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In Vitro Study of Antioxidant Activity of Carboxymethyl Chitosan derived from Silkworm (*Bombyx mori* L.) Pupa against Human Plasma Lipid Peroxidation

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ABSTRACT

The study was aimed to evaluate the lipid peroxides blockage capability of carboxymethyl chitosan (CMCTS) from silkworm pupa. It was an experimental study. CMCTS was produced from silkworm pupa. CMCTS of silkworm pupa was subjected to be analysed the toxicity through Brine Shrimp Lethality (BSL) assay, antioxidant activity through DPPH assay, and lipid peroxides inhibitory capacity in human plasma through TBARS assay. The result of our screening indicated that 24 h of treatment of CMCTS had a low impact on the percentage of *Artemia salina* L. nauplii lethality. CMCTS of silkworm pupa provided a moderate antioxidant activity and a significant inhibitory effect on the lipid peroxide level of human plasma at a concentration of more than or equal to 10 mM. These results proved that CMCTS of silkworm pupa was non-toxic substance also can be used as an alternative antioxidant source.

Keywords: *Bombyx Mori* L.; Carboxymethyl Chitosan; Antioxidant; Lipid Peroxides

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INTRODUCTION

Non-communicable diseases, e.g., cardiovascular and cancer cases, are increasing every year. (WHO 2015), stated that the incidence of world cancer in 2012 reached approximately 14 million cases and was predicted to grow 70 % in 2023? The rate of cardiovascular disease in the world reached about 17.5 million cases each year. Oxidative stress is responsible for those non-communicable diseases as it has a prominent role in non-communicable disease development (Pham-Huy et al., 2008). Oxidative stress will be occurred when the antioxidants and ROS (Reactive Oxygen Species) balance disturbed by the declining number of antioxidants and ROS accumulation in the body. One way to slow it down is by providing antioxidants for the body.

Availability of the body's antioxidant formed as enzymatic-antioxidants (naturally provided by the body) and non-enzymatic antioxidants (obtained from food intake) is known to work effectively in blocking the adverse effects of ROS (Birben et al., 2012). Due to the importance of non-enzymatic-antioxidant, many studies about bioactive substances related to antioxidants against the oxidation process were carried out. Those generally identified in the form of phenolics or alkaloids compound which commonly found in the plant parts such as stem or leaves (Masriani et al., 2020). However, there is one compound that can be obtained from the waste, namely carboxymethyl chitosan (CMCTS), which is a chitosan derivate. Naturally, chitosan is available as polysaccharide contains cationic monomer d-glucosamine bound to β (1 → 4) glycosidic linkages. Improving the solubility in water and increase the use of chitosan, a chemical modification made to obtain a variety of other functional substances. CMCTS is one crucial type of chitosan derivatives (Sun et al., 2008). Some studies showed many benefits provided by chitosan and its derivatives against health, such as antioxidants, anti-hypertensive, and anti-cancer (Ngo et al., 2015). However, the investigation on the capacity of CMCTS produced from silkworm pupa against the health still rarely done.

Mostly, CMCTS production is derived from crustaceans' shells (Yang et al., 2000). In fact, as a waste of the silk industry, pupa of silkworm is also an alternative source of chitosan as well as CMCTS (Zhang et al., 2000; Zhu and Zhang, 2016). A study showed that the degree of deacetylation chitosan of silkworm pupa produced in the laboratory scale reached 84 % (Kusharto and Koesharto, 2012). Production of silkworm cocoons in Wajo - South Sulawesi, Indonesia (a potentially silk fabric production in Indonesia) reaches 37 tons per year (Kusharto et al., 2013). Average chitosan can be produced by 2.40 % dry weight of *Bombyx mori L. multivoltine pure races* pupa (Suresh et al., 2012). Therefore, it is estimated that chitosan produced from silk manufacture wastes in Wajo, Indonesia can be reached 711 kg per year. It indicates that the waste of the silk industry is very potential to be reused as a source of CMCTS, with the result that reducing the number of disposed of wastes to the ecosystem. This research aimed to analyse the potency of silkworm pupa as a source of antioxidants CMCTS and its inhibitory capability on lipid peroxidation by *in vitro* assay using human plasma.

