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SMEDDS of *Citrus hystrix* ethanolic extract improves cardiac and hepar histopathology profile on doxorubicin-induced rats

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Abstract

Citrus hystrix D.C. (kaffir lime) peel contains several flavonoids including rutin, naringenin, hesperidin. *C. hystrix* peel ethanolic extract (ChEE) has shown its potency as cardioprotector agent in chemotherapy. However, there are limitations to the utilization of ChEE due to its poor water solubility and low oral bioavailability. Accordingly, self-microemulsifying drug delivery system (SMEDDS) formulations were developed to improve the oral absorption of flavonoids. Tween 80, Corn oil, and propylene glicol (5:1:1 ml) were combined to form ChEE-SMEDDS. The present study is to evaluated ChEE-SMEDDS for their physicochemical properties and *in vivo* using combination with doxorubicin to see blood serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), nitrit oxide (NO) activity and also cardio-hepato-histopathology of female Sprague Dawley rats. The results showed that ChEE-SMEDDS repaired cardio-hepato-histopathology profile of doxorubicin-induced rats, but did not reduce serum activity of NO, ALT and AST. These results indicated that ChEE-SMEDDS has potency to be developed and improved as cardio-hepato-protector agent in chemotherapy.

Keywords: *Citrus hystrix* D.C., SMEDDS, Cardio-hepatoprotector, Histopathology, Chemoterapy

INTRODUCTION

Doxorubicin is an anthracycline compound and highly efficacious anticancer drug that is widely used for most patients who diagnosed with neoplastic disease. However, its clinical use very often becomes strictly restricted by its toxicity on several organs (El-Sayyad, *et al.,* 2009). High cumulative administrative of doxorubicin are demonstrated to the development of cardiomyopathy, which manifest that chronic effects will eventually lead to congestive heart failure (Simůnek, *et al.,* 2009). Many patients treated with doxorubicin have been reported for having liver functions abnormalities (Henninger, *et al.,* 2012). The mechanisms of doxorubicin-induced cardiac and hepatic toxicity has been shown to be mediated through designate the involvement of free radicals generated from metabolism of doxorubicin (Brilhante Wolle, *et al.,* 2012). Free radicals may cause subcellular alteration which eventually will lead to cellular damage (Anandakumar, *et al.,* 2007).

Citrus hystrix D.C. (*C. hystrix*), commonly known as kaffir lime, consists of flavonoids including rutin, naringenin, and hesperidin (Bisset, *et al.,* 1994). A study reported that among parts of the plant, highest yield of extraction was obtained from peels (Ampasavate, *et al.,* 2010). Antioxidant activity and free radicals scavenging properties of rutin, naringenin and hesperidin have been examined by numerous researchers using various assay systems.

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Rutin reduced mitochondrial damage and prevented cardiac mitochondrial dysfunction (Punithavathi, *et al.*, 2010), naringenin suppressed lipopoly- saccharide-induced tumor necrosis factor-release and liver injury in mice (Kawaguchi, *et al.*, 2009), while hesperidin decreased ALT and AST activities on CCl_4-induced rats (Tirkey, *et al*., 2005). Previous study have reported that the combination of doxorubicin and *C. hystrix* peel ethanolic extract (ChEE) using female Sprague Dawley rats had revealed the cardioprotective and hepatoprotective effects in ameliorating most histopathological alterations induced by doxorubicin (Putri, *et al.*, 2013). However, *C. hystrix* is known to be hydrophobic due to its flavonoids that possess low bioavailability in water thus the permeability in intestinal mucouse remains low (Solanki, *et al.*, 2012). Until this point, however, there is no a formulation strategies was reported to enhance the oral absorption of ChEE further. Recently, Self-microemulsifying Drug Delivery System (SMEDDS) formulations have been applied to improve oral absorption of poor-solubility natural products.

