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Protective Effect of Cocoa Extract on Ethanol Induced Liver Injury in *Sprague-dawley* Rats

(Kesan Pelindung Ekstrak Koko untuk Kecederaan Hati Teraruh Etanol pada Tikus *Sprague-dawley*)

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ABSTRACT

Cocoa is a rich source of dietary polyphenol, highly potential antioxidant against free radicals. This study was designed to identify the effect of cocoa polyphenol extract in protecting from ethanol-induced liver injury in rats. Fifty male Sprague-dawley *rats were divided into five groups fed with or without ethanol (4 g/kg/d), cocoa extract (300 mg/kg/d) and silymarin (200 mg/kg/d) continuously for 3 weeks using an enteral feeding protocol. All treatments were given orally every day for three weeks and continuously supply food and water* ad libitum*. Results showed that cocoa extract (CE) from unfermented cocoa beans had a total polyphenol content of 335.70±27.51 mg GAE/g and 38.10±4.52 mg CaE/g. Meanwhile, analysis normal phase-high performance liquid chromatography shows CE contains 59.47±9.44 mg/g and 14.69±1.63 mg/g of epicathechin and catechin, respectively, which is three fold higher compared to commercial cocoa powder. It also contains 59.69±2.15 mg/g theobromine which also three fold higher compared to caffeine 19.87±1.37 mg/g.* In vitro *study showed cocoa extract contains high antioxidant activities by 91.9±1.00 % against superoxide scavenging system (O_2^-) and 97.7±0.15% against α-α-diphenyl-β-picrylhydrazyl radical (DPPH) systems.* In vivo *study showed increasing level in both liver function enzymes, aspartase aminotransferase (AST) and alanine aminotransferase (ALT) in ethanol intoxication by 116.80±5.23 mmol/L and 56.37±2.71 mmol/L, respectively. Ethanol intoxication was blocked by cocoa extract nearly 89.95±1.18 mmol/L and 46.75±0.74 mmol/L, respectively, and it was comparable with SDT group for both enzymes AST and ALT by 112.19±6.02 mmol/L and 42.49±0.62 mmol/L, respectively. Furthermore, ethanol groups showed significantly lower ($p<0.05$) of glutathione level by 0.29 ±0.03 µmol/g, however cocoa extract with antioxidant defense system either direct or indirectly protect liver injury by increasing glutathione level at 0.53±0.02 µmol/g. As a result, cocoa extract shows its potential as antioxidant agents to protect ethanol-induced liver injury.*

Keywords: Antioxidant activity; chronic ethanol; cocoa extract; glutathione; liver injury

