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Hepatoprotective Effect of Citrus Sinensis Peel Extract Against Isoniazid and Rifampicin-induced Liver Injury in Wistar Rats

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ABSTRACT

Tuberculosis (TB) is one of the leading causes of death in developing countries. One of the problems in controlling TB disease is that most anti-tuberculosis drugs are hepatotoxic. Citrus sinensis peel extract is the rich source of secondary metabolites with high potential effectiveness as an antioxidant. In the present study, we evaluate the hepatoprotective effect of Citrus sinensis peel ethanolic extract (CSPEE) on isoniazid and rifampicin-induced liver injury in Wistar rats. Twenty five adult male Wistar rats were divided into 5 groups of 5 each : control, (INH+RIF) (50 mg/kg bw once a day for 14 days), (INH+RIF) + various dose of CSPEE (300, 450, 600 mg/kg bw). CSPEE was given orally once a day for 14 days followed by administration of INH + RIF suspension. The measurement of serum ALT and AST were carried out on the 15th day. Histopathologic examination of the liver was also performed. The Serum ALT and AST of the rats that induced with INH + RIF were increased significantly ($P<0.001$) compare to those of control groups, and the histopathologic slides showed steatosis, vacuolation and necrosis of hepatic cells. The serum ALT and AST in groups treated with CSPEE were not significantly different ($p>0.05$) with those of control groups. The serum ALT and AST and histopathological examination of the liver of the group that administered 600 mg/kg CSPEE were closest to normal rats. Citrus sinensis peel extract exhibits hepatoprotective effect on liver injury induced with INH + RIF in Wistar rats.

Keywords: citrus sinensis peel; drug-induced liver injury; isoniazid; rifampicin; Wistar rat

INTRODUCTION

Drug-induced hepatotoxicity is the most common cause of acute liver failure in the United States (Ostapowicz et.al. 2002). Drug-induced Liver Injury (DILI) is common and almost all classes of drugs can cause liver disease. This is because the liver is responsible for metabolizing most drugs. Among hepatotoxic drugs, acetaminophen (paracetamol) is the most frequently studied. However, a variety of different pharmacological agents can cause liver damage, including anesthetics, anticancer, antibiotics, antituberculosis, antiretroviral drugs, and heart medications. In addition, a large number of traditional medical therapies and herbal medicines may also be hepatotoxic (David & Hamilton, 2010).

Tuberculosis (TB) is the ninth cause of death in the world and the leading cause of death from a single infectious agent, ranking above HIV / AIDS. It is estimated that there were 10.4 million TB sufferers in 2016: 56% are in five countries: India, Indonesia, China, Philippines and Pakistan (WHO, 2017). According to data as of January 2018, the total number of TB patients in Indonesia is 360,770 or 138 people per 100,000 people (Indonesian Health Profile Data and Information, 2018).

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Standard therapy for TB includes a combination treatment of isoniazid (INH), rifampicin (RIF), pyrazinamide, and ethambutol. INH can also be used alone for TB prevention (Depkes, 2011). One of the problems in controlling TB disease is that most of the first-line oral anti-tuberculosis (OAT) drugs are hepatotoxic (Department of Health, 2011). INH, rifampicin and pyrazinamide are hepatotoxic, whereas ethambutol and streptomycin are not (Tostman, 2007). In 10-20% of patients, aminotransferase enzymes increase up to 3 or 4 times above the normal value without clinical symptoms and do not need to stop giving OAT drugs. In 1% of patients, there are hepatitis symptoms such as loss of appetite, nausea, vomiting, jaundice and right upper abdominal pain and can be followed by death if TB treatment is not stopped temporarily (Deck & Winston, 2012). In 0.5% of patients who use INH monotherapy, there is an increase in levels of Alanine aminotransferase (ALT) (Nolan *et al.,* 1999). Research from Chowdhury et al (2001) concluded that oxidative stress arising from antituberculosis treatment can cause OAT-induced hepatotoxicity. Some studies say that the administration of drugs or substances that can fight oxidative stress can protect the liver from damage (Pal *et al.,* 2006).