SMEDDS is an isotropic mixture of oil, surfactant, and co-solvents which spontaneously forms a homogeneous, transparent/translucent, isotropic and thermodynamically stable microemulsion upon dispersion on mild agitation in the presence of water or gastrointestinal fluid with oil droplet sizes of less than 50 nm (Patel, *et al.*, 2007; Sachan, *et al.*, 2010; Hauss, *et al.*, 2007). A small droplet causes a reduction of the interfacial energy, an increase in the surface area, and a rapid release of the drug. Surfactants penetrate intestinal cell membranes and disturb lipid bilayers resulting in increased oral absorption (Gursoy and Benita, 2004). Co-solvents also assist in oral absorption by increasing the fluidity of the interface and damaging the liquid crystalline or gel structure which is a common barrier for microemulsion formations. Moreover, the inclusion of oils can increase the amount of lipophilic drug transported through the intestinal lymphatic system (Patel, *et al.*, 2007; Sachan, *et al.*, 2010).

Therefore, the aim of this study was to develop the formulations of ChEE-SMEDDS in order to enhance its oral bioavailability. The developed formulation was then observed using *in vivo* study by determining the activities of serum enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) also biomarkers of nitric oxides (NO) on doxorubicin-induced cardiotoxicity and hepatotoxicity in female rats. Further observation was conducted by analyzing the histopathological changes in cardiac and hepatic tissues.

MATERIALS AND METHODS

Animals

Thirty six female Sprague-Dawley rats weighing 80 to 170 g (about 50 days) were obtained from Unit of Experimental Animals Development, Universitas Gadjah Mada. Animals were housed with six animals per cage and adapted for one week of acclimatization period. All animals were kept under uniform managerial and standard hygienic conditions throughout the experimental period, also maintained under a constant temperature, humidity and light-controlled environment. They were allowed free access to standard pellet diet and tap water ad libitum. Body weight was recorded daily throughout the study. The animal handling protocols of this study were in accordance with the guidelines of the animal care of the Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia, and approved by the committee for animal research.

Materials

C. hystrix peels were supplied from Kaliurang, Yogyakarta. Plants were identified at Laboratory of Pharmaceutical Biology, Faculty of Pharmacy UGM. ChEE was prepared using maceration technique from dried peels with 70% ethanol for 5 days and re-maceration of residue for consecutive 2 days. ChEE was then concentrated using rotary evaporator. All of oils, surfactants and co-solvents were of reagent grade and used without further purification. Doxorubicin were purchased as ampoules (PT. Combiphar). ALT and AST kit (Bio Rad, 731) was prepared.

Identification of Flavonoid Compounds

Identification of falvonoid compounds in ChEE using Thin Layer Chromatography (TLC). TLC was performed on the 10 × 5 cm plates precoated with silica gel F 254. A volume of 2 µL of ethanolic solutions of rutin,

naringenin, hesperidin, hesperitin used as standards and sample extracts were spotted on the plates. The mobile phases for TLC analysis were chloroform: acetone: formic acid (75:16,5:8,5 v/v/v). Spots were sprayed with $AlCl_3$ and detected under UV light at 254 and 366 nm.

Solubility studies

The solubility of ChEE in various oils, surfactants, and cosolvents was studied in order to identify the suitable compositions of SMEDDS by adding 500 mg of ChEE to 1 mL of oils, surfactants, co-solvents. After that, the mixtures were then centrifuged at the speed of 1000 rpm for 20 minutes. Later, they were observed to show their solubility in oils, surfactants, and co-solvents.

Stability studies

Stability studies were conducted by mixturing ChEE SMEDDS with co-solvent: oil: surfactant by using ratios (1: 1: 3 mL), (1: 1: 4 mL), and (1:1:5 mL). Three replicate assessments were performed for each mixture. Later, the formulas were observed for 7 days to see the ratios of oil, surfactant, and co-solvent which gave the greatest formation stability while using the smallest volume were selected.

Self-emulsification studies

Visual assessment of self microemulsification ChEE-SMEDDS concentration (approximately 0.2 ml) was diluted with purified water (20 mL) and gently stirred with magnetic stirrer.

Formulation of ChEE-SMEDDS

The formulations were prepared by initially dissolving the formulation amount of 500 mg ChEE in co-solvent. Oil was then added. Later, surfactant was added and the final mixture was mixed by vortexing until a clear solution was obtained.