ABSTRAK

Koko kaya dengan sumber polifenol diet bertindak sebagai antioksida dan berpotensi untuk menyingkirkan radikal bebas. Kajian ini dilakukan bagi mengenal pasti kesan ekstrak polifenol daripada koko untuk mencegah kerosakan hati tikus yang disebabkan oleh pengambilan etanol. Sebanyak lima puluh ekor tikus Sprague-dawley *jantan, dibahagikan kepada lima kumpulan iaitu kumpulan kawalan, etanol (4 g/kg/d), ekstrak koko (300 mg/kg/d) dan silimarin (200 mg/kg/d) yang mengandungi sepuluh ekor bagi setiap kumpulan. Setiap rawatan diberikan secara oral selama 3 minggu dan pengambilan makanan dan minuman adalah secara* ad libitum*. Analisis Folin-ciocalteau ekstrak koko (CE) telah menunjukkan bahawa jumlah kandungan polifenol ialah sebanyak 335.70±27.51 mg GAE/g dan 38.10±4.52 mg CaE/g. Manakala keputusan fasa normal kromatografi cecair berprestasi tinggi (NP-HPLC) pula menunjukkan ekstrak koko mengandungi kandungan epikatekin sebanyak 59.47±9.44 mg/g dan katekin sebanyak 14.69±1.63 mg/g iaitu tiga kali ganda lebih tinggi berbanding serbuk koko komersial. Ia juga mengandungi theobromina sebanyak 59.69±2.15 mg/g, iaitu tiga kali ganda kandungan lebih tinggi berbanding kafein iaitu 19.87±1.37 mg/g. Kajian* in vitro *menunjukkan aktiviti antioksida ektrak koko adalah sangat tinggi iaitu sebanyak 91.9±1.00% terhadap radikal superoksida (O_2^-) dan 97.7±0.15% terhadap radikal α-α-difenil-β-pikrilhidrazil (DPPH). Manakala kajian* in vivo *menunjukkan berlaku peningkatan terhadap kedua-dua enzim fungsi hati iaitu enzim aspartase aminotransferase (AST) dan alanine aminotransferase (ALT) di dalam ketoksikan etanol masing-masing sebanyak 116.80±5.23 mmol/L dan 56.37±2.71 mmol/L. Ketoksikan etanol dapat dicegah dengan kahadiran ekstrak koko dengan kandungan enzim masing-masing adalah sebanyak 89.95±1.18 mmol/L dan 46.75±0.74 mmol/L, selari dengan kumpulan silimarin bagi kedua-dua enzim AST dan ALT iaitu masing-masing 112.19±6.02 mmol/L dan 42.49±0.62 mmol/L. Selanjutnya, kumpulan etanol menunjukkan penurunan yang signifikan ($P<0.05$) kandungan glutation sejenis antioksida pertahanan badan semula jadi sebanyak 0.29 ±0.03 µmol/g, walau bagaimanapun, kehadiran ekstrak koko yang bertindak samada secara langsung atau tidak langsung melindungi kerosakan hati telah meningkatkan kandungan glutation sebanyak 0.53±0.02 µmol/g. Kesimpulannya, ekstrak koko telah menunjukkan bahawa ia sangat berpotensi sebagai agen antioksida dalam melindungi kerosakan hati yang disebabkan oleh pengambilan etanol.*

Kata kunci: Aktiviti antioksida; ekstrak koko; etanol kronik; glutation; kerosakan hati

Introduction

Increase of functional foods or nutraceutical products in the global market to prevent chronic diseases has become one of the major research fields lately. Cocoa is well known for its high flavonoid content, mainly catechin and epicatechin which has been proven as a potent antioxidant that react as a highly effective chemo preventive agent against chronic diseases including cancer, heart disease, diabetes, neurodegenerative disease and ageing (Kerimi & Williamson 2015; Martin et al. 2016, 2013; Ramos 2008). In this sense, flavonoid compounds may be involved either directly or indirectly to scavenge or detoxify free radicals to prevent chronic diseases and increase human health. Indeed, direct antioxidant effects of flavonoids seemed to be partly based on their structural characteristics via hydrogen donating (radical scavenging) properties and their metal chelating antioxidant properties (Lambert & Elias 2010; Nakagawa et al. 2004; Shahidi & Ambigaipalan 2015). More importantly, flavonoids can avert free radical-induced damage indirectly by modulating several enzymes related to oxidative stress, modifying the metabolism such as conjugating enzymes (glucuronidation, sulfation, acetylation, methylation and conjugation) as well as through regulation of certain transcription factors (Martin et al. 2016).

Beneficial actions of phytochemicals in cocoa and chocolate have been recognized by their biologically active compound or synergistic effects with other compounds such as methylxanthine, mainly theobromine and caffeine (Franco et al. 2013). Methylxanthine were reacts on adenosine receptors in the central nervous system, enhance erousal, mood and concentration levels (Nehlig 2010). In fact, the psychoactive properties of these methylxanthine was proven by Smit et al. (2004) which shown its psyho-stimulant effect that give better daily life, i.e. more efficient thinking, exploring and focusing. Moreover, human and animal studies have indicated theobromine is able to suppress cough (Usmani et al. 2005) without the side effects by other antitussive drugs, such as codeine. It might be due to inhibitory effect on afferent nerve depolarization at peripheral and by blockade of adenosine receptors at molecular level and inhibition of phosphodiesterases or both (Coleman 1980).

