering array of opportunities to develop healthier food products using innovative approaches and a number of different strategies. Compared to other natural sources of bioactive ingredients, microalgae have many advantages such as their huge biodiversity, the possibility to grow in arid land and with limited fresh water consumption and the flexibility of their metabolism, which could be adapted to produce specific molecules. All these factors led to very sustainable production making microalgae eligible as one of the most promising foods for the future, particularly as source of proteins, lipids and phytochemicals (Buono *et al.*, 2014).

C. vulgaris is one of many food, which contains abundant amount of antioxidant. According to a study by Cha *et al*. (2010), there were 15 type of antioxidants within *C. vulgaris* extract. These antioxidant are including in lipophilic and hydrophilic compounds, which is capable of reducing oxidative effect of free radicals (Satish & Dilipkumar, 2015).

Study about potential of *C. vulgaris* as antioxidant to prevent kidney damage was never been performed. Therefore, this study aimed to understand the effect of *C. vulgaris* extract in reducing kidney damage, which was exposed to CCl_4. We studied the effect of *C. vulgaris* extract administration on histopathological features of wistar rat (*Rattus norvegicus)* kidney and its dose that effective in reducing kidney damage in *Rattus norvegicus* which was induced by CCl_4. Result of this study will advance the knowledge about antioxidant potential of *C. vulgaris* and open the possibilities of further study in human body.

METHODS

Animal Test and Study Ethic

Animal used in this study were 30 male white rats (*Rattus norvegicus*) wistar strain with 2-3 month of age and 100-150 g of weight from UD Wistar, Yogyakarta. Permission for using these animal has been granted by Ethical Committee of Health Research, Dr. Moewardi General Hospital, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, number 680/VII/HREC/2017.

Tools and Materials

Tools used in this study were cage (40x30x15 cm), drinking bottle, gastric probe, and a set of equipment for tissue processing and a microscope for histologic observation.

Materials used in this study were 30 male *Rattus norvegicus* wistar strain with 2-3 month of

age and 100-150 g of weight; *C. vulgaris* extract with 3 mg, 4 mg, and 5 mg of doses; rats feed, husk, CCl_4 solution, physiologic NaCl, olive oil, and a package of chemical reagents for tissue processing.

Location and Time of Study

This study was performed within 6 month in Animal House, Laboratory of Animal Physiology, and Laboratory of Animal Structure and Development of Faculty of Biology Jenderal Soedirman University. We also conducted the experiment in Laboratory of Pharmacy, Jenderal Soedirman University.

Production of C. vulgaris Extract

Production of ethanol extract was performed using maceration method (Cha *et al.*, 2010). Dry *C. vulgaris* weighed as much as 50 g and added to 100 ml of 96% ethanol in beaker glass. This solution was mixed and left for 24 hour until it was sedimented. After 24 hour, the maserate was taken and placed in clean beaker glass. Furthermore, the maserate was resolved by using 96% of ethanol (remaseration). Remaseration was performed three times in 24 hour, 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 study used experimental method with complete random design, which was consisted of 6 treatment and 4 repetition. The 6 treatment were consisted of K+, KS, K-, P1, P2, and P3. Before the experiment performed, the rats in each treatment 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 of administration.

For 30 days of experiment, the rats in KS, P1, P2, and P3 treatment groups were given *C. vulgaris* extract twice a day (before meals) with dose 2 mg/ 100 g BW, 3 mg/ 100 g BW, 4 mg/ 100 g BW, and 5 mg/ 100 g BW, respectively, using gastric probe. Induction using 0.25 ml/100 g BW of CCl_4 was administered to K-, P1, P2, and P3 treatment groups using gastric probe on day 9, 12, 16, 19, 23, and 26. Physiologic NaCl were given to rats in K+ and KS group, which were free of CCl_4 induction.

Histologic Preparation

After 30 days of treatment, on the day 31, wistar rats were decapitated using ether. Furthermore, these rats were dissected and their left kidney were extracted. The kidney was dissected and cut using surgical tools. Left kidney was isolated, washed using physiologic NaCl, and fixated in neutral buffered formalin solution for 48 hour. After 48 hour, the kidney was processed into histologic preparation using paraffin method and was stained using Haematoxylin-eosin stain (Arsad *et al.*, 2014).

Histologic Evaluation

Histologic preparation of kidney was observed using light microscope. We observed the proximal tubule in cortex and the diameter of Bowman's space in 5 field of each slice using 400 fold of magnification. Measurement of Bowman's space diameter was performed by calculated the difference between Bowman's capsule and glomerulus (Kotyk *et al.*, 2015). Calculation of damaged proximal tubule cell was performed using Modified Cell Degeneration Index Formula (Maulina, 2015) as follows:

Cell Degeneration Index of Tubule = (Amount of Cell Necrosis /Amount of Total Cell) x 100%

Data Analysis

Data of this study were consisted of cell degeneration index of kidney tubules data, Bowman's space diameters data, and histopathological image of kidney. Qualitative data of kidney histology were analyzed descriptively. Cell degeneration index data were transformed into arc. $\mathrm{Sin}\sqrt{y}$. Furthermore, kidney weight, cell degeneration index of kidney tubules, and Bowman's space diameters were analyzed using ANOVA with significant level of 5% (p=0.05). If the result show significant value, it will be analyzed with post hoc test using HSD Tukey.