In vivo study

Animals were randomly divided into six groups with six rats in each group and received treatment as follows. Group 1 (Dox-treated group) was injected with doxorubicin 5 mg/kg intraperitoneally on Day 1 and Day 8. Group 2 and Group 3 were also treated with doxorubicin on Day1and Day 8 as well as the first group, along with ChEE-SMEDDS 500 mg/kg and 1000 mg/kg (p.o.), respectively, for consecutive 13 days (Day 1 to Day 13). Group 4 was administered daily 1000 mg/kg of ChEE-SMEDDS (p.o.) for consecutive 13 days as well (Day 1 to Day 13). Later, Group 5 were treated by SMEDDS carrier 1000 mg/kg (p.o) for 13 days. Group 6 served as control group and received standard diet and tap water. At Day 14 all blood samples were collected, the animals were sacrificed and the organs (heart and liver) were fixed in 10% buffered formalin solution for further examination.

Assay of serum ALT and AST

Blood samples were collected on Day 14 and sera were separated by 3000 rpm/min centrifugation at 4°C for 15 min. Obtained sera was then separated into microtube and kept at -20°C. Effects of ChEE-SMEDDS on ALT and AST activity was analyzed as the procedure from kit manufacturer (Bio Rad, 731).

Nitric oxide (NO) scavenging activity

Blood sample were reacted with Greiss reagent (1% sulphanilamide, 2% H3PO4 and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromaphore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamme was read at 540 nm and referred to the absorbance of standard solutions of potassium nitrite treated in the same way with Griess reagent.

Haematoxylin and eosin (H&E) staining

Animals were sacrificed by decapitation at the end of experiment (Day 14). Hearts and livers were dissected, then small pieces of heart and liver tissues were immediately fixed in 10% buffered formalin solution, then embedded in paraffin wax. Tissues were then sectioned for 3-5 mm thickness and prepared for staining by H&E. Stained hepatic and cardiac tissues were observed using light microscope (Olympus® DP12 microscope digital camera system, NY) with an immersion oil lens at magnification of 100-400x.

Statistical analysis

All data are expressed as Mean±SD (n=6). Statistically significant difference was determined by analysis of variance (ANOVA) followed by post-hoc comparisons using Tuckey's significant difference test. Statistical significance was considered at $p<0.05$ (SPSS 17.0).

RESULTS

Solubility studies

In self emulsifying formulation, the important consideration in formulation is avoiding drug precipitation on dilution in the gut lumen in vivo. Therefore, the components used in the formulation should soluble. Propylene glycol, tween 80, and corn oil showed the highest solubilization capacity for ChEE. Thus, for our study we selected propylene glycol, tween 80, and corn oil as surfactant, co-surfactant, and oil respectively (Fig. 1).

Stabilities studies

The stabilities studies of selected components (Propilen Glikol : corn oil : Tween 80) were done by solubilize ChEE in each component with different ratio (1:3:1; 1:4:1; 1:5:1). The 1:5:1 ratio showed the highest stabile compared with others. The stabile parameter are no phase separation and drug precipitation. Thus, these studies confirmed the stability of the developed formulation and its compatibility with 1:5:1 (Fig. 2).

Effect of doxorubicin, ChEE-SMEDDS and their combination on ALTand ASTactivity

In this present study, the administration of doxorubicin did not increase the activities of ALT, represented by the no significant activities of ALT in Dox-treated group compared to control group. In contrast, ALT activities of both groups treated with doxorubicin and two dose of ChEE-SMEDDS have increased, indicating both lower and higher doses of ChEE-SMEDDS had no lowering effects on the AST activities throughout the study. ChEE-SMEDDS-treated group itself also showed higher activities of ALT compared to control group.

Similarly, the effects of doxorubicin on AST activities, the ChEE-SMEDDS 1000 mg/kg did increase the activites of AST significantly, compared to Dox-treated group. Any ChEE-SMEDDS administered groups also showed higher activities of compared to control and Dox-treated group (Table 1).

A B C

Figure 1. Solubilization studies for propylene glycol (A); tween 80 (B), and corn oil (C) for ChEE

A B

Figure 2. Stabilities studies of basis SMEDDS (A) and ChEE-SMEDDS (B). The highest stabile formula (ratio 1:5:1) is showed by yellow circle and the lowest stabile formula is showed by red circle

Table 1. Effect of doxorubicin, ChEE-SMEDDS and their combination on AST and ALT activity

Groups	NO (µM)
Dox	35.37±3.89
Dox + ChEE-SMEDDS 500 mg/kg bw	26.94±26.94
Dox + ChEE-SMEDDS 1000 mg/kg bw	40.76±6.41
ChEE-SMEDDS 1000 mg/kg bw	45.11±15.58
SMEDDS excipients	54.87±19.98
Control	40.65±7.41

*$p<0.05$ compared to the control

Effect of doxorubicin, ChEE-SMEDDS and their combination on NO activity

This study have shown the result of Doxorubucin and ChEE-SMEDDS administration on NO activity. Dox-trated group was lower compared to control group. Similarly, any ChEE-SMEDDS administered groups also showed no significant effects on NO activitiy compared to those in control group (Table 2).