Evidence from clinical studies by Khan et al. (2012) and Neufingerl et al. (2013) also shows theobromine from cocoa consumption significantly increase plasma HDL cholesterol levels and decreases LDL concentration, conferring to prevent cardiovascular and reduce risk of coronary heart disease. The benefits of caffeine in coffee was show reduction in the incidence of two of the most prevalent neurodegenerative disease: Parkinson's (costa et al. 2010) and Alzheimer's (Eskelinen et al. 2009; Maia 2002). Epidemiological studies also show intake of caffeine in dietary coffee during the middle age are less prone to suffer from neurological disease when they get older. With an agreement that the same methylxanthine occur in cocoa and fits it role by adenosine receptor blockade in the brain results in higher neuronal activity thereby enabling a longer life for these cells. The higher neuronal activity may be due to a regulation in the perfusion of the brain (Klaassen et al. 2013; Koppelstaetter et al. 2008; Pelligrino et al. 2010) and/or an increase in cerebral oxygen consumption (Haller et al. 2013). Another potential mechanism for neuroprotection may be an increased cerebrospinal fluid production (Han et al. 2009; Wostyn et al. 2011). However, the possible synergistic interactions between flavonoids and methylxanthines are still unclear and need further investigation.

Alcohol-induced liver disease remains one of the most common and deadly causes of chronic liver disease (Sussman et al. 2002). Alcohol abuse has increased around the world, especially among young people who began to drink early (Hingson & Howland 2002; Hingson et al. 2005). The hepatotoxicity of alcohol has often been attributed to its major metabolite acetaldehyde. In fact, acetaldehyde is capable of interacting with cellular constituents like proteins, glutathione or coenzyme A and may initiate hepatocellular damage by interacting with vital physiological functions (Szabo 2003). Elevation of enzymes alanine aminotransferase (ALT) and aspartase aminotransferase (AST) are the most sensitive indicator to hepatocellular injury (Strubelt et al. 1987) as well as non-enzymatic indicator namely glutathione thus directly reacts with free radicals (Valko et al. 2004). Previous study by McKim et al. (2002) has been shown that cocoa extract from Cocoapro cocoa (Mars, New Jersey) which composed more than 80% of flavanol epicatechin and epicatechin-based oligomeric procyanidins, protects against early alcohol-induced liver injury. Meanwhile, the purpose of this study was to identify the flavanolic content and antioxidant activity of Malaysia unfermented cocoa beans which would protect against ethanol induced liver injury in the rat enteral ethanol feeding model.

Materials and Methods

chemicals and standard solutions

All solvents were of analytical grade and supplied by Sigma-Aldrich, Chemicals, Co. (St. Louis, USA) except for acetone, *folin-ciocalteau* reagent, methanol, acetic acid and dichloromethane supplied by Merck Chemicals (Darmstard, Germany) and hexane from J.T. Baker Chemicals (New Jersey, USA).

preparation of phenolic extract

Cocoa fruits were supplied by Cocoa Research and Development Centre, Lembaga Koko Malaysia (LKM) at Hilir Perak. Cocoa fruits was breaking into two and cocoa beans was dried in an oven (Memmert, Germany) with blower at 60°C until the moisture content achieved of 4-6%. Dried cocoa beans were defatted using a Soxhlet unit (Buchi, Switzerland) with hexane and cocoa extract which were extracted with 70% acetone. Cocoa extract were

concentrated using rotary evaporator N-N-series (EYELA, USA) under partial vacuum at 70°C to remove hexane and aqueous extract were freeze-dried (LABCONCO, USA) and kept in -40°C until used.

DETERMINATION OF TOTAL PHENOLIC CONTENT USING FOLIN-CIOCALTEAU ASSAY

Total polyphenol content in cocoa extract was determined according to the *Folin-ciocalteau* method (Adam 1928). Briefly, 0.1 g defatted cocoa seeds were dissolved in 10 mL 70% (v/v) acetone and were sonicated (SONICATOR, USA) for 30 min. Samples were centrifuged (Universal 32®, Hettich Zentrifugen, Germany) at 5 000 rpm for 20 min. An amount of 100 µL of the supernatant was added with 7.9 mL distilled water followed by 0.5 mL folin-ciocalteau reagent (previously diluted 10-fold with distilled water) and allowed to stand at room temperature for 5-8 min. Then, 1.5 mL of 20% sodium carbonate (Na_2SO_3) solution was added to the mixture. Mixtures were left at 20°C for 2 h and absorbance of each mixture was determined at 765 nm using UV-vis spectrophotometer (Shimadzu, Japan). A standard calibration curve was obtained from 0, 50, 100, 150, 250, 500, 750 and 1000 mg/L gallic acid and catechin. Results were expressed as gallic acid equivalents (GAE) and catechin equivalents (CaE) in milligrams per gram extract.

