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Effects of Kersen Leaves Extract (*Muntingia calabura* L.) on SGOT and SGPT Levels of Soft Drink Induced Mice

Muhammad Nuzul Azhim Ash Siddiq[1]*, Sri Anna Marliyati[1], Hadi Riyadi[1], Wiwin Winarsih[2]
[1]Department of Community Nutrition, Faculty of Human Ecology, IPB University, Bogor 16680, Indonesia
[2]Department Veterinary Clinic Reproduction and Pathology, Faculty of Veterinary Medicine, IPB University, Bogor 16680, Indonesia

ABSTRACT

This study aimed to analyse the antioxidant activity, total phenols, and flavonoids of kersen leaves (*Muntingia calabura* L.) extract (KLE) and determine the hepatoprotective activity of KLE in soft drink induced mice. Soft drinks induction causes liver injuries which increases the serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) levels. Mice were divided into five treatment groups (n=5) and were given Tween 80 (1%) (normal control), Tween 80 (1%) and 7 ml soft drinks (negative control), KLE (1000, 1500, and 2000 mg/kg), and 7 ml soft drinks per oral once daily for 4 weeks. Antioxidant activity was measured by 2.2-diphenyl-1-picrylhydrazyl (DPPH) method. Total phenols and flavonoids were analyzed by Folin-ciocalteu colorimetric and aluminium chloride ($AlCl_3$) method. The blood samples were collected for biochemical analysis. The result showed that the KLE antioxidant activity were 11.11$\pm$0.50 ppm. Total phenols and flavonoids detected in the KLE samples were 2.19$\pm$0.12 mgGAE/g and 2.43$\pm$0.24 mgQE/g respectively. Mice treated with KLE had a significantly lower ($p<0.05$) serum SGOT and SGPT levels than negative control group. In conclusion, KLE has a potential hepatoprotective activity that works in synergy with its antioxidant activity.

Keywords: antioxidant activity, *Muntingia calabura* L., SGOT, SGPT, soft drink

INTRODUCTION

Soft drinks consumption has increased worldwide in the past three decades (Nielson & Popkin 2004). The consumption of western food categorised as processed foods and sugary drinks is closely related to the incidence of obesity, metabolic syndrome, and non-alcoholic fatty liver disease (NAFLD) (Longoto 2013). Long term soft drinks consumption can lead to increase in oxidative stress by elevating the production of reactive oxygen species (ROS) this may cause hepatic toxicity. Enhancement of ROS production is indicated by the increase in malondialdehyde (MDA) and the decrease in glutathione peroxidase (GSH-Px), glutathione reductase (GSH-R), and catalase levels, as well as the decrease in the mRNA expression levels of glutathione s-transferase (GST) and superoxide dismutase (SOD) which play an important role as endogenous antioxidants (Alkhedaide *et al.* 2016). In addition, excessive soft drinks consumption cause liver injuries that are followed by the increase in the activities of GPT and GOT (Jeroh *et al.* 2012) and resulting in an imbalance of antioxidants in the body.

Antioxidants are compounds which able to inhibit the oxidation by scavenging free radicals and highly reactive molecules that causes cell damage (Winarsi 2007). Imbalances of endogenous antioxidants require antioxidants from the outside (exogenous antioxidants). Exogenous antioxidants can be derived from naturally occurring or synthetic. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are examples of synthetic exogenous antioxidants. However, synthetic antioxidants are reported to have hepatotoxic and carsinogenic side effects. Thus utilization of natural antioxidants as a much safer alternatives is imperative. Various studies have been done on the effects of antioxidants and phytochemicals compounds extracted from various plants. Many studies indicated a reverse relationship between the antioxidant-rich plant source foods and the incidence of human disease (Zeng *et al.* 2014).