Histopathological analysis of hepar

From microscopic observation, it discovered that administration of doxorubicin induced a massive hepatotoxicity, which was indicated by severe vacuolar degeneration in hepatocytes compared to control group (Fig. 3A). Treatments with combination Dox + ChEE-SMEDDS 500 mg/kg weight body in rats markedly several karyopyknotics (Fig. 3B).

In contrast, few inflamatory were found in Dox + ChEE-SMEDDS 1000 mg/kg weight body group (Fig. 3C). Single treatment with ChEE-SMEDDS showed atrophy in cells and wider sinusoid compared to control group.

Histopathological analysis of cardiac

From observation using light microscope, the administration of doxorubicin caused cardio-myopathy and persisting with an irregular structure (Fig. 4A). These histopathological alterations clearly suggested the toxicity of doxorubicin on cardiac tissues. Two groups (Fig. 4B and 4C) treated with Dox + 500 mg/kg body weight ChEE-SMEDDS and Dox + 1000 mg/kg body weight ChEE-SMEDDS showed normal myocardial structure as seen in control group (Fig. 4F). Also, single treatment of ChEE-SMEDDS was found to be safe (Fig. 4D).

Table 2. Effect of doxorubicin, ChEE-SMEDDS and their combination on NO activity

Groups	Serum Enzyme Activity (UI/L)	
	AST	ALT
Dox	121.58±39.38	49.86±13.91
Dox + ChEE-SMEDDS 500 mg/kg bw	183.28±41.47	48.06±3.26
Dox + ChEE-SMEDDS 1000 mg/kg bw	145.64±35.22	49.62±5.11
ChEE-SMEDDS 1000 mg/kg bw	204.5±55.08	70.16±26.20
SMEDDS excipients	194.2±32.14	66.04±12.07
Control	136.46±30.18	48.30±9.63

*$p<0.05$ compared to the control

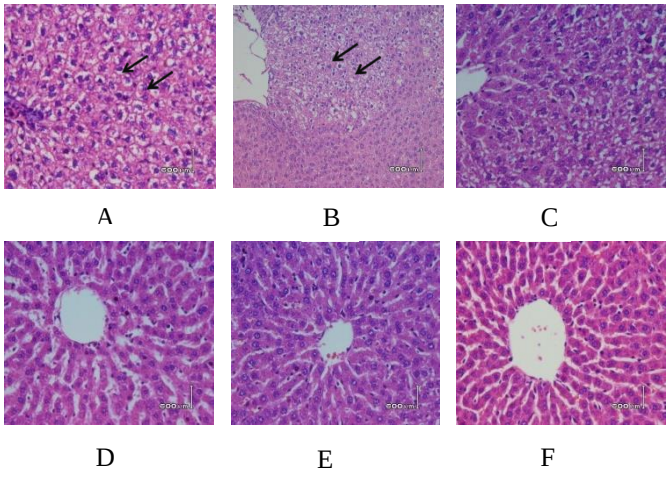

Figure 3. Histological profil of hepar sections. A: Dox; B: Dox+ChEE-SMEDDS 500 mg/kg body weight; C: Dox+ChEE-SMEDDS 1000 mg/kg body weight; D: ChEE-SMEDDS 1000 mg/kg body weight; E: SMEDDS excipients; F: Control. Black arrows point hydropic degeneration and karyopyknotic. Magnification 600x.