QUANTIFICATION OF POLYPHENOL

Identification and quantification of polyphenol compound was done according to the method of Hammerstone et al. (1999). A CLEAN-UP extraction column with C18 (500 mg/3 mL) for solid phase extraction (SPE) were purchased from UCT (Bristol, USA). The column was wetted with methanol and then conditioned with deionized water. Freeze-dried cocoa extract, approximately 100 mg were suspended in 1 mL of water with the aid of sonication. A 250 µL aliquot of the suspension was loaded onto the prewetted column, and the sugars were eluted with 20 mL of water. The phenolics were eluted from the column with 5 mL of a mixture of acetone, water and acetic acid in a ratio by column of 70:29.5:0.5, respectively. The columns were regenerated and the procedure repeated until the entire 100 mg sample had been passed through the column. The elutes containing the sugars were discarded, whereas the phenolic-containing fractions were combined, concentrated by rotary evaporation and freeze-dried.

Chromatographic analyses were performed using high performance liquid chromatography (HPLC) Hewlett Packard 1100 series (Agilent Technologies, Waldbronn Germany) equipped with an autoinjector, quarternary HPLC pump, column heater, diode array detector and HP ChemStation for data collection and manipulation. Normal-phase separations of the flavonoid were performed on a Supelco-Si column (Sigma, USA) 5 µ silica column (25 × 4.6 mm) at 37°C. UV detection were recorded at $\lambda = 280$ nm. The ternary mobile phase consisted of (A) dichloromethane, (B) methanol and (C) acetic acid and water (1:1 v/v). Results were analyzed and interpret. Quantification of theobromine, caffeine, epicatechin and catechin was carried out and used as a standard.

ANTIOXIDANT CAPACITY: SUPEROXIDE ANION (O_2^-) RADICAL SCAVENGING ACTIVITY

Superoxide radical was generated using the Xanthine/Xanthine Oxidase (X/XOD) Superoxide Scavenging assay by a modified method of Vimala et al. (2003). Nitro blue tetrazoleum (NBT) (100 mL of 4.1 mM/l) solution was prepared by adding 3.15 g tris hidro chloride (TrisHCl), 0.1 magnesium chloride ($MgCl_2$), 15.0 mg bromo-4-chloro-3-indolyl phosphate and 34.0 mg 4-nitro blue tetrazolium chloride to 100 mL distilled water. Reaction mixture (100 mL) was prepared by dissolving 0.53 g Na_2CO_3 (pH10.2), 4.0 mg ethylenediaminetetraacetic acid (EDTA) and 2.0 mg xanthine in 0.25 nMm NBT solution and kept in refrigeration at 4°C. Reaction mixture (999 mL) was transferred into micro cuvette and placed in a 25°C cell holder of a spectrophotometer. Superoxide was generated by adding 1×10^{-3} U/mL of XOD and measured at 560 nm for 120 s. Reaction mixture (979 mL) was transferred into micro cuvette and placed in a 25°C cell holder of a spectrophotometer. Superoxide dismutase (SOD) (1.16 U/mL) was added into the reaction mixture and mixed followed by XOD (1×10^{-3} U/mL) to generate oxyradicals and measured at 560 nm for 120 s at intervals of 10 s. Acetone crude extracts of cocoa beans were dissolved in the reaction mixture at a concentration of 250 mg/mL. The stock solution (5 mL) was added to 994 µl of the reaction mixture and placed in a cell holder to auto zero. XOD (1×10^{-3} U/mL) was then added and after thoroughly mixed, XOD and SOD curves were developed. Results of the scavenging effect were calculated based on the percentage of superoxide scavenged.

