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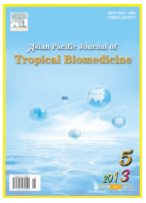

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Cardioprotective and hepatoprotective effects of *Citrus hystrix* peels extract on rats model

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PEER REVIEW

Peer reviewer

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Comments

This is a good study of exploring plants extract to be combined with doxorubicin to reduce side effects of doxorubicin. The results are interesting, especially its cardioprotective effects.
(Details on Page 374)

ABSTRACT

Objective: To observe the combination effect of doxorubicin and *Citrus hystrix* (kaffir lime's) peel ethanolic extract (ChEE) on blood serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity and cardio-hepato-histopathology of female Sprague Dawley rats. **Methods:** Doxorubicin and ChEE (5 rats per group) were administered in five groups of 3 rats each for 11 d. Group I: doxorubicin (dox) 4.67 mg/kg body weight; Group II: dox+ChEE 500 mg/kg body weight; Group III: dox+ChEE 1 000 mg/kg body weight; Group IV: ChEE 1 000 mg/kg body weight; Group V: untreated (control). **Results:** ChEE repaired cardiohistopathology profile of doxorubicin induced cardiotoxicity and hepatotoxicity rats, but did not repair neither hepatohistopathology profile nor reduce serum activity of ALT and AST. **Conclusion:** ChEE has potency to be developed as cardioprotector agent in chemotherapy.

KEYWORDS

Citrus hystrix D.C., Cardiohepatoprotector, Histopathology, Chemoterapy

1. Introduction

Most patients diagnosed with cancer receive chemotherapy regiments[1], which involves the use of drugs to fight against cancer as well as supportive drugs to reduce the possible occurring side effects[2]. Doxorubicin is a powerful anticancer drug that has been extensively used for treating several hematogenous and solid human malignancies[3,4]. Long-term application of this chemotherapeutic agent, however, has been strictly restricted by its toxicity on several organs[4]. High cumulative doses of doxorubicin are associated to the development of cardiomyopathy, which manifest that chronic effects will eventually lead to congestive heart failure[5]. Evidence of liver function abnormalities in patients treated with doxorubicin have also been reported[6].

The exact mechanisms of doxorubicin-induced cardiac and hepatic toxicity still remain unclear although various mechanisms have been postulated, including oxidative stress, inhibition of DNA and protein synthesis, myofibrillar degeneration, cardiomycyte apoptosis via caspase, mitochondrial DNA damage[7,8]. Among those diverse mechanisms, most evidence designate the involvement of free radicals generated from metabolism of doxorubicin[9,10]. Free radicals may cause subcellular alteration which eventually will lead to cellular damage[11]. Uptake of exogenous antioxidants and free radical scavengers may contribute to enhancement of antioxidant defense system, providing protection in a greater extent[12].

Citrus hystrix (*C. hystrix*), so-called kaffir lime, contains abundant naringenin and hesperidin, which are considered as flavonoid compounds[13]. A study reported that among

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parts of the plant, highest yield of extraction was obtained from peels[14]. Antioxidant activity and free radicals scavenging properties of naringenin and hesperidin have been examined by numerous researchers using various assay systems[15,16]. Naringenin could increase CD^{4+}/CD^{8+} ratio on T cell which is responsible for immune system[17], while hesperidin could enhance innate and adaptive immune system[18], and cardioprotective effect on doxorubicin induced rats[19]. Previous study on doxorubicin-induced immunosupression in Sprague Dawley rats had revealed the immunostimulatory activity of *C. hystrix* peels ethanolic extract. Therefore, the potential protective role of these compounds has prompted us to address in the current study the possible cardioprotective and hepatoprotective effects of antioxidant-rich plant, *C. hystrix* D.C.

The cardioprotective and hepatoprotective effects was investigated by determining the activities of serum enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) on doxorubicin induced cardiotoxicity and hepatotoxicity in female rats. Further investigation was conducted by observing the histopathological changes in cardiac and hepatic tissues.

2. Material and methods

2.1. Animals

Female Sprague-Dawley rats weighing 80 to 120 g (about 40-50 d) were obtained from Unit of Experimental Animals Development, Universitas Gadjah Mada. Animals were conditioned 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 diet and tap water *ad libitum*. Body weight was recorded weekly 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.