Muntingia calabura L. or locally known as *'Kersen'* or *'Talok'* is one of the common

***Corresponding Author:** tel: +6287781068148, email: nuzulazhim@gmail.com

plants available in Indonesia. The utilization of *Muntingia calabura* L. plants in Indonesia is still very rare. Many studies have proven the benefits of *kersen* leaves scientifically. Sufian *et al.* (2013), showed that Kersen Leaf Extarct (KLE) was able to act as antibacterial. Various dosage KLE shows cardioprotective effect, antidiabetic effect, antiproliferation, anti-inflammatory, and high antioxidant activity (Nivethetha *et al.* 2009; Sridhar *et al.* 2011; Zakaria *et al.* 2011; Balan *et al.* 2015). The role of KLE as a hepatoprotective agent had been reported in the Mahmood *et al.* (2014a), which indicated methanol extract of kersen leaves potentially decreased the liver damage and significantly lowered serum level hepatic enzymes SGOT and SGPT of rats induced by paracetamol. Research conducted by Murti *et al.* (2016) showed that administration of KLE with a dose of 500 mg/kgBW/day for 30 days in rats induced by soft drink with a dose of 50 ml/day showed significantly decreased in liver damage shown in miscroscopic examination.

This study was conducted to help reduce oxidative stress in the liver caused by soft drink consumption. The lack of utilization of kersen leaves, though it has been known as hepatoprotective agent, entices researcher to optimize its role. Therefore, the purpose of this study was to analyse antioxidant activities various dosage of KLE (*Muntingia calabura* L.) and examine the effect of KLE on SGOT and SGPT levels in mice induced by soft drink.

METHODS

Design, location, and time

The study design was a completely randomized design (CRD) using male mice as the experimental animals. Mice were divided into five treatments (n=5) and given Tween 80 (1%) (normal control, N), Tween 80 (1%) and 7 ml soft drinks (negative control, CN), Tween 80 (1%) and KLE (1000 mg/kg(A), 1500 mg/kg(B), and 2000(C) mg/kg) and 7 ml soft drinks per oral once daily for 4 weeks. This research was held from January until June 2018.

Materials and tools

The material used in this study was *kersen* leaves (*Muntingia calabura* L.) obtained in Cikarawang Village, Bogor Regency, West Java. The number of experimental animals was determined using the Federer formula: (t-1) (r-1) ≥15. Based on the formula, the sample size used in this study was 25 healthy male mice *Mus musculus*, DDY strain (4-5 weeks old; weight, 25-30 g). All mice were obtained from Indonesia National Agency of Drug and Food Control.

The solvents for extraction was water, while dymethyl sulfoxide (DMSO) and 2.2-diphenyl-1-picrylhydrazyl (DPPH) 125 μM were used to analyze antioxidant activity. Folin-Ciocalteu 10%, NaOH 1%, gallic acid standard solution were used to calculate total phenols. Acetone, hexamethylenetetramine (HMT), HCl, $AlCl_3$, and quercetin were used for total flavonoid assay. SGOT and SGPT serum levels were analyzed using commercial kits, based on the manufacturer's protocol.

The equipments used for *kersen* leaves extraction, antioxidant activity analysis, SGOT and SGPT serum level analysis were oven, magnetic stirrer Bellco glass.inc (Vineland, USA), ultrasonic bath (Branson 2200), vacuum evaporator, glass (Pyrex®), filter paper Whatman, micro pipet 1000 μL and 10-100 μL, reaction tube (Schott Duran), centrifuge (Kokusan, Japan), spectrophotometry u-2800, Clinical Chemistry Analyzer SELECTRA JUNIOR.

Material and equipment for animal maintenance were individual cage with cage area 33.5 x 27 x 12 cm, husk, drink bottle, digital scales, standard fed, Coca-cola, stainless-steel feeding needles & Tween 80 [1%v / v] for oral administration of KLE. Tween 80 is an especially attractive nonionic surfactant, non-toxic, environmental friendly, biocompatible, and commercially inexpensive (Kerwin 2008). Materials and equipment for blood and surgery at the end of the intervention are xylazine (0.2 ml) and ketamine (0.6 ml), hypodermic needle, eppendorf tube, centrifuge, scalpel, surgical board, hand gloves, antiseptic soap, and masks.