ANTIOXIDANT CAPACITY: FREE RADICAL SCAVENGING ACTIVITY ON A-A-DIPHENYL-B-PICRYLHYDRAZYL RADICAL (DPPH)

The antioxidant activity of cocoa extracts was measured in terms of hydrogen donating or radical scavenging ability, using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method of (Blois 1958) with slight modification. Crude acetone extracts of cocoa beans (4 mL of 0.5 mg/mL) were added to 1 mL of DPPH (1 mM in methanolic solution) in a 5 mL screw cap bottle. The solution was shaken and incubated at room temperature for 10 min and the decrease in absorbance at 520 nm was determined at the end of incubation period with a spectrophotometer. The remaining concentration of DPPH in reaction medium was calculated from a calibration curve obtained with DPPH at 520 nm. The percentage of remaining DPPH ($DPPH_R$) was calculated as following formula: $\%\ DPPH_R = [(DPPH)_T/(DPPH)_{T=0}] \times 100$, where $DPPH_T$ was the concentration of DPPH at the

time of 60 min and $DPPH_{T=0}$ was the concentration of DPPH at time zero (initial concentration). Their scavenging effect was calculated based on the percentage of DPPH scavenged.

IN VIVO ASSAYS: ANIMAL HANDLING

The present study was approved by the Ethical Committee on the use of animals for the research, Department of Medicine and Health Science, Universiti Kebangsaan Malaysia (Selangor, Malaysia) with ethic approval no. FST/2016/MOHD KHAN/18-MAY/761-MAY-2016-AUG.-2018. Fifty male *Sprague-dawley* rats (160-180g) were purchased from Institute Medical Research (Kuala Lumpur, Malaysia) and acclimatized for 7 days. During this period, food and tap water were supplied *ad libitum*. The animals were housed in plastic cages with stainless steel covers and kept in rooms maintained at temperature 24-28°C and 30-70% relative humidity with a 12-h light/dark cycle.

EXPERIMENTAL DETAILS

Rats were randomly distributed into five groups (N = 10 for each group). Group I received only normal saline for a total period of three weeks and served as control (C). Animals of group II (CE) received 300 mg/kg cocoa extract for a total period of three weeks. For groups III (EtOH), rats received normal saline for the first week and normal saline plus 4 g ethanol for the following 2 weeks. Rats in groups IV (CE+EtOH) only received 300 mg/kg cocoa extract for the first week but for the following two weeks, rats were given both cocoa extract and ethanol. Group V received 200 mg/kg silymarin in water (w/v) for three weeks and also challenged with 4 g ethanol for the last two weeks. This ethanol dosage was based on preliminary studies which shown rats treated with 4 g/kg ethanol produce liver injury after 3 days of experiments. However, rats treated with 5 and 10 g/kg ethanol (referred to protocol by McKim et al. 2002) died after 3 days of experiments. All rats were given free access to water throughout the study period and treatment given via oral gavages for total of three weeks. The rats were weighed once a week.

PREPARATION OF TISSUES AND BIOCHEMICAL ASSAYS

After 3 weeks of study, the rats were anaesthetized with thiopental (50 mg/kg body weight, intraperitoneal) and sacrificed by exsanguinations. Five mL blood was collected from intracardiac cavity and placed into a vacutainer tube (BD Diagnostic, New Jersey, USA). The liver was quickly removed, weighed and freeze-dried with liquid nitrogen for further used.

Plasma enzymes For biochemical analysis, heparinized blood was centrifuged (Universal 32®, Hettich Zentrifugen, Germany) for 10 min at 1000 g at room temperature (25°C). The supernatant was collected and plasma enzymes, AST (EC2.6.1.1) and ALT (EC 2.6.1.2) levels were measured using Chemistry Analyzer (Hitachi 902 Automatic Analyzer, Japan), as described in Roche (Roche Diagnostics GmbH, Tutzing, Germany) kit leaflet.

Antioxidant assays Hepatic GSH content was measured after the reaction with 5, 5,-dithiobis-2-nitrobenzoic (DTNB) as substrate according to the method (Ellman 1959) with slight modification. This mixture was centrifuged (Bechman X22R, Indianapolis, USA) at 14,000 rpm for 30 min at 4°C. The volumes of supernatant were recorded. Three tubes were prepared and filled with 2.9 mL buffer solution pH8.0 and 0.1 mL supernatant. Twenty µL 10 mM DTNB was also added into two tubes; one left as blank, and left for an hour in room temperature. The absorbance was read at 420 nm and the concentration of glutathione was calculated using following formula; Specific Activity of GSH = $A/E \times V_s/V_i \times V_n/g \times 1000$, where A is absorbance; E is molar absorbance coefficient (13.6×103 M cm^{-1}); V_s is supernatant volume (mL); V_i is volume of used supernatant (mL); V_n is homogenate volume (mL) and g is tissue (liver) weight (g). Results were expressed in µmol/g liver.