2.2. Materials

C. hystrix D.C. fruits were harvested from Jedigan, Trirenggo, Bantul. Plants were identified at Laboratory of Plants Taxonomy, Faculty of Biology Gadjah Mada University, Yogyakarta, Indonesia. Extract was prepared by the maceration procedure from dried peels using 70% ethanol for 5 d and re-maceration of residue for consecutive 3 d. Extract was then concentrated using rotary evaporator. Doxorubicin, purchased as ampoules (PT. Kalbe Farma, Indonesia). ALT and AST kit (Bio Rad, 731) was prepared.

2.3. Experimental design

Animals were randomly divided into five groups with three rats in each group and received treatment as follows. Group 1 (Dox-treated group) was injected with doxorubicin 4.67 mg/kg intraperitoneally on Day 1 and Day 6. Group 2 and Group 3 were also treated with doxorubicin on Day 1 and Day 6 as well as the first group, along with ChEE 500 mg/kg and 1 000 mg/kg (*p.o.*), respectively, for consecutive 11 d (Day 1 to Day 11). Group 4 was administered daily 1 000 mg/kg of ChEE (*p.o.*) for consecutive 11 d as well (Day 1 to Day 11). Group 5 served as control group and received standard diet and tap water. At Day 12 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.

2.4. Assay of serum ALT and AST

Blood samples were collected on Day 12 and sera were separated by 3 000 r/min centrifugation at 4 °C for 15 min. Obtained sera was then separated into microtube and kept at −20 °C. Effects of ChEE on ALT and AST activity was analyzed as the procedure from kit manufacturer (Bio Rad, 731).

2.5. Haematoxylin and eosin (H&E) staining

Animals were sacrificed by decapitation at the end of experiment (Day 12). 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 100-400×.

2.6. Statistical analysis

All data are expressed as Mean±SD (n=3). 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).

3. Results

3.1. Effect of doxorubicin, ChEE and their combination on ALT and AST activity

In the present study, the administration of doxorubicin increased the activities of AST, represented by the significantly higher activities of AST in Dox-treated group compared to control. AST activities of both groups treated

with doxorubicin and two dose of ChEE also elevated significantly, indicating both lower and higher doses of ChEE had no lowering effects on the elevated AST activities throughout the study. ChEE-treated group itself showed no significant changes of AST activities compared to those in control group (Table 1).

In contrast to the effect of doxorubicin on AST activities, Dox-treated group showed no significant changes in ALT activities compared to control group (Table 1). Similarly, any ChEE-administered groups also showed no significant effects on ALT activities compared to those in control group (Table 1).

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

Groups (n=3)	Serum enzyme activity (IU/L)	
	AST	ALT
Dox	186.83±12.70[a]	42.50±2.95
Dox+ChEE 500 mg/kg body weight	204.10±53.85[a]	49.10±2.72
Dox+ChEE 1 000 mg/kg body weight	217.97±8.01[a]	45.87±2.71
ChEE 1 000 mg/kg body weight	126.37±31.42	49.70±5.67
Control	124.83±19.37	43.77±5.19

[a]: P<0.05 compared to the control.

3.2. Histopathological analysis of liver

Light microscopic observation discovered that, administration of doxorubicin induced a massive hepatotoxicity, which was indicated by severe pathological alterations and few inflamatory compared to control group (Figure 1a). Treatments with both lower and higher doses of ChEE on doxorubicin induced hepatotoxicity in rats markedly restored the hepatic configuration and eliminated inflamation induced by doxorubicin. Most nuclei exhibited normal shape with edema (Figures 1b and 1c), and inflammatory changes were found. In ChEE group no histological alterations were observed compared to control (Figure 1d).