Phytochemical analysis was performed at the Biopharmaca Research Center Laboratory, IPB University. The mice were kept in the Animal Management Unit Laboratory, Faculty of Veterinary Medicine, IPB University. SGOT and SGPT serum levels were performed at the Regional Health Laboratory Bogor City. This research has obtained ethical approval from animal ethics committee FKH IPB (Faculty of Veterinary Medicine, Bogor Agricultural University) with the certificate number 088/KEH/SKE/III/2018. KLE were made at Food Chemistry and Nutrient Analysis Laboratory Department of Community Nutrition, Faculty of Human Ecology and SEAFAST Center IPB University.

Procedures

The research stages begun with the preparation of KLE and phytochemical analysis (antioxidant activity, total phenols, and flavonoids), followed by the administration of KLE and observation of its effects on animal experiments. The last stage was SGOT and SGPT serum levels analysis.

Kersen extraction and preparation. The leaves were washed using flowing water for three times and followed by aquadest for one time, then drained. Next, the leaves were dried by using oven ($\pm 40^oC$) for 7 days and processed into powder. The powder dissolved in water solvent with ratio 1:20. Subsequently, they were treated using ultrasound bath (Branson 2200) for 30 minute. Treated solutions were filtered by Whatman No. 41 and evaporated using vacuum evaporator to produce a dry extract.

Antioxidant Activity Assay. DPPH method was used to examine antioxidant activity (Salazar-Aranda *et al.* 2011). Dry extract of *kersen* leaves was re-dissolved in DMSO then homogenized using vortex homogenizer. Samples were mixed with DPPH 125 μM in micro plate at different concentration (3.90-250 ppm). The mixtures were homogenized at room temperature in darkness for 30 min. The absorbance was measured at 517 nm in Elisa micro plate reader (BioTek Instruments).

Total Phenol Assay. Total phenols were determined using Folin-Ciocalteu. Dry extract of *kersen* leaves was redissolved in methanol then mixed with Folin Ciocalteu 10%. The mixtures were kept at room temperature for eight minutes then added by NaOH 1%. They were kept in dark condition for one hour. The absorbance was measured at 730 nm in spectrophotometer (U-2800). Gallic acid was used as standard solution.

Total Flavonoid Assay. Total flavonoids were measured using $AlCl_3$ method. Samples were hydrolyzed by mixed with acetone, HMT, and HCl for 30 min. The solutions were filtered and the filtrate was separated using acetone to produce ethyl acetate fraction. $AlCl_3$ was then added and the solution was kept for 30 min at room temperature in dark conditions. The absorbance was read at 425 nm in spectrophotometer (U-2800). Quercetin was used as the standard solution.

SGOT and SGPT Serum Analysis. Blood sampling was done via intracardial syringe as much as 2 ml. After centrifugation at 3000 rpm for 20 min, blood serum were stored in eppendorf tube at 2 °C-4 °C until assays were performed. The serum SGOT and SGPT level were estimated using automated chemistry analyzer (Selectra JR). The operational procedures of the Selectra JR with each biochemical parameter were performed in accordance with the standard operating procedure. Since the standard method of collection are imperative if research testing results are mean to be translated to a clinical and diagnostic tests, the treatment and control samples were handled in the exact same treatment throughout the entire analytical process from study design and collection of samples to data analysis.

Intervention of KLE and Soft Drink. KLE were administered in experimental animals for 4 weeks. Oral feeding of KLE performed with oral gavage feeding needles for mice. The extract was dissolved in Tween 80 (1%) solution as an emulsifier for oral administration. The N treatment (normal control) was given only Tween 80 (1%). Soft drinks was given daily from 4 pm to 8 am or 60 minutes after oral feeding of KLE and replaced to standard water at 8 am every day. CO_2 bubles formed in soft drink were removed by shaking the bottles vigorously and left the bottles opened for 60 min. In the N treatment standard drink was given ad libitum, whilst the CN treatment (control negative) was given soft drinks 7 ml per day at 4 pm to 8 am and standard water at 8 am every day.