STATISTICAL ANALYSIS

All data are presented as mean ± S.E.M. (standard error mean). The data were analyzed using ANOVA test and Duncan New Multiple Range Test through Statistic Analysis System version 2.0 (SAS Institute, Cary, NC). Mean difference between groups of variables were tested with variance analysis (ANOVA). Two ended *p* values less than 0.05 were considered as statistically significant.

Results and Discussion

TOTAL POLYPHENOL CONTENT

Cocoa beans are rich in important bioactive compounds which are flavonols (catechin and epicatechin) and metylxanthines (theobromine and caffeine) generally known to be beneficial for maintenance of health (Franco et al. 2013). Study by Othman et al. (2007) showed that Malaysian cocoa beans contain higher phenolic content compared to Sulawesi, Ghana and Ivory Coast cocoa beans. The high content of phenolic substances in cocoa beans not only resulted in the purple color in cocoa beans but also may contribute to bitter taste. Table 1 shows the results for phytochemical properties and *in vitro* antioxidant potential of cocoa extract from unfermented cocoa beans from Cocoa Research and Development Centre, Lembaga Koko Malaysia (LKM) at Hilir Perak. Our findings showed that the cocoa extract contained high level of total phenolic content (335.7 mg GAE/g, 38.10 mg CaE/g) surpassing green tea which has about 117.30 mg GAE/g (Samman et al. 2001) and could be an important source of antioxidant. However, several factors may affect the levels of total phenolic content in cocoa beans such as location, growing conditions, harvesting period, cultivation, sampling and analytical procedures and thus, could also affect the antioxidant activity.

Antioxidant actions of phytochemicals have been recognized by their biologically active polyphenol

compounds such as flavonoids and phenolic acids, which possess powerful antioxidant activities. In fact, these polyphenols act as oxidation terminators by scavenging free radicals to form stable ones (Rice-Evans & Miller 1997). Here, our findings from NP-HPLC shows cocoa extract contained high epicatechin and catechin content by 59.47±9.44 mg/g and 14.69±1.63 mg/g, respectively (Table 1). In comparison with commercially polyphenol rich cocoa powder, named CocoanOX only contain 19.36 mg/g epicatechin (Cienfuegos-Jovellanos et al. 2009) which is 3-fold lower compared to cocoa extract. This high flavonoid content implies its functions as a potent antioxidant against free radicals, contributing to health maintenance through its protective properties. In addition, the existence of theobromine and caffeine has also been linked to promote health benefits and are remarkable through their physiological effect. Here, our findings show theobromine content from LKM unfermented cocoa beans which are 59.69±2.15 mg/g was 3-fold higher compared to the caffeine which is 19.87 ± 1.37 mg/g in the cocoa extract. Indeed, the higher theobromine content in cocoa is probably due to the N-methyltransferase-catalyzed metabolism of theobromine to caffeine in the four-step caffeine biosynthesis pathway (Ashihara et al. 2008). Considering the variation of methylxanthines in cocoa beans, the accumulation of these compounds occurs in the late development of the seeds as reported by Pereira-Caro et al. (2013). Psycoactive properties of methylxanthines have been revealed by their mechanisms mainly acting as adenosine receptor blockers in every cell (Orru et al. 2011).

As cocoa extract contains high phenolic content, our findings shows *in vitro* high antioxidant activities was 91.9 ± 1.00 % against superoxide scavenging system (O_2^-) and 97.70 ± 0.15 % against α-α-diphenyl-β-picrylhydrazyl radical (DPPH) systems (Table 1). In this regard, several studies show this antioxidant capabilities of flavonoids compound having direct antioxidant action from the ability to scavenge free radicals, chelate redox-active metals and attenuate other process involving reactive oxygen species (ROS) (Fraga 2007; Hollman et al. 2011). Significant amounts of flavonoid monomers (catechin and epicatechin) in cocoa extract possess as highly potential antioxidant agent that may be applied in conjunction with medicines to promote health benefits.