Oranges are the most abundant fruit harvested in the world with an annual production of around 115.5 million tons in 2012. Orange peel is a waste and a by-product of citrus fruit which contains many high-value-added compounds, including polyphenols, carotenoids and essential oils. Polyphenols and carotenoids are known to have many health benefits, mostly associated with antioxidant activity. According to Putnik (2017), the total content of polyphenols is higher in orange peel, which is generally discarded, compared to the fruit itself. Phytochemical investigations of sweet orange peel extract (Citrus sinensis L. Osbeck) found the following contents: carbohydrate, tannin, saponins, flavonoids, anthocyanins and β cyanins, quinones, phenols, coumarins, proteins and amino acids and alkaloids (Selvi *et al.,* 2013). The ethanol extract of Citrus sinensis peel contains many phytochemicals including flavonoids which have antioxidant activity and trap free radicals (Rekha and Bhaskar, 2013). Several studies on the benefits of sweet orange peel extract (Citrus sinensis) have been done such as: evaluation of anti-inflammatory, antibacterial and antioxidant properties (Omodamiro & Umekwe, 2013; Mehmood *et al.,* 2015; Shetty *et al.,* 2015; Hassan *et al.,* 2017); antidiabetic and anticholesterol activities in mice (Muhtadi *et al.,* 2015; Ernawita *et al.,* 2017); protective effect on the onset of oxidative stress and alcohol-induced ulcus gastricus in mice (Selmi *et al.,* 2017); improvement of liver functions of animal trials induced by cigarette smoke. (Setyawati & Anggraeni, 2018); and the effectiveness of Citrus sinensis peel methanolic extract as a hepatoprotector against liver damage and oxidative stress induced by CCl4 in Wistar rats (Ikeokwu, 2014).

This study aimed to examine the protective effect of citrus sinensis (L) peel ethanolic extract (CSPEE) against liver injury induced by INH and RIF in male white rats (Rattus norvegicus) Wistar strain.

METHODOLOGY

Materials

Sweet oranges were bought from the local fruit market. Plant identification was carried out at Herbarium Medanense of the University of North Sumatera. Citrus sinensis peel that has been collected and washed with running water, chopped into small pieces, drained and spread over paper until the water is absorbed, then dried in a drying cabinet. Dry peels were pulverized to a fine powder using a blender. Dried powder was weighed, and stored in an air-tight bottle. Extraction was carried out by maceration using 96% ethanol and suspended in 0.5% NaCMC (sodium carboxy methyl cellulose) solution.

Animals used for testing were male Wistar rats, obtained from the Faculty of Pharmacy of Universitas Sumatera Utara. The age of Wistar rats was approximately 90 – 120 days and weighed 150–200 g.

Methods

Extraction of Citrus sinensis peel

Extraction was done by maceration using 96% ethanol as follows.

Put the simplicia powder in a glass container, added 75 parts of the solvent, covered and left for 5 days in a place protected from sunlight while stirring occasionally.

Maserate was separated, squeezed and maceration pulp is washed with 96% ethanol until 100 parts are obtained.

Transfer to a closed vessel, leave in a cool place and protected from light for 2 days.

Extract was filtered and the filtered extract was concentrated using rotary evaporator at 40°C

Preparation of extract suspension

Three hundred mg extract was put to a mortar and 0.5% NaCMC suspension was added little by a little while crushed with a pestle until homogeneous and then poured into a 10 ml volumetric flask. 0.5% NaCMC suspension was added up to the marked line. The same procedure was carried out for making 450 mg and 600 mg extract suspension.

Qualitative analysis of phytochemicals

Test for alkaloids

0.5 g of simplicia powder + 1 ml of 2 N chloride acid + 9 ml of distilled water, heated on a water bath for 2 minutes, waiting to be dried and filtered. The filtrate was used for the following experiments:

3 drops of filtrate + 2 drops of Mayer reagent solution, observed for the formation of yellowish-white precipitation.