Data analysis

Data was processed and analyzed using Microsoft Excel 2010 and SPSS software version 21.0 for windows. The data were presented as the mean ± standard error of the mean. Analysis of Variance (ANOVA) was used to analyze differences between groups and followed by further analysis with Duncan Multiple Range Test (DMRT). Pre-test and post-test data of results were assessed by paired sample t-test to analyzed differences data between before and after intervention. $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS AND DISCUSSION

Antioxidant activity, total phenolic and flavonoid of *kersen* leaves extract (KLE)

Kersen plants (*Muntingia calabura* L.) contain antioxidants and active components. The antioxidant capacity of each part of *kersen* plant determined using 2.2-diphenyl-1- picrylhydrazyl (DPPH) were flower (87%), leaf (63%), raw fruit (34%), ripe fruit (30%), and stem (35%) (Singh *et al.* 2017). The previous research has shown

that aqueous extract of *kersen* leaves contain flavonoid, saponins, tannins, steroids, and triterpenes (Zakaria *et al.* 2007) and the flavonoid compounds can act as antioxidants, antibacterial, and anti-inflammatory agent (Arum *et al*. 2012). Tabel 1 shows the antioxidant activity, total phenolic and flavonoid of KLE.

The result of this study are in line with Adam *et al.* (2015) which showed antioxidant activity from water and methanol extract of *kersen* leaves were 22.28 ppm and 23.28 ppm respectively. Studies conducted by Siddiqua *et al.* (2010) also reported the radical scavenging activity of KLE with IC_{50} value of 22 ppm, compared to ascorbic acid, as reference drug and produced an IC_{50} value of 12 ppm. Both previous studies corroborates the results found in this study.

Total phenol and total flavonoids are important components for the antioxidant activity of *kersen* leaves. Total content of phenol obtained from this study was lower than the results of other studies conducted by Zakaria *et al.* (2011), Bodke *et al.* (2013) and Balan *et al.* (2015) which equal to 29.7±6.6 mg GAE/g, 25.00±2.52 mgGAE/g, and 4.13 mgGAE/g, respectively. Total flavonoid levels in this study was lower than Bodke *et al.* (2013) using petroleum ether, chloroform, ethanol and aqueous extract of *kersen* leaves that equal to 17.93±0.89 mgQE/g, 47.08±0.68 mgQE/g, 123.31±0.54 mgQE/g, and 44.83±0.21 mgQE/g, respectively. Ramadas *et al.* (2015) stated that total flavonoids of KLE (42.61±1.02 mgQE/g) was higher when compared to *kersen* fruits extract and *kersen* root extract respectively, 14.34±0.01, and 21.71±0.31 mgQE/g.

Many factors influence quality of herbs and these include species variation, environmental conditions, and the time of harvesting, storage, and processing (Mahmood *et al.* 2014b). According to Borges *et al.* (2013), environmental factors such as soil composition, temperature, rainfall, and ultraviolet radiation can affect concentrations of phenol components including flavonoids. In addition, the solvent is an important factor in extracting phenolic and flavonoid components. Polar solvents such as methanol and water are able to extract the flavonoid component better than ethanol due to flavonoids in plant tissues in polar glycosides (Zuraida *et al.* 2017).

In this study, the result shows that total flavonoids was lower that total phenol. It was because insensitivity the calorimetric method used in measuring total flavonoids and total phenol. Calorimetric methods can not provide a specific compound for total phenol and total flavonoids in a plant (Amorati and Valgimigli 2015; Chang *et al*. 2002). Therefore, researchers strongly recommend to use more sensitive measurement methods such as HPLC method. Wu *et al.* (2006) stated that the HPLC method was the most accurate method for compound measurements.

Mice daily water intake during the intervention

Mice were given standard feed, standard water at 8 am-4 pm, and soft drinks at 4 pm-8 am (A, B, C, CN). Soft drinks were given throughout the night in order to let the mice drank the supposed dose of soft drinks, since mice tend to be active during night time. Standard feed was given ad libitum and not measured. Standard water and soft drink which given to the mice were measured daily. Table 2 shows the total daily water intake each treatment during intervention.