BODY WEIGHT

In vivo study of the effect of cocoa extract on body weight of rats intoxicated with ethanol is shown in Table 2. The mean weight of rats in each group between days 0 and day 21 did not differ from each other ($P > 0.05$) in % normality of body weight in all groups. The minimum body weight of rats was 110 g and the maximum is 250 g. There was a pattern of increasing % normality of body weight of rats given 300 mg/kg cocoa extract and 200 mg/kg silymarin drugs by 204.17±3.00% and 240.00±5.78%, respectively. However, decreasing pattern of % normality of body weight of rats occurs but not significantly different ($P > 0.05$) in groups given 4 g/kg ethanol and mixture of 300 mg/kg cocoa extract plus 4 g/kg ethanol by 180.83±20.67% and 184.00±18.87%, respectively.

TABLE 1. Phytochemical properties of cocoa extract and its *in vitro* antioxidant potential

Biochemical Analysis	
Total polyphenol content (mg GAE /g)	335.70 ± 27.51
Total polyphenol content (mg CaE /g)	38.10 ± 4.52
Theobromine (mg/g)	59.69 ± 2.15
Caffeine (mg/g)	19.87 ± 1.37
Epicatechin (mg/g)	59.47 ± 9.44
Catechin (mg/g)	14.69 ± 1.63
Superoxide Scavenging (%)	91.9 ± 1.00
DPPH Radical Scavenging (%)	97.7 ± 0.15

Data are express as means ± SEM (n = 3-4) gallic acid equivalents (GAE) and catechin equivalents (CaE)

TABLE 2. Effect of cocoa extract on % normality of body weights of rats intoxicated with ethanol

Groups	% normality of body weights
Control (distilled water)	186.25±16.50a
300 mg/kg cocoa extract	204.17±3.00a
4 g/kg ethanol	180.83±20.67a
300 mg/kg cocoa extract + 4 g/kg ethanol	184.00±18.87a
200 mg/kg silymarin + 4 g/kg ethanol	240.00±5.77a

Data are express as mean±SEM (n=10) for each group for 3 weeks. Mean values followed by same alphabet in the same column are not significantly different at p>0.05

PLASMA ENZYMES

It has been shown that consumption of ethanol damages the cells and affects liver function, which may lead to alcoholic liver disease (ALD), characterized by liver failure (Lieber 1997; Nordman 1994). Metabolism of ethanol generated production of free radicals and plays a major role in ethanol-induced oxidative stress. Oxidative stress is an important mechanism of ethanol-induced liver injury and is associated with increased serum level of transaminase (Caballeria 2003). It is support by McKim et al. (2002) show serum ALT progressively increased in animal fed with alcohol. Similarly, our findings also show significantly increased ($P<0.05$) level of both enzymes AST and ALT in EtOH groups by 29% and 27%, respectively (Table 3) compared to control groups. However, our findings also reported the protective properties of the cocoa extract, which was able to protect the hepatic function by preventing damages due to ethanol consumption. In fact, ALT is a cytoplasmic enzyme released into circulation in response to damage to the structural integrity of the liver cells (Sallie et al. 1991), and it can also affect cell organelles as mitochondrial releasing compartment enzymes such as AST. Similarly, our findings show both enzymes AST and ALT was significantly reduced ($P<0.05$) in CE+EtOH group producing 89.95±1.18 mmol/L and 46.75±0.74 mmol/L, respectively, where, it was comparable with SD+EtOH group for both enzymes AST and ALT by 112.19±6.02 mmol/L and 42.49±0.62 mmol/L, respectively. Furthermore, consumption of cocoa extract alone showed no significant difference ($P>0.05$) compared to control group for both enzymes AST and ALT. The protective properties of cocoa extract may be due to its high phenolic content acting either direct or indirect protection against free radical produced from ethanol metabolism.