Materials and Methods

Carboxymethyl chitosan preparation

Silkworm pupa was gathered from the central silk-reeling industry in Pati, Jawa Tengah, Indonesia. This experimental study was a continuation of previous research (Fadly et al., 2017).

CMCTS was prepared from chitosan of silkworm pupa by processing in alkalization and continued into carboxymethylation. The alkalization process was conducted by dissolving 10 g of chitosan derived from a cocoon into 100 mL of isopropyl alcohol 40 ℃, stirred up for 30 minutes, followed by adding about 12 mL 10 M NaOH into the solutions, stirred up for 90 minutes. Afterward, subjected to the process of carboxymethylation by addition of 30 g monochloroacetic acid (divided into four parts) within 5 minutes, boiling at 60 ℃ for 4 hours and finished by washing using 96% ethanol and drying at 50 ℃ in an oven (Xue et al., 2009).

Copolymer characterization

Fourier Transform Infrared (FT-IR) was used in the ccharacterization of copolymer CMCTS. Initially, the dried sample obtained from the previous stage was pressed with spectroscopy grade KBr (Potassium Bromide pellets), and FT-IR was recording the FT-IR spectra within the range of 400 – 4000 cm^{-1}.

Toxicity evaluation

The toxicological evaluation was carried out through Brine Shrimp Lethality (BSL) assay (Meyer et al., 1982). It is begun by hatching *Artemia salina* L. eggs in a beaker glass 1 L containing seawater and placed under 40 watts fluorescent lights with aeration. After 48 hours, the eggs hatched into nauplii and ready for the test.

Preparing 2000 ppm sample stock solution (40 mg CMCTS in 20 mL seawater). Filled each vial with 1 mL of seawater containing ten nauplii. Sample stock solution and seawater were added to those vials; therefore, the solution concentration becomes ten ppm, 100 ppm, 500 ppm, and 1000 ppm, left to stand for 24 hours. As a control, used 4 mL of seawater, which contains 10 – 12 nauplii without sample stock addition. The mean percentage of nauplii mortality were plotted against the logarithm of CMCTS concentrations. Half maximal lethality concentration (LC_{50}) was determined by the antilogarithms linear equation calculation acquired from the curve of the relation between the CMCTS level and the mean percent mortality of nauplii.

Antioxidant activity determined by DPPH assay

The determination of antioxidant activity was conducted through DPPH (1,1-Diphenyl-2-Picrylhydrazil) assay (Molyneux, 2004). Prepared 0.4 mM DPPH solution (16 mg DPPH (MW = 394.33) into 100 mL methanol p.a, homogenized, keep in dark bottle and avoid the light exposures). Prepared blank solution (1 mL of 0.4 mM DPPH into 5 mL volumetric flask, add methanol p.a until the borderline of the volumetric flask, homogenized). The sample test solution was prepared by dissolving 10 mg sample into methanol p.a up to volume 10 mL (1000 ppm) in the closed flask, mix well-using shaker for 2 hours, separate the filtrate and residue of the sample using centrifuge 4000 rpm for 15 minutes, do the separation for many times until the filtrate totally transparent, and concentrated the filtrate. Added methanol p.a to the concentrated filtrate until volume 5 mL. Put 20 µL filtrate into a test tube, add a 1 mL solution of DPPH, and add deionized water up to 5 mL. Incubated at 37 ℃ for 30 minutes, avoid light exposure (darkroom). The wavelength absorbance was measured at a λ 516 nm of UV/vis spectrophotometer. IC_{50} was calculated by linear equations of the percent inhibition curve.

Percent inhibitory effectiveness (%) = $(1\text{-}A_{\text{sample }516\text{nm}}/A_{control\ 516\text{nm}}) \times 100$

Lipid Peroxide of human plasma determined by TBARS assay

The lipid peroxide blockage capability was determined by in vitro studies using human plasma through *Thiobarbituric Acid Reactive Species* (TBARS) level test (Anderson et al., 2001; Grzegorczyk-Karolak et al., 2015).