Table 1. Antioxidant activity, total phenolic and flavonoid of *kersen* leaves extract (KLE)

Phytochemical assay	Mean±SEM
Antioxidant activity (IC_{50})	11.11±0.50 ppm
Total phenolic	2.19±0.12 mgGAE/g
Total flavonoid	2.43±0.24 mgQE/g

SEM= standard error of the mean

Tabel 2. Total daily water intake each treatment (ml)

Treatment	8 am-4 pm	4 pm-8 am	Total
N	1.53±0.11[a]	3.53±0.41[d]	5.06±0.44[b]
CN	1.42±0.36[a]	4.25±0.51[cd]	5.67±0.48[b]
A	1.18±0.25[a]	5.46±0.68[bc]	6.63±0.89[ab]
B	1.18±0.35[a]	6.70±0.12[ab]	7.88±0.43[a]
C	1.14±0.20[a]	6.99±0.07[a]	8.13±0.19[a]

Values with different superscript letters in one column indicate the significant difference based on ANOVA test results ($p<0.05$)
N= normal control, CN= negative control, A= KLE 1000mg/kg, B= KLE 1500mg/kg, C= KLE 2000mg/kg.

The average total water intake of mice ranges from 5.06 to 8.13 ml per day (Table 2). Total water intake in this study were in line with the result of study conducted by Bachmanov *et al.* (2002) that showed the total water intake of mice was ranged from 3.9 to 8.2 ml per day. The total standard water consumed at 8 am-4 pm at each treatment showed no significant difference (p>0.05). However, different test results showed that the consumption of soft drinks in A, B, and C treatment were significantly higher than N treatment (p<0.05). Mice have the same perception of the sweet taste like human beings, so the intake of soft drink in A, B, C, and CN treatment were higher than standard water intake in N treatment at 4 pm-8 am (Bachmanov et al 2001).

Mice body weight during the intervention

Mice (*Mus musculus*) used in this study were healthy white male mice aged 4-5 weeks. The average initial body weight after the adaptation period was 29.2 grams. The body weight of individual mice was monitored on the first week and fourth week during the whole intervention period. All measurements were conducted using digital scale and performed in the morning before feding. The body weight of mice each treatment during the intervention are presented in Table 3.

The body weight before treatment (week-1) and bodyweight after treatment (week-4) showed no significant difference in A, B, C and normal control (N) treatments (p<0.05). However, there was a significant difference (p=0.03) in the CN treatment (Table 3). Based on these results, it can be concluded that the provision of KLE able to maintain the body weight of mice in A,B, and C treatment. A study conducted by Jambocus *et al.* (2017), showed that *Morinda citrifolia* L. leaf extract administration to rats given a high-fat diet and normal diet did not show any significant difference compared to control with normal diets. However, there was a significant weight gain in rats given high-fat diet without *Morinda citrifolia* L. leaf extract. Anti-obesity mecanishm in the extract did not affect food intake, so that the appetite suppression was not considered as the main mechanism. The main mechanism of the extract comes from the role of antioxidants to maintain body weight in normal diet and to prevent weight gain in high-fat diet. Other supporting studies from Abdali *et al.* (2015) stated that some natural antioxidants such as in green tea and green coffee can give a small effect on weight loss in obesity. It was because the hapotoprotective and antioxidant activity may prevent damage in liver cell thus maintain the healthy weight.

The effect of the treatment on SGOT and SGPT levels

The SGOT value of CN treatment was significantly higher than that of the N treatment (p<0.05). It shows that the provision of soft drink in animals could increase serum SGOT levels. SGOT levels of A, B, and C treatment showed a tendency to decrease following the increased

Tabel 3. The body weight of mice before and after intervention

Treatment	W-1	W-4	ΔW1&W4	p*
N	28.20±1.07^{bc}	28.20±1.56^{ab}	0.00±1.00^{a}	1.000
CN	27.25±0.75^{c}	21.00±1.54^{c}	-6.25±1.49^{b}	0.003
A	30.20±0.37^{a}	32.60±1.12^{a}	2.40±0.98^{a}	0.070
B	30.40±0.60^{a}	32.40±1.57^{ab}	2.00±1.70^{a}	0.305
C	30.00±0.41^{a}	31.25±1.25^{b}	1.25±0.85^{a}	0.239

*Paired t-test value with significant difference on p<0.05
N= normal control, CN= negative control, A= KLE 1000mg/kg, B= KLE 1500mg/kg, C= KLE 2000mg/kg
Values with different superscript letters in one column indicate the significant difference based on ANOVA test results (p<0.05)