3 drops of filtrate + 2 drops Bouchardat reagent, observed for the formation of brownish-red precipitation.

3 drops of filtrate + 2 drops of Dragendorff reagent, observed for the formation of orange precipitation.

Alkaloids were positive if precipitation was formed at least two of the three experiments above.

Test for flavonoids

0.5 g of simplicia powder + 10 ml of methanol, refluxed for 10 minutes, then filtered hot with filter paper. The filtrate was diluted with 10

ml of distilled water, waited to room temperature, then added 5 ml of ether, shaken carefully and allowed to stand. The methanol layer was taken out, then evaporated at 400 C, the residue was dissolved in 5 ml of ethyl acetate. The filtrate is used to test flavonoids in the following ways:

One ml of the solution was evaporated, the residue was dissolved in 1 to 2 ml of 96% ethanol, then added 0.5 g of zinc powder and 2 ml of 2 N hydrochloric acid, left to stand for 1 minute. Then added 10 ml of concentrated hydrochloric acid. In 2 to 5 minutes, a red coloration confirmed the presence of flavonoids.

Test for saponins

0.5 g of simplicia powder is put into a test tube, added with hot water, let it cool. Then shaken it vigorously for 10 seconds. If a solid foam of 1 to 10 cm is formed for not less than 10 minutes and does not disappear with the addition of 2 N hydrochloric acid indicating the presence of saponins

Test for tannins

0.5 g of simplicia powder was boiled in 10 ml of distilled water in a test tube and then filtered. The filtrate was diluted with water until it was colorless. 2 ml of solution is taken and added 1-2 drops of FeCl3 1% and observed for a blue or blackish green color, indicating the presence of tannins.

Test for steroids and terpenoids

1 g of simplicia powder was macerated with 20 ml ether for 2 hours then filtered. The filtrate was evaporated in a vaporizer cup. The residue obtained was dissolved in 0.50 ml of chloroform, then added 1-2 ml anhydrous acetic acid and 2 drop concentrated sulfuric acid (Liebermann-Burchard reagent). If bluish green is formed, it indicates sterols. If the results obtained are in the form of a brownish or violet ring on the border of the two solvents, indicating the presence of a terpenoids.

Preparation of the test animals

The rats were randomly divided into 5 groups of 5 each, and placed in individual cages and acclimated for 1 week for habitat conditioning.

Group 1: control, the animals were not given any treatment. Food and drinks were given ad libitum.

Group 2: test animals were given 0.5% NaCMC suspension once daily for 14 consecutive days followed by administration of a single dose of INH + RIF suspension 50 mg / kg body weight 6 hours after administration of 0.5% NaCMC suspension. Food and drinks were given ad libitum.

Group 3: the animals were administered orally CSPEE at dose of 300 mg/kg body weight once daily for 14 days followed by administration of a single dose of INH+RIF suspension 50 mg/kg body weight 30 minutes after administration of the extract. Food and drinks were given ad libitum.

Group 4: the animals were administered orally CSPEE at dose of 450 mg/kg body weight once a day for 14 days, followed by administration of a single dose of INH+RIF suspension 50 mg/kg 30 minutes after the administration of the extract. Food and drinks were given ad libitum.

Group 5: the animals were administered orally CSPEE at dose of 600 mg/kg body weight once a day for 14 days, followed by administration of a single dose of INH+RIF suspension 50 mg/kg body weight 30 minutes after the administration of the extract. Food and drinks were given ad libitum.

The Animals were anesthetized with chloroform based on laboratory procedure on the 15th day. Blood was obtained by cardiac puncture immediately, and the liver was saved for histopathological examination. The study protocol was approved by Health Research Ethics Commission (KEPK) Universitas Prima Indonesia (Surat Pernyataan Layak Etik Penelitian No.006/KEPK/UNPRI/II/2019).

ALT and AST examinations from the blood samples were done at the Clinical Laboratory of the North Sumatra Health Office.

Histopathological examination of the liver was carried out and based on work procedures applied at the Pathology Laboratory of the Faculty of Medicine, Universitas Sumatera Utara.