RESULTS AND DISCUSSION

According to the obtained result (Table 1), the tubule of rats on K- treatment group, which was inducted by CCl_4 without *C. vulgaris* extract administration, were damaged with high average value of 47.84% ± 0.04. This value was far greater than the average value of K+ treatment group, which was neither inducted by CCl_4 nor received *C. vulgaris* extract, with damage percentage of 9.96% ± 0.02. The amount of cell degeneration index of tubules in K- treatment group showed significant damage on proximal tubule due to CCl_4. The damage was happened due to

Table 1. Result of Histologic Examination of Wistar Rats Kidney

Treatment Group	Cell Degeneration Index (%) ± Deviation Standard (DS)	Bowman's Capsule (x 2,5 μm) ± DS	Glomerulus (x 2,5 μm) ± DS	Bowman's Space (x 2,5 μm) ± DS
K+	9.96 ± 0.02	35.14 ± 3.13	31.64 ± 3.7	3.50 ± 1.09
KS	9.58 ± 0.01	35.74 ± 1.88	32.53 ± 3.15	3.21 ± 0.70
K-	47.84 ± 0.04	35.59 ± 3.15	28.54 ± 1.88	7.04 ± 1.71
P1	23.17 ± 0.04	36.69 ± 1.42	31.45 ± 0.57	5.24 ± 0.86
P2	20.59 ± 0.007	36.49 ± 1.88	32.28 ± 1.99	4.21 ± 1.63
P3	14.60 ± 0.02	36.34 ± 0.84	32.33 ± 0.84	4.00 ± 1.07

accumulation of nephrotoxic compound and its biotransformation by P450 cytochrome (Tugba *et al.*, 2014). This result was showed damage on tubule cell of wistar rats after administration of 0.25 ml/100 g BW CCl_4. The study showed that the nucleus became picnotic after CCl_4 administration. Current study also showed dilatation, vacuolation, and brush border loss of tubule cell. These findings also relevant to study by Ogeturk (2005), Adewole (2007), and Ashafar (2008), which showed similar damage on tubule cell of wistar rats after CCl_4 administration.

Percentage of tubule cell damage on P1, P2, P3, and KS treatment group were 23.17% ± 0.04; 20.59 ± 0.007; 14.60% ± 0.02; and 9.58% ± 0.01, respectively. Percentage of tubule cell damage on P1, P2, and P3 treatment group were less than percentage of tubule cell damage on K- treatment group. These findings proved the ability of *C. vulgaris* extract in preventing cell damage due to CCl_4 exposure. These findings were also supported by normal histologic features of the cell with no vacuolation, no dilatation, few picnotic nucleus, and normal brush border.

Result of statistical analysis using ANOVA showed that administration of *C. vulgaris* extract produced significant effect on cell degeneration index of tubule cell ($p<0.05$). Further test using HSD Tukey (Table 2) showed similar notation value between P1 and P2 ($p>0.05$). Similar notation value was also shown on HSD Tukey test between P2 and P3 ($p>0.05$). On the other words, small range of *C. vulgaris* extract dose on P1, P2, and P3 treatment group did not produced significant difference.

Notation value of P3 treatment group also showed similar result with K+ and KS treatment group, which means the administration of 5 mg/100 g BW *C. vulgaris* extract was not capable to reduce the percentage of tubule cell damage. However, administration of *C. vulgaris* extract was capable to produce sufficient protection against oxidative stress, which was proved by significant difference of notation value between K- and other treatment group (P1, P2, and P3).

Table 2. Notation Value of Tukey Test on Cell Degeneration Index of Tubule Cell and Bowman's Space

Treatment Group	Cell Degeneration Index	Diameter of Bowman's space
K+	A	A
KS	A	A
K-	D	B
P1	C	Ab
P2	Bc	A
P3	Ab	A

Result of measurement of Bowman's Space diameter showed that wistar rats on K- treatment group had the biggest average (7.04 μm ± 1.71) compared to other treatment groups (Table 1). Average value of K- treatment group was far above average value of K+ treatment group (3.50 μm ± 1.09). Dilatation of Bowman's space and shrinkage of diameter (atrophy) of glomerulus, which is shown in Table 1, indicated degeneration and disintegration of glomerulus (Ogeturk, 2005) and Hismiogullari *et al*. (2015), which showed dilatation of Bowman's space and atrophy of glomerulus on wistar rat's kidney after administration of CCl_4. The diameter of Bowman's space on P1, P2, and P3 treatment groups were 5.24 μm±0.86, 4.21 μm±1.63, and 4.00 μm±1.07, respectively. The three of these treatment groups had smaller Bowman's space diameter than K treatment group. It means the administration of *C. vulgaris* extract as antioxidant is capable of protecting kidney from damage.