Blood plasma samples were collected from 5 subjects. The subjects must be met the prerequisite (Anderson et al., 2001; Dietrich-Muszalska and Kwiatkowska, 2014), i.e., female or male 25 -35 years old; healthy; not in medication or consuming pills or drugs; has normal blood glucose and lipid profiles (total cholesterols, triglycerides, LDL, and HDL); has normal body mass index; do not consume antioxidant supplement within the last month; do not smoke; all subjects might be from the same social-economic level. The study obtained approval by The Ethics Committee of the Faculty of Medicine, University of Indonesia, Indonesia. A signed informed consent form was obtained from all subjects after informing them about the study.

Blood taken from subjects are put into tube contain sodium citrate (up to 5 mmol/L). The blood was then centrifuged at 3000 rpm for about 15 minutes to get the plasma. About 100 µL plasma was incubated with CMCTS added (0 mM, 5 mM, 10 mM, 20 mM, and 40 mM) at 37 ℃ for 15 minutes. Afterward, all incubated plasma samples were added 2 mM of H_2O_2 at 37 ℃ for 15 minutes.

Plasma samples incubation was stopped after 15 minutes by decreased the temperature in an ice bath. Plasma samples then mixed with 15 % (w/v) cold TCA in 0.25 M HCl and 0.37 % TBA in 0.25 M HCl and submerged in boiling water (± 95 ℃). Furthermore, samples were centrifuged at 1200 rpm for 15 minutes, and the absorbencies of supernatants were measured at λ 532 nm of UV/Vis spectrophotometer.

Statistical analysis

The results were managed and processed using Microsoft Excel for Windows. Lipid peroxide inhibitory ability was statistically analyzed by SPSS System for Windows V 20.0. Analysis of variance (p = 0.05) and the means separated by Duncan's multiple range test was conducted.

Results and discussion

The utilization of silkworm had been developed since the silk road unify China and India. People use silkworm to obtain the based substances of silk, which has high value economically. In the process of silk manufacture, pupa as the waste of it turns to be an environmental problem. This part of cocoon discarded and causes a bad odor, disturb the environment and society pleasures. Hence, a waste handling mechanism is required further to enhance the utilization of pupa and decrease the number the disposal.

Characterization of copolymer

Most CMCTS was produced from crustacean's shell, whereas silkworm pupa might be a great source of it. The FT-IR spectra proved that carboxymethylation in CMCTS was successfully done. It was identified clearly by C=O, C-O, and C-O-C groups (Meyer et al., 1982; Zamani et al., 2010).

According to FT-IR spectra, which was obtained (Figure 1), the C-O-C group absorption signal of the secondary

hydroxyl group has been shifted to 1065.33 cm^{-1}. The band stretched at 1254.11 cm^{-1} indicated the C-O group. Stretching vibration at 1631,72 cm^{-1} indicated amine groups (-NH_2). The asymmetrical stretched attributed at 1740.44 cm^{-1} representing the carboxylate group (C=O). Furthermore, the band stretched at 3417.42 cm^{-1} assigned to O-H and N-H group.

Toxicity evaluation

Toxicity evaluation was conducted by BSL assay using *Artemia salina* L. as the subjects. After 72 hours of observation, the average number of *Artemia salina* L. mortality was increasing along with the increase of CMCTS concentrations (µg/mL) applied. There were four CMCTS concentration applied (Figure 2) i.e. 50 µg/mL CMCTS led 6.70 % mortalities; 100 µg/mL CMCTS led 13.30 % mortalities; 500 µg/mL led 53.30 % mortalities; and 1000 µg/mL led 73.30 % mortalities. The toxicity of CMCTS was expressed in an LC_{50} value of about 1139.50 µg/mL.