The data obtained were analyzed by One Way Anova at a significant level of 0.05 to determine differences between the treatment groups, then followed by the Tukey HSD post-Hoc test at a significant level of 0.05 to see which groups were different.

RESULT AND DISCUSSION

Qualitative test of phytochemical investigation showed positive result for alkaloids, flavonoids, tannins, saponins, and terpenoids.

The average values of serum ALT and AST of the experimental animals were as follows

ANOVA test results showed difference between groups ($p = 0.001$). The results of the Tukey HSD post-Hoc analysis showed that group 2 had a significantly higher serum ALT and AST (3-5 times greater) compared group 1 and group 3, group 4 and group 5 ($p = 0.001$).

Tabel I. Mean ± standard deviation serum ALT and AST of the experimental rats

Group	ALT (mean ± SD)	AST (mean ± SD)
1. control	42.96 ± 5.40[b]	130,66 ± 28,04[b]
2. INH+RIF	179,12 ± 41,68[a]	334,68 ± 79,16[a]
3. INH+RIF+CSPEE 300 mg/kg	71.96 ± 21.30[b]	196,2 ± 40,22[b]
4. INH+RIF+CSPEE 450 mg/kg	57.84 ± 16.03[b]	164,72 ± 41,20[b]
5. INH+RIF+CSPEE 600 mg/kg	51.40 ± 9.38[b]	147,16 ± 20,45[b]

[a] = significant (p<0,05) with group 1, 3, 4 and 5; [b] = no significant (p>0,05)

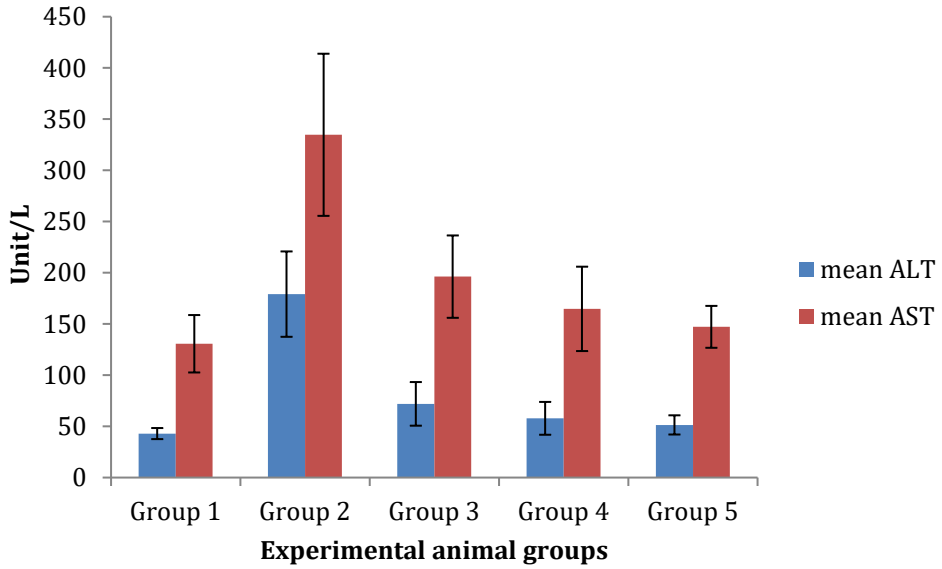

Figure 1. Bar chart of the Mean±SD of serum ALT and AST of the experimental rats.

The trasaminases values of the control group and the CSPEE treated groups were not differ statistically (p>0.05) The bar chart of the average values of serum ALT and AST of the experimental rats can be seen in the following figure 1.

Histopathological examination of the liver showed hepatocellular steatosis, vacuolation and necrosis in a group of white rats induced by INH + RIF (figure 2B). The administration of sweet orange peel extract before being induced with INH + RIF protects the extent of injury to the liver in dose dependent manner. The higher the dose of orange peel extract given, the less damage to the liver (Figures 2C, 2D and 2E).