Statistical analysis using ANOVA proved that administration of *C. vulgaris* extract produce significant effect on Bowman's space diameter with $p<0.05$. Further analysis using HSD Tukey (Table 2) showed administration of 3 mg/100 g

BW *C. vulgaris* extract on P1 treatment group was not capable to produce significant effect toward Bowman's space diameter because of its notation value is similar to K- treatment group notation value ($p>0.05$). Notation value on P2 and P3 treatment group proved that administration of 4 mg/100 g BW and 5 mg/100 g BW of *C. vulgaris* extract was capable to produce significant effect on Bowman's space diameters, which was shown by significant difference of P2 and P3 treatment group's notation value with K- treatment group.

According to the result of observation and calculation, we found that the increase of tubule cell damage percentage was reciprocal to Bowman's space diameter. Dilatation of Bowman's space was followed by decrease of glomerular diameter. However, this study was not capable to provide the actual reason, which could explained the relationship between these two parameters, using histological observation only. Therefore, we strongly recommended to perform further study about urea, creatinine, and renal malondialdehyde (MDA) on serum, which are generally used to assess renal function (Ogeturk, 2005).

The reason behind the relationship between tubule cell damage and Bowman's space has been reported on several studies. The tubule cell damage interrupts reabsorption process and slows urinary flow from renal corpuscle to tubule cell. Furthermore, renal corpuscle size is increasing due to urinary accumulation on Bowman's space. Another study reported that Bowman's space dilatation was caused by increase of Glomerular Filtration Rate (GFR) due to sodium reabsorption process and hyper filtration on glomerulus. In result, tubuloglomerular feedback will be reduced and Bowman's space will be dilated (Helal, 2012; and Henegar, 2001).

Result of histologic observation on P1, P2, and P3 treatment group showed that *C. vulgaris* extract was capable of protecting kidney from oxidative stress after administration of CCl_4, which is a nephrotoxic compound. These result was shown by tubule cell degeneration index and Bowman's space diameter. Observation result also proved that an increase of *C. vulgaris* extract dose was followed by a decrease of kidney damage due to CCl_4. This statement was supported by the fact that P3 treatment group had the smallest tubule cell degeneration index and Bowman's space diameter. This protective nature of *C. vulgaris* extract was reciprocal to a study by Blas-Valdivia *et al.* (2011), which proved that administration of *C. vulgaris* extract capable of reducing cell damage and cortical atrophy due to $HgCl_2$. Not only protecting kidney from nephrotoxic compounds, *C. vulgaris* also capable of protecting kidney damage due to diabetes mellitus (Aizzat, 2010).

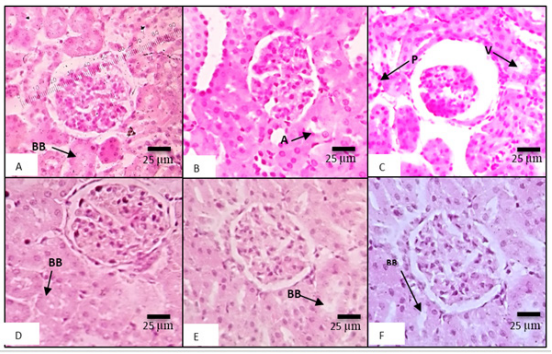

Figure 1. Micrograph Picture of Wistar Rat's Kidney after Administration of *C. vulgaris* Extract and Inducted by CCl_4 on 400 x magnification (Haematoxylin-Eosin Stain). Figure A from K+ and B from KS treatment group showed normal Brush Border (BB), glomerulus (G), Bowman's space (BS). Figure C showed picnotic of nucleus (P), vacuolation (V), diminished brush border, and dilatation of Bowman's space on K- treatment group. Treatment group of P1 (D), P2 (E), and P3 (F) showed normal condition.

The ability of *C. vulgaris* extract to provide protection against oxidative stress and free radical is related to antioxidant compound inside it. Several antioxidants in this extract are lutein, a-carotene, b-carotene, ascorbic acid, chlorophyll, and tocopherol (Cha *et al.*, 2010). According to a study by Blas-Vladivia (2011), the most important antioxidant, which had a role in preventing damage due to free radicals, is carotene. This compound can remove single oxygen and peroxide compounds through redox reaction (Jian-Ming *et al.*, 2010). The results of this study strongly support further research on immunostimulant content test *C. vulgaris* and determine the efficient dose for representative animals mammals also in humans.

CONCLUSION

According to result and discussion, can be concluded that the administration of *C. vulgaris* extract can protect white wistar rat (*Rattus norvegicus*) kidney from damage, which were picnotic cell vacuolation, brush border damage, and Bowman's space broadening due to CCl_4 exposure. The most effective dose of *C. vulgaris* extract, which gave protection for kidney was 5 mg/100g BW.

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