SPECIFIC ACTIVITY OF GLUTATHIONE (GSH)

It has been shown that normal cell activity may generate oxygen radical species called reactive oxygen species (ROS) thus play different positive roles *in vivo* such as they are used for destruction of microbes and pathogen by white blood cell and being involved in intercellular and intracellular signaling. Major sources of oxygen radicals are through energy production in the mitochondria. Oxygen becomes major concern and interest to researchers because it is highly susceptible to free radical-formation due to its electronic structure. However, excess amounts of oxygen-derived radicals are very harmful to the cells and they are also capable to generate other free radical that would be more destructive. Interestingly, our body naturally developed complex antioxidant defense system and its can act at different levels which are radical preventive (first line), radical scavenging (second line), radical induced damage repair (third line) and finally adaptation radical mechanism (fourth line) (Ighodaro & Akinloye 2018; Niki 1993).

The top first line defense antioxidant, glutathione is an endogenous antioxidant defense that can act to suppress or prevent the formation of free radicals or reactive species in cells. It can neutralize any molecule quite fast with the potential of developing into free radical with the ability to induce the production of other radicals. In turn, increased free radicals exceeding the antioxidant capacity of the cells generating oxidative stress which appears can be highly

TABLE 3. Effect of cocoa extract on liver plasma enzymes of body weights of rats intoxicated with ethanol

Groups	AST	ALT
Control (distilled water)	90.66±2.45a	44.46±1.40c
300 mg/kg cocoa extract	84.31±1.52a	45.87±0.84c
4 g/kg ethanol	116.80±5.23b	56.37±2.71d
300 mg/kg cocoa extract + 4 g/kg ethanol	89.95±1.18a	46.75±0.74c
200 mg/kg silymarin + 4 g/kg ethanol	112.19±6.02b	42.49±0.61c

Data are express as mean±SEM (n=10) for each group for 3 weeks. Mean values followed by same alphabet in the same column are not significantly different at $p>0.05$

TABLE 4. Effect of cocoa extract on specific activity of glutathione of rats intoxicated with ethanol

Groups	Glutathione
Control (distilled water)	0.52±0.04a
300 mg/kg cocoa extract	0.53±0.02a
4 g/kg ethanol	0.29±0.02b
300 mg/kg cocoa extract + 4 g/kg ethanol	0.55±0.03a
200 mg/kg silymarin + 4 g/kg ethanol	0.57±0.02a

Data are express as mean±SEM (n=10) for each group for 3 weeks. Mean values followed by same alphabet in the same column are not significantly different at $p>0.05$

damaging as they can attack biological macromolecules such as lipids, proteins and DNA (Halliwell & Gutteridge 1999; Valko et al. 2004), which may be enhanced by depletion in antioxidant defense system. Metabolism of ethanol is accompanied by generation of very reactive metabolites i.e. acetaldehyde and free radicals that can readily react oxygen radicals called superoxide anions which can inactivate catalase (Davies 1987; Davies & Delsignore 1987; Kono & Fridovich 1982; Mauch et al. 1986) or glutathione. In line with this fact, our findings show that groups of rats which received ethanol showed significantly lower ($P<0.05$) glutathione level by 0.29±0.03 µmol/g compared to control groups and groups of rats which received 300 mg/kg cocoa phenolic extract with or without 4 g ethanol and 200 mg/kg silymarin with ethanol by 0.52±0.05, 0.53±0.02, 0.55±0.03, and 0.57±0.02 µmol/g, respectively (Table 4).

Moreover, catalase and other antioxidative enzymes may be inactivated by another ethanol metabolite-1-hydroxyethyl radical (Puntarulo et al. 1999). In agreement with high phenolic content and high superoxide anions scavenging of cocoa extract in our previous result (Table 1), strong antioxidant capabilities of flavonoids react either directly or indirectly to scavenge or detoxify free radicals.

Conclusion

This study has shown that cocoa extract contains significantly high flavonoid content and strong antioxidant activity. In this sense, cocoa extract may prevent oxidation stress induced by ethanol metabolites, which react either directly or indirectly to scavenge free radicals. Moreover, reduction of plasma enzymes (AST and ALT) showed cocoa extract were able to reduce liver injury caused by ethanol consumption. Therefore, our findings suggested that cocoa extract can be a potential antioxidant agent in the prevention of liver disease associated to ethanol consumption.

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