Table I shows the average ALT and AST values of the untreated animal group were 42.96 ± 5.40 U / L and 130.66 ± 28.04 respectively. These values were in the normal range of white rats. The normal range of ALT and AST are 10-40 U/L and, 50-150 U/L respectively (Hasan *et al.,* 2017). The average ALT and AST values of groups of white rats that were induced with INH + RIF of 50 mg / kg each showed a significant increase (4-5 times above the normal upper limit) giving an indication of liver cell damage. This is consistent with research that in rats, the dose of INH which can give a hepatotoxic effect is 50 and 54 mg / kg (Pal, *et al.,* 2006, Abdel-Ghaffar, 2017). In humans, antituberculosis drugs are reported to be hepatotoxic in up to 28% of patients receiving this drug (Tostman *et al.,* 2007).

Isoniazid is metabolized in the liver, becoming mono and diacetylhydrazine and several other compounds. Hydrazine and AcetylHydrazine are thought to undergo further oxidation and produce reactive metabolics involved in hepatotoxicity. This process involve cytochrome p450 activity especially CYP2E (Delaney & Timbrell, 1995). Co-administration of drugs that increase the activity of cytochrome p450 has an additional effect: rifampicin, for example, increases the toxicity of INH (Sarma, *et al.,* 1986; Finkel *et al.,* 2009). The use of a combination of rifampicin and INH has been associated with a risk of hepatotoxicity. Rifampicin induces isoniazid

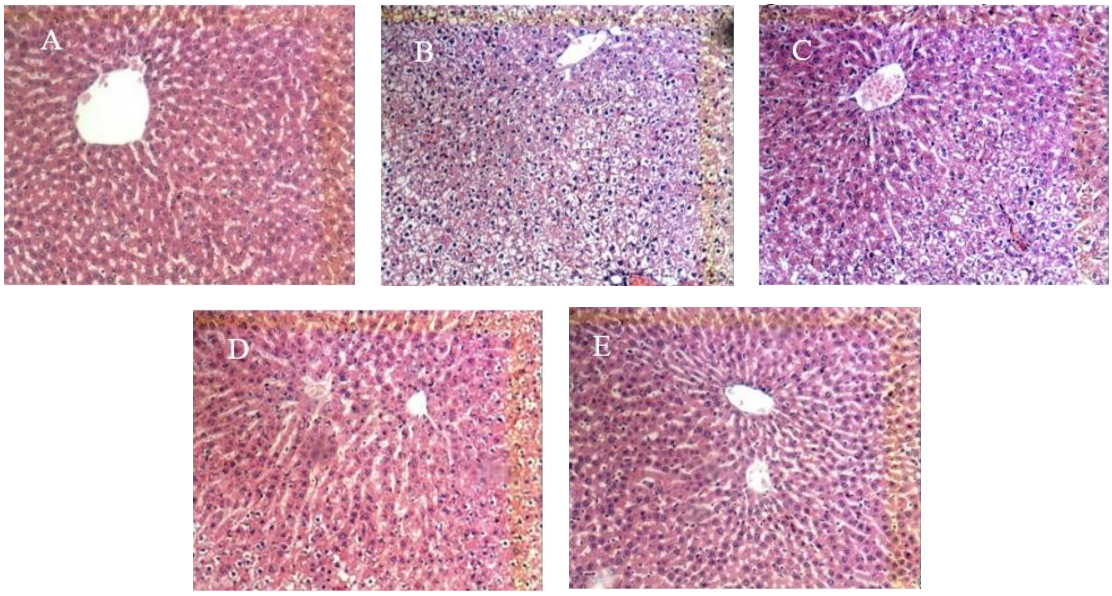

Figure 2. A. control / normal. B. Group 2. Show steatosis, vacuolation and loss of liver parenchyma pattern. C. Group 3. Vacuolation is seen in periportal and midzonal. D. Group 4. Vacuolation decreases in the peripheral zone. E. Group 5. Vacuolation hardly seen with normal liver parenchyma pattern.