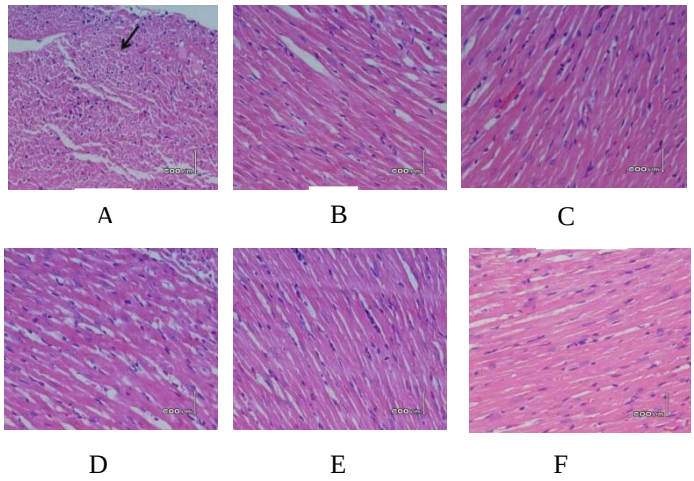

Figure 4. Histological profil of cardiac sections. A: Dox; B: Dox+ChEE-SMEDDS 500 mg/kg body weight; C: Dox+ChEE-SMEDDS 1000 mg/kg body weight; D: ChEE-SMEDDS 1000 mg/kg body weight; E: SMEDDS excipients; F: Control. Black arrows point cardiomyopathy. Magnification 600x.

DISCUSSION

The present work was aimed to explore the combination effect of doxorubicin and ChEE on blood serum ALT, AST and NO activity also cardio-hepato-histopathology of female Sprague Dawley rats. The results of this study revealed that treatment of ChEE-SMEDDS in both concentrations had no lowering effects on the elevated ALT, AST and NO activities. Histopathology profile on cardiac and hepar showed that doxorubicin caused anomalous histological changes in the cardiac and hepar tissue. However, ChEE-SMEDDS treatment prevented the changes in cardiac tissue and maintained the histological structure almost similar to that of normal control.

The existing experimental evidence suggests that doxorubicin-induced oxidative stress is due to the generation of free radicals. In biological systems, doxorubicin is enzymatically reduced to the doxorubicin semiquinone radical.This semiquinone radical directly transfers its electron to molecular

oxygen, generating free radicals, namely, superoxide and hydrogen peroxide. This free radical generation plays an important role in the cardio-toxicity of doxorubicin. Cardiac tissue is especially susceptible to free radical injury due to the lower activities of the free radical detoxifying mechanisms, such as SOD, CAT and GSH. Doxorubicin also has a high affinity for the phospholipid component of the mitochondrial membrane in cardiac myocytes, leading to selective accumulation of doxorubicin in the cardiomyocytes. Further, a previous study also stated that reactive oxygen species are able to trigger intrinsic mitochondria-dependent apoptotic pathway in cardiac myocytes. Moreover, nitric oxide also has been considered to be associated with cardiomyopathy due to high levels of nitric oxide production via inducible nitric oxide synthase (iNOS), which related to dilate cardiomyopathy and congestive heart failure. Similar to cardio-myopathy mechanism, generated free radicals also play an important role in doxorubicin-induced hepatotoxicity. Peroxidation by superoxide radicals in lipids membrane will cause alteration on hepatocytes. Generation within membrane and lipoproteins of peroxyl and alkoxyl radicals, aldehydes and other products of lipid peroxidation affected hepatocytes to a greater extent by evoking formation of high molecular mass protein aggregates within the membrane.

Those mechanisms, combining exogenous antioxidants as supportive in chemotherapy regimen seems to be very effective in an attempt to protect cells from oxidative damage due to its ability in scavenging free radicals. Flavonoids are well known polyphenolic natural antioxidants the presence of which may be responsible for the antioxidant role of ChEE-SMEDDS and protection of myocardial tissues from doxorubicin-induced oxidative injury. The highly presence of naringenin and hesperidin in C. hystrix peels showed their main role as antioxidant and free radicals scavenging agents. Biochemically, ChEE-SMEDDS did not show protective effects but looking at histological examination, it showed that ChEE-SMEDDS has potency as supportive agent to prevent doxorubicin induced cardiotoxicity and hepatotoxicity.

In conclusion, the present study using female Sprague Dawley rats had demonstrarted the cardioprotective and hepatoprotective effects of ChEE-SMEDDS alterations induced by doxorubicin. The protective effects of ChEE-SMEDDS could possibly reside most parts on its free radical scavenger activity. Hence, ChEE-SMEDDS possesses the potential to be applied by further clarification of standardization and clinical test in order to improve its therapeutic benefit.

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