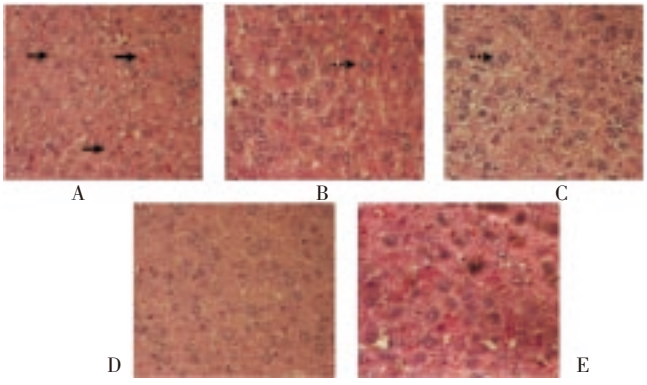

Figure 1. Histological profil of liver sections.
A: Dox; B: Dox+ChEE 500 mg/kg body weight; C: Dox+ChEE 1 000 mg/kg body weight; D: ChEE 1 000 mg/kg body weight; E: control. Black arrows point bleeding cells, while dashed arrows point edema cells. Magnification 200×.

3.3. Histopathological analysis of cardiac

Sections from control and ChEE group showed normal histological structure of the myocardial muscle cells (Figures 2d and 2e) respectively. Micrographs of Dox group showed a loss of normal myocardial structure and persisting with an irregular structure (Figure 2a). In addition, edema with inflammatory cells infiltration was showed on respective micrographs (Figure 2a). These histopathological alterations clearly suggested the toxicity of doxorubicin on cardiac tissues.

Treatment of ChEE was able to relieve most of the histopathological alterations induced by doxorubicin (Figures 2b and 2c). Micrographs of group treated with Dox +500 mg/kg body weight ChEE showed regeneration of myocardial architecture and palliates the edema that were considered to be severe in the myocardium of Dox treated group, but numerous inflammatory cells were still present (Figure 2b). Photomicrographs from Dox+ChEE 1 000 mg/kg body weight treated group were similar to that in Dox+ChEE 500 mg/kg body weight group, but with lesser infiltration of inflammatory cells (Figure 2c). In addition to its protective effects, single treatment of ChEE was found to be safe. It could be seen on sections of ChEE 1 000 mg/kg body weight group that show no alterations of myocardial structure (Figure 2d).

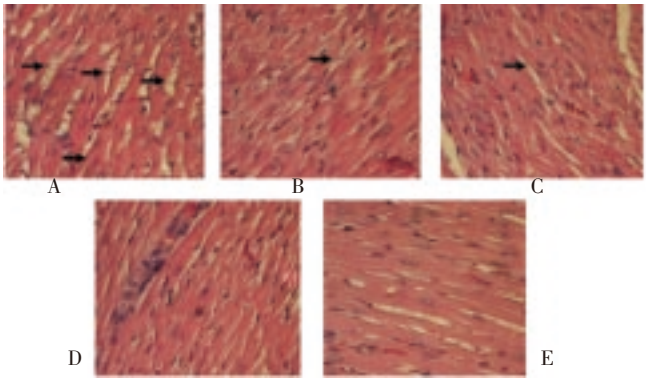

Figure 2. Histological profild of hearth sections.
A: Dox; B: Dox+ChEE 500 mg/kg body weight; C: Dox+ChEE 1 000 mg/kg body weight; D: ChEE 1 000 mg/kg body weight; E: control. Black arrow pointscell vacuola. Magnification 200×.

4. Discussion

This study explored the combination effect of doxorubicin and kaffir lime's peel extract on blood serum ALT and AST activity and cardio-hepato-histopathology of female Sprague Dawley rats. The treatment of ChEE in both concentrations had no lowering effects on the elevated ALT and AST activities. Histopathology profile on liver showed that doxorubicin induced a massive hepatotoxicity. Treatments with both concentrations of ChEE on doxorubicin induced cardiotoxicity and hepatotoxicity in rats markedly restored the hepatic configuration and

eliminated inflamation induced by doxorubicin. The effect of ChEE on heart tissues showed that ChEE was able to relieve most of the histopathological alterations induced by doxorubicin.