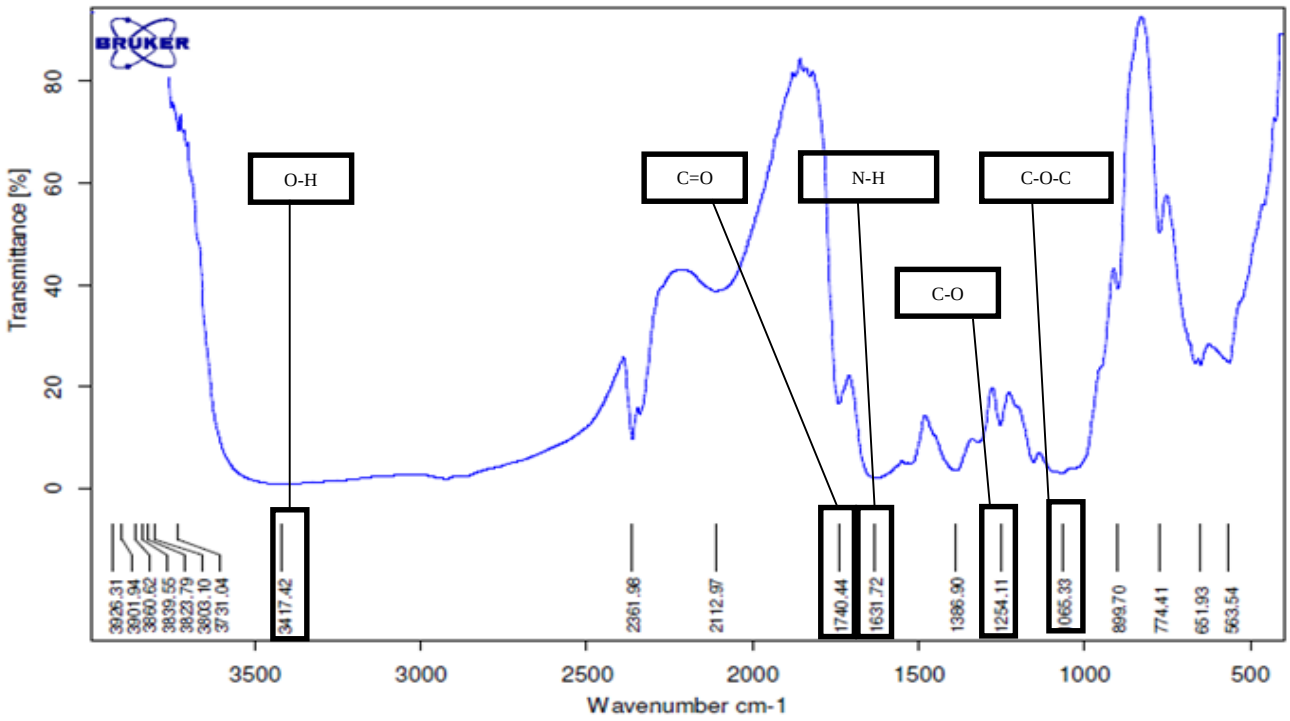

Figure 1. FT-IR Spectra of Carboxymethyl Chitosan Copolymer of Silkworm Pupa

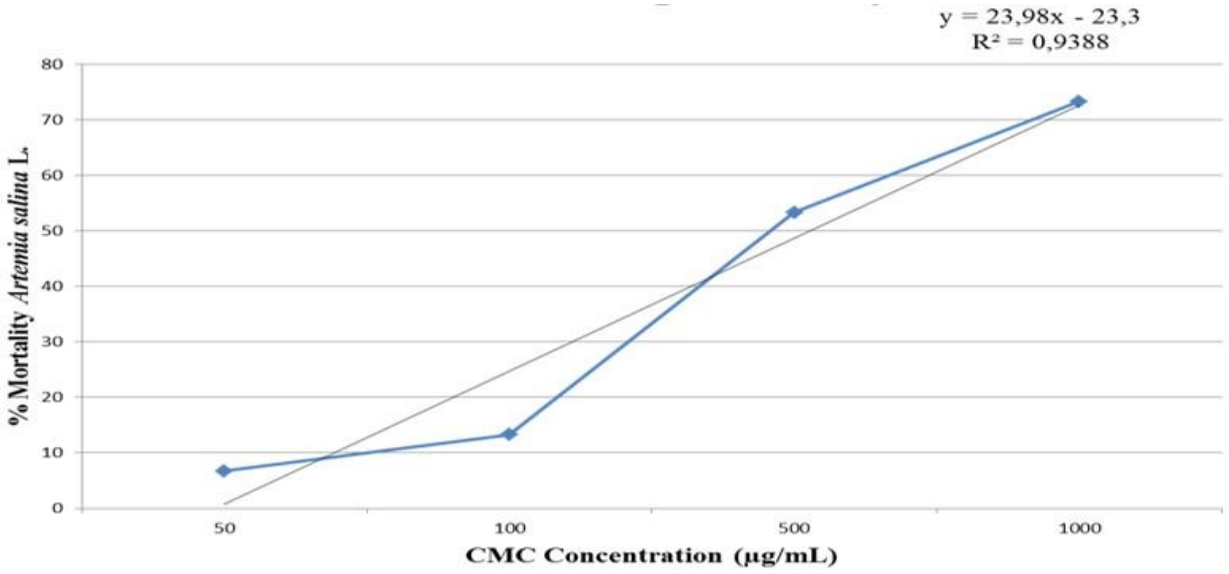

Figure 2. The average number of *Artemia salina* L nauplii mortalities.

Referring to (Clarkson et al. 2004) and (Meyer et al. 1982), that LC_{50} value about ≥ 1000 µg/mL is categorized as non-toxic. Since CMCTS derived from silkworm pupa is categorized as a non-toxic substance, it is quite safe to be used as a biomedical treatment or as a food ingredient, due to the use of *Artemia salina* L. that has a very high sensitivity toward toxic with the result that BSL assay has confidence level about 95 % (Carballo et al., 2002; Meyer et al., 1982).

Antioxidant activity

Antioxidant activity was determined by the DPPH assay. The average number of free radical neutralized by CMCTS was increasing along with the increase of CMCTS concentration was applied (Figure 3). The number of free radical neutralized about 15.08 % at the level CMCTS of 2.5 mM (0.62 mg/mL); 17.03 % free radical neutralized at the level CMCTS of 5 mM (1.25 mg/mL); 21.19 % free radical neutralized at the level CMCTS of 10 mM (2.49 mg/mL); 26.89 % free radical neutralized at the level CMCTS of 20 mM (4.98 mg/mL); and 34.39 % free radical neutralized at the level CMCTS of 40 mM (9.96 mg/mL).