Table 4. SGOT and SGPT level in serum *Mus musculus* after 4 weeks treatment

Treatment	SGOT (U/l)	SGPT (U/l)
N	108.64±17.43^{b}	43.82±6.62^{c}
CN	212.90±35.77^{a}	97.43±18.68^{a}
A	230.40±15.39^{a}	85.20±5.55^{ab}
B	132.46±28.07^{b}	51.02±13.17^{bc}
C	129.13±8.92^{b}	52.86±9.83^{bc}

N= normal control, CN= negative control, A= KLE 1000mg/kg, B= KLE 1500mg/kg, C= KLE 2000mg/kg
Values with different superscript letters in one column indicate the significant difference based on ANOVA test results (p<0.05)
U/l= units per litre

dose administered in mice (Table 4). The SGOT levels of B and C treatments were not significantly different from the N treatment ($p>0.05$). Therefore, it can be concluded that KLE able to reduce levels of SGOT in mice that have been given soft drinks. The C treatment showed the lowest SGOT score when compared with the A and B treatments, but the value was still above the N treatment.

The results of the serum SGPT in this study showed the same tendency with the serum SGOT levels. CN had significantly higher serum SGPT level than N ($p<0.05$). The KLE in treatment B and treatment C had significantly lower serum SGPT levels ($p<0.05$) than CN. These result showed that KLE was able to reduce levels of serum SGPT in mice. Study conducted by Zakaria *et al.* (2018) showed that KLE administration significantly reduced the level of serum SGOT and SGPT and also increased the activity of endogenous antioxidant enzymes in paracetamol-intoxicated rats.

Increased in serum SGOT and SGPT levels which occured in CN treatment indicated liver cell injury caused by high consumption of soft drinks. Alkhedaide *et al.* (2016) suggested that induction of soda in rats caused liver injury in mice characterized by increased activity of the enzymes SGPT and SGOT. Increased SGOT concurrent with elevated SGPT suggests hepatocellular injury (Hall & Cash 2012). Lebda *et al.* (2017), reported that excessive consumption of soft drinks is also capable of causing visceral fat accumulation, impaired glucose tolerance and blood lipids, disturbing levels of liver antioxidants/oxidants, and impaired adipogenetic cytokines. Consumption of carbonated beverages can lead to liver damage characterized by increased liver enzyme activity (Jeroh *et al.* 2012).

The purpose of soft drink induction to mice in this study was to analyze the potential hepatoprotective activity of KLE. Oxidative stress occurred as a result of soft drink consumption resulted in low activity of endogenous antioxidant. Oxidative stress has been associated with the etiology and pathogenesis of various chronic diseases and serves a vital role in the aging process. High levels of free radicals or reactive oxygen species (ROS) can cause direct damage to lipids inside cells and induce peroxidation (Alkhedaide *et al.* 2016).

KLE received by each treatment (A, B, C) was 35 mg / day, 45 mg / day, and 65 mg / day, respectively. Interestingly, based on the results of SGOT and SGPT, treatment C that had the highest concentration of extract had the lowest SGOT and SGPT levels compared to others treatments. This result indicated that treatment C, which had the highest antioxidant activity, was better able to prevent the increasing of SGOT and SGPT levels caused by induction of soft drinks. Antioxidant activity, high total content of phenols and flavonoids indicated that KLE is a potential hepatoprotective agent. According to Anjani *et al.* (2018), Quercetin as flavonoid in Okra (*Abelmoschus esculentus* L.) extract play an important role as antioxidant by scavenging ROS (Reactive Oxygen Species) and can reduced the level of MDA (malondialdehyde) as the final product of lipids peroxidation. This is supported by the study of Gupta *et al.* (2006) who reported that the combination of hepatoprotective effects and antioxidant activity synergistically prevents the process of initiation and progression of hepatocellular injury.

CONCLUSION

Kersen leaves (*Muntingia calabura* L.) extract has an antioxidant activity. Administration of the extracts with highest dosage of 65 mg/day showed the greatest effects to reduce serum SGOT and SGPT levels, however the serum SGOT and SGPT levels were still higher than normal control treatment. Further research using higher dose is needed to identify whether the effect of *kersen* leaves extract (KLE) is able to normalize SGOT and SGPT levels.

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