Doxorubicin can generate free radicals through enzymatic (semiquinone free radicals) that leads to membrane peroxidation and non enzymatic pathways that generate superoxide anion and hydrogen peroxide which are responsible for lipid peroxidation[20,21]. Free radicals generated from metabolisms of doxorubicin will enhance the cellular oxidative stress, which then induced cardiomyopathy. A previous study also stated that reactive oxygen species are able to trigger intrinsic mitochondria-dependent apoptotic pathway in cardiomyocytes[22]. Despite reactive oxygen species, nitric oxide also has been considered to be associated with cardiomyopathy. High levels of nitric oxide production via inducible nitric oxide synthase (iNOS) are related to dilated cardiomyopathy and congestive heart failure[23]. Radical interaction of nitric oxide with superoxide will produce another highly toxic oxidant, peroxynitrite anion ($ONOO^-$), which can interact with nucleic acids[24].

Similar to cardiomyopathy mechanism, generated free radicals also play a major role in doxorubicin-induced hepatotoxicity. Peroxidation by superoxide radicals in lipids membrane will cause alteration on hepatic tissues. Generation within membrane and lipoproteins of peroxyl and alkoxyl radicals, aldehydes and other products of lipid peroxidation affect hepatic tissues to a greater extent by evoking formation of high molecular mass protein aggregates within the membrane[11]. Next study of ChEE on iNOS and some enzymes related to doxorubicin metabolism is interesting to be evaluated further.

From those mechanisms described above, combining exogenous antioxidants as supportive in chemotherapy regimen seems to be very promising in an attempt to protect tissues from oxidative damage due to its ability in scavenging free radicals. Flavonoids comprise a family of compounds that possess radical scavenging and iron-chelating properties[10]. It was found abundantly in *Citrus* species. Naringenin and hesperidin are flavonoids that present highly in *C. hystrix*[14], concentrated in peels[15]. Powerful antioxidant and free radicals scavenging activity of these compounds have been noted[10,18]. The study of antioxidant effect of ChEE need to be explored more details.

Further confirmation of biochemical alterations on doxorubicin induced rats showed that both doses of ChEE were able to protect cardiac tissues of the rats based on histopathology study. Even though these histopathological results were not concomitant with those in enzymatic study, there are no exact values of the biomarker enzymes that directly correlate to severity of injured cardiac or hepatic tissues. Biochemically, ChEE did not show protective effects but direct histological examination showed that ChEE is a very potential supportive agent to prevent doxorubicin induced cardiotoxicity and hepatotoxicity. However, biomarkers of superoxides and nitric oxides were not yet analyzed in this study, and need to be clarified further.

In conclusion, the present study using female Sprague Dawley rats had revealed the cardioprotective and hepatoprotective effects of *C. hystrix* peels ethanolic extract (ChEE) in ameliorating most histopathological alterations induced by doxorubicin. The protective effects of ChEE could possibly reside most parts on its free radical scavenger activity. Thus, ChEE possesses the potential to be applied clinically in order to improve the therapeutic benefits of doxorubicin.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Long term usage of doxorubicin in cancer therapy induced cardiotoxicity and hepatotoxicity. Free radicals of doxorubicin may cause cellular alterations which eventually lead to cellular damage. Previous study explored antioxidant activity of flavonoids on citrus peels. Thus, application of peels extract containing antioxidant in doxorubicin induced cardiotoxicity and hepatotoxicity rats was addressed to heal free radical effects on heart and liver.

Research frontiers

Previous study showed immunostimulatory activity of *C. hystrix* peels ethanolic extract. Therefore, the potential protective role of the extract has prompted the authors to address in the current study the possible cardioprotective and hepatoprotective effect in the same *in vivo* model.

Related reports

Studies was conducted on doxorubicin induced cardiotoxicity and hepatotoxicity female rats, by examining activities of serum enzymes ALT and AST, as well as morphological changes on heart and liver tissues.

Innovations and breakthroughs

The data showed improvement on heart histopathology of doxorubicin induced cardiotoxicity and hepatotoxicity rats, but did not repair neither hepatohistopathology profile nor reduce serum activity of ALT and AST.

Applications

Free radical of doxorubicin might also induce cellular toxicity in several organs. It might be significant to know the effect of citrus hystrix peels extract on kidney.

Peer review

This is a good study of exploring plants extract to be combined with doxorubicin to reduce side effects of doxorubicin. The results are interesting, especially its cardioprotective effects.

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