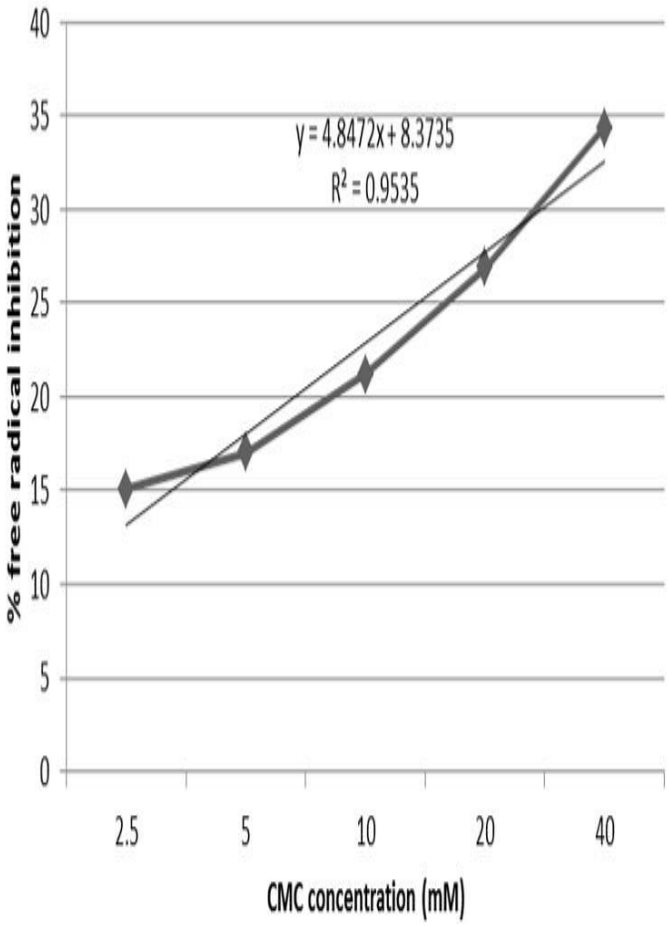

Figure 3. Free radical inhibition

Result of the observation shown that CMCTS of silkworm pupa had an IC_{50} value of about 2.14 mg/mL. Referring to (Omisore et al., 2005), CMCTS produced from silkworm pupa is classified as a moderate antioxidant. Antioxidant activity may be measured by DPPH free radicals scavenging effect. DPPH is a component containing free radicals that will capture the proton of the other free radicals. Antioxidants are the scavenging effect of DPPH free radicals triggered by the ability of antioxidants to donate H^+. CMCTS has the number of H^+ more than chitosan that will improve the amount of H^+ donations. Therefore, CMCTS has a higher antioxidant activity than chitosan (Zhao et al., 2011). In addition, the scavenging activity is closely related to the energy of unbound O-H or N-H and radical stability formation. Chitosan, which is the basic molecules before it is produced into CMCTS, has a firm intra-molecular and inter-molecular hydrogen bonds. In the form of chitosan, OH and NH_2 were challenging to remove and react to ˙OH. As a result, CMCTS has better antioxidant activity than chitosan (Alexandrova et al., 1999).

Lipid peroxide blockage in human plasma

In vitro analysis of the antioxidant activity of CMCTS against lipid peroxide in human plasma was determined by applying CMCTS with concentration were 0 µL (control), 10 µL, 20 µL, and 40 µL to the plasma. The observation was conducted through the exposure of CMCTS to the lipid peroxide in the human plasma of 5 chosen subjects. The subject had characteristics that met the qualification needed (Table 1.). The results of the observation of lipid peroxides in human plasma after CMCTS exposure are presented in Figure 4.

In this study, lipid peroxide inhibition on human plasma by in vitro observation had a very significant differences ($p<0.01$) between control (0 mM CMCTS) and 10 mM CMCTS; between 0 mM CMCTS and 20 mM CMCTS; between 0 mM CMCTS and 40 mM CMCTS. Lipid peroxide inhibition in human plasma had no significant differences ($p>0.01$) between 10 mM CMCTS and 20 mM CMCTS; 10 mM CMCTS and 40 mM; and 20 mM CMCTS and 40 mM CMCTS. Based on the observation, the dose improvement of CMCTS will decrease the lipid peroxide in human plasma.

In the control group, the number of lipid peroxides in human plasma was 1.263 ± 0.028 nmol/mL CMCTS. In the group of 10 µL CMCTS, the amount of lipid peroxide in human plasma was 1.236 ± 0.024 nmol/mL or 2.18 % lower than the control group. In the group of 20 µL CMCTS, the number of lipid peroxides in human plasma was 1.226 ± 0.016 nmol/mL or 2.94 % lower than the control group. In the group of 40 µL CMCTS, the amount of lipid peroxides human plasma was 1.219 ± 0.16 nmol/mL or 3.53 % lower than the control group.

The higher concentration of CMCTS may decrease more lipid peroxides. In the control group, the number of lipid peroxides in human plasma was 1.263 ± 0.028 nmol/mL CMCTS. In the group of 10 µL CMCTS, the amount of lipid peroxide in human plasma was 1.236 ± 0.024 nmol/mL or 2.18 % lower than the control group. In the group of 20 µL CMCTS, the number of lipid peroxides in human plasma was 1.226 ± 0.016 nmol/mL or 2.94 % lower than the control group. In the group of 40 µL CMCTS, the amount of lipid peroxides human plasma was 1.219 ± 0.16 nmol/mL or 3.53 % lower than the control group.