hydrolase, increasing hydrazine production, especially in slow acetilators (Tostman, *et al.,* 2007). Previous studies showed that antituberculosis combinations can cause: (1) damage to the liver cell membrane resulting in leakage of ALT, ALP and bilirubin enzymes so their levels increase in the blood; (2) imbalance in endogenous oxidant-antioxidant defenses via increased lipid peroxidation and glutathione homeostasis; (3) enhanced the CYP2E1-mediated bioactivation mechanism (Tasduq, *et al.,* 2007; Eminzade, 2008)

Histopathological examination showed extensive liver damage with liver cell steatosis and lipid vacuolation and loss of normal liver parenchyma pattern (figure 2B). This is in accordance with what was described by previous researchers that in experimental animals, hydrazine causes liver cell steatosis and vacuolation and glutathione depletion; vacuolation of lipids and swelling of the mitochondria were in periportal and midzonal hepatocytes (Tostman *et al.,* 2007).

The average serum ALT and AST of groups of white rats that were induced with INH + RIF and also received CSPEE treatment were significantly lower than those of INH+RIF induced rats. This effect is dose dependent. The higher the dose, the lower the ALT and AST level. This result was also supported by histopathological examination (figure 2C,2D, and 2E). This result proved the hepatoprotective effect of the ethanol extract of citrus sinensis peel on liver damage induced with antituberculosis isoniazide and rifampicin. The ethanol extract of citrus sinensis peel contains many phytochemicals including flavonoids which have antioxidant activity and trap free radicals (Rekha and Bhaskar, 2013).

Antioxidant activity and free radical scavenging can protect the liver cells damage caused by oxidation. Previous research proved the existence of free radical scavenging activity of ethanol extract of citrus sinensis. The antioxidant activity of methanol and ethanol extracts of *C. sinensis* peel showed a significant free radical scavenging activity generated by ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (55.8% and 60.7%) as compared to other solvent extracts i.e. chloroform and diethyl ether. Similar results were also found by DPPH (2,2-diphenyl-1-pikrihydrazyl) assay. The methanolic and ethanolic extracts of *C. sinensis* peel showed 70 to 80% DPPH scavenging activity based on its capability as hydrogen donator (Mehmood, *et al.,* 2015).

Other researcher on sweet orange peel aqueous extract obtained IC50, the dose needed to trap 50% of DPPH and superoxide (O2-) free radicals, which were 188.49 and 198.4 µg/mL respectively, indicating strong antioxidant activity of citrus sinensis peel aqueous extract compared to other herbal extracts. This might be caused by the high phytochemical phenol content (169.94 ± 1.13

GAE (Gallic acid equivalence)/gram) and flavonoids (87.84 ± 1.59 mg QtE (quercetin equivalent)/gram) (Selmi, 2017). Besides that citrus sinensis peel extract can increase the activity of liver enzyme catalase (an antioxidant produced endogenously by liver cells), so it can reduce the effects of oxidation by free radicals which can damage liver cells and thus decrease ALT and AST levels in dose dependent manner (Ikeokwu, 2014).

The average ALT and AST values in group-3 (CSPEE 300 mg/kg) have shown significant differences (p = 0,000) with the mean ALT and AST values in group-2 (INH+RIF induction group). This means that at dose of 300 mg/kg, CSPEE can provide a hepatoprotective effect.

The average value of ALT and AST group-3 (CSPEE 300 mg/kg), group-4 (CSPEE 450 mg / kg), and group-5 (CPSEE 600 mg/kg) even though it shows a declining value, but not significantly different (p> 0.05), and also not significantly different from the control/normal group (p> 0.05). However, from those three treatment groups, the average ALT and AST values and histopathological features closest to the control group was group-5. So it can be concluded that the optimal dose of ethanol extract of citrus sinensis peel to protect the liver against INH and RIF induced liver injury were 600 mg/kg.

CONCLUSION

Citrus sinensis peel extract 600 mg/kg exhibits hepatoprotective effect on liver injury induced with INH + RIF in Wistar rats.

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