Table 1. Characteristics of Subjects

Subject Characteristics			
Sex		: Male, n = 3	
		: Female n = 2	
Age		: 25 - 32 years	
Last education level		: undergraduate	
Current job		: Students	
Monthly food expense		: USD 62.37 - 77.86	
Monthly non-food expense		: USD 29.70 – 96.57	
Smoke		: No	
Drugs / medicinal usage and consumption		: No	
Supplement / antioxidant consumption		: No	
Health characteristics		**Value**	**Category**
Body mass index (kg/m^2)		18.51 – 22.99	Normal
Blood parameter check up	Total cholesterol (mg/dL)	174 – 182	Normal
	HDL (mg/dL)	38 – 41	Normal
	LDL (mg/dL)	113 – 117	Normal
	Trigliceride (mg/dL)	120 – 128	Normal
	Blood glucose (mg/dL)	89 – 97	Normal
Specific illness	Heart disease	No	Healthy
	Diabetes mellitus	No	Healthy
	Urinary tract disease	No	Healthy
	Hypertension	No	Healthy
	Stroke	No	Healthy
	Cancer	No	Healthy
	TBC	No	Healthy
	Alzheimer	No	Healthy
	Gout	No	Healthy

The reaction mechanism in free radicals consists of initiation, propagation, and termination phases. The initiation phase is the stage where free radicals are formed. Free radicals are formed, which need a lot of energy. Therefore, oxygen, heat, UV radiation, and metal are required as a catalyst. The propagation phase is the stage where the bonding occurs at free radicals because it reacts with other stable molecules to form new free radicals. This phase continues and causes the formation of more free radicals. The last phase is the termination phase, which is the stage where two free radical molecules react with each other to form a stable (non-radical) molecule. But this is very rare because of the low number of radical species and the little possibility of two radicals colliding with each other. The CMCTS may decrease the lipid peroxide in human plasma.

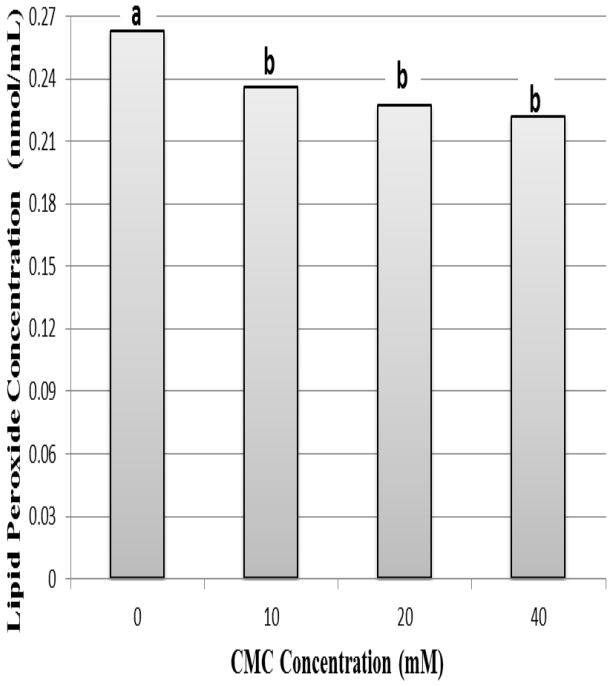

Figure 4. Lipid peroxide inhibition on human plasma

Commonly, the antioxidants in foods interfered the lipid auto-oxidation through a quick hydrogen atoms donation to lipid-free radical (Boskou and Elmadfa, 2019). In the analysis of TBARS levels, the addition of antioxidants will stop the propagation stage since the radical antioxidants have low energy and do not react with the other antioxidants radicals to form a stable molecule (Lobo et al., 2010). Improving the concentration of CMCTS would decline the number of lipid peroxides in human plasma. The CMCTS of silkworm pupa had a significant impact on human plasma lipid peroxides based on in vitro study. CMCTS had a substantial influence at the level of ≥ 20 µL. The reduction effect of CMCTS to the number of lipid peroxides was associated with the ability of CMCTS as an antioxidant. CMCTS, which has a high number of H^+ will become a donor of H^+ to the radical hydrogen ions without altering the antioxidant into reactive radicals after donation.

Conclusion

The result of our screening indicated that the CMCTS of silkworm pupa is a non-toxic substance with an LC_{50} value was 1139.50 µg/mL CMCTS. Antioxidant activity of CMCTS was expressed in half maximal inhibitory concentration, with an IC_{50} value of 2.14 mg/mL CMCTS and classified as a moderate antioxidant. CMCTS of silkworm pupa provided a significant inhibitory effect on the lipid peroxide level of human plasma at a concentration ≥ 20 µL.

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